

# **Nutrigenomics and Proteomics in Health and Disease**

## **Food Factors and Gene Interactions**

*Nutrigenomics and Proteomics in Health and Disease: Food Factors and Gene Interactions*  
Edited by Yoshinori Mine, Kazuo Miyashita and Fereidoon Shahidi  
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# **Nutrigenomics and Proteomics in Health and Disease**

## **Food Factors and Gene Interactions**

### **Editors**

Yoshinori Mine  
Kazuo Miyashita  
Fereidoon Shahidi

Functional Food Science and Technology Series

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Fereidoon Shahidi *SERIES EDITOR*

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# Preface

Recent advances in the areas of functional foods, nutraceuticals, and natural health products have been culminated by those in modern molecular nutrition. Thus, the advent of nutrigenomic, proteomic, and metabolomic has resulted in a leap toward individualized nutrition, hopefully in the near future. In this connection, the present book on nutrigenomic and proteomic is expected to provide links and information relevant to health promotion and disease risk reduction. Many of the bioactive components present in foods or produced upon ingestion or upon processing under conditions mincing digestion are found to improve health status related to cardiovascular diseases, certain types of cancer, inflammatory disorders and immune response, diabetes, gastrointestinal tract conditions as well as various psychological problems, and the metabolic syndrome. The techniques used to study such benefits have improved over the recent years and unique tools have now become available

that facilitate undertaking of challenges thought impossible only a decade ago.

This book provides a state-of-the-art compilation of the most recent developments in the exciting field of nutrigenomics and proteomics. It is of special interest to nutritionists, food scientists, biochemists, pharmacologists and biologists, among others. This book serves as a reference compendium for scientists in academia, industry, and government laboratories. It may also be used as a text for senior undergraduate and graduate students in multidisciplinary areas listed. We are indebted to world-renowned scientists for their excellent contributions that made the publication of this book possible.

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# **Section I**

## **Introduction**

# 1

## Nutrigenomics and Proteomics in Health and Disease: An Overview

*Yoshinori Mine, Kazuo Miyashita, and Fereidoon Shahidi*

Association between diet and chronic diseases has long been recognized through epidemiological studies. Modern molecular nutrition focuses on health promotion, disease risk reduction, and performance improvement through diet and lifestyle considerations (Kussmann and Blum 2007; Ronteltap et al. 2008). New genomic, proteomic, and metabolomic techniques are now enabling us to find out more about the basis of these associations through examination of the functional interactions of food with the genome at the molecular, cellular, and systemic levels (Corthésy-Theulaz et al. 2005; Kato 2008; Mariman 2006). The human genome is estimated to encode over 30,000 genes and to be responsible for generating more than 100,000 functionally distinct proteins. While traditional nutrition research has dealt with providing nutrients to nourish populations, nowadays it focuses on improving health of individuals through diet. Modern nutritional research is aiming at health promotion and disease risk reduction and on performance improvement (Trujillo et al. 2006). Nutrigenetics questions as to how individual genetic disposition, manifesting as single nucleotide polymorphisms, copy-number polymorphisms, and epigenetic phenomena, affects susceptibility to diet. Nutrigenomics addresses the inverse relationship; that is, how diet influences gene transcription, protein expression, and metabolism. Metabolomics is a diagnostic tool for metabolic classification of individuals. A major methodological challenge and first prerequisite of nutrigenomics is integrating genomics (gene analysis), transcriptomics (gene expression analysis), proteomics (protein expression analysis), and metabolomics (metabolite profiling) to define a “healthy” phenotype (Kussmann et al. 2006; Milner 2004). The long-term deliverable of nutrigenomics is personalized nutrition for maintenance of indi-

vidual health and prevention of disease (Fay et al. 2008; Kaput 2008; Ronteltap et al. 2008). “Nutrigenomics” may offer a new approach for understanding the beneficial effects of dietary compounds on the development of severe polygenic diseases, such as cardiovascular disease, diabetes, and hypertension (Keusch 2006).

This book aims to compile current science-based nutrigenomics and proteomics in food and health. The book comprises four sections: (I) Introduction, (II) Genomics and Proteomics in Health and Diseases, (III) Food Factors–Gene Interactions, and (IV) Advanced Analytical Techniques for Nutrigenomics and Proteomics.

Chapter 1 summarizes aims and scope as well as overall highlights of this book. Chapter 2 consists of introductory *omics* in nutrition and health research. Nutrigenomics contains the three *omics* disciplines gene, protein, and metabolite profiling (transcriptomics, proteomics, and metabolomics) as applied to the field of nutrition and health. Furthermore, nutrigenomics forms the scientific basis for developing nutrition adapted to the specific needs of (rather large) consumer groups, be they healthy, at risk, or diseased. The three *omics* platforms are introduced in this chapter that also describes their application in nutritional research. Microarray-based gene expression analysis is the most mature genome-wide profiling platform. Consequently, transcriptomics in nutritional studies is widely applied when it comes to basic and preclinical research in either cell culture systems or animal models. Proteomics has evolved as an analog to genomics, from identifying all proteins present in a given sample at a given time to a global molecular analysis platform addressing functional aspects of biological systems. Comparing such variations in the proteome enables the discovery of key proteins and

the identification of modulated pathways involved, for example, in specific nutrition-related processes. Over the last two decades, proteomics has developed into an established technology for biomarker discovery, clinical applications, disease profiling and diagnostics, and the study of protein interactions and of the dynamics of signaling pathways. Metabolites represent the endpoints of metabolism and can provide information on the molecular events associated with the adaptations of the body to increased or decreased fluxes of nutrients through metabolic pathways. Metabolomics in nutrition addresses the challenge of characterizing food-related metabolic modulations. Moreover, individual metabolites such as cholesterol, glucose, and homocysteine are considered as markers for health or disease status. Nutrigenomics and nutrigenetics are key science platforms to promote health and prevent disease through nutrition that better meets the requirements and constraints of consumer groups with specific health conditions and particular lifestyles. Section II comprises three chapters on the impact of nutrigenomic and proteomic interventions on health and diseases. Chapter 3 deals with personalized nutrition and medicine. The concept of personalizing nutrition and medicine—and therefore healthcare—emerged from the human genome and haplotype projects. The results of these large-scale, international initiatives offered the hope that nutrition and medicine could be tailored to the individual. The significant advances in understanding complex biological processes relied on reductionistic approaches: hold all variables but one constant. While this strategy was successful for certain monogenic phenotypes, understanding complex systems requires analytical approaches that incorporate rather than avoid complexity. The key challenge for personalizing healthcare then is not the complexity of the data sets, but acquiring those data sets in a manner to reduce noise and increase true signals. This might best be accomplished by preselecting phenotypes based on quantitative data, or alternatively, preselecting genotypes that maximize differences in allele frequencies of candidate genes involved in nutrient metabolism or other physiological traits. The integrative whole system analyses of the data sets and new visualization methods such as shown with network analysis tools provide a path not only to perform these complex experiments, but also to develop biological insight into the outcomes. The development of nutrigenomics and genetics and the application of this knowledge will provide strategies for maintaining health and improving medical treatment of chronic diseases.

Chapters 4 and 5 discuss obesity and nuclear receptors and inflammatory genes involved in obesity-

induced inflammatory responses and pathologies. Obesity is the state of excessive formation of adipose tissues. Recent research has clarified the differentiation of adipocytes, the level of subsequent fat accumulation, and the secretion of the biologically active adipocytokines by adipocytes). In particular, it has been clarified that adipocytokines secreted by adipocytes play a significant role in the pathogenesis of diseases such as diabetes and cardiovascular diseases and are closely associated with the pathogenesis and exacerbation of ailments arising from obesity. This chapter discusses obesity and the metabolic syndrome and then describes the nuclear receptors that are most important in adipocyte differentiation and the mechanism underlying the expression of function of adipocytes affecting obesity from the viewpoint of nutrigenomics. Obesity is also a low-grade systemic chronic inflammatory condition, characterized by abnormal cytokine production, increased acute phase proteins, and other inflammatory mediators. Obesity-induced inflammation consists of a set of inflammatory immune components and inflammatory signaling pathways similar to those involved in classical inflammation, such as inflammatory cells like macrophages, inflammatory mediators like cytokines and chemokines, as well as inflammatory signaling molecules. Obesity-induced inflammation is considered to serve as the potential mechanism linking obesity to obesity-related pathologies such as insulin resistance, type 2 diabetes, fatty liver disease, atherosclerosis, some immune disorders, and several types of cancer. Chapter 5 specifically focuses on obesity-induced inflammatory components, linking to obesity-related pathologies. Adipose tissue-derived inflammatory genes/proteins such as adipocytokines and signaling molecules and the inflammatory cross-talk within adipose tissue cells through adipocytokine. Allergies affect almost 20% of the population in the developed world and allergies can be life-threatening. Individuals may be allergic to a variety of natural or synthetic molecules, such as foods, drugs, chemicals, dust, pollen, and metals. Genomic and proteomic methods are powerful techniques for the identification, characterization, and *in vitro* diagnosis of allergies. Chapter 6 describes molecular mechanisms of allergy and gene interactions and susceptibility to allergic responses. It also reports on recent therapeutic approaches for allergies using recombinant DNA techniques.

Section II includes various food factors—gene interactions and their impact in health and diseases. This section consists of 16 chapters that cover lipids, proteins/peptides/amino acids, carotenoids, phytochemicals, and probiotics. Chapters 7 and 8 deal with the beneficial effects of conjugated linoleic

acid (CLA) and regulation of gene transcription by fatty acids. CLA has been shown to exert various physiological functions, other than antimutagenicity, such as anticarcinogenic and antiobesity (reduction of body fat mass) activities, prevention of atherosclerosis, enhancement of immune function, and suppression of blood pressure, despite the fact that physiological properties of CLA are still limited. The physiological effects of CLA are also described along with potential health benefits of conjugated linolenic acid. Dietary fat is an important macronutrient required for the growth and development of all organisms. Excessive levels of dietary fat or imbalance in its composition (saturated versus unsaturated fat) have been related to the onset or development of several chronic diseases such as coronary artery disease, obesity, and type 2 diabetes as well as certain types of cancer. The biological functions of lipids are mainly carried out by fatty acids and/or derived signaling molecules such as ceramides, diacylglycerols, eicosanoids, and coenzyme A thioesters (acyl-CoA). The last two decades have provided evidence that major (glucose, fatty acids, amino acids) or minor (iron, vitamin, etc.) dietary constituents regulate gene expression in a hormone-independent manner. The molecular mechanisms by which fatty acids and/or their metabolites control the transcription of genes involved in their own metabolism or in carbohydrate metabolism are also described. These effects are mediated either by direct binding on transcription factors such as PPARs, LXR, HNF-4, RXR, etc. (each belonging to the nuclear receptor superfamily) or alternatively through modifications in nuclear abundance and/or activity of numerous transcription factors such as SREBP-1c, ChREBP, and NF- $\kappa$ B.

Chapter 9 focuses on amino acid biological functions as nonnutrient. Although amino acids are widely known as the building blocks of proteins, their functions in living organisms are vast as they can interact with the endocrine, neuronal, and immune systems to influence the balance between health and disease. These systems, particularly in diseased states, affect the amino acid availability and may induce pathways to alter protein synthesis. The underlying mechanism of the regulation of the biological functions is partially due to amino acid control of gene expression. This chapter reviews the importance of amino acid balance and the consequences of amino acid imbalance at the genetic level. Health and disease implications through amino acid deficiency and supplementation was explored. Many amino acid studies have reported health benefits during diseased states, such as cancer, inflammatory disorders, diabetes, gastrointestinal disorders, and muscular wasting diseases. Understanding the mechanism of amino

acid control of genes, both singly and in unison, may provide its involvement in disease progression and prevention. Many researchers have reported that food proteins and their peptides express a variety of functions in the body, including a reduction of blood pressure, antimicrobial activity, antioxidative, anti-inflammatory, antisatiety, anticancer, antiobesity, anti-allergy, modulation of immune cell functions, and regulation of nerve functions. Bioactive peptides are peptide sequences present in the intact protein that under normal circumstances do not have biological properties, but when they are released as peptides *in vitro* or *in vivo*, they exert biological activities. There is increasing commercial interest in the production of bioactive peptides from various sources such as egg, milk, cereal, and fish proteins. Chapter 10 summarizes recent advances of food-derived bioactive peptides–gene interactions and their mechanisms of actions. Although their properties and physiological effects have not been completely explored, bioactive peptides can broadly be divided into two categories: (1) peptides that exert their effects by direct physical interaction with another molecule, and (2) peptides that interfere with gene expression. Bioactive peptides that alter gene expression can do so by (1) epigenetic modification of the proteins that attach to the DNA, (2) alteration of the cell's primary signaling ligand to indirectly influence transcription factor activity, and (3) interference with cell signaling and gene expression via direct binding of peptide ligand to receptor. Understanding the behavior of dietary proteins and peptides in the intestine is also important for designing functional foods with physiological functions.

Carotenoids represent a large group of isoprenoid structures with many different structural characteristics and biological activities. To date, a wide range of carotenoids have been isolated, identified, and quantified from the extracts of fruits and vegetables commonly consumed in the world. The best known biological function of carotenoids is their established role as pro-vitamin A. Chapter 11 describes the nutrigenomic study on the anti-obesity effect of allenic carotenoids from seaweeds and vegetables, with special reference to their regulations on relative gene and protein expressions. Fucoxanthin and neoxanthin are the major carotenoids present in chloroplasts of brown seaweeds and higher plants, respectively. Fucoxanthin is the most abundant of all carotenoids, accounting for >10% of the estimated total natural production of carotenoids. The key for success of fucoxanthin will be induction of uncoupling protein 1 in white adipose tissue (WAT) and downregulation of adipokines such as TNF $\alpha$ . The regulatory effect of fucoxanthin on PPAR $\gamma$  and  $\gamma_3$ -AR in WAT is

correlated with its antiobesity and antidiabetic effects. Furthermore, the relationship between carotenoid structure and suppressive effect on the differentiation of 3T3-L1 adipose cells shows that carotenoids containing an allene bond and an additional hydroxyl substituent on the side group may show the characteristic antiobesity activity.

Chapter 12 deals with the control of systemic inflammation and chronic diseases by the use of turmeric and curcumenoids. Numerous plant-derived, but also microbially derived, substances, often referred to as chemopreventive agents, have documented anti-inflammatory effects and are believed to reduce the rate of aging and prevent degenerative malfunctions of organs and also development of acute and chronic diseases. Among these are various curcumenoids, the active ingredients in turmeric and curry-containing foods, and thousands more of hitherto little or totally unexplored substances. This chapter focuses on documented experimental and clinical effects of supplementation of turmeric, various curcumenoids, and pure curcumin. The Food and Drug Administration (FDA) has approved a health claim for soy-based food products for health benefits primarily based on epidemiological data indicating that high soy consumption is associated with a lower risk of cardiovascular diseases. Soy isoflavones also show a beneficial role in obesity, diabetes, coronary artery disease, and osteoporosis in postmenopausal women. Soy isoflavones have been shown to inhibit carcinogenesis and cancer cell growth *in vivo* and *in vitro*. It has also been found that soy isoflavones lower total cholesterol and low-density lipoprotein cholesterol, suggesting the effect of isoflavones on cardiovascular disease risk reduction. Chapter 13 presents gene expression and proteomic profiling by soy isoflavones. It has been found that soy isoflavones regulate the expression of genes that are related to estrogen regulation, organ differentiation, and fat and bone metabolism in normal cells. Soy isoflavones also inhibit the growth of cancer cells through the modulation of genes, which control cell proliferation, cell cycle, apoptosis, oncogenesis, transcription regulation, and cell signal transduction system. In this chapter, current evidence on the molecular effects of soy isoflavones as documented by nutrigenomic and nutriproteomic research is provided.

Over the last two decades more than five thousand peer-reviewed articles and tens of thousands of news articles have provided evidence for enhanced health benefits of tea consumption. At present, multiple evidences have proven the involvement of tea beverages in health promotion that are directly linked to its polyphenol content. Green tea has firmly es-

tablished its powerful strength in reducing oxidative stress, suppressing cancer-related risks, cardiovascular disease, neuronal damage, and hepatic disorders, among others. Epidemiological and clinical studies have also proven that individuals consuming tea or many form of tea polyphenols benefit from a lower incidence of cancers and other lifestyle-related diseases such as diabetes, obesity, and cardiovascular disease, among others. However, the question that is duly continued to be answered is how green tea polyphenols exert their health beneficial effect? Chapter 14 explores how green tea polyphenols modulate genome functions for protective health benefits. Is it a simple site-specific activity or alteration of a pathway that ultimately lead to altered activity of one or more secondary molecules required to maintain normal cell function or enhancing the meaningful roles of the molecules to maintain cell machinery systems? This chapter reviews how green tea polyphenols modulate genome function, gene repair, protecting genes, and exerting the roles considered auspicious, that even remained unknown until a decade ago. This chapter also lists the latest evidences in accordance with the enhanced physiological functions. Reactive oxygen species are generated ubiquitously in aerobic organisms. When these cytotoxic agents overwhelm endogenous antioxidant defense systems, serious oxidative stress and damage occur as reflected by the oxidative modification of macromolecules such as lipid, protein, and DNA. Thus, it is critical that cells maintain optimal antioxidant defenses in order to reduce oxidative damage. Dietary supplementation and therapeutic use of antioxidants are emerging measures to prevent and treat oxidative stress-induced diseases. Chapter 15 describes oat avenanthramides as novel antioxidants. Oat (*Avena sativa*), although consumed in considerably lower quantities worldwide than wheat and rice, has a highly edible quality and contains high nutritional value compared to other minor grains. Over the past decade, interest of restoring oat as a natural antioxidant additive in food has been on the rise. Other than tocopherols, tocotrienols, and flavonoids, oat contains a unique group of approximately 40 different types of polyphenolic compounds called avenanthramides (AVA) that consist of an anthranilic acid derivative and a hydroxycinnamic acid derivative linked by an amide bond similar to those found in peptides. There is strong evidence that AVA are potent inhibitors of cell proliferation and inflammatory processes, especially in the endothelial cells and smooth muscle cells of blood vessels. These effects have been shown to be mediated by its inhibition of proinflammatory cytokine production and signaling. AVA have also been reported

to modulate endogenous antioxidant defense such as increasing plasma glutathione level and upregulating tissue superoxide dismutase activity, the mechanisms of which remain to be elucidated. Chapter 16 reviews cancer-preventive effects and molecular actions of anthocyanins. Anthocyanins are naturally occurring polyphenolic compounds that confer an intense color to many fruits and vegetables. A few population-based investigations have highlighted the potency of anthocyanins or anthocyanin-containing mixtures on cancer prevention or cancer risk reduction. Studies on animal models have revealed that high intake of anthocyanins or anthocyanin-containing mixtures protects against tumorigenesis of colon, skin, and mammary glands. Extensive studies in cancer cell lines have shown the inhibitory effects of anthocyanins or anthocyanin-containing mixtures on the growth of cancer cells derived from malignant human tissues including vulva, stomach, colon, lung, breast, leukemia, uterus, mouth, and prostate. Recent molecular data have demonstrated that anthocyanins could modulate oncogenic cellular signaling transduction pathways (MAPK and EGFR), transcriptional factor activations (AP-1, NF- $\kappa$ B, p53), and downstream gene expressions (COX-2, iNOS, Bax). These molecular actions are involved in the processes of cell transformation, inflammation, and apoptosis, which provide molecular basis for the cancer-preventive effects of anthocyanins.

Chapter 17 deals with how food components activate capsaicin receptor, transient receptor potential vanilloid subtype 1 (TRPV1). Capsaicin is a pungent principle of hot pepper. Capsaicin exerts several biological activities such as causing burning sensation, stimulating primary afferent neurons conducting chemical pain or hotness, enhancing energy metabolism, showing protection against stomach mucosa, inducing apoptosis in some cancer cells, and so on. Many of them are exerted through capsaicin receptor activation. Because obesity is one of the serious factors on lifestyle-related diseases such as hypertension, stroke, diabetes, and hyperlipemia, this chapter focuses on the thermogenic action or body fat lowering effect of capsaicin. Thermogenic action of capsaicin is thought to be exhibited through activation of TRPV1. From the discovery of TRPV1 gene in 1997, food components activating TRPV1 have been vigorously investigated. There are lists of capsaicinoids of hot pepper, piperine of black pepper, eugenol of clove, ginsenosides of Asian ginseng, and evodiamine of *Evodia rutaecarpa*, among others. Capsiate inhibits accumulation of body fat in humans. Anthocyanins are the largest group of water-soluble pigments in the plant

kingdom. In the human diet, they are derived primarily from a wide variety of plant sources including crops, beans, fruits, vegetables, and red wine, and their effects are also diverse and important to health promotion. Chapter 18 focuses on blackcurrant (*Ribes nigrum* L.) anthocyanins because blackcurrant is rich in it and blackcurrant is consumed in many countries. This chapter provides a review of the newly discovered effects of anthocyanins including their antiobesity effect, antidiabetes effect, and vision improvement. Chapter 19 describes various biological activities of licorice. Licorice, the root of the leguminous *Glycyrrhiza* plant species, is one of the most useful and popular plants in both Asia and Europe, and the history of its consumption as a traditional medicine and food goes back to over 4,000 years to the era of ancient Mesopotamia and Egypt. Licorice contains triterpenes and phenolic constituents such as glycyrrhizin, a well-known typical active constituent of licorice, and the species-specific constituents glabridin, glycy coumarin, and licochalcone A in *G. glabra*, *G. uralensis*, and *G. inflate*, respectively. In *G. glabra*, the species specific compound is glabridin. Various studies have shown the biological effects of glabridin, licorice, or its extracts. These include antioxidative, estrogen-like, anti-inflammatory and anti-*Helicobacter pylori* activities. Hydrophobic flavonoids from *G. glabra* are extracted and concentrated, and the resulting extract is referred to as licorice flavonoid oil (LFO). DNA microarray analysis suggests that the antiobesity effects of LFO are attributable to suppressed fatty acid synthesis and activated fatty acid catabolism in the liver. LFO has also received FDA approval as a new dietary ingredient in the United States in 2006. Therefore, further studies that elucidate the mechanism of LFO containing licorice hydrophobic flavonoids would contribute to the efficient application of LFO in the treatment of metabolic syndrome. Isopentyl diphosphate and its isomer dimethylallyl diphosphate are the universal five-carbon precursors of isoprenoids. Isoprenoids are contained in many herbal plants, and several isoprenoids have been shown to be available for pharmaceuticals, for example, artemisinin and taxol as malaria and cancer medicines, respectively. Various isoprenoids are contained in many plants not only for herbal use but also for dietary consumption. Chapter 20 reports on several bioactive isoprenoids, contained in herbal or dietary plants, which have possibilities to ameliorate metabolic disorders via activation of ligand-dependent transcription factors, that is, nuclear receptors. Chapter 21 reviews anti-inflammatory and anticarcinogenic potential of citrus coumarins and

polymethylated flavonoids. Citrus fruits are well known to contain an array of secondary metabolites in terms of their chemical structures and biological activities, which biosynthesize monoterpenes (*d*-limonene, etc.), triterpenes (limonoids), flavonoids (nobiletin, hesperidin, etc.), coumarins (auraptene, bergamottin, etc.), and carotenoids ( $\beta$ -carotene,  $\beta$ -cryptoxanthin, etc.). Ample evidence obtained from *in vitro* and *in vivo* experiments as well as epidemiological surveys indicates that frequent intake of citrus fruits is beneficial to human health. These citrus compounds are hydrophobic and thus tend to localize in gastrointestinal mucosa in rodents as compared to general polyphenols present. Thus, abundant data have revealed both auraptene and nobiletin to be highly promising citrus components with anti-inflammatory and anticancer activities, with notable action mechanisms and effects on metabolism. One of the distinct characteristics of citrus fruits, as compared with other foods, is the variety of active constituents in terms of chemical characteristics and bioactivities. Thus, combination studies using different types of citrus components for enhancing each efficacy are warranted, such as combining nobiletin (targeting COX-2 transcription) and auraptene (targeting COX-2 translation) to determine their additive or even synergistic effects.

Food and Agricultural Organization of the United Nations and the World Health Organization define *probiotics* as “live microorganisms which when administered in adequate amounts confer a health benefit on the host.” The majority of probiotics are strains of lactobacilli or bifidobacteria and they are administered in food products such as yogurt, milk drinks, and cheese, as well as capsules and tablets. The effect that beneficial microbes have on health maintenance is becoming more and more recognized, given the realization that so many organisms reside in the human body. The reintroduction of beneficial organisms (probiotics) to the host has mostly been via food and dietary supplement products, and thus relevant to nutrigenomics. Chapter 22 discusses some examples of how probiotic microbes and their proteinaceous and other by-products contribute to health. As more human and microbial genomic information emerges, it will become clearer under what conditions probiotic organisms interface with the host in an optimal way.

Section IV highlights recent advances in analytical techniques for nutrigenomic and proteomic research in food and health. Chapter 23 describes microarray as a powerful tool for studying the functions of food and its nutrients. Microarray is a high-throughput genomic tool. It can be used for profiling and monitoring the expression levels of tens and thousands

of genes (entire genomes). It can also be used to determine the influence of food nutrients and/or bioactive compounds (food factors) on metabolic pathways and to understand how food nutrients and factors maintain homeostatic control of gene expression levels. Microarray technology is a “nutrigenomics” tool and can be used to investigate the levels of transcripts in particular. Typically, food is a complex and variable mixture of nutrients and other components. Most food factors are weak dietary signals and must be considered in the context of chronic exposure. Microarray analysis clearly indicates the effects exerted by food factors and nutrients on metabolic pathways via transcriptome modifications. Moreover, the results of microarray analysis suggest that food factors and nutrients influence the metabolome because alterations in the transcriptome cause changes in the metabolome. Therefore, microarray analysis is one of the most convenient tools for inferring the proteome and metabolome. This technology will enhance understanding of the manner in which food and nutrition influence metabolic pathways and how these factors maintain homeostasis under normal conditions or diet-related or non-diet-related disease conditions. Chapter 24 highlights challenges and current solutions in proteomic sample preparation. Proteomics is a discipline of relatively short history, but it holds great promise in elucidating biochemical information via quantitative determinations of the whole collection or representative proteins. One of the common objectives in proteomic studies is the discovery of biomarkers. Although biological systems are extremely complex, and the technology challenges are still many, hundreds and thousands of biomarker candidates are being discovered with advancements made in proteomic technologies. One of the major hurdles in proteomics is the identification of true biomarkers via analytical and clinical validation studies. This chapter reviews some critical aspects of biomarker determination using proteomic methods and some examples of new developments in the proteomic sample preparation techniques, particularly the “pressure cycling technology.” In the past few years, many high-throughput techniques have been developed and applied in biological studies. These techniques such as “next generation” genome sequencing, chip-on-chip, and microarray, among others, can be used to measure gene expression and gene regulatory elements in a genome-wide scale. Moreover, as these technologies become more affordable and accessible, they have become a driving force in modern biology. Traditionally, biologists described these relationships between a limited number

of genes or proteins using a descriptive language. With the huge amount of data produced by high-throughput techniques, biologists have to deal with thousands of biological relations in a single experiment. In this situation, the traditionally descriptive ways for biological relations are not sufficient to deal with the huge number of relations under study. The only way to deal with a large amount of relations is through mathematical representations and computations by researchers in biological sciences. Chapter 25 first introduces basic computational concepts and then illustrates the procedures and computational techniques for high-throughput data analysis, using examples from cancer research. Proteomics is central to nutrigenomics and has the potential to explain many of the physiological changes associated with nutritional stimuli. In proteomics, all proteins expressed in a cell or tissue are analyzed to identify the presence or absence of some key proteins that provide information about the early stages of disease or different conditions. However, a comprehensive analysis of peptides and small proteins of a biological system corresponding to the respective genomic information was missing in proteomics. Chapter 26 introduces the concept of peptidomics. The term *peptidomics* was first introduced as a subset of proteomics for the description of peptides as gene products in February 2000 at the ABRF conference “From Singular to Global Analysis of Biological Systems.” This was coined as a short version of “peptide proteomics” and was defined as the technology for comprehensive qualitative and quantitative description of peptides in a biological sample. Studies of peptidomics cover peptides with low-molecular-weight and small proteins (0.5–15 kDa), since peptides among the families of hormones, cytokines, and growth factors play a central role in many physiological processes. In addition, application of peptidomics knowledge to the nutrient effect may yield potential information about the diet-induced peptide changes and may act as good biomarkers. However, the field of peptidomics is relatively new and has potential to progress in future with the advent of high-throughput mass spectrometry-based technologies coupled with bioinformatics and genomic databases.

Completion of human genome project coupled with the advancement in “omic” technologies enabling researchers to analyze the complex interplay of metabolism, gene expression, and function, and more broadly, genetic diversity within and between human populations. Nutrition science has broadened to the new discipline of nutrigenomics, which allows an in-depth understanding of metabolism, health, and pathophysiology of disease that ultimately could be

used to prevent or treat diseases. The major goal of this book is to comprehensively understand the response of the body’s genes to diets and food factors through various omics technologies such as transcriptomics, proteomics, and metabolomics. This will contribute to the development of new preventive and therapeutic strategies for both pharmacological and nutritional interventions (Bauer et al. 2004; Mariman 2006; Milner 2007).

The editors have succeeded in bringing together many renowned international experts in nutrigenomics and proteomics in health and diseases. We are grateful to all the authors for their state-of-the-art compilation of recent rapid development in this field. We believe that this book certainly deserve a broad readership in the disciplines of nutrition, pharmacology, nutraceutical/functional foods, food science, biology, biochemistry, biotechnology, and life science. This book could also be used as a reference book by senior undergraduate and graduate students as well as nutraceutical and pharmaceutical industry.

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## 2

# Omics in Nutrition and Health Research

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## INTRODUCTION

Nutrients and genomes interact. Nutrition is the most important lifelong environmental impact on human health status. While nutrigenetics addresses how an individual's genetic makeup predisposes for susceptibility for dietary intake, nutrigenomics rather asks how nutrition influences the expression of a given genome.

Nutrigenomics contains the three *omics* disciplines—gene, protein, and metabolite profiling (transcriptomics, proteomics, and metabolomics)—as applied to the field of nutrition and health. Together, they are a prerequisite for nutritional systems biology; that is, the understanding of the dynamic interaction between food components and the entire diet with cells, organs, and the whole body. Nutrigenomics furthermore forms the scientific basis for developing nutrition adapted to the specific needs of (rather large) consumer groups, be they healthy, at risk, or diseased. This chapter introduces the three omics platforms and describes their application in nutritional research. We also discuss current limitations, recommend future developments, and highlight the opportunities for omics integration and correlation with genetics in a nutritional context.

## TRANSCRIPTOMICS IN NUTRITION AND HEALTH RESEARCH

Microarray-based gene expression analysis is the most mature genome-wide profiling platform. Consequently, transcriptomics in nutritional studies is widely applied when it comes to basic and preclinical research in either cell culture systems or animal models. The mRNA profiling bears the potential to

identify specific transcript changes as a response to the administration of a nutrient or non-nutrient compound, or to a treatment or dietary intervention in a well-defined experimental setting. The observed changes in mRNA level are not necessarily causal markers; they might rather represent a pattern of expressed transcripts that changes in a characteristic and reproducible way. Gene expression profiling has the character of a screening process covering thousands of potential indicators of the (changed) metabolic status and, therefore, it often also reveals unexpected findings.

### MICROARRAY-BASED GENE EXPRESSION PROFILING

Although microarrays are not the only available technology for genome-wide gene expression profiling, it has established itself by far as the most widely deployed in research. This is mainly due to a range of commercially available platforms and the meanwhile high level of standardization. Today's microarray platforms are based on either single long or multiple short oligonucleotides as probes. They have different manufacturing procedures and use different labeling methods. The arrays display probes that hybridize with high sensitivity and specificity with their counterparts from the sample. Commercial platforms such as those provided by Affymetrix (Barone et al. 2001) and Agilent (Wolber et al. 2006) rely on *in situ* synthesis of the probes. Affymetrix oligonucleotide arrays consist of 25-mer probes, whereas those of Agilent use longer 60-mer probes. Multiple short oligonucleotides per gene can better discriminate between related sequences, but the longer probes provide better sensitivity. Illumina introduced in 2004 a new microarray technology for quantitative

gene expression profiling on the basis of randomly assembled arrays of beads with each bead carrying a gene-specific probe sequence but multiple copies of each sequence-specific bead in an array (Kuhn et al. 2004). This new platform seems to provide an increased sensitivity compared to the other major commercial microarray platforms, with a larger number of detected genes, and it only needs 25 ng of RNA as starting material for analysis.

Depending on not only the platforms and technologies but also on the RNA source, numerous problems can arise in transcript profiling approaches. One more general challenge is the signal-to-noise ratio, which can be improved with multiple short probes per gene or the same long probe per gene present in multiple copies on the same array. In order to improve interlaboratory comparability, standards for reporting microarray data have been established under MIAME (minimum information about a microarray experiment) (Brazma et al. 2001). This standard contains information required to consistently describe microarray data so that the results derived from its analysis can be independently verified.

Compromised reproducibility of expression profiles is a severe problem that has been meanwhile addressed by a number of studies. However, different conclusions were drawn ranging from good concordance of results across analysis platforms to poor comparability between platforms and laboratories. Recent comparisons of different array platforms (Barnes et al. 2005; Bosotti et al. 2007) revealed that the signal concordance significantly improved with increasing amount of expressed transcript. The concordance was excellent when probes on different platforms could be identified as likely to target the same set of transcripts of a given gene. It appears now that the main factors contributing to result variability are the natural differences between biological samples, rather than the techniques per se. However, variations in sample preparation have been observed when experiments are conducted by different operators. Therefore, the use of automated systems should be envisaged to reduce the “human factor” in technical variability (Raymond et al. 2006).

The challenge of performing microarray studies has today moved from data generation to analysis and interpretation. Analyses restricted to lists of significantly expressed genes with *p* values and fold changes are insufficient to fully understand the underlying biology of metabolic adaptations. A single highly regulated gene does not necessarily have an important biological meaning by itself. Therefore, understanding the biological meaning of the many

observed gene changes requires their assembly to motifs of regulation. This can be achieved either via cluster analysis as a data-driven approach or by knowledge-based annotation analysis.

Cluster analysis uses statistical algorithms to organize genes according to their similarity in pattern of expression. The result is displayed graphically in an intuitive form for biologists and the highlighted patterns can be interpreted as indications of the cell status. Moreover, coexpression of unknown genes with well-characterized genes can bring suggestions on the functions of genes that are not well described yet.

Annotation-based analysis refers to a set of structured and precisely defined vocabularies, called ontologies, used to characterize genes and gene products. This kind of analysis aims at finding out how genes are involved in different molecular functions, biological processes, and cellular components based on the gene annotation. Thus, ontologies are structured in a form that represents a network of linked terms. Cluster analysis may miss subtle changes, whereas annotation-based analysis appears frequently too restrictive. This is why the combination of both analyses is often used to better interpret a gene expression data set.

A large number of tools are available to the science community for microarray data analysis. Classical software for gene expression experiments usually provides cluster and annotation-based analysis. A new generation of tools also offers metabolic and regulatory pathway analysis based on literature-derived information, enabling to interpret data in a larger context (Joyce and Palsson 2006; Khatri and Draghici 2005; Weniger et al. 2007; Yi et al. 2006). Despite the fact that the basic standards for reporting microarray analyses are set under MIAME and that a large variety of commercial and public domain software tools for data interpretation are available, there are still limitations in microarray data analysis.

A more specific problem in nutritional applications is the often low signal-to-noise ratio: in contrast to pharmaceutical or toxicological studies in which usually a limited number of target genes show rather robust changes, in nutrition studies transcript levels typically change more subtly, but the number of affected genes is often surprisingly high and this renders interpretation challenging. Therefore, a sound interpretation of the data needs independent confirmation by assessing protein levels by classical techniques or proteomics (see next part of this chapter) in combination with physiological readouts.

### MICROARRAY-BASED TRANSCRIPTOMICS IN STUDIES ON HUMAN NUTRITION AND HEALTH

Applications of the different omics technologies appear unlimited when utilizing cells in culture or model organisms, but they are constrained when it comes to studies in humans. Expression profiling at the mRNA level is restricted by the limited availability of vital cells or tissues for analysis. Although tissue samples may be obtained via biopsies, especially in nutrition research, these invasive techniques are restricted in use and require ethical approval in every study—in other words, it is very difficult to obtain a biopsy from a control sample.

Different types of blood cells or even whole blood is therefore generally an interesting source of biological material in human transcriptomic studies. Blood cells respond to dietary intervention, and more interestingly, they have different lifetimes, exhibit different gene expression profiles, and can reach and occupy different body compartments. In particular, peripheral blood mononuclear cells (PBMCs) are sampled for microarray-based identification of candidate mRNA markers in human studies in response to nutritional factors. However, attention should be paid to the result interpretation because PBMCs comprise different kinds of cells (B and T lymphocytes and monocytes), each showing a cell-type-specific gene expression signature. From a technical point of view, care needs to be taken for sample storage and preparation, particularly when using peripheral blood cells for transcriptome analysis. It has been demonstrated that sample handling and prolonged transportation significantly alters gene expression profiles (Debey et al. 2004) and these procedures have to be highly standardized for across-site comparisons. More recently, also whole-blood RNA samples are used for profiling purposes. These require the depletion of globin mRNA in order to detect low-abundance transcripts. Various protocols have recently been written to enhance sensitivity and quality of mRNA detection from whole-blood samples (Field et al. 2007; Ovstebo et al. 2007) but, so far, this has not yet been applied to human nutritional studies.

### MICROARRAY-BASED TRANSCRIPTOMICS IN HUMAN NUTRITIONAL INTERVENTION STUDIES

Whole-genome gene expression analysis is increasingly being deployed to assess the efficacy and safety of food ingredients and to evaluate the molecular outcomes of dietary interventions.

Nutrients and genomes interact. Human genetic variation influences nutrient bioavailability and bio-

efficacy. An individual's genome predisposes the organism with regard to the use of nutrients, and—vice versa—the nutrients can significantly alter the expression of the genome. The next-generation transcriptomics technologies, that is, the sequencing-based gene expression analyses are promising means to better determine the molecular mechanisms underlying these interactions and their modification by genetic variation: these techniques enable both the analysis of transcript abundance and its variation among individuals. Nutrigenetics and nutrigenomics are the disciplines addressing these interactions and form the scientific basis for the development of specific diets that could prevent or delay disease and promote health and well-being. This is especially envisaged in chronic diseases because of the lifelong impact of nutrition.

Consequently, a substantial part of these chronic disease-related nutrigenomic/nutrigenetic studies focused on cardiovascular disease, type 2 diabetes, or gastric disorders. While many of the early studies had assumed that single nucleotide polymorphisms (SNPs) were the main source of human genetic variability, an increasing body of evidence suggests the importance of additional layers of variability, including copy number polymorphisms (CNPs) and epigenetic regulation such as DNA methylation. Many complex diseases like irritable bowel disease (Crohn's disease and ulcerative colitis) have been shown to be related to SNPs on particular chromosomal regions, but are also associated with copy number variation of certain other genes (McCarroll and Altshuler 2007; Shelling and Ferguson 2007). Such discoveries suggest that a detailed description of the genetic background of complex diseases is a challenging but necessary objective in order to better prevent pathological development by, for example, adapted diets.

DNA methylation appears to provide a format for long-term dietary imprinting of the genome (Waterland and Jirtle 2003). Some evidence shows that chronic diseases present in adults are due to persistent adaptations to early-life nutrition. DNA methylation would therefore be directly influenced by dietary methyl supplementation, suggesting that nutritional supplementation may have unexpected adverse consequences on the gene regulation in human, and that well-adapted diets applied already at pre- and postnatal stage may exert a fundamental and long-lasting impact.

Even cognitive development seems to be amenable to genetically counseled nutritional intervention. Studies have shown that nutrients, such as n-3 long-chain polyunsaturated fatty acids (n-3 LC-PUFAs),

can affect brain development and, therefore, the cognitive function. A recent publication showed that the association between breast-feeding and IQ is moderated by a genetic variant in a gene involved in the control of fatty acid pathways (Caspi et al. 2007).

Some gene expression profiling approaches are also used to understand the bioactivity of specific food-derived components and to complement previous epidemiological studies that suggested a potential health benefit. Thus, transcript profiling has been used in nutritional interventions to assess the effect of nutrients. Such studies dealt, for example, with antioxidants with the aim of mimicking the benefits of caloric restriction (Lane et al. 2007); with plant-derived flavonoids like green tea catechins (McLoughlin et al. 2004; Vittal et al. 2004); and with soy isoflavones and flavones (Fuchs et al. 2005a, b; Herzog et al. 2004), which have been shown to provide remarkable biological effects as important as cancer-preventive activity. Other health-beneficial nutrients like polyunsaturated fatty acids (Kitajka et al. 2004; Lapillonne et al. 2004) or micronutrients like zinc (Kindermann et al. 2005; tom Dieck et al. 2005) and vitamin E (Johnson and Manor 2004) have also been studied by transcriptomics to describe their effect on the metabolism. The aim here is to identify an affected set of genes whose regulation illustrates a metabolic adaptation. This type of fundamental discoveries can then be used as a basis for the development of adapted diets focusing on particular (pathological) states of the organism.

Given the degree of complexity of genetic research, it appears evident that a combination of genetics and gene expression experiments applied to the same subjects in the same studies can confer added value, assuming that the analysis tools are ready to integrate the related results. Therefore, the deployment of microarray-based gene expression analyses should and will be more and more complemented by SNP, CNP, or epigenetic studies. Nevertheless, the number of human studies in which only transcript profiling is applied to assess the biological effects of nutritional intervention or to identify markers of health continues to grow.

## PROTEOMICS IN NUTRITION AND HEALTH RESEARCH

Proteomics has evolved as an analogue to genomics, from identifying all proteins present in a given sample at a given time to a global molecular analysis platform addressing functional aspects of biological systems (Wilkins et al. 1996). In contrast to the genome, the proteome is highly dynamic and con-

stantly changing in response to the environment of a cell or an organism. Comparing such variations in the proteome enables the discovery of key proteins and the identification of modulated pathways involved, for example, in specific nutrition-related processes. Over the last two decades, proteomics has developed into an established technology for biomarker discovery (Lescuyer et al. 2007; Schrattenholz and Groebe 2007), clinical applications (Mischak et al. 2007), disease profiling and diagnostics (Marko-Varga et al. 2005; Vitzthum et al. 2005), the study of protein interactions (Gingras et al. 2007), and of the dynamics of signaling pathways (Scholten et al. 2006). Nutritional proteomics is an emerging field in which these technologies are applied to nutritional research. It holds great promise to (a) profile and characterize dietary and body proteins, digestion, and absorption, (a) identify biomarkers of nutritional status and health/disease condition, and (c) understand functions of nutrients and other dietary factors in growth, reproduction, and health (Wang et al. 2006a).

In the following, we briefly summarize the main technologies deployed for protein separation, identification, and quantification. Then, we review proteomic studies with a specific focus on nutritional interventions.

## PROTEOMICS TECHNOLOGIES

Numerous reviews of proteomic technologies and applications have been published. Most recently, *Nature Methods* dedicated a special section on mass spectrometry (MS) in proteomics that gives an excellent overview on topics such as large-scale data generation, analysis, and validation (Nesvizhskii et al. 2007), elucidation of cellular networks of protein interactions (Kocher and Superti-Furga 2007), mass spectrometric imaging (Cornett et al. 2007), “top-down” analysis of intact proteins (Siuti and Kelleher 2007), and clinical research perspectives (Beretta 2007).

The proteomics workflow essentially consists of sample preparation and protein/peptide (pre)separation, identification, and quantification. The latter two encompass the complex interface between data generation and processing/validation. Despite tremendous progress at all levels of this workflow, the term “proteome” remains—in contrast to the genome—a theoretical entity, because proteomic studies have to date never revealed an entire proteome. Recent efforts, for example, in *Drosophila melanogaster* research, catalogued up to 63% of the predicted proteome (Brunner et al. 2007). Coverage in higher organisms, however, rarely reaches

more than 10%, with the numbers for quantified proteins being even smaller (Bantscheff et al. 2007). Nevertheless, open databases (e.g., PRIDE (<http://www.ebi.ac.uk/pride>) (Jones et al. 2008) or PeptideAtlas (<http://www.peptideatlas.org>) (Desiere et al. 2006)) have been established to convert data and results from proteomic experiments into publicly accessible information. The most recent addition of this collective effort to standardize and share protein data is the Human Proteinpedia (<http://www.humanproteinpedia.org>) (Mathivanan et al. 2008). This portal provides an integrated view of the human proteome, allowing users to contribute and edit proteomic data similar to the online encyclopedia Wikipedia (Giles 2005). Human Proteinpedia can accommodate data from diverse platforms, including yeast two-hybrid screens, MS, peptide/protein arrays, immunohistochemistry, western blots, co-immunoprecipitation, and fluorescence microscopy-type experiments.

For nutritional studies, in vitro samples like cells as well as ex vivo samples such as tissues and body fluids may be suitable. Cultivable primary cells, that is, nontransformed cell lines, should be chosen over cancer cell lines as those have a number of deregulated pathways as compared to normal cells as recently demonstrated by a systems biology-oriented approach integrating transcriptomic and proteomic analysis of buccal epithelial tumor cells (Staab et al. 2007). Cell cultures, however, offer the advantage of virtually unlimited protein supply but the in vitro models may be far from the in vivo situation. Therefore, intestinal epithelial cell lines have been compared at proteomic level to ex vivo recovered gut cells in different cellular stages (Lenaerts et al. 2007).

Ex vivo proteomic samples encompass tissue sections from gut (Lopes et al. 2008; Marvin-Guy et al. 2005), liver (Edvardsson et al. 2003), biliary tract (Kristiansen et al. 2004), and muscle (Gelfi et al. 2006) obtained by resection of biopsies. An important constraint of proteomic sampling for any nutritional study is the demand of being minimally invasive or noninvasive. Therefore, less invasively sampled body fluids like blood plasma (Anderson et al. 2004) and urine (Adachi et al. 2006) are attractive. The plasma proteome is characterized by the highest complexity and the widest dynamic range, but the proteins in blood are by nature relatively soluble. The urinary proteome has revealed an astonishingly high number of intact proteins and is therefore an information-rich proteome source; however, truncation and degradation of urinary proteins add to the complexity.

Extensive sample preseparation, depletion, and enrichment strategies are required due to the complexity of a proteome and the technical limitations of modern MS.

Two-dimensional gel electrophoresis enabled for the first time to separate, visualize, and quantify many proteins simultaneously in one image and paved the way for proteomics (Gorg et al. 2004). The current state-of-the-art methodology is 2D-DIGE, which stands for differential imaging gel electrophoresis (Tonge et al. 2001): the control and case “proteomes” are labeled each with a specific fluorescent dye, then mixed and co-separated, and subsequently analyzed for fluorescent color ratios in a similar way as DNA microarrays. Despite the remarkable improvements of gel-based proteomics thanks to DIGE (Sellers et al. 2007), the gel approach still suffers from (a) difficult automation and thus limited throughput, (b) relatively narrow dynamic range, and (c) a discrimination of proteins with extreme physicochemical properties (size, pI, hydrophobicity).

Therefore, chromatography-based techniques have been developed for protein and peptide (pre)separation. The established term in this context is multidimensional protein identification technology (MudPIT) (Motoyama et al. 2006), also called “shotgun proteomics” approach (reviewed by Wu and MacCoss 2002). Typically, two-dimensional chromatography (ion exchange followed by reversed phase) is online coupled to electrospray ionization (ESI) MS (see below). The approach is based on “proteome” digestion upstream in the workflow and peptide-level separation.

Often, protein and peptide preseparation alone is insufficient for dealing with proteome-scale sample complexity. Therefore, the most abundant proteins may have to be depleted from the sample, that is, specifically be removed by affinity chromatography without affecting the remaining protein composition, especially when analyzing human plasma (Gong et al. 2006) or serum (Bjorhall et al. 2005). The “Equalizer Technology” was described recently (Righetti et al. 2006): a combinatorial library of ligands bound to beads was shown to reduce the concentration differences in human plasma and urine, essentially by binding less of the abundant and more of the rare proteins.

A complementary strategy of gaining access to low-abundant proteins is enrichment of the latter, for example, when targeting subproteomes such as the phospho- or glycoproteome. Various chemical scavengers have been developed to capture phosphopeptides and proteins, such as IMAC (immobilized metal affinity capture), titanium dioxide resins, or alumina

particles. Recently, Reinders et al. thoroughly compared the performance of these techniques (Reinders and Sickmann 2005). Glycosylated peptides and proteins can be enriched by lectin affinity (Vosseller et al. 2006) or different trapping reactions such as hydrazide chemistry (Sun et al. 2007). Nandi et al. have developed a so-called tagging-via-substrate (TAS) approach for global identification of *O*-GlcNAc-modified proteins, enabling *O*-glycosyl enrichment (Nandi et al. 2006).

Key characteristics of a modern mass spectrometer are sensitivity (today femto- to attomolar), mass accuracy (high to low ppm), mass resolution (10,000 to millions), and speed of MS and MS/MS acquisition. MALDI (matrix-assisted laser desorption/ionization) (Tanaka 2003) and ESI (Fenn 2003) are the most popular and powerful methods to produce gas phase ions of proteins and peptides. Different mass analyzers are used in proteomics experiments, such as triple quadrupole (QqQ) instruments, especially for targeted multireaction monitoring (MRM) experiments to, for example, simultaneously quantify dozens of plasma proteins (Anderson and Hunter 2006). Combined with time-of-flight (ToF) or ion-trap (IT) analyzers, they form hybrid systems such as Q-ToF and Q-IT instruments. Fourier transform ion cyclotron resonance mass spectrometers (FT-ICRs) (Nielsen et al. 2005) and the more recently introduced orbitrap system (Makarov et al. 2006) represent the high-end proteomics MS space. FT-ICR instruments offer ultimate resolution (>100,000) and low-ppm mass accuracy that enables “top-down” analysis of intact proteins as opposed to the more frequently employed “bottom-up” approach (Siuti and Kelleher 2007).

Protein quantification can be achieved through staining with protein dyes, and as discussed above, currently the most advanced technology at protein level is 2D-DIGE (Sellers et al. 2007). A further option is to incorporate stable isotopes into proteins and/or peptides in a differential manner (heavy versus light isotope) and to quantify the proteins/peptides by mass spectrometric comparison of the signals derived from the light- and heavy-isotope-labeled sample. These methods have been summarized by the Regnier group (Julka and Regnier 2004) and assessed in real-life scenarios by Wu et al. (2006) and the Heck team (Kolkman et al. 2005). Introduction of the mass labels can also be achieved by metabolic labeling (Beynon and Pratt 2005; de Godoy et al. 2006). This approach is advantageous because of its minimal interference with the biological system. Metabolic labeling is routinely performed with cultured cells ranging from bacteria and yeast to mam-

malian cells. This has been demonstrated in multicellular organisms such as *Caenorhabditis elegans* and *D. melanogaster* (Krijgsveld et al. 2003) and very recently even in rats (McClatchy et al. 2007). Chemical or enzymatic methods must be applied to label ex vivo recovered tissues or fluids. The chemical tagging concept has been introduced by Aebersold et al. under the name ICAT (isotope-labeled affinity tag) (Gygi et al. 1999). The iTRAQ (isotope tags for relative and absolute quantification) method (Ross et al. 2004) offers quadruplex (and soon eight-plex) analysis, that is, four conditions can be compared in one experiment. In view of multiple chemical tagging methods typically performed post-digestion and targeting one amino acid side chain at a time, our group has come up with a new concept termed AniBAL (aniline-benzoic acid labeling): the same tag is introduced into all amino and carboxyl functions already at the protein level (Panchaud et al. 2008). This approach minimizes sample bias, optimizes proteome coverage, and is based on a simple and symmetric chemistry. Bowman et al. have extended the stable-isotope concept to quantitative glycomics by developing a quadruplex derivatization scheme amenable to mass spectral readout (Bowman and Zaia 2007). Label-free approaches have been developed more recently, based on highly reproducible LC-MS conditions, which allow comparative peptide analysis of complex samples (Old et al. 2005; Ono et al. 2006).

All quantification approaches discussed so far deliver relative quantitative information. Absolute quantification (AQUA) of proteins/peptides was described by Gerber et al. (2003) employing the classical isotope-labeled internal standard approach. The concept of proteotypic peptides takes this strategy to the proteome level by selection of the “best flying” peptide for each protein as a unique identifier (Mallick et al. 2007). Recently, targeted quantification of more than 50 plasma proteins was demonstrated with this approach (Anderson and Hunter 2006). However, design and production of such isotope-labeled reference peptides still needs further improvement for optimal exploitation (Mirzaei et al. 2008).

MS data analysis requires sophisticated software to acquire, store, retrieve, process, validate, and interpret these data and to eventually transform them into useful biological information. While peptide and protein identification and database search programs like Mascot (Perkins et al. 1999), Sequest (Yates et al. 1995), or Phenyx (Colinge et al. 2004) have a long and successful standing (reviewed by Nesvizhskii et al. 2007), entirely new software infrastructures for data processing and validation have been built, such

as the SBEAMS architecture (<http://sbeams.org/>) housing the Trans-Proteomic Pipeline and microarray modules that manage both gene and protein expression data. The idea behind such resources is to provide the researcher with means to assess the quality of the data in a data-set-dependent manner and to control the trade-off between false positives (specificity) and false negatives (sensitivity) (Urfer et al. 2006) on the basis of extensive statistical evaluation. Apart from the progress in proteomics data processing, software platforms enabling cross-correlation with other omics sources and supporting pathway interpretation are emerging and maturing rapidly. The success of proteomics will largely depend on the ability of converting data into proper biological information.

### PROTEOMICS IN NUTRITIONAL INTERVENTION

Proteomics in nutritional science has the potential to deliver biomarkers for nutritional intervention and individual disposition, assess nutritional status at the molecular level, and discover bioactive food peptides and proteins (Fuchs et al. 2005d). We have recently published several reviews summarizing nutritional proteomic applications such as nutritional intervention (Kussmann et al. 2007), elucidation of immune-related gut disorders (Kussmann and Blum 2007), characterization of functional ingredients such as probiotics or milk and soy proteins (Kussmann et al. 2005), or the investigation of perturbed energy metabolism like in diabetes and obesity (Kussmann and Affolter 2008). In the following, an overview on some recent developments and findings is given with regard to the application of proteomics in nutritional interventions.

Soy-based diets have been shown to promote a cardioprotective effect, which is attributed to soy isoflavones as well as soybean proteins. Fuchs et al. (2005c) studied the effect of soy isoflavones, with genistein as the most abundant compound, with regard to the protective activity against atherosclerosis. They demonstrated that genistein at low and high concentrations reversed the stressor-induced decrease of anti-atherogenic proteins. The same group also identified biomarkers in peripheral blood mononuclear cells (PBMCs) (Fuchs et al. 2007) that respond to a dietary intervention with isoflavone-enriched soy extract in postmenopausal women. Twenty-nine proteins have been identified that showed significantly altered expression in PBMCs, including a variety of proteins involved in anti-inflammatory response, thus suggesting that soy isoflavones increase anti-inflammatory activity in

PBMCs. Very recently, Astle et al. (2007) reviewed proteomic and metabolomic responses to dietary factors (i.e., soy isoflavones, polyphenolic substances, carotenoids) and supplements and concluded that powerful proteomic tools such as top-down MS and protein microarrays will critically contribute to the characterization of subtle proteome changes resulting from the variability in consuming dietary factors and supplements.

Proteomic investigations of the enteric nervous system (ENS), the nervous system of the gastrointestinal tract (Hansen 2003), have the potential to deliver insights into gut functionality. It is, for example, recognized that early life events (such as neonatal maternal separation) predispose adults to develop visceral pain and enhanced colonic motility in response to acute stress. In order to better understand the molecular basis for these functional gut disorders induced by environmental stress, our group has established a proteomic catalogue of the rat intestine (Marvin-Guy et al. 2005) and assessed stress effects on intestinal protein expression (Lopes et al. in press). Barcelo-Batllo et al. (2002) have investigated implications of cytokine-induced proteins in human intestinal epithelial cells (hIECs) that are related to the inflammatory bowel syndrome (IBS) (Wood et al. 1999). The motivation behind this study was that cytokine-regulated proteins in intestinal epithelial cells (IECs) had been associated with the pathogenesis of inflammatory bowel disease (Hansen 2003).

Dietary components seem to be important determinants of cancer risk and tumor behavior. Functional proteomic studies will therefore be essential to comprehend the relationship between dietary interventions, proteome changes, and cancer (Milner 2006). As dietary modifications and interventions have the potential to significantly lower cancer risk and its associated complications, validated biomarkers will be invaluable tools for this research (Davis and Milner 2007). Four studies have employed proteomics, either as such or combined with gene expression analysis, to address biomarkers for protection against cancer. Breikers et al. (2006) identified 30 proteins differentially expressed in the colonic mucosa of healthy mice upon increased vegetable intake. Six proteins identified with altered expression levels could be brought into the context of a protective role in colorectal cancer. The second study integrated DNA microarrays with proteomics to investigate the effects of nutrients with suggested anticancer properties and to develop a colon epithelial cell line-based screening assay for such nutrients (Stierum et al. 2001). Tan et al. (2002)

assessed sodium butyrate effects on growth inhibition of human HT-29 cancer cells in vitro by employing a 2DE-MS-based proteomic strategy. Butyrate treatment altered the expression of various proteins, in particular those of the ubiquitin–proteasome pathway: a result suggesting that proteolysis could be an important mechanism by which butyrate regulates key proteins in the control of cell cycle, apoptosis, and differentiation.

Combining gene and protein expression profiling in colonic cancer cells, Herzog et al. (2004) identified the flavonoid flavone, present in a variety of fruits and vegetables, as a potent apoptosis inducer in human cancer cells. Flavone displayed a broad spectrum of effects on gene and protein expression that related to apoptosis induction and cellular metabolism.

Not only the presence but also the absence of a particular nutrient can have a marked effect on an organism. The consequences of nutrient deficiency were studied by force-feeding rats with a zinc-deficient diet and analyzing the hepatic transcriptome, proteome, and lipidome (tom Dieck et al. 2005). Prime metabolic pathways of hepatic glucose and lipid metabolism and their changes under zinc deficiency could be identified by this combined omics analysis, which cause liver lipid accumulation and hepatic inflammation. In the same context, Fong et al. (2005) showed that alleviation of zinc deficiency by zinc supplementation resulted in an 80% reduction of COX-2 mRNA, a key enzyme involved in inflammation.

Food deprivation results in metabolic, structural, and functional changes in the small intestine. Consequently, it influences gut mucosal integrity, epithelial cell proliferation, mucin synthesis, and other processes (Lenaerts et al. 2006). A comparative proteomics study in mice identified intestinal proteins whose expression was changed under different starvation conditions. The results of this study provide novel insights into the intestinal starvation response and may contribute to improve nutritional support during conditions characterized by malnutrition.

Proteomic analysis of depleted human serum samples from a dietary intervention study with supplemented vegetables (broccoli) provided biomarkers for evaluating dietary exposure (Mitchell et al. 2005). MALDI-TOF analysis allowed the identification of the B-chain of  $\alpha$ 2-HS-glycoprotein (fetuin), a serum protein previously found to vary with diet and to be involved in insulin resistance and immune function.

Food components may not only alter gene and protein expression but also target posttranslational modifications (Davis and Milner 2004). Diet-induced protein modifications can ideally be assessed by proteomic techniques. For instance, the protein phos-

phorylation status of the ERK protein changes after exposure to diallyl disulfide, a compound present in processed garlic, an effect resulting in cell cycle arrest (Knowles and Milner 2003). Another example is the modification of thiol groups in the cytoplasmic protein Keap1 (Dinkova-Kostova et al. 2002). This alteration of the protein redox status affects its binding to the protein Nrf2 that acts as a transcriptional regulator.

In summary, nutritional proteomics is an emerging field of proteomics and its success will depend on numerous factors. First, the technology platforms will profit from further improvements including advanced protein/peptide separation techniques, better depletion and enrichment methods, and more sensitive and specific mass analysis techniques. Second, bioinformatic tools to assess data quality and to convert data into interpretable information are advancing rapidly. New systems will be able to support the reconstruction of pathways and regulatory networks even in the presence of fragmentary data (Staab et al. 2007), thus helping to integrate incomplete omic data sets. Third, intelligent focusing on proteome subsets (at the level of cell organelles and protein subclasses, or the mass spectral level, that is, proteotypic peptides and multiple-reaction monitoring) will provide deeper insights into molecular networks. Fourth, addressing protein turnover at proteomic scale, that is, interpreting protein abundance changes as a result of protein synthesis and degradation (Doherty and Beynon 2006) will add value to nutritional intervention studies performed with stable-isotope-labeled amino acids, peptides, and proteins. Furthermore, such data should enable an improved correlation between transcript, protein, and metabolite data, as an increased protein synthesis rate is more reliable in the biological context as just the change at transcript level. Lastly, dietary changes in humans represent rather subtle interventions, resulting in many small rather than a few big molecular changes, rendering data interpretation challenging. Therefore, improved definition of human cohorts undergoing dietary interventions through proper genotyping can be expected to deliver clearer readouts from omics applications.

As nutrition science develops into a holistic molecular science with systems biology character, all intervention studies including those that use proteomic approaches should be based on standardized diets and ingredients, stratified cohorts, and ideally follow the double-blinded, placebo-controlled crossover design. The standardization initiatives in the omics community (Brazma et al. 2001; Lindon et al. 2005; Orchard et al. 2007; Taylor et al. 2007) have yet to be met by similar efforts in the nutrition field that

define rules for providing sufficient experimental information and for best practices in nutritional intervention studies. One such attempt has been launched within the European Nutrigenomics Initiative, NuGO ([www.nugo.org](http://www.nugo.org)) (Astley and Elliott 2007).

## METABOLOMICS IN NUTRITION AND HEALTH RESEARCH

Metabolites represent the endpoints of metabolism and can provide information on the molecular events associated with the adaptations of the body to increased or decreased fluxes of nutrients through metabolic pathways. Metabolomics in nutrition addresses the challenge of characterizing food-related metabolic modulations (Rezzi et al. 2007a). Moreover, individual metabolites such as cholesterol, glucose, and homocysteine are considered as markers for health or disease status.

The metabolomics concept arose mainly from advances in analytical techniques that enable simultaneous measures of hundreds of metabolites in biological matrices. The derived profiles can be explored to characterize metabolic phenotypes and homeostatic processes under normal or pathophysiological conditions (Nicholson et al. 1999). These modulations are expected to be more subtle than the ones caused by toxicological insults or pharmaceutical interventions. Moreover, biological effects in nutrition cannot be reduced to the action of a single molecule; they rather result from the interaction between the consumer metabolome and the food metabolome, the latter encompassing nutrients and non-nutrient compounds. The metabolic response reflects the simultaneous modulation of many metabolic pathways, which are *a priori* more difficult to target through a nutritional intervention as compared to a single mechanism or target protein in pharmacological approaches. Metabolomics in nutrition deals with a complex interplay between (a) the mammalian host genome and the genomes of its gut microflora, and (b) environmental cues determined by food habits, diet composition, and other lifestyle components (Gavaghan et al. 2000; Nicholson et al. 2004; Nicholson and Wilson 2003; Rezzi et al. 2007a).

### METABOLOMICS TECHNOLOGIES

#### Nuclear magnetic resonance spectroscopy and mass spectrometry

Nuclear magnetic resonance (NMR) spectroscopy (Glassbrook and Ryals 2001) and MS (Watkins and German 2002) are the “working horses” in current metabolic profiling. MS is generally and

NMR is increasingly coupled to upfront LC or GC preseparation of the analytes. Apart from “classical” MALDI- and ESI-based techniques (see section on proteomics), FT-ICR MS (Buchanan and Hettich 1993; McIver et al. 1994) has advanced metabolomics due to its superior mass accuracy (low ppm) and resolution (100,000+). Combined with the inherent sensitivity of MS *per se*, FT-ICR can decipher complex metabolite mixtures, even without extensive preseparation (Aharoni et al. 2002).

NMR usually relies on measuring proton resonances ( $^1\text{H-NMR}$ ) because this classical one-dimensional proton resonance measurement is most sensitive. However, other nuclei are also used in NMR-based metabolomics, such as  $^{19}\text{F}$ : in one case, vitamin C homeostasis was specifically monitored in a diabetes model (Nishikawa et al. 2003). MS is superior in terms of sensitivity and structural elucidation, whereas NMR is *per se* quantitative (signal is proportional to the analyte amount independent of the analyte nature). Moreover, as NMR analytes do not physically interact with the instrument and because chemical shifts are measured rather than mass-over-charge ratios, NMR platforms are more robust and stable over time, and therefore, interplatform and/or between-laboratory comparisons are easier (Keun et al. 2002). Due to this complementarity, modern metabolomic laboratories take advantage of some LC preseparation followed by a sample split: the larger part of the sample is preconcentrated for NMR purposes and the smaller amount is directly infused to MS. The sensitivity of NMR-based metabolomics can be enhanced by sample preconcentration through reversed-phase capture and by utilization of NMR cryoprobes. A specific advantage of NMR is the option of profiling metabolites directly in intact tissues by magic angle spinning (MAS) (Shockcor and Holmes 2002): a few milligrams of tissue are placed in a rotor and spun rapidly at  $54.7^\circ$  relative to the applied magnetic field within the bore of the magnet. Doing so, the line broadening, characteristic of solid-state NMR, can be dramatically reduced, and consequently, resolution is greatly enhanced.

### Chemometrics

Chemometrics can be understood as a mathematical toolbox for chemistry (Lavine and Workman 2004) and differs in that sense from bioinformatics, which represents storage, retrieval, and analysis of computer-derived information (rather than raw data) in a biological context (Bains 1996). Chemometrics started as a discipline with the effort

to use an entire spectrum as source of information rather than only the assignable peaks (Holmes and Antti 2002; Stoyanova et al. 2004). When applied to NMR- and MS-based metabolomics, chemometrics encompasses spectral processing, peak alignment, outlier detection, normalization, and so forth.

Software tools for metabolomic data analysis are mainly based on multivariate statistical methods (Lindon et al. 2001). Depending on the mathematical model building, the statistics can be divided into nonsupervised and supervised techniques. Non-supervised approaches such as principal component analysis (PCA) are applied to explore the overall statistical variance with the goal of clustering the metabolic profiles and detecting outliers: large amounts of mass spectral or NMR spectroscopic data are understood as a multivariate statistical problem and the metabolite concentrations represent the true variables. Spectra are divided into “bins” of discrete spectral width (ppm in the case of NMR,  $m/z$  in the case of MS), and the areas under the curve (AUC) in these “bins” are integrated and serve as pseudovariables. PCA therefore reduces a large number of (usually correlated) “true” variables into a smaller number of (uncorrelated) variables, the so-called principal components. PCA results in the decomposition of raw data into “scores,” which reveal the relationship between samples, and into “loadings” that show the relationships between the variables. The first PC explains the greatest variability in the data, the second PC (independent of/orthogonal to the first) explains it second best, and so on.

As a metabolic response associated with intake of specific foods or individual ingredients is expected to be subtle on the background of global metabolic variability, the application of supervised statistical techniques, for example partial least square discriminant analysis (PLS-DA), is often needed in order to maximize the identification of metabolites responsible of a class-specific metabolic fingerprint (Rezzi et al. 2007a; Trygg and Wold 2002).

### METABOLOMICS IN PRACTICE

An asset of metabolite profiling is the noninvasive nature of monitoring metabolic endpoints in humans (German et al. 2003). Metabolomics has been employed in preclinical and clinical research, for environmental, biomedical application, and in toxicology (Robertson 2005). More recently, it has developed a strong impact in nutritional research (Whitfield et al. 2004) and applications of metabolomics in various fields of nutrition have recently been reviewed (Rezzi et al. 2007a).

As it applies to other omics disciplines, metabolite profiling, identification, and quantification face the challenge of high interindividual variability. Because of the rather subtle metabolic effects, biological and environmental confounding factors and interindividual variability need to be considered and an experimental design with well-defined inclusion and exclusion criteria is a prerequisite. In this perspective, standards for execution and reporting of metabolic analyses have been recently recommended by the standard metabolic reporting structures (SMRS) working group (Lindon et al. 2005). This initiative finds its analogues in the transcriptomic domain (MI-AME) (Brazma et al. 2001) and in the proteomic society (MIAPE, minimal information about a proteomic experiment) (Taylor et al. 2007).

In animal trials, the genetic homogeneity of the studied strains decreases the variability of the basal metabolism, but environmental conditions and the gut microflora activity contribute significantly to metabolic disparities among subjects (Martin et al. 2007; Nicholson et al. 2004). Applications of metabolomics to human nutrition trials are even more challenged by metabolic variability due to (epi)genetic differences and the wide panel of eating patterns determined by physiological, psychological, behavioral, cultural, and socioeconomic factors. The standardization of the diets (both for intervention and during a run-in phase of a study) is recommended to reduce metabolic variations that are not linked to the intervention itself. Hence, the control of sources and amounts of macro- and micronutrients consumed is a must (Lenz et al. 2004; Rezzi et al. 2007b; Stella et al. 2006).

The type of samples to be analyzed and the frequency of their sampling depend on the scientific question. In most cases, urine and plasma samples will serve the purpose. Metabolic profiles of urine provide a time-averaged representation of recent homeostatic metabolic changes and also encrypt information on the metabolic activities of the gut microbiome (Dumas et al. 2006a; Nicholson and Wilson 1989). Plasma reveals more instantaneous metabolic events and reveals insights into the dynamics of nutrient and metabolite flow between the organs. Metabolomics is increasingly applied to study fecal matter, which may carry information on metabolic gut health, digestive efficiency, and the activity of the gut microbiota (Marchesi et al. 2007). The sample preparation methods depend on the selected analytical techniques, that is, MS and/or NMR. NMR is the less-demanding platform, because virtually any biofluid or tissue is amenable to analysis in its intact form.

### Metabolomics to assess nutritional intervention

Metabolomics in nutrition attempts to elucidate metabolic alterations in response to a food ingredient, food product, or diet. Metabolomic applications in nutrition-oriented research have recently been reviewed (Rezzi et al. 2007a). Targeted metabolic analysis is generally dominated by MS in combination with particular sample preparation and chromatographic separation (Fiehn et al. 2000; Watkins and German 2002). With this approach, metabolite pools such as amino acids (Matsuzaki et al. 2005; Noguchi et al. 2003) or lipids (Watson 2006) are profiled for quantitative assessment of their changes in response to nutritional stimuli. For example, in order to examine protein intake and its consequences for human health at molecular level, metabolomics was recently suggested as an assessment tool for adequacy and safety of amino acid intake (Watkins et al. 2003).

Lipid profiling (also termed lipidomics) delivers insights into lipid storage, signaling, cellular membrane architecture, and intercellular interactions in response to nutritional inputs and other environmental factors (Watkins et al. 2003; Watson 2006). The concept of screening selected metabolites in biological systems was also extended to stable-isotope studies under the term “tracer-based metabolomics” (Lee and Go 2005): this approach complements the metabolomics-derived knowledge on multiple concentration changes of metabolites with the calculation and the analysis of the molecular flux distribution within biochemical reaction networks.

For biomarker discovery, holistic metabolic profiling is preferred because it is more suited to exploit unexpected metabolic variations in complex organisms. Selman et al. (2006) have, for instance, contributed to the understanding of the molecular processes underlying caloric restriction in a mouse model using NMR analysis of plasma in combination with whole genome RNA transcript profiling in various biological compartments. Metabolic changes associated with caloric restriction were further explored in a recent study in dogs (Wang et al. 2007). Metabolomics was also deployed to assess effects of active ingredients such as polyphenols (Solanki et al. 2003b), of stress stimuli (Wang et al. 2006b), and of aging processes (Williams et al. 2005) in rat models.

Metabolomic platforms increasingly serve to monitor dietary interventions. Flavonoid consumption via tea drinking has been attributed to potential health benefits like cancer prevention, anti-inflammatory action, and cardioprotectant activity. <sup>1</sup>H-NMR and PCA-based metabolomics were applied to investigate the bioavailability and metabolic responses in

rats to a single dose of epicatechin (EC) (Solanki et al. 2003b). Both EC bioavailability and bioactivity were demonstrated via metabolic effects and excretion of EC metabolites.

The diversity of cellular actions of isoflavone antioxidants supports their protective effect in a variety of experimental and human diseases such as renal and cardiovascular disease. <sup>1</sup>H-NMR spectroscopy followed by chemometrics was employed to follow the administration of soy isoflavones in healthy postmenopausal women under controlled environmental conditions (Solanki et al. 2003a). Differences in plasma lipoprotein, amino acid, and carbohydrate profiles were observed after the soy intervention, suggesting a soy-induced change in energy metabolism.

Two thought-to-be-beneficial diets enriched in different long-chain polyunsaturated fatty acids (LC-PUFAs) were tested in a rat nutritional intervention model. The lipidomic/transcriptomic study revealed stearoyl-CoA desaturase as an enzyme target for an arachidonate-enriched diet (Mutch et al. 2005).

### Metabolomics to elucidate host-microbiome cross talk

Metabolic microbiota–host interactions are increasingly addressed in metabolomic studies in mammals (Dumas et al. 2006a; Martin et al. 2007). Nicholson et al. (2005) emphasized the role of the gut microbiota in the systemic response to drugs and toxins. An extension of this concept was presented by Nicholls et al. (2003) who globally monitored urinary metabolites as axenic rats adapted to normal gut microbiota under laboratory conditions. In another recent paper, Martin et al. (2007) described the modeling of transgenic metabolic effects following the inoculation of the mouse intestine with nonadapted human fecal flora: metabolic fluctuations in diverse biological matrices, that is, plasma, urine, liver tissue, and fecal and cecal contents were assessed by holistic NMR-based metabolomics complemented by targeted MS analysis of bile acids. The study revealed that the bile acid profile was affected by the conventional microbiota, while the nonadapted human flora was relatively unable to metabolize mouse bile acids. The relative inability of the nonadapted human flora to deconjugate the mouse bile acids resulted in an impaired absorption of dietary fats with subsequent effects on fat accumulation and fat peroxidation in the liver.

The compositional differences of the intestinal microbiota and variable dietary intakes of humans account for much of the metabolic variability observed

between individuals (Dumas et al. 2006b; Lenz et al. 2004; Stella et al. 2006; Walsh et al. 2006). Kochhar et al. (2006) have characterized the metabolic signatures related to gender, age, and body mass index to better understand the role of these confounding variables in human nutrition trials. Walsh et al. (2006) showed in healthy subjects that a standardized diet renders the urinary metabolic profiles of these individuals much more similar. Stella et al. (2006) reported metabolic fingerprints associated with vegetarian, low-meat, and high-meat diets. Lenz et al. (2004) investigated the metabolic differences between fish-based and low-carbohydrate diets, as obtained in Swedish and British populations, respectively.

Dietary preferences and habits are predominantly of cultural, socioeconomic, psychological, and behavioral origin, although some biological determinants might also have an influence (Breen et al. 2006; Drewnowski 1997, 1999). Very recently, the metabolome associated with food preferences, as exemplified with chocolate consumption in humans, has been characterized (Rezzi et al. 2007b). In 22 healthy male volunteers, stratified according to their chocolate-eating habits with a specifically designed questionnaire, a 1-week double crossover trial including a controlled diet was performed in which portions of chocolate and bread (placebo) were given to the subjects. The chocolate preference was associated with a specific metabolic signature imprinted in the metabolism even in the absence of the chocolate stimulus. The metabolites contributing to this “chocolate preference signature” derive from differences in basal energy metabolism and host–microbiome interactions in the gut.

In summary, metabolomics in nutritional sciences, employing NMR and/or MS platforms, captures changes in metabolite concentrations and profiles to derive marker metabolites and signatures that can be linked to dietary intakes, health status, and host–microbiome interactions. A key issue of further metabolomics research is to dissect the metabolomes of the host and the microbiota.

## CONCLUSIONS AND PERSPECTIVES

Nutrigenomics is a dynamically evolving field. It will further advance, thanks to technological progress at all three omics levels and profit from increasing correlation and integration of transcript, protein, and metabolite data. The latter requires thorough understanding of the timing of the events of gene transcription, protein expression, and metabolite generation, as well as it takes the further maturation of informatic tools for integrative data analysis.

Nutrigenomics, with its potential to deliver rather dynamic biomarkers for nutritional and health status as well as ingredient activity or efficacy, is increasingly being linked to nutri(epi)genetics, which can furnish rather static or long-term biomarkers for individual disposition toward diet, and may improve study cohort definition. The standardization initiatives launched in the omics community will be complemented more and more by equally indispensable efforts of harmonizing dietary interventions in terms of, for example, standardized diets with defined micro- and macronutrient content and origin.

As diet is the most prominent lifelong environmental impact on human health and as—with prolonging life span and changing life style in developed countries—chronic diseases become more prevalent, nutrigenomics and nutrigenetics are key science platforms to promote health and prevent disease through nutrition that better meets the requirements and constraints of consumer groups with specific health conditions and particular life styles.

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## Section II

# Genomics and Proteomics in Health and Diseases

# 3

## Toward Personalized Nutrition and Medicine: Promises and Challenges

*Baitang Ning and Jim Kaput*

### INTRODUCTION

The concept of personalizing nutrition and medicine—and therefore healthcare—emerged from the human genome and haplotype projects. The results of these large-scale, international initiatives offered the hope that nutrition and medicine could be tailored to the individual. The current paradigm, even if unacknowledged, is based almost exclusively on calculating the average biological response or the average incidence of a phenotype of a group or population. For nutrients, these epidemiological methods associate the intake of a nutrient with a phenotype—usually a disease state. Similarly, genetic studies associate the presence or absence of a gene variant with disease incidence of a group. Converting these population averages to individuals is a challenge, and indeed may not be possible given current experimental strategies. Any individual's genetic makeup, their lifestyle, or the interactions between a person's unique genetic makeup and their environment may not be represented in the study population. Novel research strategies may need to be developed that specifically examine an individual as opposed to a population group. This is a daunting challenge given the well-proven research strategies of the past. However, current methods do not usually account for genetic heterogeneity among study participants, complexity of environmental influences, and the intricacies of gene–environment interactions that differ among individuals. We review these challenges in light of the overarching challenge of developing personalized healthcare.

### GENETIC DIVERSITY AND GENOMIC VARIATION OF HUMAN GENOME

The completion of Human Genome Project (Lander et al. 2001; Venter et al. 2001) and subsequent haplotype analyses (Consortium 2003; Consortium 2004; Frazer et al. 2007) have revealed the minimal extent of human genome sequence variations among different individuals. The HapMap project showed that Africans (Yoruba, Nigeria) have the greatest while Asians (Han Chinese, Japanese) have the least genetic diversity (Hinds et al. 2005; Jorde and Wooding 2004) consistent with the historical migratory population of humans. Although 10–15% of genetic variation is unique to a given ancestral group (Hinds et al. 2005; Jorde and Wooding 2004), differences in frequencies of alleles exist among populations because genetic subsets of a population migrated to new environments. Any two given individuals are expected to have approximately 0.1% difference—or 3 million nucleotides—between their DNA sequences. More recently, the complete genomic sequences of three individuals, Venter, Watson, and Maryolein Kriek, have been completed. The two haploid genomes that contribute to Venter's genetic makeup were found to have more than 4 million genetic variants between maternal and paternal chromosomes (Levy et al. 2007), suggesting a greater variation (~0.5%) than previously predicted. In addition to single nucleotide polymorphisms (SNPs) other variations occur in the genome.

### SINGLE NUCLEOTIDE POLYMORPHISMS

Among many kinds of genetic variations, SNPs are the most common: a single base is replaced by another, and both versions exist in the population with frequencies greater than 1%. Some SNPs may alter phenotypes of the allelic carriers due to the changes in protein structure or function. Since the majority of genetic variations are located in intronic region within genes and intergenic regions between genes, they may affect RNA splicing, structure, or stability, but proving that these polymorphisms have direct consequences on a phenotype is challenging. These variations are often used as genetic markers for gene mapping and linkage analysis in population genetics and evolutionary studies (Hacia et al. 1999). In the coding region of a protein, SNPs may change amino acids of a translated protein (nonsynonymous) and thus alter the structural and/or functional characteristics of the protein. Polymorphisms in the noncoding region, upstream of the coding sequence, may alter gene expression via a direct change of context of *cis*-acting elements where transcriptional factors alter regulatory processes (Orphanides 2002), or in *trans* where a variant transcription factor alters regulatory processes. Many studies also suggested that polymorphisms occurring in the 3'-UTR of transcripts could alter the corresponding protein expression (Hesketh 2004; Mandola et al. 2004). Functional SNPs may be an important source of interindividual variation in benefit–risk analyses of responses to nutrients and food.

### STRUCTURAL VARIATIONS IN THE HUMAN GENOME

Structural variations in chromosomes, such as insertions or deletions, chromosomal segment rearrangements and variation, and increases or decreases in the number of genes (i.e., copy number variants (Feuk et al. 2006)), were known prior to the characterization of SNPs. However, due to challenges in characterizing these variants and lack of standards in integrating analytical methods, structural variations have not been exploited or fully explored in pharmacogenomics or nutrigenomics applications (Scherer 2007). However, the advent of sequence data and improved methodology has shown that gene copy number variations (CNV) may be associated with common diseases such as in autism spectrum disorders, bipolar disorders, rheumatoid arthritis, type 1 diabetes, Crohn's disease, and Alzheimer's disease (Estivill 2007). Alterations in copy number can occur for genes involved in nutrient metabolism. Individuals

whose ancestors were exposed to either high-starch or low-starch environments differed in the number of amylase genes (Novembre et al. 2007; Perry et al. 2007). In addition to demonstrating copy number variants for genes involved in nutrient metabolism, structural variation occurred within the same ancestral population (i.e., several groups within central Africa).

### PISTATIC INTERACTIONS

Epistasis is a phenomenon that the function of a given gene is modified (either masked or enhanced) by one or other genes, commonly known as gene–gene interactions. As a result, gene–gene interactions (Carlborg and Haley 2004; Hartman et al. 2001; Moore 2003, 2004) may alter genetic expression of a gene variant (Cheverud et al. 2001; Togawa et al. 2006; Yang 2004) including some genes affected or involved in nutrient metabolism (Chiu 2006). Evolutionary theory and developmental biology suggested that the epistasis is beneficial to stabilize phenotypes by buffering against the effects of mutations (Moore 2005). Epistatic interactions occur in underlying genetic architectures that are composed of networks of genes and proteins, since pathways are interconnected by sharing metabolites or having direct physical interactions. An example of epistasis effect is that different patients with phenylketonuria have variable onsets and severities (reviewed in Scriver 1988, 2007). More recently, a common polymorphism in ghrelin (GHRL) abolished the association of an allele of its receptor (GHSR) with myocardial infarction and cardiovascular disease (Baessler et al. 2007).

Epistatic interactions may partially explain why different ethnic groups have different risks regarding effects of genetic variants, by comparing effects of a gene variant among ancestral populations (Klos et al. 2005) or in admixed populations (Suarez-Kurtz 2005; Suarez-Kurtz and Pena 2006). HapK, a haplotype consisting of four SNPs in the leukotriene 4 hydrolase gene (*LTA4H*), increases the population risk from 1.35-fold in Europeans (the origin of the HapK allele) for cardiovascular disease (CVD) and myocardial infarction (MI) to almost fivefold in African Americans who carry the same haplotype. The epistatic interaction occurs (a) because *LTA4H* interacts differently with one or more gene variants in either African versus European chromosomal regions, resulting in increased effect of LTH4 activity in African Americans, (b) because different environmental factors alter the influence of *LTA4H* on myocardial infarction (Helgadottir et al. 2006), or (c) because of a combination of epistatic and gene–environment

interactions (Klos et al. 2005). LTA4H participates in leukotriene and prostaglandin metabolisms that are linked to dietary fatty acid intake (Kelley 2001).

While epistasis may be most noticeable for recently admixed populations such as African Americans or Latino-Americans, population structure exists even within seemingly homogenous groups such as in Iceland (Helgason et al. 2005). European populations are stratified north to southeast (Campbell et al. 2005; Sladek et al. 2007) and east to west (Bauchet et al. 2007).

#### GENE-ENVIRONMENT INTERACTIONS

Archibald Garrod reintroduced the concept of gene-environment interactions in the modern era (1902), which was first stated by Hippocrates (~2,500 years before present: let food be your medicine and medicine your food). With the exception of certain diseases caused by mutations, many human diseases result from the interactions of genetic susceptibility and modifiable environmental factors (Hunter 2005; Kaput 2004; Kaput et al. 1994). A scientific colloquialism is that genotype loads the gun and environment pulls the trigger. An individual may inherit a predisposition for a phenotype (such as a disease), yet the onset of the phenotype (disease develops or not) and the magnitude of the phenotype (severity and outcome of a disease) may be modified by the exposure to the appropriate environmental factors. The overall concept is simply stated: an organism metabolizes food chemicals and other xenobiotics (toxins, pollutants, drugs) and expression of genetic information is influenced by those same xenobiotics. These chemicals may alter gene expression, chromatin structure, DNA repair processes, or other regulatory processes and thus ultimately influence phenotypes, including diseases such as cancer, diabetes, allergies, and infertility (Edwards 2007). Asthma is a classic example that demonstrates environmental factors triggering a genetic susceptibility. In the last two decades, gene association and genome-wide linkage studies for asthma in different ethnic groups were carried out to identify candidate disease makers. Some chromosomal regions, including 2q, 5q, 6q, 11q, 12q, and 13q, have been repeatedly reported to be associated with asthma, indicating that one or more genes in these regions may be involved in the disease (Bierbaum 2007). Several studies show that SNPs on chromosome 5q interact with environmental tobacco smoke to develop the phenotype (asthma) in young children. Several candidate gene approach studies have suggested that interactions of immunity genes CD14 and Toll-like receptor 4 with exposure to envi-

ronmental microbes promotes asthma development. Polymorphisms in glutathione S-transferase (GST) genes may also interact with environmental tobacco smoke and air pollutant exposures, playing an important role in the etiology of asthma development (Koppelman 2006). Association analysis only based on genetic factors is therefore futile because environmental factors may influence the expression of the gene (or SNP) linked to a specific phenotype, and could also influence the genes that interact with it.

#### EPIGENESIS

The term “epigenetic” refers to the stable changes in DNA structure without altering its sequence and thereby alters the maintenance or expression of genetic information. The two most widely described and characterized epigenetic mechanisms are chromatin remodeling through histone (and other chromatin protein) alterations and DNA methylation (Delaval and Feil 2004; Dolinoy et al. 2007; Esteller 2007; Fowler and Alarid 2004; Jiang et al. 2004; Jirtle and Skinner 2007; Morgan et al. 2005). Epigenetic mechanisms are based on covalent modifications of DNA and/or protein molecules.

DNA methylation is an enzymatic process in which the 5 position of the cytosine ring is methylated by DNA methyltransferases, usually at CpG dinucleotides using *S*-adenosylmethionine (*S*-AM) as a carbon donor (Mason 2003; Sneider et al. 1975). *S*-AM is generated in the one-carbon pathway and the substrates choline and methionine, and cofactors folate, vitamin B12, vitamin B6, and riboflavin are derived from the diet. Deficiencies in these nutrients alter one-carbon metabolism, impair DNA methylation, and have been linked to increased risk of neural tube defects, cancer, and cardiovascular diseases (Stover and Garza 2002).

Posttranslational modifications of histones are essential for structure remodeling of chromatin. Methylation, acetylation, and biotinylation of histones each alters chromatin structure and plays a role in many biological processes including gene silencing, DNA damage repair, cell proliferation, and cell apoptosis (Bishop and Guarente 2007; Oommen 2005). The substrate for histone biotinylation is biotin, a water-soluble B-complex vitamin, known as vitamin H or B7.

Chromatin remodeling is a dynamic process of structural changes to the chromatin that results in decreased or increased “packing” of DNA chains, which is essential for accessibility for transcriptional machinery, thus influencing the gene expression, DNA replication and repair, chromosome

condensation, and segregation and apoptosis (Baroux 2007; Wang 2007). Chromatin remodeling could alter expression over short time intervals, but may also be altered permanently through changes in nutrient availability during key developmental windows (Esteller 2007; Gallou-Kabani and Junien 2005; Jirtle and Skinner 2007; Simmons 2007; Sutton et al. 2006). In addition to nutrient levels, total caloric intake has also been linked to chromatin remodeling (Bishop and Guarente 2007; Geiman and Robertson 2002). Energy sensing occurs, in part, through the NADH:NAD<sup>+</sup> (reduced nicotinamide adenine dinucleotide:nicotinamide adenine nucleotide) ratio (reviewed in Blander and Guarente 2004). *SIRT1* (sirtuin 1), an NAD<sup>+</sup>-dependent histone deacetylase (HDAC), appears to be a key regulator of chromatin structure (Blander and Guarente 2004). Acetylation and deacetylation of chromosomal proteins alter their interactions, resulting in changes in structure and regulation of gene expression (Cho et al. 2004; Eberharter and Becker 2002; Hsiao et al. 2002). Many, if not most, human studies (for drugs, genetic association, or others) assume similar if not identical developmental paths to adulthood. Strategies for dissecting early environmental influences and the corresponding causative genes and pathways will be essential for understanding how to maintain health and prevent disease in adulthood.

### HETEROGENEITY OF DISEASE: THE TYPE 2 DIABETES MELLITUS EXAMPLE

Health and chronic diseases are complex traits that are influenced by many normally functioning genes. Unlike Mendelian single-gene diseases, such as Huntington's chorea and sickle cell anemia, most chronic diseases, such as cancers and hypertension, are multifactorial traits resulting from the interaction of multiple environmental factors acting on multiple genetic pathways. The phenotypes, genetic complexity, and gene–environment (nutrient) interactions of type 2 diabetes mellitus (T2DM) provide a model for these concepts.

#### HETEROGENEITY OF SYMPTOMS AND TREATMENT

T2DM is a metabolic disorder that is characterized by insulin resistance, relative insulin deficiency, and hyperglycemia. Complex and multifactorial metabolic changes during the disease process usually result in various organ damages and functional impairments.

Hence, T2DM presents with considerably different clinical symptoms, disease severity, and outcomes among individual patients (Stumvoll et al. 2005). One classification schema described four levels (low, moderate, high, and very high), each of which consists of six clinical measurements: glycemic control (Hb1Ac, hypoglycemia, ketosis), cardiovascular risk factors, peripheral neuropathies, eye disease, renal disease, and autonomic neuropathies (Rosenzweig et al. 2002). This complexity results in “personalizing” clinical management of T2DM by lengthy trial-and-error methods of changing dosages and medications.

The first option for early stage or less severe cases of T2DM is to modify diet and lifestyle to attain glycemic control. Only ~20% of patients responded to this treatment (Koro et al. 2004). More severe cases are treated with one or more of the six classes of drugs that target different pathways and organs: insulin secretion by the pancreas (sulfonylurea, meglitinides, exenatide), glucose absorption by the intestines ( $\alpha$ -glucosidase inhibitors), glucose production in the liver (biguanide = metformin), and insulin sensitivity in adipose and peripheral tissues (thiazolidinediones, e.g., rosiglitazone and pioglitazone—reviewed in Kaput et al. 2007a). Approximately 50% of T2DM patients take oral medications only, about 11% take combinations of oral agents with insulin, and the remainder take no medications (20%) or insulin alone (16.4%) (Koro et al. 2004). Since these drugs treat different pathways in different organs, T2DM is better considered a collection of metabolic–genetic diseases with aberrant glucose regulation as the common feature.

#### HETEROGENEITY OF GENETIC COMPONENTS

Identifying the genes that cause abnormal responses in these molecular pathways would contribute to the development of diagnostic tests for sorting individuals into treatment groups at the initial visit. A recent study demonstrated that gene-based diagnostics could aid in drug selection: permanent neonatal diabetes caused by either L213R or I1424V mutations in *ABCC8* (*SUR1*) could be treated with glyburide (a sulfonylurea) rather than usual treatment with insulin (Babenko et al. 2006). However, the identification of causative variants has met with limited and uneven success. For example, with the recent genome-wide association studies (GWAS; Frayling et al. 2007; Sandhu et al. 2007; Saxena et al. 2007; Scott et al. 2007; Sladek et al. 2007; Zeggini et al. 2007) identifying eight new loci and potential candidate genes contributing to T2DM, a total of 11 candidate genes are associated with increased

**Table 3.1.** Candidate T2DM genes from genome-wide association studies.

Candidate	Chr	rs	Risk/ref	Freq	Odds	Reference
CDKAL1	6	rs7754840	C/G	0.31	1.12	(Saxena et al. 2007)
CDKN2B	9	rs10811661	T/C	0.83	1.20	(Saxena et al. 2007)
CDKN2B	9	rs564398	C/T	—	1.12	(Zeggini et al. 2007)
FTO	16	rs8050136	A/C	0.38	1.17	(Zeggini et al. 2007)
HHEX	10	rs1111875	C/T	0.53	1.13	(Saxena et al. 2007)
IGFBP2	3	rs4402960	T/G	0.29	1.14	(Saxena et al. 2007)
IGFBP2	3	rs1470579	C	0.30	1.17	(Saxena et al. 2007)
KCNJ11	11	rs5219	T/C	0.47	1.14	(Saxena et al. 2007)
PPARG	3	rs1801282	C/G	0.86	1.14	(Saxena et al. 2007)
SLC30A8	8	rs13266634	C/T	0.65	1.12	(Saxena et al. 2007)
TCF7L2	10	rs7903146	T/C	0.26	1.37	(Saxena et al. 2007)
	11	rs9300039	C/A	0.89	1.25	(Scott et al. 2007)

Recent genome-wide association studies found new loci associated with T2DM. Candidate genes for these loci were identified (see text for details). Chr is chromosome and rs is the SNP ID number. Risk/ref denotes the allele associated with T2DM and the reference allele. Freq is the frequency of the risk allele in the European population. Odds are the average odds ratio—usually referred to as risk factor—from the referenced article. The last line of the table indicates an allele with no readily recognizable gene nearby.

population risk to T2DM (Table 3.1). Genes that had previously been considered excellent candidates on the basis of reproducibility or significance (Florez et al. 2003), including the glucagon receptor (*GCGR*), glucokinase (*GCK*), glucose transporter 1 (*SLC2A1*), and the aforementioned *ABCC8*, were not identified in this large genome-wide screen.

#### GWA APPROACH: A SIMPLE SOLUTION FOR COMPLEX DISEASES?

GWAS are an evolutionary step toward understanding the genetic contribution to complex, low-penetrant diseases. GWAS allows interrogation of the relationships between genetic variation and biological phenotypes in the whole genome. Previous association studies were criticized because they could not be replicated, did not appropriately match cases to controls, and were underpowered—that is, the sample size was small (e.g., Cardon and Bell 2001; Hirschhorn et al. 2002; Ioannidis 2005; Lander and Kruglyak 1995; Newton-Cheh and Hirschhorn 2005; Risch 1997; Tabor et al. 2002). These recent GWAS attempted to address those limitations by increasing the number of cases (range: 1,215–2,938) and controls (range: 1,258–3,550), which was possible because the cost of arrays has decreased. The Wellcome Trust Case Control Consortium analyzed 14,000 cases of seven common diseases (bipolar, coronary artery disease, Crohn's disease, rheuma-

toid arthritis, and type 1 and type 2 diabetes) with 3,000 shared controls (The Wellcome Trust Consortium 2007). Several of the studies replicated results in separate populations or shared samples. Collectively, over 18,000 individuals were analyzed for T2DM with about 14,000 controls. To reduce population stratification and concomitant epistatic interactions, samples for individual studies came from defined populations (Finland, Sweden, England/Ireland, France, Ashkenazi Jews). A method developed to test for stratification was used in some studies (Devlin and Roeder 1999), and the data were analyzed by a variety of algorithms for potential population substructures (e.g., Saxena et al. 2007). Collectively, these GWAS identified 50 candidate loci associated with type 1 and type 2 diabetes, Crohn's disease, autism, cancer, immune function, and other diseases and provide testable hypothesis for the involvement of candidate genes involved in genetic predispositions of common traits (McCarthy 2008).

While these large studies had some success, the eight new candidates collectively “explain” between 0.5 and 2.4% of the population attributable fraction (PAF) for T2DM (Saxena et al. 2007). Several limitations contributed to the number of genes identified and their small PAF. The various GWAS used different criteria for including individuals in the case population. In some of the experimental designs, cases included newly diagnosed (not yet on medications) as well as those on any type of T2DM medication

regardless of the molecular pathway targeted by the drug (see heterogeneity of health and disease section). Combining all the patients as T2DM regardless of disease subtype (on the basis of medication) reduced (or averaged) the contributions of causative genes in different pathways. Hence, GWAS may produce false-positive and false-negative results because of subject ascertainment (McCarthy et al. 2008).

In addition, regardless of matching the overall genomic architecture of individual cases to individual controls, epistatic interactions may still occur in seemingly homogeneous populations (Campbell et al. 2005; Sladek et al. 2007) as described above because distinct chromosomal regions (and not average total genomic structure) may differ among cases and controls. Li et al. (2008) failed to replicate the association of FTO (fat mass and obesity-associated gene) to adiposity (and therefore T2DM) in Han Chinese from Shanghai and Beijing—an example consistent with differences in gene–gene interactions, or alternatively, differences in disease development in different ancestral groups (see below). New mapping strategies that are designed specifically for analyzing epistasis in association studies may provide a means to account for confounding of gene–gene interactions (e.g., Chiu 2006; Motsinger and Ritchie 2006; Togawa et al. 2006).

A significant limitation of these GWAS is that they did not assess nutrient intakes (Pearson 2008) even though diet–gene interactions are major contributors to the control of gene expression and could influence associations among genes–phenotype and dietary intake (Kaput 2004; Kaput and Rodriguez 2004; Ordovas and Corella 2004, 2006). Differences in intake of calories, dietary fat, high glycemic index carbohydrates, and certain micronutrients are linked to type 2 diabetes (Biesalski 2004; Bonnefont-Rousselot 2004; Hung et al. 2003; Neff 2003; Nestel 2004) and other chronic diseases (Wahlqvist 2005). Nutrients and nonnutrient components of foods regulate pathways and genes involved in maintaining health or producing disease (Kaput 2004; Kaput et al. 1994), although the specific gene–nutrient associations have not yet been identified. For some genes or transcription factors, however, the mechanisms of how they are activated by nutrients are known. For example, the peroxisome proliferator-activated receptor gamma 2 (PPAR- $\gamma$ ), a target of thiazolidinediones (TZD), is activated by the dietary lipids linoleic, linolenic, arachidonic, and eicosapentaenoic acid (Chambrier et al. 2002; Nosjean and Boutin 2002) and their metabolites (Kaput et al. 2007b). Hence, measures of nutrient intakes would improve gene–phenotype association studies, as demonstrated by

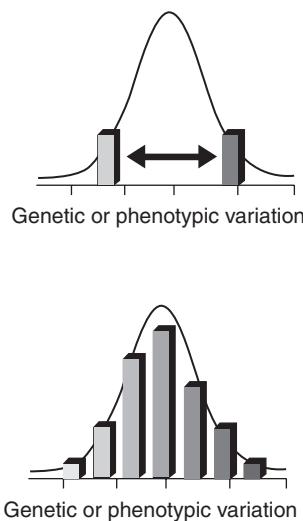
associations between certain nutrients and individual genes involved in cardiovascular disease (e.g., Corella et al. 2006, 2007; Lai et al. 2006; Tai et al. 2005).

These GWAS and other association approaches yield the PAF (Karp et al. 2007; Levine 2007; Rockhill et al. 1998). While these estimates are the best available for guiding preventive and treatment strategies, population-based risk fractions have limited utility for individuals: Data derived from one ancestral group (e.g. Europeans, African, or Asian) may not apply to individuals from other ancestral populations or to other individuals within the studied ancestral group because of uncharacterized epistatic or gene–environment interactions. Increasing the size or diversity in the study groups—for example, including African Americans, Latino-Americans, and European Americans—may reduce the size of the biological response by averaging high and low responders, by gene–gene interactions (epistasis) and gene–environment interactions.

The methodological challenges for genetic association studies and nutritional epidemiology can be addressed by (a) developing and using uniform nutrient intake measurements that can be compared within populations and among populations, (b) standardizing measures of relevant physiological parameters, and (c) testing for genetic ancestry within chromosomal segments, rather than overall genetic makeup.

## A PROPOSED PATH TO PERSONALIZATION

The challenges for nutrigenomics and genetics research are to account for genetic, environmental, and phenotype diversity and to develop a path for creating personalized nutrition and healthcare. We have previously proposed a concept of identifying and creating “metabolic groups”—such as fast, medium, slow, or requiring different levels of macronutrients—within the continuous phenotypes found in the total human population (Kaput 2008). This approach would not be “individualized,” yet would group individuals of like metabolism on the basis of nutrient–gene interaction data. Specifically, current omic technologies can be used to identify and group individuals with common phenotypes and analyze the genetic differences between them (Figure 3.1). Alternatively, individuals can be selected on the basis of variations in genes (and not just variants used for genetic mapping) and phenotypes can be compared (Figure 3.1). The first groups tested would be those most different in phenotypes or genetic makeup—that is, to determine



**Figure 3.1.** Comparative phenotype/comparative genotype strategy. The steps for defining the diversity of variation in the human population is to (i) pre-select and bin individuals of different genotypes or phenotypes with known nutrient intakes (i.e., based on genotype X environment interactions), (ii) compare genotypes of different phenotypes and nutrient intakes or phenotypes with known nutrient intakes of different genotypes, and (iii) fill in intermediate bins of known genotype, phenotype, and diet interactions. Number of bins would be defined by clusters of similar genotype X environment interactions.

the largest range of variation within the human population. Instead of selecting subjects of one ancestral population (Europeans, Asians, or Africans) who are either healthy or have disease, analyses should be done between genetic or metabolic groups, members of whom are healthy or with the same subtype of disease. Nutrient assessments are a required component of this experimental strategy since nutrient–gene interactions may alter membership in the metabolic group.

The key feature of this approach is to predetermine the subjects on the basis of some quantitative and reproducible phenotype or genotype with the inclusion of environmental assessments. Members of each group would have like genotypes or metabolic phenotypes, but between-groups differences would be large. Once maximum differences of differing phenotypes or genotypes are determined, groups between the extremes can be determined. While it is expected that most biological traits are continuous with no discrete breaks in the phenotypic or genetic continuum, such “binning” is a standard for medi-

cal practice that uses clinical measurements to group individuals into treatment options and for statistics that rely on tertiles, quartiles, quintiles, and so on, to determine structure within experimental data. The difference from these standard approaches is that binning is done prior to physiological analyses if the genetic variation is predetermined or prior to genetic analyses if different phenotypes are identified rather than after experimental data are acquired.

### CONCEPTUAL EXAMPLES

This comparative phenotype/comparative genotype concept is based on many laboratory animals studies since phenotypic differences among inbred strains (i.e., different genotypes) are utilized for genetic mapping (Brown et al. 2005; Kobayashi et al. 2006; Kumar et al. 2007; Togawa et al. 2006), but also for analyzing differences caused by gene–nutrient interactions (Akhi et al. 2005; Brown et al. 2005; Clee et al. 2006; Cheverud et al. 2004; Kaput et al. 1994; Paigen et al. 1990; Park et al. 1997; Smith Richards et al. 2002; Takeshita et al. 2006). We (Kaput et al. 2007a, b) and others (Lohmueller et al. 2006) proposed that patients be grouped on the basis of clinical measurements (see clinical heterogeneity section). A successful application of this idea was preselecting women with the phenotype of early onset (< 35 years of age) versus late onset of breast cancer that allowed the identification of 17q21 (Hall et al. 1990) and subsequent identification of BRCA1 gene (Szabo and King 1995).

### EXAMPLES OF ANCESTRY GROUP DIFFERENCES IN HUMAN PHYSIOLOGY

Ample evidence exists for different metabolic groups in different ancestral populations.

- Amylase copy number varies among different populations (Novembre et al. 2007; Perry et al. 2007) on the basis of long-term adaptation to carbohydrate abundance in the local environment. These differing environments may be located on the same continent.
- Physiological and genetic differences have been found in adaptation to high altitudes by Tibetan and Andean populations (Beall 2007).
- The body mass index for diabetes onset is 25 or greater in individuals of European ancestries but 21–23 in Asian and Native North Americans (Deurenberg-Yap et al. 2002; Naser et al. 2006; Razak et al. 2007).

- Subphenotypes of diabetes differ among ancestral populations (Lorenzo 2006).
- Differences among ancestral groups can be explained by the effect of common genetic variations in world populations on gene expression (Spielman et al. 2007).

### DISEASE PHENOTYPE ANALYSES

To analyze genetic differences underlying different phenotypes, more complete analyses of phenotypes and nutrient assessments will be needed. In many studies, phenotyping is limited to one class of molecules (e.g., serum lipids for cardiovascular disease, some measure of glucose or insulin levels for T2DM). However, aberrations in one pathway or mechanism may have consequences for other physiological processes. For disease states, the compare phenotype concept requires a schema for sorting individuals into groups. Different effectiveness of T2DM drugs (Kaput et al. 2007b) or detailed disease classification methods (Rosenzweig et al. 2002) can be used to establish phenotypic groups for case-case, case-control, or cross-sectional studies. An additional variable for such analyses is creation of similar cases in different ancestral backgrounds that will allow the comparison of genetic ancestry on specific metabolic parameters or classification criteria. Do individuals of Indian ancestry successfully treated with metformin have the same gene variants associated with that response as Native Americans successfully treated with metformin?

In addition to analyses of disease, this comparative strategy can be applied to assess the healthy phenotype. The “healthy” state may be just as metabolically diverse among individuals as disease states. The challenge homeostasis model being developed by the European nutrigenomic community (Elliott et al. 2007) may be used to develop groups differing in health processes. The oral glucose tolerance test is an example of a homeostatic challenge. While there are three existing “bins” for this test (normal, impaired glucose tolerance, diabetic; Waugh et al. 2007), additional groups could be identified by assessing other metabolic systems (e.g., amino acid level differences) that are altered by the glucose bolus. Such changes occur because amino acid metabolism is linked to glucose metabolism. Additional nutritional challenges for fat intake, vitamin response, or other nutritional variables may be developed and the various omic technologies now permit these more detailed analyses at ever-reducing costs. Complete nutrient intake assessments would, of course, be required to completely analyze base-

line phenotypic data as well as data derived from homeostatic challenges.

### GENETIC “BINNING”

While phenotypic binning is conceptually easier to consider, a comparative genomic approach is also possible. In these cases, screening of individuals for similar allele frequencies, regardless of ancestry, would permit the identification of individuals who differ in phenotype (either disease or health), who may respond similarly to homeostatic challenges, or who may respond differently to long-term diets. An example of this approach was done by analyzing frequencies for 43 autosomal loci, including 35 genes in Asian populations: individuals of similar allele frequencies could be clustered among nine different “countries” (Kim et al. 2005). While the intent was to identify and sort individuals into genetic ancestry groups, the method demonstrated that not only different genetic groups exist within a population but also individuals with similar allele frequencies can be found distributed among the different Asian populations. Such grouping can be done by selecting SNPs in coding or regulatory regions of genes and testing with genotyping arrays. The comparative genetic “binning” step does not require knowledge of allele frequencies in various populations, since individuals would be assigned to genetic groups following SNP and copy number variant analyses. Following the binning step, detailed phenotypes and nutritional variables could be analyzed in conjunction with “health” or “disease” phenotypes; for example, using existing criteria (fasting triglycerides, insulin, glucose levels) or through selected challenges to homeostasis (oral glucose tolerance test, lipid challenge, etc.). Initially, phenotype analyses would focus on the two most different genetic groups to define the full range of human variation.

### APPLICATIONS

Although the goal of nutrition, nutrigenomics, and pharmacogenomics is personalized nutrition and healthcare, the path to personalization may require that individuals first be placed into metabolic groups on the basis of gene-nutrient interactions. While the exact number of different phenotypes cannot be predicted *a priori*, it is likely that there may be several groups that define a range of physiological responses. For example, heart health of certain individuals may require a low-fat diet (e.g., ~10%) or others medium fat (~20%) (Dreon et al. 1999, 2000) and perhaps a small subset may require high fat for optimum health. The specifications for defining the

bins and the number of these metabolic bins will be empirically determined following phenotypic and genetic analyses.

### SUMMARY OF THE STEPS TO PERSONALIZATION

The significant advances in understanding complex biological process relied on reductionistic approaches: hold all variables but one constant. While this strategy was successful for certain monogenic phenotypes, understanding complex systems requires analytical approaches that incorporate rather than avoid complexity. Genes interact with nutrients and nutrients alter genetic expression—analyzing one and ignoring the other results in incomplete analyses. The key challenge for personalizing health care, then, is not the complexity of the data sets, but acquiring those data sets in a manner to reduce noise and increase true signals. This might best be accomplished by preselecting phenotypes on the basis of quantitative data, or alternatively, preselecting genotypes that maximize differences in allele frequencies of candidate genes involved in nutrient metabolism or other physiological trait. The integrative whole-system analyses of the data sets and new visualization methods, such as shown with network analysis tools, provide a path to not only perform these complex experiments, but also develop biological insight into the outcomes. The development of nutrigenomics and genetics and the application of this knowledge will provide strategies for maintaining health and improving medical treatment of chronic diseases.

### DISCLAIMER

This work includes contributions from, and was reviewed by, the FDA. This work has been approved for publication by this agency, but it does not necessarily reflect official agency policy.

### CONFLICT OF INTEREST

The author declares there is no conflict of interest.

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# 4

## Obesity and Nuclear Receptors: Effective Genomic Strategies in Functional Foods

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and Nobuyuki Takahashi*

### INTRODUCTION

Modern people have a tendency to overeat owing to stress and loosening of self-control. Moreover, energy expenditure varies greatly among individuals. In most modern people, the conscious expenditure of energy by exercise rather than nonconscious basal metabolism varies greatly from individual to individual and conscious energy expenditure is insufficient for most people. Scientific reduction of obesity is important under these circumstances. Furthermore, recent research has clarified the differentiation of adipocytes, the level of subsequent fat accumulation, and the secretion of the biologically active adipocytokines by adipocytes (Shimomura et al. 1996). In particular, it has been clarified that adipocytokines secreted by adipocytes play a significant role in the pathogenesis of diseases such as diabetes and cardiovascular diseases, and are closely associated with the pathogenesis and exacerbation of diseases arising from obesity. Adipose tissues and obesity have become the most important target for the prevention and treatment of many lifestyle-related diseases (Figure 4.1). In this chapter, we first discuss obesity and the metabolic syndrome and then describe mainly the nuclear receptors that are most important in adipocyte differentiation and the mechanism underlying the expression of function of adipocytes affecting obesity from the viewpoint of nutrigenomics.

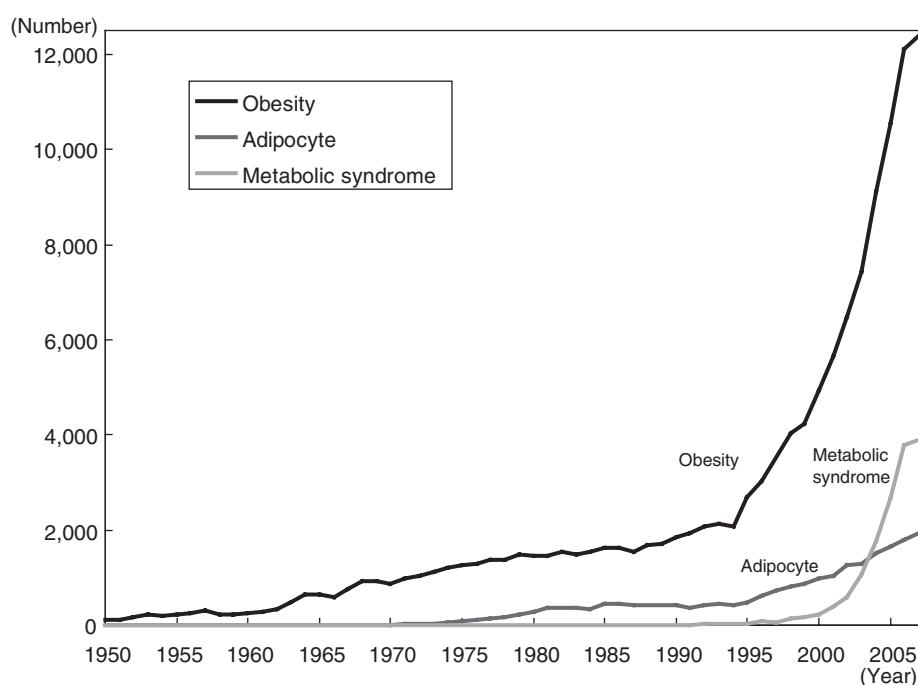
### ROLE OF BODY FAT AND CAUSE OF OBESITY

Generally, body fat, which causes obesity, has a negative image. However, body fat is an essential tissue in the body of animals including humans. Overly

thin people with 15% body fat or lower, except some athletes, have poor adaptability to environmental changes such as changes in temperature and low resistance to infectious diseases. Body fat has the following five main functions: (1) storage and supply of energy for daily life activities, (2) heat insulation for maintenance of body temperature, (3) maintenance of position of internal organs (thin people often have gastropathy), (4) secretion of sex hormones, particularly female hormones (excessive dieting causes menstrual disorders), and (5) secretion of adipocytokines (recently also called adipokines) (Figure 4.2).

Adipocytokines belong to a group of recently discovered proteinaceous biologically active substances secreted by adipocytes. Adipocytokines with desirable properties include adiponectin exhibiting antidiabetic and antiarteriosclerotic effects and leptin exhibiting feeding regulatory effects. Adiponectin, a novel adipose-tissue-specific, collagen-like protein acts as a hormone, has been noted as an important antiatherogenic and antidiabetic protein, or as an anti-inflammatory protein. In addition, adipocytokines with undesirable properties have been identified, such as tumor necrosis factor-alpha (TNF- $\alpha$ ) causing diabetes and PAI-1 causing arteriosclerosis. Body fat is a double-edged sword depending on the amount and site of accumulation. The concept and term of adipocytokines originated from Japanese obesity researchers, mainly those belonging to Osaka University Graduate School of Medicine in Japan (Matsuzawa 2006; Shimomura et al. 1996).

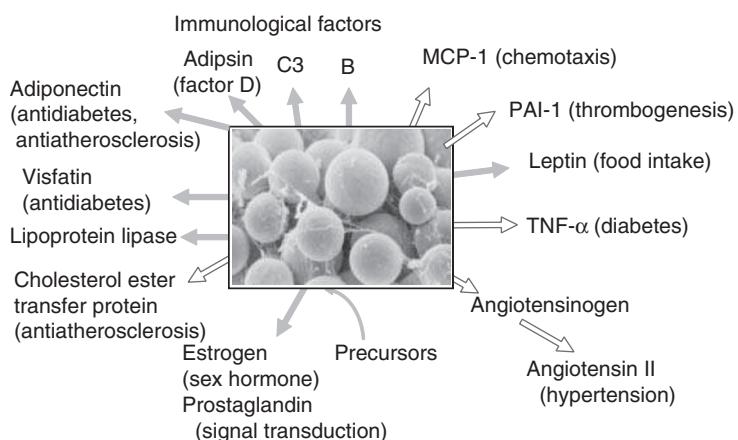
Obesity is determined by the balance between the intake and expenditure of energy (energy balance). When energy intake exceeds energy expenditure, surplus energy is accumulated as body fat, which leads



**Figure 4.1.** How many obesity-related researches are reported every year in the world? Obesity, adipocyte, and metabolic syndrome are used as key words in obesity-related research on SciFinder database accessed at January, 2008.

to obesity. In a healthy adult, the energy balance is well maintained and a certain body weight is maintained for a long period. However, this balance is disrupted when energy intake increases excessively due to overeating or in the absence of overeating energy expenditure decreases due to lack of exercise, resulting in surplus energy accumulated as body fat.

The main causal factors (etiologies) of obesity are the following: (1) overeating, (2) abnormal eating pattern, (3) heredity, (4) lack of exercise, and (5) heat production disorder. It is considered that obesity is not caused by a single factor but rather by a complex mixture of these factors. Overeating, including abnormal eating patterns, and the lack of



**Figure 4.2.** Adipose tissue as a secretory organ. Adipose tissue secretes various cytokines, chemokines, and fat-soluble vitamins. The black arrows show good fatness factors and the white arrows show bad fatness factors.

exercise are the most common causes of obesity in modern lifestyle. In addition, it has been found that genetic predisposition such as gene single nucleotide polymorphism (SNP) observed in an individual or a family is significantly associated with these causal factors (described later).

## REALITY OF GOOD FATNESS AND BAD FATNESS

### DISTINCTION BETWEEN FATNESS AND OBESITY

“Fatness” is a condition in which excessive body fat is accumulated in the body and it is not necessarily a disease. There are people who are fat but do not have any health problem. Sumo wrestlers are a good example. Active sumo wrestlers have a significant amount of subcutaneous fat; however, as long as a certain or higher level of exercise is maintained in “keiko” (practice), they rarely develop diseases commonly associated with fatness. Fatness is a condition in which the amount of body fat is high in the body. In 2001, the Japanese Society for the Study of Obesity set the criteria for the diagnosis of fatness, which needs to be treated by weight reduction as in the case of “obesity,” from the medical viewpoint. Obesity is determined on the basis of two aspects: (1) obesity with clinical conditions (10 conditions such as diabetes, abnormal glucose tolerance, hyperlipidemia, and abnormal lipid metabolism) that can be improved or stabilized by weight reduction, and (2) obesity with excessive accumulation of visceral fat (the area of visceral fat is 100 cm<sup>2</sup> or more on a CT image at the umbilical level). Medically treatable obesity has recently been redefined as metabolic syndrome following the establishment of clear criteria for diagnosis (scientific quantitative diagnosis), which had previously been unclear. New drugs (antiobesity drugs) are now being developed targeting medically treatable obesity.

### SOCIAL BACKGROUND OF OBESITY AND HEALTH ECONOMICS

Not only the basic and clinical aspects of obesity but also the social background is important in research on obesity. Because obesity is closely associated with the development of lifestyle-related diseases and metabolic syndrome and is medically treatable, the high medical cost becomes a social problem. According to a recent estimate, 388 million people worldwide will die within 10 years (2007–2017) because of lifestyle-related diseases, resulting in an economic loss of 33 billion dollars in the United Kingdom and 558 billion dollars in China (King and

Thomas 2007). On the other hand, another estimate showed that most cases of early death can be prevented by appropriate health care management and drug treatment, and that the death of at least 36 million people can be prevented by 2015 if necessary measures are taken simultaneously throughout the world (King and Thomas 2007).

## OBESITY AND HEREDITY

Genetic factors are highly associated with the development of obesity. However, this does not mean that the characteristic of having an obese body is heritable but rather that the high capacity of storing body fat is heritable. A person having a high capacity “to become fat” (it has an advantage in times of starvation and genes encoding; such a capacity is called thrifty genes) develops obesity only when he or she consumes excessive energy by overeating or does not exercise sufficiently, resulting in a decrease in energy expenditure. The  $\beta$ 3-adrenoceptor gene with a mutation is a typical thrifty gene. One in three Mongoloids living in Asian countries has this gene mutation. This gene mutation likely causes obesity and diabetes when the carrier has the problem of overeating. More than 120 types of gene mutation associated with obesity have been reported by 2005 (Rankinen et al. 2006). Table 4.1 shows the identified candidate genes associated with obesity. Helpful information is available at the obesity gene map database on the Web site of Pennington Biomedical Research Center (<http://obesitygene.pbrc.edu/>) (Weisberg et al. 2003).

The etiological factors for obesity are roughly divided into “30% hereditary and 70% environmental,” showing that environmental factors such as lifestyle account for a large proportion. Lack of exercise not only decreases energy expenditure but also changes physical metabolisms (constitution) so that the basal metabolism decreases and energy tends to be stored in the body as fat. Moreover, food preferences of children, such as that for fast food, and family lifestyle are significant factors for the development of childhood obesity, and become a major social issue in economically advanced countries and in countries achieving rapid economic development.

## FORMATION OF ADIPOCYTES AND CONTROL OF DISEASE DEVELOPMENT

### CHARACTERISTICS OF ADIPOCYTES

Body fat is triacylglycerol stored in white adipocytes in adipose tissues. Surplus energy (mainly blood glucose and blood triacylglycerol) after a meal is rapidly

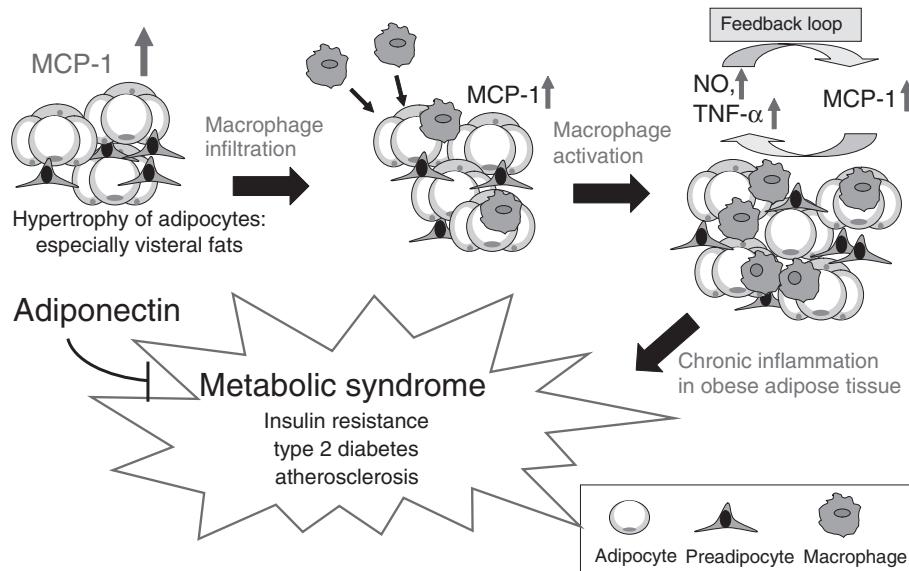
**Table 4.1.** Major candidate genes with obesity-related phenotypes.

Gene	Phenotype
ACE (angiotensin I converting enzyme (peptidyl-dipeptidase A) 1)	Overweight
ADRB3 (adrenergic $\beta$ 3 receptor)	Obesity, BMI, abdominal visceral fat
APOA1 (apolipoprotein A-1)	BMI (in type 2 diabetics)
APOB (apolipoprotein B)	BMI, abdominal fat
FABP2 (fatty acid binding protein 2, intestinal)	BMI, abdominal fat
HSD11B1 (hydroxysteroid (11- $\beta$ ) dehydrogenase 1)	BMI (in children)
IL6 (interleukin 6 (interferon, $\beta$ 2))	BMI (in men)
IL6R (interleukin 6 receptor)	Obesity (in women)
INS (insulin)	Obesity, BMI, waist-to-hip ratio (in obese women)
IRS2 (insulin receptor substrate 2)	BMI
LDLR (low-density lipoprotein receptor)	BMI, obesity
LEP (leptin)	Body weight, BMI
LEPR (leptin receptor)	Body weight, BMI, abdominal adipocyte size
LIPE (hormone-sensitive lipase)	Obesity, BMI, percent body fat, fat mass
LPL (lipoprotein lipase)	BMI (in women)
MC3R (melanocortin 3 receptor)	BMI, percent body fat, respiratory quotient
MC4R (melanocortin 4 receptor)	BMI, percent body fat, fat mass (in females)
NPY (neuropeptide Y)	BMI
NPY5R (neuropeptide Y receptor Y5)	Obesity
NR3C1 (glucocorticoid receptor)	Obesity, abdominal visceral fat
PPARA (peroxisome proliferative activated receptor-alpha)	BMI (in type 2 diabetics), percentage of body fat
PPARG (peroxisome proliferative activated receptor-gamma)	Obesity, BMI, abdominal visceral fat
PPARGC1 (peroxisome proliferative activated receptor-gamma, coactivator 1)	BMI, fat mass, adipocyte size
TNRC11(THR-associated protein)	Obesity
UCP1 (uncoupling protein 1)	Body weight, BMI, resting metabolic rate

taken into adipose tissues and becomes body fat. The number of adipocytes is approximately 30 billion in an adult (40–60 billion in an obese person) and a maximum of approximately 0.9–1.0  $\mu$ g fat is accumulated in an adipocyte. Because there is a limit in the amount of fat accumulated in an adipocyte, the absolute amount of accumulated fat is increased by the proliferation of new adipocytes. Animals, which have always been facing “starvation” since birth, have achieved this very ingenious system during the course of biological evolution.

Naturally, animals have a physiological mechanism for survival, by which they store energy as fat in their body and do not easily discharge the stored energy. Such a mechanism is closely associated with the development of obesity in humans, that is, the ability to form adipose tissue. Recently, it has been discovered that adipocytes secrete undesir-

able disease-causing adipocytokines, such as TNF- $\alpha$ , causing insulin resistance, and PAI-1, associated with clot formation. However, they also produce and secrete good adipocytokines such as adiponectine, which exhibit antiarteriosclerotic and antidiabetic effects. Adipocytes are highly associated with various lifestyle-related diseases. Furthermore, it has been found that macrophages invade adipose tissues in obesity and produce cytokines and chemokines such as TNF- $\alpha$ , MCP-1, and NO, which cause chronic inflammatory reactions resulting in the exacerbation of the metabolic syndrome (Figure 4.3) (Xu et al. 2003; Yu et al. 2006). Adipose tissues not only store fat but are the major tissues controlling the development of diseases. It is crucial in terms of preventive medicine and health science to study the characteristics and properties of adipose tissues in detail and control them (Rodriguez et al. 2005).



**Figure 4.3.** The development of inflammatory reactions controlled by cytokines and chemokines between adipocytes and macrophages in adipose tissue.

#### FORMATION OF ADIPOCYTES AND REGULATORY MECHANISM

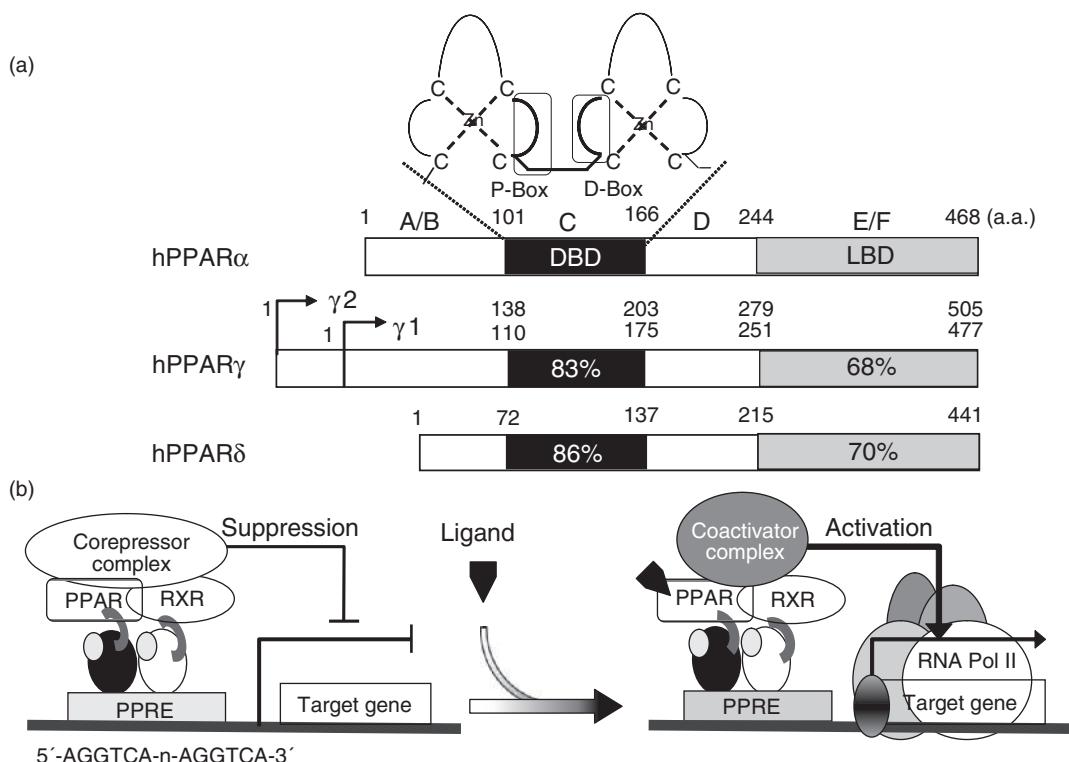
The formation of adipocytes is roughly divided into the following six steps: (1) determination of stem cells that become lipoblasts achieving the basic properties of adipocytes, (2) proliferation of lipoblasts, (3) cell proliferation arrest, (4) terminal differentiation of preadipocytes to immature adipocytes, (5) maturation of immature adipocytes along with accumulation of fat, and (6) division and proliferation of mature adipocytes. Genes specific to each process in adipogenesis from multipotent mesenchymal stem cells to mature adipocytes is now actively pursued (Rosen 2005).

Recently, the analysis of mechanisms regulating the expression of genes associated with the differentiation and development of adipocytes has been progressing rapidly. As a result, it was found that peroxisome proliferator-activated receptors (PPARs) as shown in Figure 4.4, which are the receptor-type nuclear transcription factors with long-chain fatty acids and its metabolites as the ligand, are the master regulators interacting with other transcription factors and forming a network. It is interesting that fatty acids themselves regulate the transcription of genes required for the formation of adipocytes (Freedman 1999; Takahashi et al. 2002b).

#### OBESITY AND METABOLIC SYNDROME AS TARGET OF NUTRIGENOICS

##### DEFINITION OF METABOLIC SYNDROME

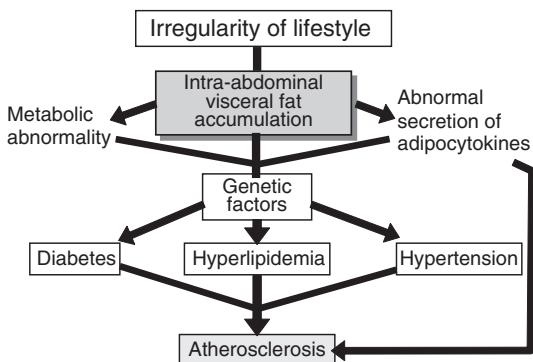
Arteriosclerotic diseases (such as myocardial infarction and cerebral infarction) develop suddenly in people in their prime and largely affect the patients, their families, and their places of work. These diseases also cause a major problem in medical economics. Conventional preventive measures for such diseases have focused on the control of hypercholesterolemia and have dealt with other risk factors individually. However, there has been a recent increase in the number of people with multiple risk factors for obesity (including mild obesity) against the background of food satiation and lack of exercise. It has been found that arteriosclerotic diseases are also observed in many people with multiple risk factors. According to a report by WHO in 2002, arteriosclerotic diseases (such as myocardial infarction and cerebral infarction) surpassed cancer and topped the list of the global causes of death (WHO: The World Health Report 2002). It has been found that multiple-risk-factors syndrome, characterized by hyperlipidemia, hypertension, obesity, and diabetes, is closely associated with the etiology of arteriosclerotic diseases. In such global circumstances, the concept and criteria



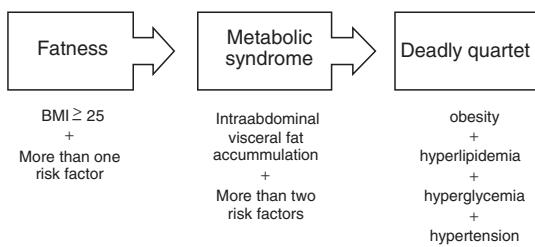
**Figure 4.4.** Structural and transcriptional features of human PPARs. (a) Structure and functional domain of human PPARs. A/B, C, D, and E/F indicate N-terminal A/B domain containing a ligand-independent transactivation domain, DNA-binding domain (DBD), hinge region, and ligand-binding domain (LBD), respectively. (b) PPAR/RXR heterodimers bind to a PPAR response element (PPRE) located in the promoter of target genes through the DBD. PPAR without ligand associates with the corepressor complex. In the presence of ligand, the ligand-bound LBD associates with the coactivator complex.

of metabolic syndrome were set in Japan and worldwide in April 2005, based on the concept of visceral fat accumulation as the essential criterion.

Metabolic syndrome is defined as “multiple-risk-factors syndrome” with visceral fat accumulation, which inevitably occurs in highly automated societies with food satiation. The syndrome is complicated by two or more of the following: insulin resistance, abnormal glucose metabolism, abnormal lipid metabolism, and hypertension; a clinical state susceptible to arteriosclerosis (Figure 4.5). The Committee set the criteria for metabolic syndrome in Japan to evaluate diagnostic standards for the syndrome in April 2005. In addition to the complications described above, the mechanism underlying the development of vascular diseases directly caused by visceral fat accumulation also underlies the development of arteriosclerosis. It was reported that the risk of arteriosclerotic diseases increases approximately



**Figure 4.5.** Primary role of visceral fat accumulation on the crises of obesity-related diseases and metabolic syndrome. (Committee to Evaluate Diagnostic Standards for Metabolic Syndrome 2005).



**Figure 4.6.** Flowchart from fatness and metabolic syndrome to deadly quartet (Matuzawa Y, Funahashi T. 2005).

35-fold among people with metabolic syndrome in Japan.

#### SIGNIFICANCE OF ESTABLISHING THE CONCEPT OF METABOLIC SYNDROME

In most of the present management strategies for multiple-risk-factors syndrome, the target is the improvement of the most prominent abnormality, whereas comorbid clinical conditions are left untreated or several drugs are used to treat each clinical condition. The establishment of the concept of metabolic syndrome with an extremely high risk of arteriosclerosis clearly shows the importance of the active improvement of lifestyle (particularly the promotion of exercise) in reducing “accumulated visceral fat,” which plays a key role in metabolic syndrome. This active improvement reduces multiple risks due to visceral fat accumulation and promotes effective preventive medicine for arteriosclerotic diseases. Figure 4.6 shows the relationship among obesity, metabolic syndrome, and the “deadly quartet” (Matuzawa and Funahashi 2005). It clarifies the stepwise relationship between the quality and level of obesity and the onset of clinical conditions associated with obesity and provides concrete measures for prevention and treatment of such diseases.

It is also expected that a drug that reduces multiple risks comprehensively and prevents arteriosclerosis will be developed and used instead of the antidiabetes, antihyperlipidemic, and antihypertensive drugs currently used for treating each clinical condition. Specific (quantitative) criteria were established for the development of drugs, which cannot be realized on the basis of the definition of “obesity” alone. The new concept is applied to food as well as drugs; there is a possibility that a new strategy will be proposed by the food industry concerned with re-

search on nutrigenomics and food for specified health uses.

## NUTRIGENOMICS IN ANTIOBESITY AND ANTIMETABOLIC SYNDROME STRATEGY

### HEAT PRODUCTION AND ANTIOBESITY STRATEGIES

Obesity is defined as the state of excessive accumulation of adipose tissues in the body. Adipocytes within adipose tissues are divided into brown adipocytes and white adipocytes in terms of their function. Brown adipose tissues are “heat-producing tissues” that oxidize and break down accumulated fat and release the obtained energy as heat. Brown adipocytes are small cells with diameters of 20–50  $\mu\text{m}$  localized in the interscapular area or the area around the kidneys and have multilocular fat droplets. Brown adipocytes contain many mitochondria that express uncoupling protein (UCP) 1, the protein involved in heat production. UCP1 promotes heat production by oxidizing its substrate, fatty acids, and uncoupling ATP synthesis. Heat production is mainly controlled by sympathetic nerves. Thus, brown adipose tissues are considered to maintain body temperature in a cold environment and diffuse and expend excess energy as heat.

Enhancement of the function of heat-producing brown adipose tissues seems to be an effective measure from the viewpoint of antiobesity. It has been considered that brown adipose tissues do not exist in humans except during the neonatal period, in contrast to laboratory animals such as rodents. However, recently, it has been found by positron emission tomography (PET)-CT using radiolabeled fluorodeoxyglucose (FDG) in combination with X-ray CT that brown adipose tissues are widely distributed in adult humans (Saito et al. 2007). UCP1 in brown adipocytes is stimulated as mediated by  $\beta$ -receptors. An agonist of  $\beta$ 3 receptors, which are frequently expressed in adipocytes, induces the expression of UCP1 genes and increases the number of mitochondria when it is administered chronically. Moreover, it induces the ectopic expression of UCP1 in white adipose tissues and skeletal muscles where UCP1 is rarely expressed under normal conditions (Nagase et al. 1996), which leads to a decrease in the amount of body fat and an increase in oxygen consumption (Kato et al. 2001). Considering these effects, it is highly expected that an agonist of  $\beta$ 3 receptors exerts antiobesity effects in humans.

### PPARs AND ANTIOBESITY STRATEGY

It is assumed that hypertrophy of adipocytes occurs first in the development of obesity and then the number of adipocytes increases when the state of energy excess continues. Therefore, clarification of the mechanism underlying fat accumulation is equivalent to clarifying the mechanism underlying hypertrophy, proliferation, and differentiation of adipocytes.

The ligand-dependent receptor-type transcription factor PPAR $\gamma$  functions as the master regulator of adipocyte differentiation. PPAR $\gamma$  is a receptor of a thiazolidine derivative, an insulin sensitizer, and is associated with various diseases. The increase in body weight due to consumption of high-fat diet is controlled and insulin sensitivity was good in PPAR $\gamma$  heterodeficient mice (Kubota et al. 1999). Considering these findings, the inhibition of the action of PPAR $\gamma$  is expected to have antiobesity and anti-insulin resistance effects. The effects of the decrease in body weight and the improvement of metabolic disorder were observed at the individual level for some PPAR $\gamma$  antagonists (Rieusset et al. 2002). Moreover, similar effects were observed for an inhibitor of the formation of a heterodimer of PPAR $\gamma$  and RXR (Yamauchi et al. 2001).

PPARs consist of three subtypes: PPAR $\gamma$  described earlier, PPAR $\alpha$ , and PPAR $\delta$ . PPAR $\alpha$  and PPAR $\delta$  also play a significant role in the regulation of glucose and lipid metabolism in the body and are considered to be associated with obesity.

PPAR $\alpha$  is frequently expressed in the liver, skeletal muscles, and brown adipose tissues where active fatty acid catabolism occurs; PPAR $\alpha$  controls the expression of genes related to fatty acid metabolism. Fibates, which are PPAR $\alpha$  agonists, improve hypertriglyceridemia. It was also reported that PPAR $\alpha$  agonists control the increase in body weight in mice (Tsuchida et al. 2005). There is a possibility that the antiobesity effect of PPAR $\alpha$  agonists is triggered by their function of inducing an increase in the expression of  $\beta 3$  receptors and UCP1 in brown adipose tissues (Tsuchida et al. 2005). Moreover, oleylethanolamide, the ethanolamide of oleic acid, has an affinity for PPAR $\alpha$  1,000-fold higher than that of oleic acid. Furthermore, oleylethanolamide has an antifeeding effect, which is mediated by PPAR $\alpha$  activation (Jin et al. 2003). These findings indicate that PPAR $\alpha$  activation induces an antifeeding effect and that PPAR $\alpha$  agonists exhibit an antiobesity effect.

Although PPAR $\delta$  is ubiquitously expressed in tissues, its activation in adipocytes increases the expression levels of UCP1 and  $\beta$ -oxidizing genes and

promotes lipolysis (Wang et al. 2003). It was also reported that the administration of PPAR $\delta$  agonists enhances  $\beta$ -oxidation in skeletal muscles and improves obesity and insulin resistance (Tanaka et al. 2003).

PPAR $\gamma$ , which directly regulates the differentiation of adipocytes, and PPAR $\alpha$  and PPAR $\delta$ , which enhance fat oxidation, are highly associated with the level of fat accumulation and obesity and are a very interesting group of target receptors in nutrigenomics.

### EFFECTIVE FOOD INGREDIENT STRATEGY AGAINST OBESITY AND METABOLIC SYNDROME

A disturbance of food balance is the major cause of the development of obesity. Obesity can be prevented or improved by adapting good eating habits. Food contains nutrients and components showing various biological regulatory functions. This indicates that obesity can be prevented or improved by not only antiobesity measures, such as the control of high-calorie food intake through dietary portion control, but also the active intake of food containing components effective for controlling obesity. Food components effective for controlling obesity have been searched on the basis of scientific evidence and some have been identified.

The following are the functions and effects of food components effective for controlling obesity and metabolic syndrome: (1) inhibition of absorption of glucose and fat, which become energy substrates, (2) increase in body heat production, (3) inhibition of food intake, and (4) qualitative improvement of obesity. Food components having a pancreatic lipase inhibitory or alpha glucosidase inhibitory effect are found to have effect (1) (McDougall and Stewart 2005; Nakai et al. 2005), and among such components, tea polyphenol has already been commercialized. As for effect (2), food components that increase body heat production through the sympathetic nervous system have been discovered (Morimoto et al. 2005; Tsuboyama-Kasaoka et al. 2006; Wolfram et al. 2006). Alpha-lipoic acid, which has already become commercially available as a food-based supplement, has effect (3). It has been found that alpha-lipoic acid inhibits food intake by inhibiting adenosine monophosphate (AMP)-activated protein kinase (AMPK) in the hypothalamus (Kim et al. 2004). Different from effects (1) to (3), effect (4) does not reduce body weight but qualitatively improves obesity owing to the inhibition of visceral fat accumulation. Food components having effect (4) have also been searched in various

food products. Here, we introduce our search for such effective components as an example.

Nuclear receptors are a group of transcription factors activated by lipophilic low-molecular-weight compounds as the ligand and, owing to their properties, directly or indirectly regulate glucose and lipid metabolism in the body. Therefore, it has been found that the regulation of the activity of nuclear receptors plays a significant role in abnormal glucose and lipid metabolism associated with obesity. Food components or natural products that control the activity of nuclear receptors have been identified (Chawla et al. 2001). Nuclear receptors are considered to be a useful target in the search for food components effective for lifestyle-related diseases associated with obesity. Regulators of the activity of nuclear receptors have been found in natural products used as spices or traditional herbal remedies for diseases such as abnormal glucose and lipid metabolism. Examples and details on the regulation of the activity of nuclear receptors have been clarified as the regulatory effect of food ingredients and herbs in terms of functional food science and pharmacology (Table 4.2) (Lazar 2004; Takahashi et al. 2002a).

Our research group also focused on the characteristics of nuclear receptors described above and has searched for components in natural products, including food ingredients, that qualitatively improve obesity by controlling the function of adipocytes. PPAR is a nuclear receptor of fatty acids. It plays a significant role in glucose and lipid metabolism (Ricote and Glass 2007). There are three subtypes of PPAR,  $\alpha$ ,  $\gamma$ , and  $\delta$ , and the activator of each subtype improves abnormalities in metabolism. We fo-

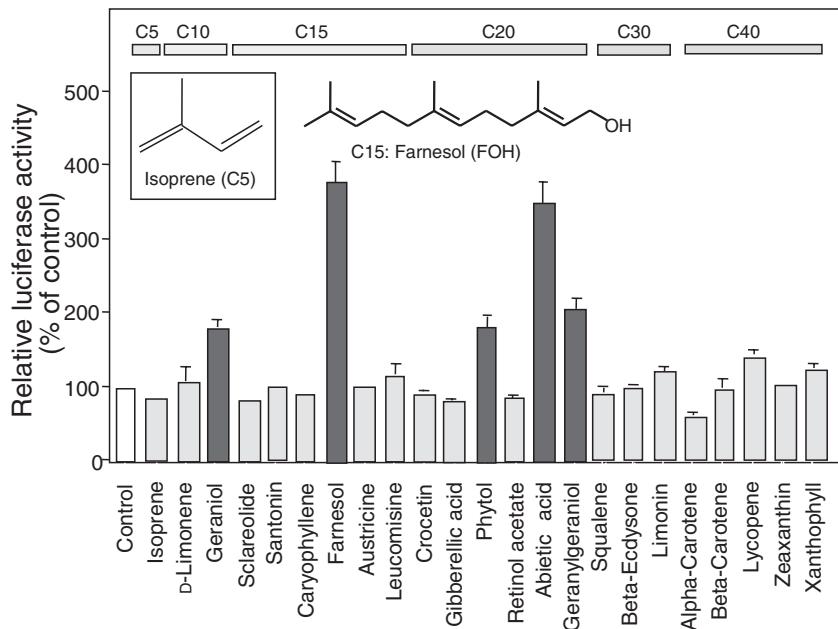
cused on PPAR $\gamma$ , which is the master regulator of the differentiation of adipocytes and is frequently expressed in adipose tissues. We developed a sensitive ligand assay system and searched for food-based activators of PPAR $\gamma$ . As a result, we found that some isoprenoids activate PPAR $\gamma$  (Figure 4.7) (details in Chapter 28; Goto et al. 2005; Kang et al. in press; Kuroyanagi et al. 2008; Park et al. 2004; Takahashi et al. 2002a, 2003). Moreover, some of these isoprenoids decrease blood glucose level and increase insulin sensitivity in obesity and diabetes animal models, indicating that these substances are effective components for lifestyle-related diseases associated with obesity. The ligand-binding site of PPARs is highly hydrophilic and shows higher ligand diversity than that of other nuclear receptors. Food components other than isoprenoids that become ligands of PPARs have been identified. Isohumulone, the bitter component of hops, shows the stimulatory effect on PPAR $\alpha$  and PPAR $\gamma$ , which was proved to be effective for patients with type 2 diabetes (Yajima et al. 2004).

Capsaicin, the pungent component of chili peppers, has anti-inflammatory effects. Capsaicin suppresses inflammation in adipose tissues in obesity and can mitigate diseases associated with obesity (Kang et al. 2007). Pungent components of other spices and phytochemicals also show similar effects (Hirai et al. 2007; Woo et al. 2007). Currently, research is progressing on the suppression of inflammatory reactions generated in adipose tissues in obesity as the treatment target for the prevention and improvement of metabolic syndrome and lifestyle-related diseases (details in Chapter 6;

**Table 4.2.** Herbal ligands for nuclear receptors and their related diseases.

Compound (Origin)	Disease	Receptor
Resveratrol (Red wine)	Cardiovascular	ER, PPAR $\alpha$ , $\gamma$
Isoprenols (Herb)	Glucose/lipid disorders	PPAR $\alpha$ , $\gamma$
Abietic acid (Pine rosin)	Glucose/lipid disorders	PPAR $\gamma$
Capsaicin (Hot pepper)	Glucose/lipid disorders	PPAR $\gamma$
Phytol (Chlorophyll)	Lipid disorders	PPAR $\alpha$
Auraptene (Citrus fruit)	Proinflammation in obesity	PPAR $\alpha$ , $\gamma$
Isohumulone (Humulus lupulus hop)	Glucose/lipid disorders	PPAR $\alpha$ , $\gamma$
Guggulsterone (Guggul tree rosin)	Lipid disorders	FXR
Genistein (Soy)	Menopause	ER, AR, PR
Diosgenin ( <i>Dioscorea villosa</i> )	Menopause	PR
Ginsenaside-Rg1(Ginseng)	Stress	ER
Hyperforin ( <i>Hypericum perforatum</i> )	Depression	PXR

ER, estrogen receptor; PPAR, peroxisome proliferator-activated receptor; FXR, farnesyl X receptor; AR, androgen receptor; PR, progesterone receptor; PXR, pregnane X receptor.



**Figure 4.7.** Effects of various natural isoprenoids compounds on activation of PPAR $\gamma$  in luciferase ligand assay system using GAL4/PPAR chimera protein. Each sample was measured at 50  $\mu$ M. Farnesol composed of 3 units of isoprene (C15), which effectively induced PPAR $\gamma$  activation.

Hirai et al. 2007; Kang et al. in press). Further development in nutrigenomics is expected to realize practical applications of food components and ingredients for the prevention and improvement of obesity and metabolic syndrome on the basis of the reduction of visceral fat accumulation and qualitative improvement of obesity.

## CONCLUSION

Obesity is the state of excessive formation of adipose tissues. The basic regulatory mechanisms underlying the differentiation and formation of adipocytes have gradually been clarified. It has also been found that the difference in the region where adipose tissues develop is closely associated with the pathogenesis of diseases and that the development of visceral adipose tissues existing in the portal vein system such as mesenteric fat leads to diabetes, hypertension, and arteriosclerotic diseases. Namely, these adipose tissues can be responsible for metabolic syndrome. Therefore, the main research subject concerning obesity and metabolic syndrome in the future is the clarification of the mechanism underlying the regulation of metabolism specifically in adipose tissues that leads to the development of diseases. Moreover, analyses

of biofactors and food components associated with the metabolic properties of organs for energy storage and expenditure such as adipose tissues and with the development of diseases should be carried out, and the development of practical applications of such components is desired.

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# 5

## Inflammatory Genes Involved in Obesity-Induced Inflammatory Responses and Pathologies

Rina Yu

### INTRODUCTION

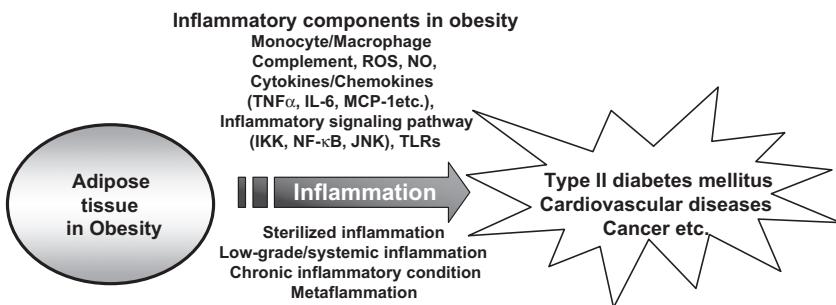
Obesity is a low-grade systemic chronic inflammatory condition, characterized by abnormal cytokine production, increased acute-phase proteins, and other inflammatory mediators. In general, inflammation serves a protective function in controlling infections and promoting tissue repairs; however, acute inflammation or chronically prolonged inflammation can cause tissue damage and plays a crucial role in the development of various inflammatory pathologies. Interestingly, obesity-induced inflammation consists of a set of inflammatory immune components and inflammatory signaling pathways similar to those involved in classical inflammation, such as inflammatory cells like macrophages, inflammatory mediators like cytokines and chemokines, as well as inflammatory signaling molecules (Das 2001; Hotamisligil 2003, 2005, 2006; Lehrke and Lazar 2004; Shoelson et al. 2006; Xu et al. 2003) (Figure 5.1). Obesity-induced inflammation is considered to serve as the potential mechanism linking obesity to obesity-related pathologies such as insulin resistance, type 2 diabetes, fatty liver disease, atherosclerosis, some immune disorders, and several types of cancer.

This chapter focuses on obesity-induced inflammatory components, linking obesity to obesity-related pathologies. Adipose tissue-derived inflammatory genes/proteins such as adipocytokines and signaling molecules, and the inflammatory cross talk within adipose tissue cells through adipocytokines are discussed. In addition, the beneficial effects of anti-inflammatory phytochemicals against obesity-induced inflammatory responses and pathologies are introduced.

### INFLAMMATORY COMPONENTS IN ADIPOSE TISSUE

#### ADIPOCYTOKINES IN ADIPOSE TISSUE

Adipose tissue not only stores fat, but also functions as an active endocrine and paracrine organ for secreting a variety of biologically active proteins, collectively referred to adipocytokines or adipokines. Adipose tissue-derived adipocytokines modulate adipocyte functions and energy metabolism, thereby playing a crucial role in obesity-related pathophysiological processes such as insulin resistance, and the development of diabetes and atherosclerosis (Hausman et al. 2001; Matsuzawa et al. 2003). Adipocytokines include tumor necrosis factor-alpha (TNF- $\alpha$ ), interleukin-6 (IL-6), plasminogen activator inhibitor-1 (PAI-1), angiotensinogen, tissue factor, transforming growth factor- $\beta$  (TGF- $\beta$ ), leptin, adiponectin, resistin, and certain chemokines such as monocyte chemoattractant protein-1 (MCP-1). Among adipocytokines, TNF- $\alpha$ , IL-6, MCP-1, and adiponectin are strongly associated with obesity-induced inflammation and obesity-related pathologies (Ahima 2006; Aldhahi and Hamdy 2003; Fontana et al. 2007; Weisberg et al. 2006). The circulation levels of TNF- $\alpha$ , IL-6, and MCP-1 positively correlate with the levels of inflammatory markers such as the C-reactive protein (Kim et al. 2006; Vozarova et al. 2001), whereas that of adiponectin negatively correlates with them (Matsuzawa et al. 2004). It appears that the dysregulation of adipocytokine release results in the chronic inflammatory condition observed in obesity, triggering the development of obesity-related pathologies.



**Figure 5.1.** Inflammatory components in obesity and related pathologies.

#### DYSREGULATION OF ADIPOCYTOKINES

TNF- $\alpha$ , a major proinflammatory cytokine involved in both acute and chronic inflammatory responses, is a representative adipocytokine related to obesity-induced metabolic syndrome. TNF- $\alpha$  acts as a powerful inducer of other proinflammatory adipocytokines such as IL-6, MCP-1, leptin, and PAI-1 (Shimomura et al. 1996), and thus contributes to inflammatory conditions in obesity. The chronic treatment of TNF- $\alpha$  decreases the expression of the insulin-sensitive glucose transporter 4 (GLU4) and insulin receptor substrate-1 (IRS-1), suppresses the tyrosine phosphorylation of IRS-1, and enhances the serine phosphorylation of IRS-1, thereby resulting in impairing insulin signaling (Feinstein et al. 1993; Hotamisligil 2003; Hotamisligil et al. 1993). The adipose tissue expression level of TNF- $\alpha$  increases in obese rodents as well as in obese patients, and the increased TNF- $\alpha$  is directly associated with obesity-induced insulin resistance (Hotamisligil et al. 1995). Subsequently, it has been shown that the neutralization of TNF- $\alpha$  protein and/or TNF- $\alpha$  gene deficiency can improve obesity-induced insulin resistance (Hotamisligil et al. 1993; Uysal et al. 1997). These findings support the inflammation theory linking obesity to obesity-related pathologies (Hotamisligil 2006).

As with TNF- $\alpha$ , IL-6 is a multifunctional cytokine that regulates immune response, hematopoiesis, acute phase response, and inflammation, and plays a role in the pathogenesis of insulin resistance. IL-6 inhibits insulin signal transduction in hepatocytes by modulating the suppressor of cytokine signaling-3 pathway (Senn et al. 2003). Circulating IL-6 level leads to increase in human obesity and insulin resistance (Vozarova et al. 2001), and weight loss results in a decrease in IL-6 level in both adipose tissue and serum (Bastard et al. 2000). IL-6 enhances the

release of adhesion molecules by the endothelium and the hepatic release of fibrinogen, as well as exerts procoagulant effects on platelets (Yudkin et al. 2000). These findings suggest that IL-6 action has a role in the development of obesity-related pathologies such as insulin resistance and atherosclerosis.

MCP-1 is a CC chemokine that exhibits chemotactic properties on inflammatory cells through the generation of local concentration gradients. MCP-1 is implicated in various inflammatory disorders including atherosclerosis and insulin resistance. For example, MCP-1 decreases insulin-stimulated glucose uptake rate and the expression levels of adipogenic genes (Sartipy and Loskutoff 2003). MCP-1-deficient mice develop no atherosclerosis (Gu et al. 1998) and exhibit improved insulin sensitivity (Kanda et al. 2006; Weisberg et al. 2006). Recently, the involvement of MCP-1 in obesity-induced inflammatory responses has been suggested (Weisberg et al. 2003; Xu et al. 2003; Yu et al. 2006). The level of MCP-1 mRNA expression, protein content, and the amount of protein released in adipose tissues from the obese mice significantly increased compared with those from the nonobese mice. Mesenteric adipose tissue produces the highest levels of MCP-1 protein among the four different fat depots (e.g., mesenteric, epididymal, renal, and subcutaneous adipose tissues). Mesenteric adipose tissue-conditioned medium induces the highest degree of macrophage migration and strongly induces macrophages to produce proinflammatory mediators such as nitric oxide and TNF- $\alpha$ . The neutralization of MCP-1 in an adipose tissue-conditioned medium significantly inhibits the migration and activation of macrophages. Adipose tissue-derived MCP-1 plays a crucial role in adipose tissue inflammatory response by activating and inducing the infiltration of macrophages into adipose tissues (Weisberg et al. 2003; Xu et al. 2003;

Yu et al. 2006). MCP-1 is closely associated with visceral obesity-related complications and thus may be a useful therapeutic target for modulating visceral obesity-related diseases (Figure 5.2).

Unlike other inflammatory adipocytokines, adiponectin has anti-inflammatory properties and attenuates atherosclerosis development by suppressing the expression of adhesion molecules in vascular endothelial cells, and inhibiting the accumulation of monocyte/macrophage-derived foam cells in the vascular wall (Ouchi et al. 2003), as well as improves insulin sensitivity by upregulating the expression of IRS-1 in skeletal muscles (Kadowaki et al. 2006).

#### ACTIVATION OF INFLAMMATORY SIGNALING MOLECULES

Some kinases such as c-Jun amino-terminal kinase (JNK), the inhibitor of nuclear factor- $\kappa$ B (NF- $\kappa$ B) kinase- $\beta$  (IKK), and protein kinase (PKC) play a crucial role in triggering inflammatory gene expression through the activation of activator protein-1 (AP-1) complexes and NF- $\kappa$ B. It has been shown that obesity-related factors such as inflammatory cytokines and free fatty acids can activate inflammatory signaling molecules such as JNK, IKK, PKC, NF- $\kappa$ B, and that more importantly, their activities markedly increase in obese adipose and liver tissues (Hirosumi et al. 2002; Hotamisligil 2006). Metabolic endoplasmic reticulum (ER) stress induced by overnutrition has been suggested to trigger the activation of the inflammatory signaling molecules, thereby resulting in insulin resistance (Hotamisligil 2006; Wellen and Hotamisligil 2005).

Toll-like receptors (TLRs), a family of pattern-recognition receptors that has a crucial role in the innate immune system, may be another potential candidate linking obesity-induced inflammation to obesity-related pathologies. In particular, TLR4 is expressed in macrophages, adipocytes, liver, and skeletal muscle, and the binding of TLR4 ligands such as LPS to TLR4 has been shown to activate the NF- $\kappa$ B pathway, thereby enhancing the transcription of inflammatory gene expression such as inflammatory cytokines (e.g., TNF- $\alpha$ , IL-6, MCP-1) and other inflammatory mediators. It has been shown that saturated fatty acids (i.e., C14:0, C16:0, and C18:0) can activate the IKK/NF- $\kappa$ B pathway and stimulate the macrophage production of TNF- $\alpha$  and IL-6 by acting as a ligand for TLR4 (Shi et al. 2006). TLR4 deficiency normalizes insulin-stimulated IRS-1 tyrosine phosphorylation, and TLR4 deletion partly prevents obesity-induced insulin resistance in mice

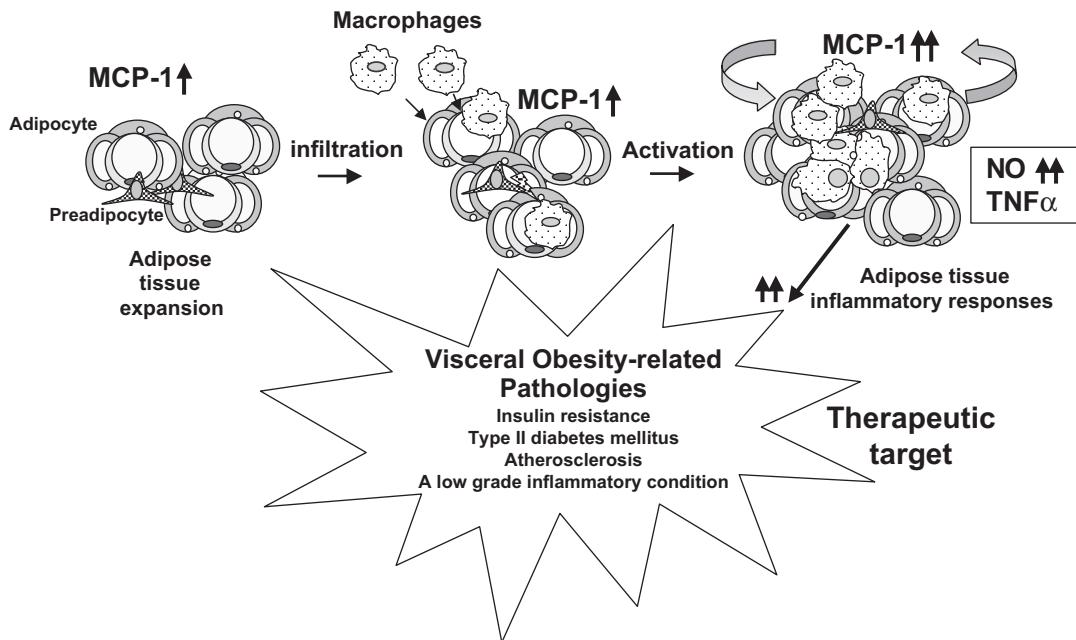
fed a high-fat diet, indicating that TLR4 mediates the lipid-induced activation of inflammatory signaling and insulin resistance in peripheral tissue (Shi et al. 2006). In this context, increased amounts of free fatty acids in circulation and tissue in obesity may directly contribute to the obesity-induced inflammation by inducing TLR4-mediated signaling in macrophages, adipocytes, and liver, which activates inflammatory signaling molecules such as IKK, JNK, and PKC, causing impaired insulin signaling and action.

#### INFLAMMATORY CELLS IN ADIPOSE TISSUE

Adipose tissue is composed of adipocytes and stromal vascular cells containing various cell types such as preadipocytes, endothelial cells, fibroblasts, and numerous immune cells. Interestingly, macrophage infiltration into adipose tissue is prominent in both obese humans and rodents, and the number of macrophages in adipose tissue correlates with body mass index, adipocyte size, and the total amount of body fat (Weisberg et al. 2003; Xu et al. 2003). It has been suggested that adipose tissue-derived MCP-1 is the key factor for inducing macrophage infiltration into adipose tissue. The level of MCP-1 released from adipocytes is significantly greater in obese mice than in nonobese mice, and are markedly increased when adipocytes are cocultured with macrophages (Bruun et al. 2005; Fain et al. 2004; Yu et al. 2006). Moreover, MCP-1 can activate macrophages to produce inflammatory mediators such as TNF- $\alpha$ , IL-6, and MCP-1, further exacerbating adipose tissue inflammation (Yu et al. 2006). MCP-1 from hypertrophic adipocytes in obese adipose tissue can also trigger macrophage infiltration into adipose tissue, and subsequently activates macrophages to release inflammatory mediators (Yu et al. 2006). This indicates that adipocytes themselves play a major role in initiating inflammatory responses in obese adipose tissue, whereas macrophages promote the inflammatory condition in adipose tissue through the cross talk between adipocytes and macrophages through inflammatory mediators such as MCP-1 (Suganami et al. 2005; Yu et al. 2006) (Figure 5.2).

#### VISCERAL FAT CAUSES ATROPHY OF LYMPH NODES

Obesity, aside from causing metabolic complications, impairs the immune system. For example, obese patients or animals are more susceptible to infectious diseases as well as to several types of



**Figure 5.2.** Adipose tissue-derived MCP-1 induces macrophage infiltration and activation and thereby augments adipose tissue inflammatory responses in obesity.

cancer (Chandra 1981; Ikejima et al. 2005; McTieran 2005; Smith et al. 2007). Visceral fat exhibits more enhanced lipolytic activity and inflammatory phenotypes than other fat depots (Wajchenberg 2000; Yu et al. 2006). Since mesenteric fat tissue, which is representative of a visceral fat depot with particular vicious phenotypes, is anatomically associated with mesenteric lymph nodes, mesenteric adipose tissue-derived factors may directly affect the mesenteric lymphoid cell system by a local interaction or cross talk, thereby affecting the immune system in obesity. Indeed, it has been found for the first time that the weight of mesenteric lymph nodes and the number of lymphoid total cells in obese mice significantly decrease compared with those in control mice; however, no change has been observed in the weight of inguinal lymph nodes (Kim et al. 2008). The numbers of CD4+ and CD8+ T cells in the mesenteric lymph nodes of obese mice significantly decrease compared with those of control. Enhanced T cell activation and apoptosis are observed in the mesenteric lymph node cells of obese mice. These suggest that visceral fat accumulation with a high-fat diet can cause the atrophy of mesenteric lymph nodes by enhancing activation-induced lymphoid cell apoptosis (Kim et al. 2008).

Thus, visceral fat accumulation may be crucial for obesity-related immune dysfunction.

### PHYTOCHEMICALS TARGETING OBESITY-INDUCED INFLAMMATORY RESPONSES AND PATHOLOGIES

Since obesity-induced inflammation can trigger the development of obesity-related pathologies, targeting obesity-related inflammatory components may be a useful strategy to prevent or ameliorate the development of obesity-related pathologies. It has been shown that several anti-inflammatory phytochemicals can modulate inflammatory responses in adipose tissue and therefore improve obesity-related pathologies such as insulin resistance (Kang et al. 2007; Woo et al. 2007). For example, capsaicin, a spicy ingredient of hot peppers, generates not only metabolic potentials to induce thermogenesis and fat oxidation (Kawada et al. 1986; Westerterp-Plantenga et al. 2006), but also anti-inflammatory properties (Kim et al. 2003). Interestingly, in the adipose tissue/adipocyte culture system, capsaicin inhibits the

expressions of IL-6 and MCP-1 mRNAs and protein release from the adipose tissues and adipocytes of obese mice, whereas it enhances the expressions of the adiponectin gene and protein (Kang et al. 2007). The action of capsaicin is associated with NF- $\kappa$ B inactivation and/or PPAR $\gamma$  activation (Kang et al. 2007). Moreover, capsaicin suppresses not only macrophage migration induced by an adipose tissue-conditioned medium, but also macrophage activation to release proinflammatory mediators. Moreover, capsaicin supplementation *in vivo* improves obesity-induced insulin resistance (Kang et al. 2007). Aside from capsaicin, other spice-derived components (e.g., diallyl disulfide, aryl isothiocyanate, piperine, zingerone, and curcumin) and others (e.g., naringenin chalcone and citrus auraptene) also suppress inflammatory responses in obese adipose tissue by suppressing inflammatory adipocytokine release and macrophage infiltration into adipose tissue (Hirai et al. 2007; Kuroyanagi et al. 2008; Woo et al. 2007), indicating that such phytochemicals are beneficial against obesity-induced inflammatory responses and obesity-related pathologies.

## CONCLUSION

It is becoming clear that obesity-induced inflammation plays an important role in the development of obesity-related pathologies such as insulin resistance, type 2 diabetes, atherosclerosis, and cancer. Obesity-related inflammatory components consist of adipocytes, inflammatory immune cells, adipocytokines, cytokines/chemokines, and inflammatory signaling molecules, and the cross talk among these inflammatory components play a critical role in the development of obesity-related pathologies. All the described associations between inflammatory components and obesity-related pathologies suggest that obesity-associated inflammatory components provide an attractive therapeutic option for the management of obesity-related pathologies.

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# 6

## Genomics and Proteomics in Allergy

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### INTRODUCTION

The word “proteomics” was coined by Marc Wilkins in 1994 (Wilkins et al. 1996). Interestingly, the word “genomics” is thought to have been formed in reference to the word chromosome. Genomics refers to the analysis of all genes and transcripts in an organism. The recent completion of the haplotype map of the human genome is expected to accelerate the discovery of genes associated with complex diseases, including allergies (Montpetit and Chagnon 2006). Microarray technology is the most powerful technique in genomics that can be used for identifying novel biomarkers in the diagnosis of allergies through allergen microarrays, for identifying therapeutic targets against allergies and for understanding mechanisms of allergy (Izuhara and Saito 2006). Comparative genomics allows the survey of closely related allergens and epitope orthologues (Bowyer et al. 2006).

Proteomics refers to the analysis of the complete set of proteins or proteome, and is used in the identification of allergens (Akagawa et al. 2007; Chassaigne et al. 2007; Gautam et al. 2007; Petersen et al. 2006; Vassilopoulou et al. 2007), in determining allergens in transgenic and nontransgenic products (Batista et al. 2007), in the identification of allergy-associated proteins (Park et al. 2007a), and in the identification of genes involved in phenotypes that increase one’s risk for developing allergies (Reilly et al. 2007). Two-dimensional gel electrophoresis, isoelectric focusing, immunoblotting, matrix-assisted laser desorption/ionization tandem time-of-flight mass spectrometry are some of the techniques employed in proteomics (González-Buitrago et al. 2007; Kussmann et al. 2006; Zhang et al. 2004). The development of protein microarrays has allowed the profiling of IgE

antibodies in the diagnosis of type-1-related allergic diseases (Harwanegg and Hiller 2006). More than 120 distinct protein families are represented as allergens in the protein database (Chapman et al. 2007).

Bioinformatics involves the integration of computers, software tools, and databases in an effort to address biological questions in the “omics,” including genomics, proteomics, transcriptomics, and metabolomics. Systems biology involves the integration of genomics, proteomics, and bioinformatics information to create a whole-system view of a biological entity (Raj et al. 2007). T-cell epitope mapping falls within the realm of immunomics, a new field that addresses the interface between the host and the allergen, bridging informatics, genomics, proteomics, immunology, and clinical medicine (De Groot 2006). Similar to and yet different from DNA microarray is immunomic microarray technology, the new functional immunomics—a spatially addressable large-scale technology for measurement of specific immunological response. Immunomic data has been successfully used to identify biological markers of allergies and as a tool in systems biology of cellular immune responses by means of immunomic regulatory network models (Braga-Neto and Marques 2006).

### ALLERGIC DISEASES

Allergies affect almost 20% of the population in the developed world and allergies can be life threatening. Individuals may be allergic to a variety of natural or synthetic molecules, such as food, drugs, chemicals, dust, pollen, and metals. Genomic and proteomic methods are powerful techniques for the identification, characterization, and *in vitro* diagnosis of allergies. Proteomic evaluation of milk from different

mammalian species is useful in recommending suitable feeding in cases of cows' milk allergy and also gives new insight into the background to allergic reactions caused by milk proteins (D'Auria et al. 2005; Natale et al. 2004). Sera of patients with shrimp allergy were found to have *Penaeus monodon* (Pen m 2) allergen, a novel cross-reactive Crustacea allergen with arginine kinase activity, and which could be useful in allergy diagnosis and treatment of Crustacea-derived allergic disorders (Yu et al. 2003). Cor a 9 belongs to the 11S globulin seed storage protein family that comprises known food allergens in peanut and soybean (Beyer et al. 2002a). Application of technologies such as proteomics and metabolomics to assess unintended changes, and the development of predictive methods to evaluate allergenicity are some of the avenues to address public concern of genetically modified foods (Schilter and Constable 2002). Sesame allergy is becoming increasingly prevalent probably due to its use in international fast food and bakery products. Detection of conserved IgE-binding epitopes in common food allergens might be a useful tool for predicting cross-reactivity to certain foods (Beyer et al. 2002b). A new isoform that may be relevant for the diagnosis or therapy of cherry allergy shows diverging IgE-binding properties (Reuter et al. 2005).

Ant sting allergy in Australia is predominantly due to the *Myrmecia pilosula* species complex. The venom was found to primarily consist of peptides with molecular weight <10 kDa, six high-molecular-weight proteins between 26 and 90 kDa, an 8-kDa dimer named pilosulin 5, and a variant named pilosulin 4 (Wiese et al. 2006). Screening of dust mite extracts through fluorogenic substrates and peptide nucleic acid-encoded inhibitor library, followed by other proteomic methods, led to the identification of Der p 1 as one of the proteases contributing to dust mite allergic response (Harris et al. 2004). Expressed sequence tagging strategy has facilitated the study of the major species of dust mites associated with allergic diseases, the identification of polymorphic forms of the allergens, the investigation of differential gene expression under various environmental conditions, analysis of protein level expression profiling, understanding the phylogenetic relationships between mites, and isolation of gene products crucial for life processes so that mite control strategies can be more effectively devised (Angus et al. 2004).

XLAAD is an X-linked recessive immunological disorder characterized by multisystem autoimmunity, particularly early-onset type 1 diabetes mellitus, associated with manifestations of severe atopy including eczema, food allergy, and eosinophilic

inflammation (Chatila et al. 2000). Using a positional approach, mutations were detected on Xp11.23 in JM2, a gene that encodes a forkhead domain-containing protein, and skewing of the patient T lymphocytes toward the Th2 phenotype was observed.

## GENE INTERACTIONS AND ALLERGIC RESPONSES

### MOLECULAR BASIS OF ALLERGIES

Gene-environment interactions underlie almost all human diseases, including atopy (allergy) and asthma (Cárdaba et al. 2007; Moffatt and Cookson 1998). Both genetic and environmental factors contribute to TCR-V beta gene expression and the development of a specific T-cell response in allergy (Beyer et al. 1999).

### Genetic Factors

Advances in genome science have facilitated strategies for studying the genetic basis of disease, namely, systematic analysis of gene expression profiles and comprehensive analysis of gene variations, such as polymorphisms (Shiojima and Tsujimoto 2001). Several studies suggest that there is a strong genetic component in the pathogenesis of IgE-mediated diseases, wherein epidemiologic studies have identified a number of genes that carry single-base changes associated with parameters of allergy (Vercelli 2002a). Genetic variation on the regulation of CD14 influences the pathogenesis of allergy. It is the combination of variations in the same gene and/or in genes arrayed along one functional pathway that might eventually lead to dysregulation strong enough to cause disease. Genetic factors have been shown to provide evidence that epithelial surfaces are active in the induction of allergic diseases. A number of genes and chromosomal regions have been identified that consistently show linkage to asthma and its related phenotypes. Known loci modify the strength of the atopic response, and ability to respond to particular allergens. Eczema has been shown to be due to a different set of genetic loci that are shared with other skin diseases such as psoriasis (Cookson 2002). Patients with IgE-mediated food allergy differed from healthy individuals with regard to genotype of the polymorphic enzyme *N*-acetyltransferase 2 (NAT2) in that there was a significant increase in the proportion of homozygous slow acetylators and no homozygous fast acetylators. The risk of development of the allergy was almost threefold greater in slow acetylators than that in healthy subjects, suggesting that the

slow acetylation genotype may be an important factor of individual susceptibility to IgE-mediated food allergy (Gawronska-Szklarz et al. 2001).

### **Environmental Factors**

The environment plays an essential role in determining the functional outcome of genetic variation. Improving existing environmental risk factors to better prevent or treat diseases such as allergies along with applied genomic research for environmentally caused diseases is important because it could help stratify disease risks and differentiate interventions for achieving population health benefits, identify new environmental risk factors for disease, or confirm suspected environmental risk factors, in addition to aid our understanding of disease occurrence at the population level (Khoury et al. 2005).

### **ALLERGIC RESPONSES**

The clinical manifestations of allergy can involve any of the different symptoms, such as anaphylaxis, asthma, rhinitis, eczema, abdominal pain, hives, angioedema, contact dermatitis, and rhinoconjunctivitis. Atopy or allergy refers to IgE-mediated responses to common allergens, and elevated levels of total and specific IgE in the serum. Mendelian (single-gene) disorders, such as Netherton's disease, develop symptoms of atopic disease (hay fever, food allergy, urticaria, and asthma) and high levels of serum IgE due to mutations and polymorphisms (Glu → Lys) in the gene encoding a serine protease inhibitor SPINK5 or LEKTI (Walley et al. 2001). The SPINK5 protein expressed in the outer epidermis and containing 13 protease inhibitor domains provides a polyvalent action against multiple substrates suggesting that allergy may result for the failure to inhibit environmental proteases such as those that arise from allergens. Similarly, many of the epidermal differentiation complex (EDC) genes that encode small proline-rich proteins (SPRPs), S100A calcium-binding proteins, and late envelope proteins (LEPs) on chromosome 1q21 have shown increased expression in atopic dermatitis and psoriasis (Nomura et al. 2003).

### **Asthma**

Microarray analysis has been used to dissect the genomics of asthma. IL-13-induced gene expression changes in airway epithelial cells of asthmatic patients have been studied using microarray analysis with traditional gene linkage and association mapping to identify key pathways or candidate genes

that are involved in the asthma response (Celedon et al. 2007; Zhen et al. 2007). A "cherry-picking" approach and a global "systems biology" approach to analyze microarray experiments have been conducted (Studer and Kaminski 2007) in combination with differential gene expression plus topological characteristics of the interaction network, providing enhanced understanding of allergic responses (Xin et al. 2007). There is compelling evidence on how environmental insults can result in DNA methylation and epigenetic phenomenon, a process that may be involved in predisposing susceptible humans to asthma (Hu et al. 2005). Polymorphism in the CD14 gene that encodes a receptor for bacterial lipopolysaccharide is also associated with asthma, perhaps providing some of the structural explanation for the hygiene hypothesis. Other environmental genes include intracellular pattern recognition receptors NOD1 and NOD2.

Eleven full genome screens have been reported for asthma, and regions of linkage have been identified. Asthma consistently shows linkage to the major histocompatibility complex (MHC), and also overlaps with loci for other diseases, such as ankylosing spondylitis on chromosomes 1p31–36, 7p13, and 16q23; type 1 diabetes on 1p32–34, 11q13, and 16q22–24; and multiple sclerosis or rheumatoid arthritis on 17q22–24 (Cookson 2002). This suggests that susceptibility to different diseases arising from these loci is influenced by individual genes in various forms (alleles), or disease susceptibility may be modified by physical clusters of genes that have a variety of effects on immune responses. It is of interest that other genes such as DPP10 (Allen et al. 2003), orphan G protein-coupled receptor for asthma susceptibility (GPRA) (Laitinen et al. 2004), PHF11 (Zhang et al. 2003), and ADAM33 (van Erdewegh et al. 2002) underlying asthma have been identified. ADAM33 expressed in bronchial smooth muscle is thought to alter the hypertrophic response of bronchial smooth muscle to inflammation (a component of a process called airway remodeling). PHF11 encodes a nuclear receptor that is part of a complex containing a histone methyl transferase (SETDB2), a regulator of HDAC (RCBTB1), and a nuclear transport molecule (karyopherin  $\alpha$ 3). DPP10 encodes a prolyl dipeptidase, which may remove the terminal two peptides from certain inflammatory chemokines. The plasma protein patterns of pregnant women with and without asthma were found to be different using surface-enhanced laser desorption/ionization time-of-flight mass spectrometry, which has the potential as a tool of monitoring disease progression in situations such as pregnancy (Murphy et al. 2006).

In a mouse model of allergic asthma, among 15 proteins that have been identified by proteomic analysis, 9 have been linked to asthma-related symptoms, oxidation, or tissue remodeling, suggesting that these proteins may prove useful as surrogate biomarkers for quantitatively monitoring disease state progression or response to therapy (Jeong et al. 2005). The combination of genomics, proteomics, and focused transgenic models provides a powerful approach for analyzing the contributions of specific mediators and cell types and for focusing on a limited number of genes associated with specific pathophysiologic aspects of asthma. One such study was the IL-13 Epi (IL-13 overexpressing transgenic mice with STAT-6 expression limited to epithelial cells) focused transgenic mouse, which isolates the effects of a single mediator, IL-13, on a single cell type, the airway epithelial cell. The mice develop airway hyperreactivity and mucus overproduction but not airway inflammation (Kuperman et al. 2005). Glucocorticoids have been shown to inhibit the expression of cytokines IFN- $\beta$  and GM-CSF and chemokines, such as RANTES and IL-8. Functional analysis indicated that the selective effects of glucocorticoids are mediated through activation of the transcription factor C/EBP (Zhang et al. 2007).

### **Hay Fever**

Hay fever is determined by an interaction of environmental and genetic factors and biologically characterized by an imbalanced Th1 and Th2 immune response and elevated IgE levels against inhalant allergens. Genotypes were determined for 15 polymorphisms in 13 genes of subjects with hay fever. The data suggested an association of genetic variants in IL-6 and IL-2 with hay fever, confirming a role of polymorphisms in IL-4R, IL-13, and IL-18 for the elevated IgE phenotype (Nieters et al. 2004).

### **Allergic Rhinitis**

Comparative proteomics has been demonstrated to identify protein changes associated with allergic rhinitis and for revealing posttranslational modifications of new potential markers of allergic inflammation. During the pollen allergy season, six sialylated isoforms of palate lung nasal epithelial clone (PLUNC) and six isoforms of von Ebner's gland protein (VEGP), including an *N*-linked glycosylation form and a cystatin S form, were at lower levels in patients than in controls. In contrast, the levels of an acidic form of alpha-1-antitrypsin were higher in patients, and eosinophil lysophospholipase (Charcot-

Leyden crystal protein/galactin 10) was found in all patients but not in the controls (Ghafouri et al. 2006). In a different study by Nakamura et al. (2004), anti-beta tubV autoantibodies were detected in 52% of the tested patients with allergic rhinitis.

### **Atopic Dermatitis**

Significant linkage of atopic dermatitis (AD) genes has been identified. These regions do not overlap with asthma linkage regions, but are closely coincident with psoriasis susceptibility loci (Bowcock and Cookson 2004), suggesting that particular genes or families of genes have general effects on immune reactions in the skin. The first genome-wide link of a study of atopic dermatitis in an Asian population identified novel loci on chromosomes 15q21 and 1q24 linked to atopic dermatitis. The study used high-density single nucleotide polymorphism (SNP) genotyping assay and the Illumina BeadArray linkage mapping panel (Enomoto et al. 2007). Altered protein expression in primary cultured fibroblasts from atopic dermatitis patients was observed using 2-D gel image analysis and real-time (RT)-PCR (Park et al. 2006). Acetaldehyde dehydrogenase 1 can be a dermal biomarker for atopic dermatitis (Park et al. 2007a,b).

### **Experimental Allergic Encephalomyelitis**

Experimental allergic encephalomyelitis (EAE) is a prototypic T-cell-mediated autoimmune disease associated with allergy. Immunodominant and nonencephalitogenic myelin basic proteins competed with an encephalitogenic peptide in an in vitro T-cell response restricted by MHC class II products, and prevented the development of EAE (Sakai et al. 1989).

### **Allergic Inflammation**

Platelet-activating factor (PAF) is a potent lipid mediator of allergic inflammation through its interaction with eosinophils (Izumi et al. 1995). Using cDNA cloning, the PAF receptor expressed in eosinophilic leukemia (EoL-1) cells was identified as Transcript 1, one of the two transcripts that were previously reported from human genomic analysis. The differentiation of the EoL-1 cell line by sodium *n*-butyrate is associated with the expression of the PAF receptor. Using a proteomics approach, matrix metalloproteinases were shown to promote egression of lung inflammatory cells through the airway, thus providing a molecular mechanism to explain enhanced

clearance and prevent lethal asphyxiation (Greenlee et al. 2006).

### Atopic Eczema

Atopic eczema is a chronic inflammatory allergic skin disorder with an increasing prevalence in industrialized countries. Several hundred new genes and partial DNA sequences have been identified by microarrays. Transcriptome analysis using skin lesion, CD4+ T cells, monocytes, and eosinophils derived from atopic eczema patients identified some differentially expressed genes (Saito 2005).

## GENES FOR SUSCEPTIBILITY TO ALLERGIES

The search for genes for susceptibility to allergies is motivated by the fact that identification of genes will lead to design of new anti-inflammatory compounds (Toda and Ono 2002). Molecules such as cytokines, cytokine receptors, chemokines, chemokine receptors, MHC molecules, and transcription factors could provide candidate genes of allergy. However, identification of susceptibility genes is not straightforward due to participation of various molecules in distinct phases of the allergic reaction, multiple environmental factors, and multiple clinical phenotypes (asthma, allergic rhinitis, atopic dermatitis, allergic conjunctivitis). Genome-wide screening and candidate gene approach have been used to identify susceptibility genes. In genome-wide screening, the presumptive location (using highly polymorphic microsatellite repeats with known chromosomal locations) of the target gene is defined, and then the target gene is sequenced to determine the polymorphisms contributing to disease susceptibility. According to the Collaborative Study on the Genetics of Asthma (CSGA), the human chromosomes 5, 6, 11, 12, and 14 have been implicated in asthma and bronchial hyperresponsiveness (CGSA 1997). In the candidate gene approach, the pathogenesis of allergy is analyzed first and then the protein potentially involved in the development of the disease is identified. The gene for the protein is sequenced and controls are found in order to investigate the relationship between DNA mutations and the disease or trait. Cookson et al. (1989) showed that there is striking linkage between chromosome 11q and IgE response underlying asthma and rhinitis and named the allergic susceptibility gene an “atopy gene.” Atopy is defined as a general predisposition to develop an allergic reaction to an innocuous antigen.

## IMMUNOGLOBULINS AND IMMUNOGLOBULIN RECEPTORS

### IgA

Secretory IgA is the predominant antibody secreted by mucosal tissues and plays an important role in survival, gene expression, and effector functions of colocalized eosinophils. Gene expression by microarray along with other techniques showed that soluble secretory IgA without multivalent antigens may regulate survival (cytokine production) and gene expression of eosinophils, while immobilized secretory IgA stimulated degranulation and superoxide release (Bartemes et al. 2005).

### Fc $\epsilon$ R1- $\beta$

Cookson et al. (1989) found that the atopy gene was transmitted through maternal inheritance and was located in 11q13 in proximity to the gene encoding the  $\beta$  subunit of the high-affinity receptor Fc $\epsilon$ R1 expressed on the mast cells and eosinophils. Point mutations in the  $\beta$  subunit of Fc $\epsilon$ R1 have been reported to be linked to asthma in some ethnic populations. Genetic heterogeneity and multiple mechanisms may be responsible in predisposing individuals to develop allergic diseases. Polymorphisms in Fc $\epsilon$ R1- $\beta$  on chromosome 11q13 are associated with asthma, allergy, bronchial hyperresponsiveness, and atopic dermatitis. Fc $\epsilon$ R1- $\beta$  is a high-affinity IgE receptor stabilizing the expression of the receptor on the mast cell surface. Employing exon shuffling, Nissim et al. (1991) expressed chimeric epsilon-heavy-chain genes composed of a mouse (4-hydroxy-3-*itrophenyl*) acetic acid (NP)-binding VH domain, and human C epsilon in which various domains were replaced by their murine counterparts. This enabled them to show that the third domain of IgE was the principal region involved in the interaction with the Fc $\epsilon$ RI.

## CYTOKINES AND CYTOKINE RECEPTORS

Differentiation of naïve T cells into Th2 cells producing a specific pattern of cytokines is tightly controlled and regulated by transcription factors. Hence, downregulation of mRNA levels of a single transcription factor leads to a “knockdown” of several mediators simultaneously (Sel et al. 2006). Using a genomic and functional approach, the molecular mechanisms underlying dendritic cell–T cell interaction during an allergic immune response to grass pollen were shown to involve upregulation of Th2 genes important for homing, adhesion, signaling, and transcription

(Lindstedt et al. 2005), and similar to many types of allergic responses.

The cytokine cluster, including IL-4, IL-5, IL-13, granulocyte macrophage colony-stimulating factor (GM-CSF),  $\beta$  subunit of IL-12, and IL-9 on chromosome 5q31–5q34, influences atopic processes, with IL-4 and IL-13 being strong candidates of allergic susceptibility genes. The coding polymorphisms Arg130 → Gln shows the strongest effect (Graves et al. 2000). In addition, the T-cell and airway phenotype regulator (Tapr) has been identified as an allergy/asthma susceptibility locus on 5q33, encodes T-cell membrane proteins (TIMs), and controls the development of airway hyperreactivity and T-cell production of IL-4 and IL-13.

#### IL-4

Linkage between the markers within the IL-4 gene and elevated IgE concentrations were first detected in Amish families in 1994. Several polymorphic nucleotides in the IL-4 promoter region were found to affect the affinity of transcription factors for their cognate *cis*-elements, thus mediating the overexpression of the gene. Variants such as –34C/T, –590C/T, and +33C/T have been reported in allergic individuals, of which –590C/T seems to be an important variant. However, it remains unclear whether it plays a direct role in the allergic condition. Proteomics study to identify IL-4-induced differences during the initial stages of human T helper cell differentiation have shown that IL-4 inhibits caspase activity by regulating expression of several key players in the Fas-induced pathway (Rautajoki et al. 2007). One of the most abundant proteins in the airway surface liquid of asthma allergy was found to be gelsolin released by epithelial cells treated with IL-4. This was possible due to a proteomics approach and suggests that gelsolin might improve the fluidity of airway surface liquid in asthma by breaking down filamentous actin that may be released in large amounts by dying cells during inflammation (Candiano et al. 2005). A proteomics approach to investigate alterations in global protein expression of bronchoalveolar lavage fluid in allergic airway inflammation of BALB/c mice challenged with ovalbumin revealed significantly high levels of proteins, including lungkine (a recently described chemokine), a family of chitinases (Ym1, Ym2, and acidic mammalian chitinase), gob-5 (a protein that mediates mucus secretion), and surfactant protein D (a C-type lectin capable of modulating inflammatory responses). These proteins may be useful as surrogate biomarkers for asthma (Zhou et al. 2005).

#### IL-13

Studies in mice and humans using SNPs show that IL-13 is a central regulator of allergic inflammation (Vercelli 2002b). Functional genomics highlight mechanistic pathways that may link genetic variation in IL-13 and the allergy. IL-13 shares a receptor component and signaling pathway with IL-4, and meets biological effects similar to IL-4 in IgE production and IgE-based mucosal inflammation. It also induces the pathophysiological features of asthma through the stimulation of bronchial epithelial mucus secretion and smooth muscle hyperreactivity in an IL-4-independent manner. Among the several multiple promoter variants and coding region variants, Gln110Arg variant seems to be important.

#### IL-4R $\alpha$

IL-4R $\alpha$  is a shared component of the receptor for both IL-4 and IL-13 and is also associated with asthma and atopy. Splicing of IL-4R $\alpha$  is associated with different asthma-associated traits. The IL-4R is composed of  $\alpha$ - and  $\beta$ -chains. The gene encoding the  $\alpha$ -chain is localized on chromosome 16p11–12, which is a site associated with asthma and is found in high IgE levels in ethnically different populations. Upon stimulation of IL-4 or IL-13, Janus tyrosine kinase-1 and 3 are activated followed by activation of the signal transducer and activator of transcription 6 (STAT-6) that has a central role in IgE production. Chatila et al. (2000) identified a variant Arg576Glu in an American and a Japanese population with allergic inflammatory disorder. Functional analysis indicated that the variant induced higher expression of CD23 and impaired the binding of the negative regulator protein tyrosine phosphatase SHIP1 by human IL-4 stimulation. However, the variant did not induce a significant effect on IL-4 signal transduction, such as STAT-6 activation. Linkage of the variant with allergy is controversial. Several variants in different populations have been identified, suggesting that each mutant in the gene of IL-4 receptor  $\alpha$ -chain affects IL-4 signaling and IgE production through different mechanisms and that multiple mutations in one gene may change the structure of the receptor leading to altered signal transduction.

#### IL-5

IL-5 is a key mediator of eosinophilic inflammation and its role in allergic airway inflammatory response is widely demonstrated in guinea pigs. Genomic and proteomic studies reveal that guinea pig recombinant

IL-5 binds to guinea pig IL-5 receptor with high affinity, similar to that seen with the human ligand–receptor pair. However, guinea pig IL-5 receptor and human IL-5 receptor do not distinguish between guinea pig, human, and mouse IL-5 orthologs, whereas mouse IL-5 receptor has restricted specificity for its cognate ligand (Scott et al. 2000).

### TGF- $\beta$

RT-PCR analysis of TGF- $\beta$  along with other techniques has shown that TGF- $\beta$  is biologically active in the intestinal mucosa and enhances oral tolerance, suggesting that TGF- $\beta$  might serve as a potential strategy to prevent food allergy (Ando et al. 2007). Intragastric administration of ovalbumin (OVA)-secreting *Lactococcus lactis* led to active delivery of OVA at the mucosa and suppression of local and systemic OVA-specific T-cell responses in OVA T-cell receptor transgenic mice. The suppression was mediated by induction of CD4+CD25+ regulatory T cells that function through a TGF- $\beta$ -dependent mechanism. Foxp3 and CTLA-4 were upregulated while there was a decrease in IFN- $\gamma$  and increase in IL-10 production (Huibregtse et al. 2007). TGF- $\beta$  is known to promote Foxp3 expression (Fantini et al. 2004; Marie et al. 2005) and inhibit the Th1-driving transcription factor T-bet (Gorelik et al. 2002) and the Th2-driving transcription factor GATA-3 (Gorelik et al. 2000; Heath et al. 2000).

Correlation between four genes IL-4, IL-4 receptor, Fc $\epsilon$ R1- $\beta$ , and STAT-6 in connection with IgE production, the role of IL-10 as regulatory cytokine of allergy, and the severity of food allergy and atopic eczema in 220 Japanese children were studied along with environmental factors such as patients' attitude and indoor environment. Results showed that the combination of SNP, data, and environmental factors appeared to determine severity (Negoro et al. 2006).

### CHEMOKINES AND CHEMOKINE RECEPTORS

Platelets are known to contain several factors, including functional chemokine receptors CCR1, CCR3, CCR4, and CXCR4, as shown by genomics and proteomics methods (Clemetson et al. 2000). CC chemokines participate in the activation and recruitment of granulocytes, monocytes, and T-cell subsets, and play a crucial role in allergic inflammation. The cluster is located on chromosome 17p12–17p11.2. RANTES is one of the most extensively studied CC chemokines in allergic disease, and attracts Th2 cells via chemokine receptors CCR1, CCR3, and CCR5.

A functional mutation in the proximal promoter of the RANTES gene, A401G, creates a near consensus-binding site for the GATA transcription factor family. This promoter polymorphism of RANTES gene was associated with atopic dermatitis or skin test positivity but was not associated with asthma and IgE levels in a German group, while another promoter polymorphism of the RANTES gene, A403G, was also found in Caucasians and associated with an increased susceptibility to asthma and atopy characterized by high serum IgE levels and skin test positivity. Functional analysis using transfectants showed significantly higher transcriptional activity of the mutant promoter with A401G, although biological function of the variant remains unknown. More recently, a promoter polymorphism of MCP1 (monocyte chemoattractant protein-1) was found in Hungarian children with asthma.

A positional candidate gene approach in identifying CC chemokine gene polymorphisms and their functional correlates was used to search for genetic factors contributing to susceptibility to atopy and asthma. An SNP was found in the RANTES proximal promoter region, and a high degree of sequence variation was identified in the 3'-untranslated region of the eotaxin gene. Using functional genomics, differentially expressed genes were identified in a panel of allergen-specific human Th2 cells and antigen-induced hyperreactive murine airways (Nickel et al. 1999). Eosinophils are attracted to sites of allergic inflammation by a number of chemoattractants, including the chemokine, eotaxin 1 that is secreted from epithelial cells and fibroblasts after IL-4 and TNF- $\alpha$  stimulation in a synergistic fashion through a STAT-6-mediated pathway (Hoeck and Woisetschläger 2001).

A gene cluster of CC chemokine receptors is located on chromosome 3p21–24. Multiple variants of CCR receptor, especially CCR5, have been found in human immunodeficiency virus-infected patients and normal subjects. One of the variants of CCR5, the CCR-delta 32-deletion polymorphism in allergic patients, was not found to have significant linkage to atopy, although functional evidence suggests that CCR5 is a good candidate gene of allergy. Multiple CCR5 such as CCR1, CCR3, and CCR5 are involved in the activation and recruitment of inflammatory cells in the late-phase reaction.

### MAJOR HISTOCOMPATIBILITY COMPLEX MOLECULES

The MHC is the longest studied locus influencing atopy. The human leukocyte antigen (HLA)-DR

alleles restrict the IgE response to particular allergens. The MHC class II-associated TNF-308 promoter SNP shows robust associations with asthma, independently of association to particular allergens (Moffatt and Cookson 1997). TCR- $\alpha/\delta$  but not TCR- $\beta$  is linked to the ability to react to particular allergens, and HLA-DR and TCR- $\alpha/\delta$  alleles interact in susceptibility to house dust mite allergens.

The MHC comprises a family of highly polymorphic genes encoding a set of transmembrane proteins that present peptide epitope to a specific antigen receptor on T cells. The MHC-encoded protein polymorphism defines the repertoire of an antigenic determinant of which each of us is capable of responding. Hence, human leukocyte antigen genes are major contributors to the genetic susceptibility underlying human diseases, not only immune diseases such as allergic disease but also cancer and infectious diseases. Genome-wide searches for an asthma susceptibility gene in ethnically diverse populations have provided evidence for linkage between 6p21.3–23 and asthma or its associated traits. A number of studies have reported the association of HLA class II (HLA-DR DQ or DP) alleles with IgE responsiveness to a number of different allergens, while other studies have shown to have a protective effect on the development of a sensitization to allergen, as that of other allergies. Conflicting results have been attributed to ethnic background, phenotype definition, or HLA-typing methods; a large allergenic contains multiple T-cell epitopes, and T-cell responsiveness and IgE reduction to specific allergen are influenced by the presence and concentration of allergen.

#### TRANSCRIPTION FACTORS

Sequencing of the Foxp3 gene, quantification of Foxp3 mRNA by RT-PCR and protein expression in peripheral blood lymphocytes of allergic patients identified a 1,388-base pair deletion of the Foxp3 gene. The new mutation within an upstream noncoding region of Foxp3 results in (very few CD4+CD25+Foxp3 cells and CD4+CD25+ with little regulatory function) a variant of IPEX syndrome associating severe immunoallergic symptoms (Torgerson et al. 2007). STAT-6, which maps to chromosome 12q in a region previously linked with total serum IgE concentration and atopy in different populations, is a key transcription factor involved in IL-4 and IL-13-mediated biological responses, such as allergies (Tamura et al. 2001; 2003). STAT-6 gene polymorphisms of a Japanese population were genotyped by PCR-FLP and PCR-SSCP. In combination, the dinucleotide repeat polymorphism of the STAT-6

exon 1 and the 2964A variant could be useful markers for predicting allergic diseases. The frequency of a SNP in the STAT-6 3'UTR in nut allergy patients was examined using PCR-RFLP and compared to healthy individuals (Amoli et al. 2002). The G allele was most frequent, and GG homozygosity was associated with increased risk of severe reaction. STAT6 3'UTR polymorphism is associated with susceptibility and severity in nut allergic patients.

Investigation of the promoter region necessary for IL-13 transcription has revealed that direct interaction between activator protein (AP-1) and GATA proteins play an important role in IL-13 transcription in mast cells (Masuda et al. 2004). Alleles of lineage commitment factors or transcription actors that bias T cells to the Th2 phenotype are potential allergy susceptibility genes. Genome-wide analysis for susceptibility genes of allergic conjunctivitis has provided evidence for association between the c-maf locus and this disease. CP2, a factor homologous to *Drosophila* Efl-1, is important for IL-4 proper-driven transcription. The human CP2 gene maps to the susceptibility locus for allergic conjunctivitis. Other transcription factors that may serve as candidate genes are BCL-6, STAT-6, GATA-3, and T-bet. STAT-6 is important for IL-4 and IL-13 signal transduction. The STAT-6 gene is located on chromosome 12q13–14, another site of genetic linkage to asthma. BCL-6 expressed in both B cells and CD4 T cells is known to encode a zinc finger transcriptional repressor and contributes to a hyperimmune Th2 cell response. BCL-6-deficient mice developed multiple organ inflammation characterized by eosinophilia and an elevation of IgE levels. A variant of BCL-6 was associated with atopy. The function in the variant of STAT6 and BCL 6 remain unclear.

T-bet, a transcription factor in Th1 differentiation, transactivates the IFN- $\gamma$  gene in Th1 cells and has the unique ability to redirect fully polarized Th2 cells into Th1 cells. GATA-3, a transcription factor in Th2 differentiation, regulates the expression of IL-5 and IL-13 genes in Th2 cells. While T-bet expression is downregulated in human asthmatic airways, GATA-3 expression is increased. Although variants are not found, these are strong candidates for allergy susceptibility genes.

#### MECHANISMS OF ALLERGIES

##### T-CELL-MEDIATED ALLERGY

Among metal-associated diseases, T-cell-mediated allergy to nickel represents the most common form of human contact hypersensitivity. Of the

22 nickel-interacting proteins that have been identified by proteomics, 9 belong to stress-inducible heat shock proteins or chaperonins suggesting a new function of these molecules in human nickel allergy, linking innate and adaptive immune responses (Heiss et al. 2005).

### Oxidative and Hierarchical Oxidative Stress

Reactive oxygen species and reactive nitrogen species produced by epithelial and inflammatory cells are key mediators of the chronic airway inflammation of asthma. Early downstream catalase inactivation and multiple oxidative reactions leading to nitration of proteins in asthmatic airways following allergen challenge were identified by proteomics (Ghosh et al. 2006). Oxidative stress is the key biological event in causing particulate-matter-induced airway inflammation and airway hyperreactivity due to enhanced IgE production and increased Th2 cytokine production (Nel et al. 1998). When exposed to particulate matter, macrophages and epithelial cells respond by producing reactive oxygen species in various cellular locations. Superoxide production in lung microsomes occurs through the action of NADPH-dependent P450 reductase as well as through damage to the mitochondrial inner membrane along with the induction of proinflammatory and cytotoxic effects and the induction of cytoprotective responses, including the expression of an antioxidant enzyme, heme oxygenase 1 (HO-1), suggesting a hierarchy of oxidative stress effects (Li et al. 2003; Xiao et al. 2003). Proteomics allows the display of oxidative modification of proteins and to look at newly expressed proteins and thus enables the testing of the premise of a three-tiered incremental oxidative stress response. The first and most sensitive responses are the induction of antioxidant HO-1 and phase 2 drug-metabolizing enzyme NADPH quinone-oxidoreductase (NQO1), both being induced by the transcription factor Nrf-2 that operates on the antioxidant response element (ARE) in the promoter of these genes. The second and third tiers include initiation of inflammation and programmed cell death, respectively.

### THERAPIES FOR ALLERGIES

The current population-based, guideline-driven approach of treating allergies needs to be tailored to individual behavior, environment, and genetic makeup in order to improve treatment outcomes (Morrow 2007).

### RECOMBINANT HYPOALLERGENS

The total cost of treatment of hay fever (allergic rhinitis) around the world was valued at over \$8 billion. The current leading treatment is cetirizine HCL anti-histamine, with global sales of \$1.5 billion in 2006. The world's first oral grass allergy vaccine offers a cure rather than just relief to millions of hay fever patients around the world (<http://www.drugresearcher.com/Emerging-targets/The-changing-face-of-allergy-therapeutics>).

### RECOMBINANT VACCINES

Successful targeting of the molecules responsible for allergies and developing therapeutic immunization to prevent allergies can be implemented through recombinant vaccines with viral properties which is a more rational approach to effective, better defined, and safer vaccines (Jennings and Bachmann 2007).

### ANTI-INFLAMMATORY COMPOUNDS

$\beta$ -Adrenergic drugs are the first line of treatment for asthma, and act through the  $\beta$ -adrenergic receptor. A variant within the promoter of the 5-lipoxygenase gene predicts the response of asthmatics to antileukotriene therapy. A polygenic genomics approach in optimizing therapy for individual allergic patients might be achieved by focusing on receptor(s) at which the drug binds directly, signal transduction cascades or downstream proteins and as in the case of asthma, and proteins involved in the relaxation and constriction of the airway smooth muscle (Bhatnagar et al. 2006).

### EXTRACTS

Extracts from dust mites produced under pharmaceutical conditions involving proteomic methods contain all the relevant allergens for desensitization purposes and in vivo diagnosis (Batard et al. 2006).

### GENE EXPRESSION

Functional genomics offers modern therapeutic methods for manipulation of gene expression in allergic diseases. These include antisense oligonucleotides, ribozymes, DNA enzymes, and RNA interference triggered by small interfering RNAs. Anti-mRNA drugs for allergic disorders may be targeted to cell surface receptors (adenosine A1 receptor, high-affinity receptor Fc-epsilon RI-alpha, cytokine receptors), adhesion molecules and ligands (ICAM-1,

VLA-4), ion channels (calcium-dependent chloride channel-1), cytokines and related factors (IL-4, IL-5, IL-13, CF, TNF- $\alpha$ , TGF- $\beta$ 1), intracellular signal transduction molecules (tyrosine-protein kinases Syk, Lyn, Btk; serine/threonine-protein kinases p38 alpha MAPkinase, Raf-1), nonkinase signaling proteins (RasGRP4) and transcription factors involved in Th2 differentiation and allergic inflammation (STAT-6, GATA-3, NF- $\kappa$ B) (Popescu 2005).

## RESOURCES

The Allergen Nomenclature Sub-Committee of the International Union of Immunological Societies (IUIS) maintains a list of “certified” protein allergens (King et al. 1995). Bioinformatics tools are available for prediction of allergenicity, allergen cross-reactivity, and T-cell epitopes, while structural analysis tools help identify structural properties of proteins, such as secondary structure or tertiary structure that may impart allergenicity (Brusic et al. 2003). Multiple bioinformatic tools and applications are used in the study of specific steps in the allergic cascade.

### NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES

The National Institute of Allergy and Infectious Diseases (NIAID) offers advances in technology that allow the analysis of immune responses at multiple levels from intracellular signaling networks, to individual cell behavior, to the functioning of a tissue, organ, and even the whole organism. The large amounts of data collected are organized in a manner that facilitates comprehension of the way in which the immune system works. Quantitative models to predict the behavior of complex biological system and to explain the mechanisms underlying physiological responses to susceptibility or resistance can then be applied to design better therapies or recombinant hypoallergens or vaccines. Proteomics allows the determination of protein number, binding affinities, posttranslational modification, other qualitative and quantitative aspect of protein expression and behavior necessary for computer modeling and simulation, mass spectrometry, and microfluidic-based multiplexed binding assays. Genomics emphasizes on system-wide analysis of transcription factors and epigenetic control of gene expression, effects of allelic polymorphism on gene expression and function, quantitative measurement of gene expression, role of noncoding regions and transcripts such as miRNAs in regulating gene/gene product expression patterns,

high-throughput analysis of gene transcription, transcription factor binding site identification, analysis of epigenetic modifications, and analysis of gene regulatory circuits.

### ALLERGEN DATABASES AND REPOSITORIES

More than a dozen important allergen databases and data repositories have been developed to date. Bioinformatics allows allergen characterization, assessment of allergenicity, and identification of allergic cross-reactivity, which in turn support the development of vaccines and therapies for allergic disease (Brusic 2006). An informal platform for immunologists to exploit “omics” data is underway at RIKEN RCAI (Riken Research Center for Allergy and Immunology). mRNA profiling experiments and 2-D gel-based proteomic analysis of various types of immune cells are carried out to generate transcriptomic and proteomic data for the immunogenomics reference database. Several bioinformatics microarray analysis tools are also mined. RefDIC (Reference genomic Database for Immune Cells) includes (1) the capability to cross-reference from transcriptomic data to proteomic data and vice versa, and (2) functions for data sharing. A microscopic platform for real-time monitoring of biomolecular interactions is being implemented. The World Health Organization (WHO) and the Food and Agriculture Organization (FAO) provide a guideline to identify allergenicity of a novel protein (Li et al. 2004). The guideline states that a query protein is potentially allergenic if it has either an identity of at least six contiguous amino acids or a minimum of 35% sequence similarity over a window of 80 amino acids when compared with known allergens (Aalberse 2000). A support vector machine (SVM)-based tool to detect homology using semisupervised iterative learning (SVM-HUSTLE) significantly outperforms existing methods such as BLAST, PSI-BLAST, COMPASS, PROF.SIM, RANKPROP, and their variants (Shah et al. 2008). A number of immunochemical, biochemical, and immunological methods (IgE immunosorbent assays using patient sera), human skin prick tests, basophil histamine release, and animal models are used to identify potential allergens. Bioinformatics tests offer an expedient method to screen for potential allergens in food products and foods, such as genetically modified foods. A new level of accuracy in computational detection of allergenic proteins using detection based on filtered length-adjusted allergen peptides (DFLAP) (Soeria-Atmadja et al. 2006) or EVALLER (Barrio et al. 2007) successfully discriminates between allergens

and nonallergens, and is best suited in detecting cross-reactive allergens. WebAllergen is a web server that predicts allergenic proteins by evaluating similarities in the underlying physicochemical properties between the query protein and the allergenicity-related protein motifs (Riaz et al. 2005).

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# **Section III**

## **Food Factors–Gene Interactions**

# 7

## Beneficial Effects of Conjugated Linoleic Acid

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### INTRODUCTION

Polyunsaturated fatty acids, including essential fatty acids, found most abundantly in foods, have a methylene base between the carbon double bonds ( $-\text{C}=\text{C}-\text{C}-\text{C}=\text{C}-$ ), with all double bonds having a *cis* configuration. Conjugated fatty acids, on the other hand, have conjugated double bonds ( $-\text{C}=\text{C}-\text{C}=\text{C}-$ ) in multiple positional (8 and 10, 9 and 11, 10 and 12, or 11 and 13) and geometric (*cis* or *trans*) isomers (Figure 7.1). In nature, there are many kinds of conjugated fatty acids, such as conjugated linoleic acid (CLA), conjugated linolenic acid (CLN), and conjugated eicosapentaenoic acid (CEPA). Among them, a well-studied representative conjugated fatty acid is CLA.

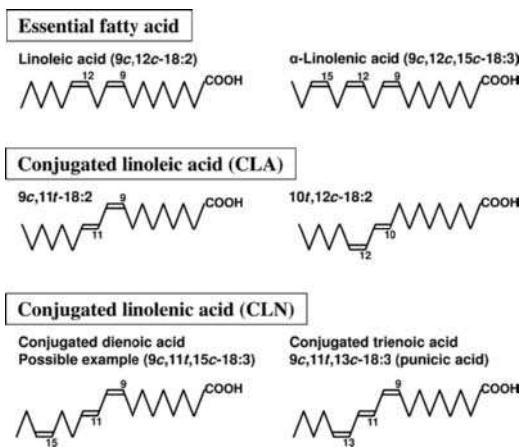
In the 1980s, Pariza and Hargraves (1985) first demonstrated that extracts of grilled ground beef exert antimutagenic activity; subsequently, Ha et al. (1987) found that the responsible antimutagen is CLA. Since then, attention has been drawn to the physiological function of CLA; now, CLA has been shown to exert various physiological functions other than antimutagenicity such as anticarcinogenic and antiobesity (reduction of body fat mass) activities, prevention of atherosclerosis, enhancement of immune function, and suppression of blood pressure (Nagao and Yanagita 2005; Pariza et al. 2001). On the other hand, the number of reports on physiological properties of conjugated fatty acids other than CLA is still limited. The limited evidence, however, indicates potential benefits of these fatty acids such as CLN. In this chapter, we describe the physiological effects of CLA and also briefly describe the potential health benefits of CLN.

### PHYSIOLOGICAL PROPERTIES OF CLA

#### SOURCE AND PREPARATION OF CLA

CLA is generally found in ruminant animals (e.g., cows, sheep, goats, and camels) and dairy products. CLA has been shown to be produced by the ruminant bacteria *Butyrivibrio fibrisolvens* as an intermediate in the process of biohydrogenation of linoleic acid into stearic acid (Chin et al. 1992; Kepler et al. 1966). In this case, 9*c*,11*t*-18:2 is the dominant CLA isomer. The content of CLA in animal meats and dairy products can be dependent on the diet of the ruminant animals; the content is generally higher if those animals are grazing on fresh, green pasture rather than feeding on a grain. However, the content of CLA is very low, in the range of 2–5 mg/g of total fat (Dhiman et al. 1999). Exceptionally, CLA is also found in nonruminant kangaroo meat (Engelke et al. 2004), but the CLA content at most is 8 mg/g of total fat (Fritsche et al. 1999). Therefore, these foods are not likely to be a significant source of CLA at the present time.

For mass production, CLA is synthetically prepared from linoleic acid (e.g., a high linoleic type of safflower oil) by alkaline isomerization (Reaney et al. 1999). The commercially available CLA usually is prepared by this method. This product is mainly a 1:1 ratio of 9*c*,11*t*-18:2 and 10*t*,12*c*-18:2. In the past two decades, many studies used synthetic CLAs that contained these isomers. It has been reported that lipases from *Geotrichum candidum* and *Candida rugosa* selectively esterify the 9*c*,11*t*-18:2 isomer; furthermore, enzymatic methods that use these



**Figure 7.1.** Chemical structure of essential fatty acids and conjugated fatty acids.

lipases to separate the 9c,11t-18:2 and 10t,12c-18:2 isomers are now available (McNeill et al. 1999; Nagao et al. 2002, 2003a). To date, it is known that different CLA isomers exert different health effects (Ip et al. 1999; Li et al. 2006; Pariza et al. 2001). The naturally available 9c,11t-18:2 can exhibit limited functions such as anticarcinogenicity, whereas the other isomer, 10t,12c-18:2, exhibits various functions including antiobesity.

There are some other potential ways to prepare CLA. It can be isomerized from linoleic acid by lactic acid bacteria, such as *Lactobacillus plantarum* (Kishino et al. 2002, 2003). With this method, 9c,11t-18:2 and 9t,11t-18:2 can be selectively produced. CLA can also be prepared by using gene-modification technology. Genes that encode enzymes that convert linoleic acid to 9c,11t-18:2 and 10t,12c-18:2 were cloned from the anaerobic bacteria *L. reuteri* and *Propionibacterium acnes*, respectively (Rosson et al. 2001). Recently, the latter gene was introduced into the rice plant by *Agrobacterium*-mediated transformation, and transgenic rice containing 10t,12c-18:2 in the seeds was successfully harvested (Kohno-Murase et al. 2006). These methods are not necessarily efficient enough for CLA mass production at the present time, and efforts to produce higher level of CLAs are expected. These methods may contribute to the preparation of a specific CLA isomer with maximal physiological functions in the near future.

#### ANTICARCINOGENIC EFFECT

In the 1980s, it was demonstrated that CLA derived from grilled ground beef exerts antimutagenicity (Ha

et al. 1987; Pariza and Hargraves 1985). Since then, the anticarcinogenic activity of CLA has been examined in a chemically induced rat mammary tumor model. CLA has been demonstrated to interfere with the growth of breast and prostate tumors (Ip et al. 1991, 1994). Evidence indicates that CLA inhibits the initiation and incidence of mammary tumors in rodents. In an *in vitro* study, CLA has also been shown to inhibit the growth of various human cancer cell lines (Schonberg and Krokan 1995; Shultz et al. 1992). In other *in vitro* studies using 9c,11t-18:2, 10t,12c-18:2, and a mixture of the isomers, CLA appeared to exert an anticancer activity irrespective of the isomer used (Chujo et al. 2003; Yamasaki et al. 2002). Although few clinical studies that show an anticancer effect for CLA are available, results from a recent study suggest that dietary CLA could protect against breast cancer in humans (Chajes et al. 2003).

One mechanism by which CLA exerts anticarcinogenesis could be dependent on activation of peroxisome proliferator-activated receptors (PPARs). CLA was reported to act as an activator of PPAR $\alpha$  (Moya-Camarena et al. 1999a, b) and PPAR $\gamma$  (Houseknecht et al. 1998). It was shown that ligand activation of PPAR $\gamma$  induced apoptosis and inhibited proliferation of prostate (Kubota et al. 1998), breast (Mueller et al. 1998), colon (Sarraf et al. 1999), and gastric cancer cells (Takahashi et al. 1999) *in vitro* and *in vivo*. In addition, activators of PPAR $\alpha$  and PPAR $\gamma$  can also inhibit activation of nuclear factor (NF- $\kappa$ B and activator protein 1. It was demonstrated that CLA decreased cellular proliferation and inhibited NF- $\kappa$ B and activator protein 1 activation in cancerous prostate epithelial cells (Ohtsu et al. 2005).

#### ANTIOBESITY (WEIGHT MANAGEMENT)

A number of studies demonstrate that CLA exerted antiobesity and hypolipidemic effects in animals, including mice, rats, and pigs (Pariza et al. 2001; Park et al. 1997). It should be noted that the ability of CLA to reduce body fat has also been observed in humans. In light of the evidence observed in *in vivo* and *in vitro* studies, the effects are considered to be attributed to increased lipolysis in adipocytes and enhanced fatty acid  $\beta$ -oxidation in adipocytes and skeletal muscle cells (Pariza et al. 2000; Park et al. 1999). In a different study, CLA was reported to increase fatty acid  $\beta$ -oxidation, whereas it suppressed fatty acid synthesis in the liver (Rahman et al. 2001). In addition, CLA was found to enhance fatty acid  $\beta$ -oxidation in muscle and brown adipose tissues, as well as oxygen consumption and energy expenditure in obese rats (Nagao et al. 2003b; Rahman et al. 2001).

The antiobesity effect of CLA could also be due to activation of PPARs. It has been demonstrated that CLA is a potent ligand and activator of PPAR $\alpha$  (Moya-Camarena et al. 1999a). CLA can induce PPAR $\alpha$ -regulated lipolytic genes, including carnitine palmitoyl transferase, acyl coenzyme A (CoA) oxidase, and uncoupling of protein in liver, muscle, and brown adipose tissue, where  $\beta$ -oxidation of fatty acids occurs (Inoue et al. 2006; Rahman et al. 2001; Wang et al. 2006). Therefore, PPAR $\alpha$  seems to be involved in the regulation of lipolytic gene expression by dietary CLA. However, dietary CLA was reported to induce these lipolytic genes even in PPAR $\alpha$ -null mice (Peters et al. 2001), suggesting that some mediator mechanisms other than PPAR $\alpha$ , such as PPAR $\beta/\delta$ , could also exist (Khan and Vanden Heuvel 2003; Moya-Camarena et al. 1999b). On the other hand, it was demonstrated that dietary CLA induced apoptosis in mouse adipose tissues, concomitant with downregulation of PPAR $\gamma$  (Tsuboyama-Kasaoka et al. 2000).

The antiobesity and hypolipidemic effects of CLA have been attributed to the 10 $t$ ,12 $c$ -18:2 rather than the 9 $c$ ,11 $t$ -18:2 isomer in an obese rat model (Nagao et al. 2003b; Park et al. 1999; Wang et al. 2006). The mechanism underlying transcriptional regulation of lipogenic genes by CLA isomers seems to be complex and is still not clear. It was reported that dietary 10 $t$ ,12 $c$ -18:2, but not 9 $c$ ,11 $t$ -18:2, lowered the concentration of hepatic triglycerides and cholesterol, concomitant with reduction of hepatic levels of fatty acid synthase and expression of messenger RNAs (mRNAs) of sterol regulatory-element binding protein 1 (SREBP1) in obese Otsuka Long-Evans Tokushima fatty (OLETF) rats (Wang et al. 2006). Also, the 10 $t$ ,12 $c$ -18:2 isomer was reported to decrease the expression of stearoyl-CoA desaturase (SCD) mRNA in mouse liver and adipocytes (Choi et al. 2000; Lee et al. 1998). SCD is a key enzyme in lipogenesis, and inhibition of this enzyme activity is considered to depress fat synthesis. In another study, 9 $c$ ,11 $t$ -18:2 reduced hepatic SREBP1c expression, whereas it increased adipose tissue SREBP1c and improved abnormalities of lipid and glucose metabolism in obese *ob/ob* mice (Roche et al. 2002). In addition, dietary CLA was reported to induce hepatic lipogenic genes, such as FAS, acyl-CoA carboxylase, and SCD, through upregulation of hepatic SREBP1c during the development of lipodystrophy in C57BL/6 mice (Takahashi et al. 2003).

In clinical studies, dietary supplementation with CLA was reported to reduce body fat, particularly abdominal fat, to change serum total lipids and to decrease whole-body glucose uptake (Blankson et

al. 2000; Smedman and Vessby 2001; Thom et al. 2001). In humans, however, the effects are not always unequivocal, and CLA had no effect on body composition in many cases (Atkinson 1999; Zambell et al. 2000), which is not the same trend observed in animal studies. The unequitable results in the clinical study could depend on many factors, such as dosage, duration of CLA exposure, and degree of obesity. Blankson et al. (2000) examined the relationship between dose level of CLA and body fat mass in obese and overweight subjects; they concluded that dietary supplementation with CLA (75% purity; mixture of 1:1 ratio of 9 $c$ ,11 $t$ - and 10 $t$ ,12 $c$ -18:2) at 3.4 g/day for 3 months was required to reduce body fat in obese and overweight subjects. It was also observed that CLA supplementation (3.4 g/day in triglyceride form) for 1 year resulted in a significant decrease in body fat mass in healthy overweight humans (Gaullier et al. 2004).

There is a known, wide, animal species-specific difference in the magnitude of CLA's ability to reduce body fat. Dietary CLA exhibits a prominent response in mice, whereas a much lower response is seen in humans. The effect in rats is intermediate between mice and humans. In mice, therefore, it was reported that dietary supplementation with CLA extremely decreased the weight of adipose tissue and caused lipodystrophy (Tsuboyama-Kasaoka et al. 2000). Lipodystrophy resulted in the lack of adipocytokines such as leptin and adiponectin, causing insulin resistance. Although humans are a less-sensitive species to the effects of dietary CLA, it was reported that treatment with dietary 10 $t$ ,12 $c$ -18:2 at 3.4 g/day for 3 months caused isomer-specific insulin resistance in obese men (Riserus et al. 2002).

An appropriate approach to increase the potential benefits of CLA without increasing the dosage seems significant in avoiding the undesirable effects of CLA. One such potential approach is the combination of CLA and other dietary components, which can stimulate fatty acid  $\beta$ -oxidation. It has been reported that CLA's potential for reducing body fat could be enhanced by combining it with soybean protein, sesamin, and chromium picolinate (Akahoshi et al. 2004, 2005; Proctor et al. 2007; Sakono et al. 2002; Sugano et al., 2001). It has also been reported that supplementation of CLA with docoshexaenoic acid alleviates the fatty liver induced by excess amounts of CLA in C57BL/6N mice (Yanagita et al. 2005).

#### ANTIDIABETIC EFFECT (TYPE II DIABETES)

There is evidence that CLA may have the ability to normalize glucose metabolism. In studies with

obese, diabetic rats, a 1.5% CLA diet normalized impaired glucose tolerance in 2 weeks; the effect of CLA was similar to that of the pharmaceutical agent troglitazone (Houseknecht et al. 1998). Because CLA has been reported to act as an agonist of PPAR $\gamma$ , the antidiabetic effect of CLA could be attributable to activation of PPAR $\gamma$ , which is similar to the effect of troglitazone (Houseknecht et al. 1998). Activation of PPAR $\gamma$  could increase plasma concentration of adiponectin and alleviate hyperinsulinemia (Nagao et al. 2003c). It has been demonstrated that adiponectin stimulates phosphorylation and activation of 5'-AMP-activated protein kinase (AMPK) in skeletal muscle (Yamauchi et al. 2002) and that activated AMPK caused GLUT4 translocation and increased glucose uptake in skeletal muscle (Kurth-Kraczek et al. 1999). In another study, it was suggested that the antidiabetic effects of CLA are attributable to the specific action of 10 $t$ ,12 $c$ -18:2 (Ryder et al. 2001). In a human study, diabetic subjects took CLA (9 $c$ ,11 $t$ -18:2 (37%) and 10 $t$ ,12 $c$ -18:2 (39%)) or safflower oil at 8.0 g/day for 8 weeks; results showed that plasma levels of 10 $t$ ,12 $c$ -18:2, but not 9 $c$ ,11 $t$ -18:2, were inversely associated with changes in body weight and serum leptin levels (Belury et al. 2003). This finding strongly suggests that the 10 $t$ ,12 $c$ -18:2 isomer could be the bioactive isomer of CLA that influences the body weight changes observed in subjects with type 2 diabetes. This isomer's antidiabetic effect combined with its effect in reducing body fat makes 10 $t$ ,12 $c$ -18:2 an attractive candidate for a possible role in weight management in humans. However, there is also growing evidence that CLA may worsen blood glucose control. In overweight people without diabetes, CLA might promote insulin resistance and thus decrease insulin sensitivity, creating a prediabetic state (Larsen et al. 2003; Moloney et al. 2004; Riserus et al. 2002, 2004).

#### ANTIATHEROGENESITY

Antiatherogenic effects of CLA have been observed in rabbits and hamsters. In these studies, the area of an aortic fatty streak was reduced in rabbits fed with 0.5 g/day of CLA for 22 weeks (Lee et al. 1994) and in hamsters fed with a 1% CLA diet for 12 weeks (Nicolosi et al. 1997). One major risk factor for coronary heart disease is a high level of blood cholesterol, particularly low-density lipoprotein (LDL) cholesterol. In rabbits, dietary CLA was found to reduce LDL, total cholesterol, and triglyceride levels in the blood (Kritchevsky et al. 2000).

In rats, moreover, dietary CLA decreased cholesterol concentration in the liver (Sakono et al. 1999). Among different CLA isomers, 10 $t$ ,12 $c$ -18:2 has been shown to be more effective in lowering the serum concentration of total cholesterol and triglyceride, whereas it raises the concentration of high-density lipoprotein (HDL) cholesterol (Akahoshi et al. 2003). In addition, both isomers of CLA (9 $c$ ,11 $t$ -18:2 and 10 $t$ ,12 $c$ -18:2) have been shown to inhibit platelet aggregation and the formation of proaggregatory cyclooxygenase product, TXA<sub>2</sub> (Truitt et al. 1999). These findings suggest that CLA, especially the 10 $t$ ,12 $c$ -18:2 isomer, can lower serum lipoproteins and reduce early aortic atherosclerosis in experimental animals. In humans, a small double-blind trial has found that CLA can reduce blood cholesterol levels in healthy human subjects (Noone et al. 2002). Although the mechanism underlying CLA's antiatherogenic effect is not clear, the effect could occur through enhancement of the level of adiponectin in circulation. Adiponectin in circulation could inhibit the expression of macrophage scavenger receptors, which play a major role in lipid accumulation, and the formation of foam cells of macrophages by taking up oxidized LDL (Ouchi et al. 2001). Also, adiponectin was reported to prevent apoptosis of endothelial cells in which apoptosis could be accelerated in atherosclerosis-prone regions (Kobayashi et al. 2004). Moreover, adiponectin was reported to increase anti-inflammatory interleukin-10 and subsequently increase tissue inhibitor of metalloproteinase 1 in human monocyte-derived macrophages, an effect that is considered to be crucial for antiatherogenicity (Kumada et al. 2004).

#### IMMUNE FUNCTION

CLA can enhance the immune system. Feeding CLA to chicks provided partial protection against the catabolic effects of endotoxin (Cook et al. 1993). Feeding of a 1% CLA diet improved the age-related decrease in the ability of T cells to proliferate in mice when the immune system was challenged by an antigen or polyclonal T-cell mitogens (Hayek et al. 1999). In addition, in another study, feeding of a 1% CLA diet significantly increased the splenic levels of immunoglobulin A (IgA), IgG, and IgM, whereas it significantly decreased those of IgE (Sugano et al. 1997). In yet another study, a significant increase in the IgA, IgG, and IgM productivities of rat spleen lymphocytes was observed at 0.05% CLA supplementation (Yamasaki et al. 2000).

### LINOLEIC ACID METABOLISM AND EICOSANOID SYNTHESIS

CLA is known to affect metabolism of fatty acids and eicosanoid. Because both CLA and linoleic acid are considered to share the same enzyme system for desaturation and chain elongation, increased dietary intake of CLA may interfere with metabolism of linoleic acid to arachidonic acid (Sebedio et al. 1997; Sugano et al. 1999). An *in vitro* study that used a transformed yeast system demonstrated that CLA inhibited linoleic acid metabolic enzymes such as  $\Delta 6$ -desaturase,  $\Delta 5$ -desaturase, and elongase (Chuang et al. 2001a, b, 2004). It has also been demonstrated that  $10t,12c$ -18:2 suppresses the  $\Delta 6$ -desaturase in HepG2 cells (Eder et al. 2002). In addition to competitive inhibition, CLA may affect linoleic acid metabolism through transcriptional regulation. Results from animal feeding studies indicate that CLA can reduce hepatic expression of SREBP1c (Roche et al. 2002; Wang et al. 2006). SREBP1c has been shown to regulate  $\Delta 6$ -desaturase gene expression (Nakamura and Nara 2003).

CLA is also considered to interfere with eicosanoid synthesis. Arachidonic acid metabolized from linoleic acid is incorporated into membrane phospholipids. CLA can also be incorporated into membrane phospholipids; therefore, replacement of arachidonic acid in the cell membrane with CLA interferes with eicosanoid synthesis, because CLA reduces the availability of arachidonic acid for production of eicosanoids such as PGE2 and LTB4 (Liu and Belury 1998; Sugano et al. 1998). Modulation of eicosanoid production by CLA is possibly involved in the spectrum of biological responses of CLA, such as carcinogenesis, immune functions, and atherogenesis (Banni et al. 1999) as well as bone metabolism (Li and Watkins 1998; Li et al. 1999).

### ANTIHYPERTENSIVE PROPERTY

Recently, the antihypertensive properties of CLA have also been reported (Yanagita et al. 2006). A diet of a synthetic CLA mixture and the  $10t,12c$ -18:2 isomer was observed to prevent the development of obesity-induced hypertension in obese, diabetic Zucker rats and obese OLETF rats (Nagao et al. 2003c, d). Feeding of CLA was also observed to prevent the development of essential hypertension in nonobese spontaneously hypertensive rats (Inoue et al. 2004). This observation could be due to the property of CLA to modulate the production of physiologically active adipocytokines, such as adiponectin, leptin, and angiotensinogen (Inoue et al. 2004; Nagao et al. 2003c, d).

### POTENTIAL HEALTH BENEFITS OF CONJUGATED FATTY ACIDS OTHER THAN CLA

#### SOURCE

In nature, at least five CLN isomers have been found in plant seeds:  $\alpha$ -eleostearic acid ( $9c,11t,13t$ -18:3), punicic acid ( $9c,11t,13c$ -18:3), catalpic acid ( $9t,11t,13c$ -18:3), calendic acid ( $8t,10t,12c$ -18:3), and jacaric acid ( $8c,10t,12c$ -18:3) (Kohno et al. 2002; Suzuki et al. 2001). All these CLN isomers contain two conjugated double bonds; therefore, they are conjugated trienoic acid (Figure 7.1). In many cases, the CLN in plant seeds consists of a single isomer.  $\alpha$ -Eleostearic acid is found in bitter gourd (*Momordica charantia*) and tung (*Aleurites fordii*) seeds at the level of 40–60% and approximately 70%, respectively, of the total fatty acids in their seed oils. Punicic acid is found in pomegranate (*Punica granatum*) and *Trichosanthes kirilowii* seeds at the level of 70–80% and approximately 40%, respectively. Catalpic acid is found in catalpa (*Catalpa ovata*) seeds at the level of 30–40%. Calendic acid and jacaric acid are found in pot marigold (*Calendula officinalis*) and jacaranda (*Jacaranda mimosifolia*) seeds, respectively, at the level of 30–40% for each. Therefore, these plant seed oils are suitable sources to investigate physiological functions of individual CLN isomers. Investigators have also found that red seaweed, *Ptilota filicina*, contains CEPA ( $5t,7t,9t,14c,17c$ -20:5 and  $5c,7t,9t,14c,17c$ -20:5) (Lopez and Gerwick 1987) and that *Bossiella orbigniana* contains  $5c,8c,10t,12t,14c$ -20:5 (Burgess et al. 1991). Green seaweed, *Anadyomene stellata*, contains  $4c,7c,9t,11t,13c,16c,19c$ -20:7 (Mikhailova et al. 1995).

CLN, CEPA, and conjugated docosahexaenoic acid (CDHA), like CLA, are reported to be prepared by alkaline isomerization (Koba et al. 2002; Tsuzuki et al. 2005, 2006b), but the synthetic product contains a number of isomers. In the case of CLN, the synthetic CLN product contains many isomers that consist of conjugated dienoic and trienoic acids (Figure 7.1). Recently, genetically modified rapeseed oil that contained punicic acid was also produced on an experimental basis (Koba et al. 2007).

#### ANTICARCINOGENIC EFFECT

Evidence shows that synthetic CLN derived from perilla oil rich in  $\alpha$ -linolenic acids delayed the development of mammary tumors induced by a chemical carcinogen in rats (Futakuchi et al. 2002). In

in this case, CLN is considered to inhibit formation of the carcinogenic chemical DNA adduct. In an *in vitro* study, synthetic CLN exhibited a cytotoxic effect on various human tumor cells (Igarashi and Miyazawa 2000a). Interestingly, the cytotoxic effect was more evident in the synthetic CLN product that contained more conjugated trienoic acids than dienoic acids. In another *in vitro* study, synthetic CEPA and CDHA that contained a conjugated triene structure also exhibited cytotoxic actions in several cancer cell lines, such as colorectal, hepatoma, lung, breast, and stomach (Igarashi and Miyazawa 2000b). Cytotoxicity of naturally available plant seed oils that contain conjugated trienoic acids was also examined in mouse tumors and human monocytic leukemia cells (Suzuki et al. 2001). The investigators found that the cytotoxic effect of tung oil (9c,11t,13t-18:3), pomegranate oil (9c,11t,13c-18:3), and catalpa oil (9t,11t,13c-18:3) was much stronger than that of pot marigold oil (8t,10t,12c-18:3), suggesting that the position of the double bond could be an important determinant for the cytotoxicity of CLN. In *in vitro* studies, 9c,11t,13t-18:3, 9c,11t,13c-18:3, and 9t,11t,13c-18:3 were also suggested to exert anticarcinogenic activity in rats (Kohno et al. 2002, 2004; Suzuki et al. 2006). Because both 9c,11t,13t-18:3 and 9c,11t,13c-18:3 are considered to be metabolized to 9c,11t-18:2 (Noguchi et al. 2001; Tsuzuki et al. 2003, 2006a), the effects of these CLNs could be attributed, in part, to the effect of the metabolite CLA. Although the mechanism underlying the effect is not clear, induction of apoptosis, modulation of cytokines, and inhibition of cancer cell proliferation can be involved (Belury 2002a, b); these effects are known to exert efficient anticarcinogenic activity.

#### ANTIOBESITY

Evidence suggests that conjugated fatty acids other than CLA also exert an antioesity effect. Dietary supplementation with synthetic CLN prepared by alkaline isomerization reduced the weight of epididymal and perirenal adipose tissues by enhancing hepatic fatty acid  $\beta$ -oxidation in rats (Koba et al. 2002). In addition, dietary synthetic CEPA and CDHA prepared from eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) were found to suppress fat accumulation in the liver and epididymal adipose tissue by decreasing synthesis of hepatic fatty acids and enhancing  $\beta$ -oxidation in rats (Tsuzuki et al. 2005, 2006b). Interestingly, the magnitude of antioesity effect of these synthetic conjugated fatty acids seemed to be stronger than that of synthetic CLA.

The effect of naturally available individual CLN isomers in reducing body fat was also examined with the use of bitter gourd oil (9c,11t,13t-18:3), pomegranate oil (9c,11t,13c-18:3), catalpa oil (9t,11t,13c-18:3), and pot marigold oil (8t,10t,12c-18:3) (Koba et al. 2006). In this study, the investigators found that dietary punicic acid (9c,11t,13c-18:3) in pomegranate, but not in the other CLNs, exhibited a significant reduction in the weight of perirenal adipose tissue, which was associated with an increase in fatty acid  $\beta$ -oxidation in the liver. This finding suggested that punicic acid (9c,11t,13c-18:3) could be at least one of the factors responsible for reducing the weight of adipose tissue in rats. The punicic acid molecule, rather than its metabolite CLA, is likely responsible for this effect, because both 9c,11t,13t-18:3 in bitter gourd oil and 9c,11t,13c-18:3 in pomegranate oil can be converted to 9c,11t-18:2, but only 9c,11t,13c-18:3 showed a reduction in adipose tissue weight. Recently, genetically transformed rapeseed oil containing punicic acid was prepared on an experimental basis (Koba et al. 2007). Feeding of the transformed oil as well as pomegranate oil decreased the weight of perirenal and epididymal adipose tissue, and the concentration of liver triglycerides in mice. Interestingly, the magnitude of the effects was greater in the transformed oil than in pomegranate oil. The reason for this finding is not clear, but it may be partly due to the difference in triglyceride molecule structure between the two oils; the punicic acid in the transformed oil exclusively occupies the sn-2 position, whereas that in pomegranate oil occupies all positions of the triglyceride molecule (Koba et al. 2007). The punicic acid content of genetically modified rapeseed oil is as low as 2.5% at the present time. Efforts to produce a transformed oil that contains a higher level of punicic acid are expected. Although the technology needs advancing and there are some issues underlying genetically transformed products, such as safety concerns and negative stereotypes for modification techniques, the findings may show the possibility and advantage of gene-modification technology.

#### CONCLUSION

CLA exerts many potential health benefits as described previously. According to the latest information, CLA is a generally safe nutritional substance for humans (Whigham et al. 2004). To date, commercially available synthetic CLA contains mainly 9c,11t-18:2 and 10t,12c-18:2, but the physiological properties of these CLA isomers differ. The mechanism underlying the multiple functions of CLA is still

being investigated. Different isomer-specific CLA products may be provided for more efficient benefits.

Evidence concerning other conjugated fatty acids is also accumulating. CLN is expected to exert anticarcinogenic and antiobesity activities. CLN may also affect immune function (Yamasaki et al. 2006). Although evidence of these effects in humans is lacking, the results observed *in vitro* and in experimental animals are promising. More studies to evaluate the physiological functions of conjugated fatty acids are required. In contrast to CLA, oils rich in CLN are naturally available. Some of these plant seed oils may also be useful as a new type of functional oil.

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# 8

## Regulation of Gene Transcription by Fatty Acids

*Jean-Paul Pégorier*

### OVERVIEW

The last two decades provided evidence that major (glucose, fatty acids, amino acids) or minor (iron, vitamin, etc.) dietary constituents regulated gene expression in a hormonal-independent manner. This chapter focuses on molecular mechanisms by which fatty acids and/or their metabolites control the transcription of genes involved in their own metabolism or in carbohydrate metabolism. These effects are mediated either by direct binding on transcription factors such as PPARs, LXR, HNF-4, and RXR (each belong to nuclear receptor superfamily), or alternatively through modifications in nuclear abundance and/or activity of numerous transcription factors like SREBP-1c, ChREBP, and NF- $\kappa$ B. Knowledge of the mechanisms that govern fatty acid-induced gene expression will provide insight into the role that dietary fat plays in physiology and health, especially in humans.

### INTRODUCTION

The high energetic value (9 kcal/g versus 4 kcal/g for the glucose) coupled with low storage bulk (as anhydrous shape in lipid droplets in white adipose tissue, liver, and muscles) make fatty acids a major source of energy for the organism. Stored fatty acids arise from either the diet or de novo synthesis from dietary glucose (lipogenesis). Dietary fat is an important macronutrient for the growth and development of all organisms. Excessive levels of dietary fat or imbalance in its composition (saturated versus unsaturated fat) have been related to the onset or development of several chronic diseases such as coronary artery disease (Kris-Etherton et al. 2002), obesity and type 2 diabetes (Kelley et al. 2002), or certain

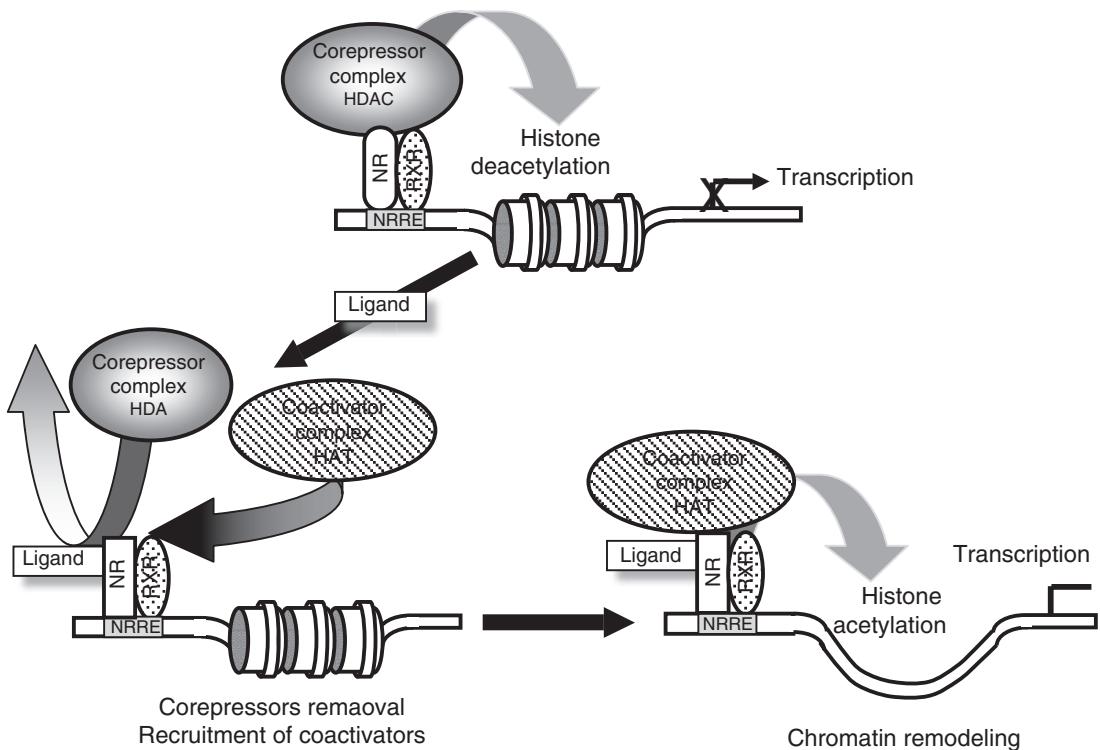
forms of cancer (Kushi and Giovannucci 2002). The biological functions of lipids are mainly carried out by fatty acids and/or derived signaling molecules like ceramides, diacylglycerol, eicosanoids, or coenzyme A thioesters (acyl-CoA). A large number of cellular systems and functions are affected by these bioactive macromolecules including regulation of ion channels or pumps, membrane trafficking and composition, protein acylation and sorting, control of enzyme activities and immune process, and the regulation of gene expression and energy metabolism (Faergeman and Knudsen 1997).

This chapter is focused on the transcriptional effect of fatty acids mainly in the liver because this organ plays a central role in whole-body lipid metabolism.

### REGULATION OF GENE EXPRESSION BY FATTY ACIDS

#### ROLE OF CHAIN LENGTH AND DEGREE OF SATURATION

When most of the studies emphasized the role of fatty acid chain length (more than 12 carbons) in the regulation of gene expression, a growing number of studies reported an effect of short-chain fatty acids (4–6 carbons) in the control of gene expression. For instance, in colorectal cancer cell lines or in immortalized colon cells, butyrate affected the expression of a number of genes involved in the regulation of cell proliferation/differentiation/apoptosis (Iacomino et al. 2001; Tabuchi et al. 2002). Similarly, butyrate induced the transcription of calcitonin gene in cultured human medullary thyroid carcinoma (Nakagawa et al. 1988) and stimulated the expression of plasminogen activator inhibitor type 1 (PAI-1) in HepG2 cells



**Figure 8.1.** Schematic representation of the mechanism of action of nuclear receptors. In the unliganded state, nuclear receptors (NR) are bound to their specific responsive element (NRRE) generally as heterodimer with *cis*-retinoic acid receptor (RXR). In this condition, heterodimers are associated with a multiprotein corepressor complex that contains histone deacetylase activity (HDAC). The deacetylated status of histones keeps the nucleosome in a conformation in which transcription is inhibited. Once a ligand binds to the receptor, the corepressor complex dissociates and a coactivator complex containing histone acetyltransferase activity (HAT) is recruited to the heterodimer. Acetylation of histone induces chromatin remodeling, a major event in the activation of gene transcription.

(Smith et al. 1996). Finally, it was suggested that the decreased expression of mitochondrial hydroxymethyl glutaryl-CoA synthase (mHMG-CoA synthase) in the colon of germfree rats (Cherbuy et al. 1995) could be due to the absence of butyrate, the most abundant short-chain fatty acid produced by fermentation of dietary fibers. Indeed, the transcriptional effect of butyrate has been demonstrated in human colon cancer cell line, on *WAF1/Cip1* gene promoter (Nakano et al. 1997), a gene encoding a protein that inhibits the G<sub>1</sub>–S phase transition. The transcriptional effect of butyrate seems mainly due to its inhibitory effect on histone deacetylase activity (Sanderson 2004; Sanderson and Naik 2000) that alters chromatin structure and transcription rate (see Figure 8.1). To get more insight into the regulation of gene transcription by short-chain fatty acid, the author recommends reading recent reviews in

this field (Davie 2003; Mei et al. 2004; Sanderson 2004).

The rest of the present chapter is focused on the transcriptional effect of long-chain fatty acids (more than 12 carbons).

Four classes of long-chain fatty acids are typically encountered in the diet: saturated fatty acids, *n*-9 monounsaturated fatty acids, and *n*-3 and *n*-6 polyunsaturated fatty acids (PUFA). Dietary fish oil PUFA from *n*-3 series are actually considered to have protective effects on cardiovascular diseases, diabetes, cancer, and neurological diseases (Seo et al. 2005). Among the pleiotropic effects responsible for these benefit actions of PUFA, the decrease in circulating concentration of very low-density lipoprotein (VLDL) and chylomicrons plays a central role (Seo et al. 2005). This mainly results from a decrease in the activity of hepatic lipogenic enzymes due to an

inhibition of gene transcription and/or modifications in mRNA maturation and/or stability (Jump 2004). Interestingly, it seems that downregulation of gene expression by fatty acids is restricted to fatty acid having more than 18 carbons and at least two double bonds (Clarke et al. 1997), whereas upregulation of gene is independent of the degree of saturation of the carbon chain of fatty acids.

#### **METABOLITE(S) RESPONSIBLE FOR THE EFFECT OF LONG-CHAIN FATTY ACIDS**

Fatty acids are delivered to cells either as complex lipoproteins (VLDL, chylomicrons) or as nonesterified fatty acids (NEFA). Triglycerides in chylomicrons and VLDL are hydrolyzed by the action of a lipoprotein lipase and NEFA enter cells via fatty acid transporters (Hajri and Abumrad 2002). Once in cells, NEFA are rapidly converted to fatty acyl-CoA thioesters by acyl-CoA synthetase (ACS) specific for the carbon chain length. At least six different ACSs have been characterized. While each isoform can activate a wide range of fatty acids, they have tissue-specific expression, subcellular distribution, and specific spectrum of activity. For instance, ACS-1, 4, and 5 are expressed in the liver: ACS-1 activates C12 to C20 fatty acids, whereas the activity of ACS-4 is restrained to arachidonic and eicosapentaenoic acids (Lewin et al. 2001). The intracellular location of ACS-1 and 4 inside the endoplasmic reticulum is in agreement with their involvement in triglyceride synthesis. Conversely, ACS-5 is located in outer mitochondrial membrane and thus plays a crucial role in the regulation of  $\beta$ -oxidation (Lewin et al. 2001). Once activated by ACS, fatty acyl-CoA is metabolized in many different metabolic pathways ( $\beta$ -oxidation, elongation, desaturation, triglyceride or cholesterol synthesis, prostanoïd or leukotriene synthesis, etc.) where each intermediate metabolite or end product can be responsible for the transcriptional effect of long-chain fatty acids (Jump 2004). For instance, it was shown that NEFA itself, long-chain acyl-CoA, lipoxygenase-derived metabolite leukotriene B4, prostacyclins, 15-deoxy- $\Delta^{12,14}$ -prostaglandin J2, and so on, are potent regulators of transcription depending on the gene considered (Louet et al. 2001; Pégrier 1998). This suggests that fatty acids can control gene transcription by different mechanisms according to the cell-specific context and the target gene.

The regulation of gene transcription by fatty acids seems to be due to two different mechanisms: first, the direct binding of the fatty acid or its metabolites onto transcription factors such that all, in our actual

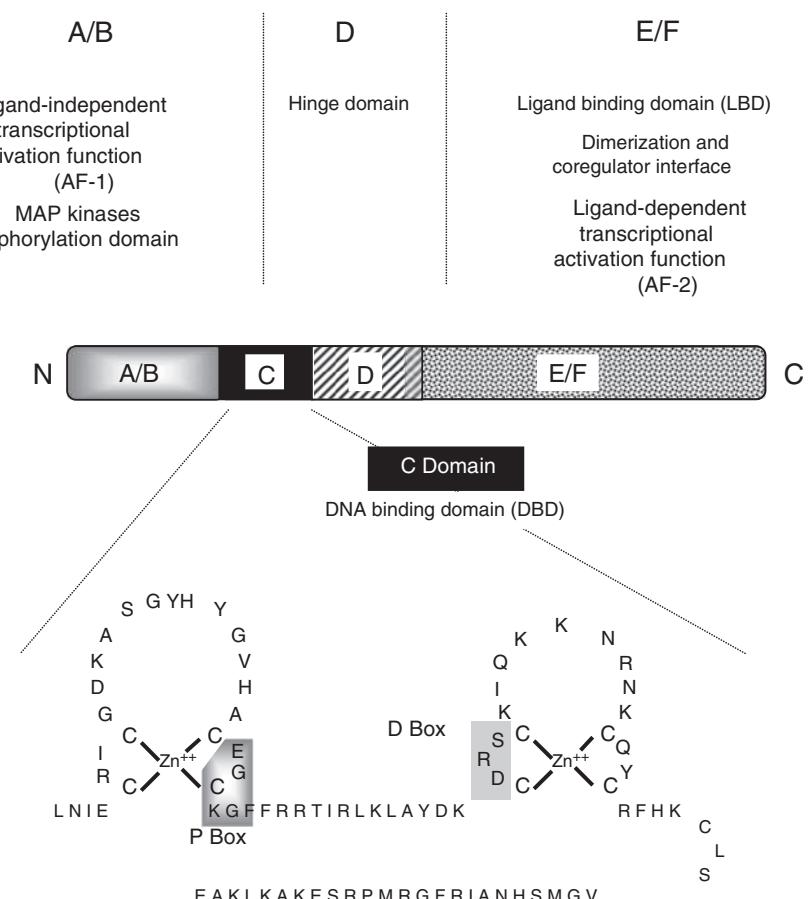
knowledge, belong to the nuclear receptor superfamily; second, indirect effects of fatty acids through changes in the activity or abundance of transcription factors such as SREBP (sterol regulatory element-binding protein), ChREBP (carbohydrate responsive element-binding protein), or NF- $\kappa$ B (nuclear factor- $\kappa$ B).

#### **ROLE OF NUCLEAR RECEPTORS IN THE REGULATION OF GENE EXPRESSION BY FATTY ACIDS**

##### **GENERAL STRUCTURE AND BASIC MECHANISM OF ACTION OF NUCLEAR RECEPTORS**

The 48 nuclear receptors that have been identified in the human and mouse genomes share a common structure of four main domains named A/B, C, D, and E/F (Figure 8.2; Desvergne and Wahli 1999; Khan and Vanden Heuvel 2003). Key functions have been assigned to each of these domains. The N-terminal A/B domain harbors a ligand-independent transcriptional activation function (AF-1). The sequence and length of the A/B domain are highly variable between receptors and among receptor subtype (Desvergne and Wahli 1999). The C domain, or DNA-binding domain (DBD), is formed by two zinc fingerlike motifs folded in a globular structure that can recognize a nuclear response element (NRE) present on target genes (Figure 8.2).

Nuclear receptors (NRs) bind to NRE as monomers, homodimers, or heterodimers (generally with RXR, 9-cis retinoic acid receptor), depending on the class of receptor (Desvergne and Wahli 1999). Three distinct sequences for NRE have been described: (1) direct repeats (DR $x$  : AGGTCA- $Nx$ -AGGTCA), where  $x$  represents the number (from 0 to 10) of any nucleotide ( $N$ ) between the two hexanucleotides; (2) everted repeats (ER $x$  : ACTGGA- $Nx$ -AGGTCA); and (3) inverted repeats (IR $x$  : AGGTCA- $Nx$ -ACTGGA). The D or hinge domain permits protein flexibility due to conformational changes induced by ligand binding. This region also contains the carboxy-terminal extension of the DBD domain that seems to be involved in recognition of the 5'-extension of the NRE (see section "Peroxisome Proliferator-Activated Receptors"). Finally, nuclear receptors present a large carboxy-terminal domain, the E/F domain, commonly named ligand-binding domain (LBD). The structure of the E/F domain varies substantially between NRs, but they all share a common sequence of 10 to 13  $\alpha$ -helices organized around the hydrophobic



**Figure 8.2.** Schematic structure of nuclear receptors.

binding pocket. In addition to the ligand binding, this domain encompasses the NR's dimerization and coregulator (corepressor and coactivator of transcription) interfaces and a strong ligand-dependent transcriptional activation function (AF-2; Figure 8.2). Briefly, upon ligand binding, the NRs undergo a conformational change that coordinately dissociates corepressors and facilitates recruitment of coactivator proteins to enable transcription of target genes (Figure 8.1).

Finally, it must be noted that in addition to ligand-induced activation of NR, some of them including estrogen (ER), androgen (AR), progesterone (PR), vitamin D (VDR), retinoic acid (RAR), and peroxisome proliferator-activated (PPAR) receptors are target of several kinases that modulate their transcriptional activity through phosphorylation mainly in the A/B domain (Burns and Vanden Heuvel 2007; Diradourian et al. 2005; Rochette-Egly 2003).

#### PEROXISOME PROLIFERATOR-ACTIVATED RECEPTORS

Among the fatty acid-regulated nuclear receptors, PPARs are the most extensively characterized. Three isoforms of PPAR have been cloned: (1) PPAR- $\alpha$  is mainly expressed in liver, digestive tract, and kidney; (2) PPAR- $\beta$ , - $\delta$ , or NUC-1 (respectively cloned in *Xenopus*, mouse, and human) are ubiquitously expressed; and (3) PPAR- $\gamma$  ( $\gamma$ 1,  $\gamma$ 2, and  $\gamma$ 3 arising from an alternative splicing of a single gene) is mainly expressed in adipose tissues and in macrophages (Desvergne and Wahli 1999). Initially characterized for their capacity to be activated by peroxisome proliferators (fibrates, xenobiotics, etc.) it was later shown that fatty acids (saturated fatty acids and PUFA) and some eicosanoids (15 deoxy-D $^{12,14}$ -prostaglandin J2, leukotriene B4) are potent ligands of PPARs (Desvergne and Wahli 1999). Structural analysis of PPARs reveals that the hydrophobic

binding pocket of these receptors is greater than most other members of this family (around 1,400 Å<sup>3</sup>; Escrivá et al. 2000). Although many fatty acids can bind PPARs in vitro, they do not have the same potency to activate PPAR. This was particularly well demonstrated in hepatic cells in which oleate (C18:1n-9) or eicosapentaenoate (C20:5n-3) bind to PPAR $\alpha$  (the predominant hepatic isoform) with quite similar affinity, but only eicosapentaenoate or docosahexanoate (C22:6n-3) activate PPAR $\alpha$  (Pawar and Jump 2003). Binding of ligands leads to an active conformation of the receptor through the stabilization of the AF-2 region of the LBD (see section “General Structure and Basic Mechanism of Action of Nuclear Receptors”) (Xu et al. 1999a). This conformational change leads to the removal of corepressor complex from the PPAR/RXR heterodimer and the recruitment of the coactivator complex (Feng et al. 1998; Nolte et al. 1998); (Figure 8.1) essential for the interaction with the transcriptional machinery. The recruitment of the transcriptional machinery can occur either directly (Kee et al. 1996) or in response to the chromatin remodeling (histone acetylation, Figure 8.1; Xu et al. 1999c). The modulation of gene transcription is due to the binding of the heterodimer PPAR/RXR to a consensus sequence (peroxisome proliferator response element, PPRE, a DR1 element, see section “General Structure and Basic Mechanism of Action of Nuclear Receptors”). Moreover, the 5'-extension (AACT) is essential for the polarity of PPAR/RXR heterodimer binding (DiRenzo et al. 1997), PPAR interacting with the 5' repeat, and RXR binding to the 3' motif (Jpenberg et al. 1997).

Using cDNA microarray technology, it was shown that dietary fish oil PUFA play a major role in the regulation of an extensive network of genes involved in hepatic fatty acid metabolism (Takahashi et al. 2002). Despite most of these genes have a PPRE in their promoter region, this does not mean that these effects are dependent on PPAR $\alpha$  activation. Until recently, this observation had led to the established dogma that regulated genes containing one or more PPRE sequences in their promoter respond to fatty acid via PPAR activation. However, a growing number of reports show that the regulation of gene expression by fatty acids is certainly more complex than the simple acceptance of this dogma. For instance, apo-AII and FAT-CD36 genes do not respond to fatty acids despite the presence of PPRE sequences in their promoter (Berthou et al. 1995; Duplus et al. 2000). In the liver of PPAR $\alpha$ -null mice (PPAR $\alpha$ <sup>-/-</sup>) the inhibitory effect of PUFA on the expression of genes encoding regulatory proteins of lipogenesis (acetyl-CoA carboxylase, ACC; fatty acid synthase, FAS; spot 14) or

glycolysis (L-pyruvate kinase, L-PK) is still present despite the absence of PPAR $\alpha$  receptor (Pan et al. 2000; Ren et al. 1997). PUFA inhibit the transcription of Δ5 and Δ6 desaturase genes, whereas PPAR agonists stimulate the transcription of these genes (Cho et al. 1999a, b). These results suggest that transcription factors different from PPAR are involved in the regulation of gene expression by fatty acids.

## LIVER X RECEPTORS

Two liver X receptor (LXR) isoforms have been described: LXR $\alpha$  mainly expressed in liver, kidney intestine, adipose tissue, and adrenal, and LXR $\beta$  that is ubiquitously present (Peet et al. 1998). Oxysterols (22-R-hydroxycholesterol, 24,25-epoxycholesterol, etc.) are natural ligands of LXRs, which are commonly known as cholesterol sensors. These receptors regulate the expression of genes involved in hepatic bile acid synthesis (7α-hydroxylase CYP7A), cholesterol reverse transport (ATP-binding cassette genes), lipogenesis (see section “Sterol Regulatory Element-Binding Protein”), and fatty acid and glucose uptake (Khan and Vanden Heuvel 2003; Li and Glass 2004) secondarily to their binding to DR4 (see section “Peroxisome Proliferator-Activated Receptors”) regulatory element (LXRE) as heterodimer with RXR. Recently, it was shown that monounsaturated fatty acids and PUFA bind to LXR $\alpha$  (Ou et al. 2001; Pawar et al. 2002) and antagonize oxysterols binding, leading to an inhibition of LXR $\alpha$  transcriptional activity (Ou et al. 2001). While it seems clear that LXR $\beta$  is insensitive to fatty acid antagonism (Pawar et al. 2002), it must be underlined that the antagonist effect of PUFA on LXR-regulated genes is not always found, especially in liver (Pawar et al. 2003).

In addition to PPAR and LXR receptors as fatty acid sensors, another nuclear receptor has been involved in fatty acid-mediated gene expression. For instance, the gene encoding CYP7A, the rate-limiting protein in bile acid synthesis, is upregulated by oxysterols (via the activation of LXR; Lu et al. 2001) and fatty acids (Cheema and Agellon 2000) but downregulated by fibrates (PPAR $\alpha$  agonists; Marrapodi and Chiang 2000; Patel et al. 2000). Indeed, analysis of the promoter region of CYP7A gene reveals the presence of a DR1 sequence (see section “General Structure and Basic Mechanism of Action of Nuclear Receptors”) that specifically binds hepatic nuclear factor (HNF)-4 $\alpha$  receptor but not PPAR/RXR heterodimer (Marrapodi and Chiang 2000; Patel et al. 2000). The relative contribution of these two receptors in the control of CYP7A gene transcription has been clearly demonstrated by phenotypic analysis of

PPAR $\alpha$  (Marrapodi and Chiang 2000) or HNF-4 $\alpha$  (Hayhurst et al. 2001) knockout mice. The next section on HNF-4 $\alpha$  may provide some clues to explain the apparent contradictory effects of fatty acids and fibrates on CYP7A gene.

#### HEPATIC NUCLEAR FACTOR-4 $\alpha$ —

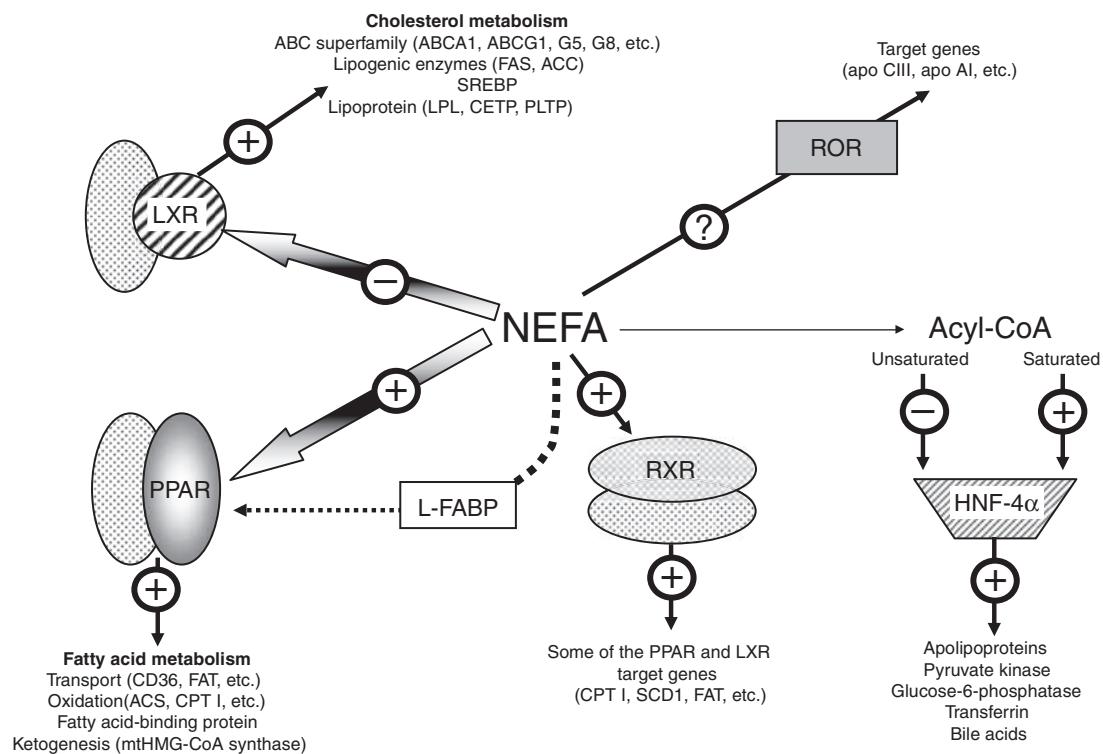
The HNF-4 class of nuclear receptor contains two subtypes in human, HNF-4 $\alpha$  ( $\alpha 1, \alpha 2, \alpha 4$ ) and HNF-4 $\gamma$  that differ in the A/B and F domains (see section “General Structure and Basic Mechanism of Action of Nuclear Receptors”). Originally identified in liver, HNF-4 $\alpha$  is also present in kidney, intestine, and pancreatic islets (Miquerol et al. 1994; Sladek et al. 1990). The expression pattern of HNF-4 $\gamma$  is more ubiquitous but high levels are also detected in liver and islets (Plengvidhya et al. 1999). Unlike PPAR and LXR receptors, HNF-4 is not able to bind native fatty acids but has a great affinity for fatty acyl-CoA (Hertz et al. 1998). While binding of saturated acyl-CoA (C14:0, C16:0) stimulates the transcriptional activity of HNF-4 $\alpha$ , binding of PUFA acyl-CoA (C18:3, C20:5, C22:6) inhibits the effects of HNF-4 $\alpha$  on gene transcription (Hertz et al. 1998). The recent x-ray crystallographic analysis of HNF-4 $\alpha$  (Bogan et al. 2000; Dhe-Paganon et al. 2002) and HNF-4 $\gamma$  (Wisely et al. 2002) revealed that although the receptor was crystallized in the absence of ligand, the LBD was occupied by C14–C18 fatty acids interacting with Arg226 through the carboxyl group (Dhe-Paganon et al. 2002; Wisely et al. 2002). These fatty acids do not exchange with exogenously added fatty acids (Dhe-Paganon et al. 2002; Wisely et al. 2002), a situation that markedly differs from PPAR and LXR that show binding and displacement of ligand typical of nuclear receptor. These results suggest that the unliganded HNF-4 receptor is not sufficiently stable for crystallization and that bound fatty acids lock the receptor in an active configuration. Finally, it was shown that the volume of the ligand-binding pocket of HNF-4 ( $370 \text{ \AA}^3$ ) is much smaller than the estimated volume for fatty acyl-CoA (around  $850 \text{ \AA}^3$ ; Bogan et al. 2000). Altogether, these observations underlined the necessity of additional work to understand the role of fatty acids in the function of endogenous HNF-4. The phenotypic analysis of CRE-Lox conditional HNF-4 $\alpha$ -null mice has shown that this nuclear receptor controls either directly or indirectly the expression of several hepatic genes. This included genes encoding proteins of lipoprotein metabolism (apo-CII, CIII, AII, AIV; reviewed in Jump and Clarke 1999), iron metabolism (transferrin; Hertz et al. 1996), carbohydrate metabolism (-L-PK, glucose-6-phosphatase,

phosphoenolpyruvate carboxykinase; Pan et al. 2000; Rajas et al. 2002), and bile acid synthesis (CYP7A; Hayhurst et al. 2001). Another difference between the previously described PPAR or LXR receptors and HNF-4 $\alpha$  is that HNF-4 $\alpha$  binds DR1 sequences as homodimer, making it as a competitor of PPAR/RXR heterodimer for binding to these DNA motifs (Hertz et al. 1996; Marrapodi and Chiang 2000; Pan et al. 2000). This has been recently demonstrated for the gene encoding glucose-6-phosphatase whose transcription is inhibited by PUFA as a loss of HNF-4 $\alpha$  binding to Glc-6-Pase gene promoter (Rajas et al. 2002). Finally, it was reported that fibrates could be converted to CoA thioesters (as fatty acids) and bind to HNF-4 $\alpha$ , leading to an inhibition of its transcriptional activity (Hertz et al. 2001). This dual level of competition with PPAR receptors (binding to DR1 motifs, fibrate-CoA binding) could explain, in part, the complex regulation of CYP7A gene by fatty acids and fibrates.

#### OTHER FATTY ACID SENSORS

In addition to these three main fatty acid sensors (i.e., PPAR, LXR, and HNF-4), it was shown that at least two other nuclear receptors are able to bind fatty acids. First, the dimerization partner of most nuclear receptors, RXR, binds monounsaturated fatty acids (oleic acid, C18:1) and PUFA (arachidonic acid, C20:4 and DHA, C22:6) as evidenced by both crystal structure (Bourguet et al. 2000) and electrospray mass spectrometry (Lengqvist et al. 2004) experiments. Moreover, these fatty acids have been shown to activate RXR in various cells or organs (brain, heart, testis, colonocytes; de Urquiza et al. 2000; Fan et al. 2003; Goldstein et al. 2003), suggesting that fatty acid ligands have the potential to exert important effects on RXR-mediated gene transcription. Loss of RXR $\alpha$  in the liver perturbs multiple metabolic pathways mediated by LXR $\alpha$  and PPAR $\alpha$ , and also those mediated by other partner of dimerization such as CAR $\beta$  (constitutive active receptor) or FXR (farnesoid X receptor; Pinaire and Reifel-Miller 2007). As discussed above, fatty acids control the expression of numerous genes through PPAR/RXR or LXR/RXR heterodimers. The recent reports showing that fatty acids are able to bind and activate not only PPAR and LXR but also RXR provide interesting clues to determine (1) the possibility that fatty acids act as heteroligands and (2) the permissive and/or synergistic consequences in the regulation of gene transcription.

The second additional nuclear receptor that binds PUFA is ROR $\beta$  (retinoic acid-related orphan receptor) (Stehlin et al. 2001). ROR $\beta$  is exclusively



**Figure 8.3.** Schematic overview of fatty acid sensor proteins. Nonesterified fatty acids (NEFA) or their respective CoA thioesters regulate the transcription of target genes through direct activation of some nuclear receptors, PPAR (peroxisome proliferator-activated receptor), LXR (liver X receptor), HNF-4 $\alpha$  (hepatic nuclear factor 4 $\alpha$ ), or RXR (cis-retinoic acid receptor). In addition, fatty acids were also found in the ligand pocket of ROR (retinoic acid-related orphan receptor) but the contribution of this receptor in the regulation of gene transcription by fatty acids remains to be determined. Finally, L-FABP (fatty acid-binding protein) could be involved in the regulation of gene expression by fatty acids. ABC, ATP-binding cassette; FAS, fatty acid synthase; ACC: acetyl-CoA carboxylase; SREBP, sterol regulatory element-binding protein; LPL, lipoprotein lipase; CETP, cholestrylo ester transport protein; PLTP, phospho-lipid transfer protein; FAT, fatty acid transporter; ACS, acyl-CoA synthetase; CPT I, carnitine palmitoyl transferase I; mtHMG-CoA synthase, mitochondrial hydroxymethylglutaryl-CoA synthase; SCD1, steroyl-CoA desaturase 1.

expressed in the area of the central nervous system, whereas two other isoforms  $\alpha$  and  $\gamma$  are expressed in many tissues (Boukhtouche et al. 2004; Hirose et al. 1994). ROR $\alpha$  plays a central role in the regulation of lipid metabolism as evidenced by its stimulating effect on intestinal apo-CIII, liver apo-AI, and skeletal muscle CPT I gene expression (Lau et al. 2004; Raspe et al. 2001; Vu-Dac et al. 1997). Recent studies reveal that cholesterol is a natural ligand of ROR $\alpha$  (Boukhtouche et al. 2004). Whether fatty acids can bind this isoform and interfere with natural ligand remains to be determined.

A general overview of fatty acid-regulated genes by nuclear receptors is depicted in Figure 8.3.

In addition to their direct effects as ligands, fatty acids affect the nuclear abundance and/or activity of

many transcription factors that control the expression of genes involved in lipid or glucose metabolism. This last part of this chapter describes three examples of such kind of regulation.

## EFFECT OF FATTY ACIDS ON NUCLEAR ABUNDANCE AND ACTIVITY OF TRANSCRIPTION FACTORS

### STEROL REGULATORY ELEMENT-BINDING PROTEIN

Three SREBP isoforms have been cloned; SREBP-1a and 1c (derived from alternative transcription start

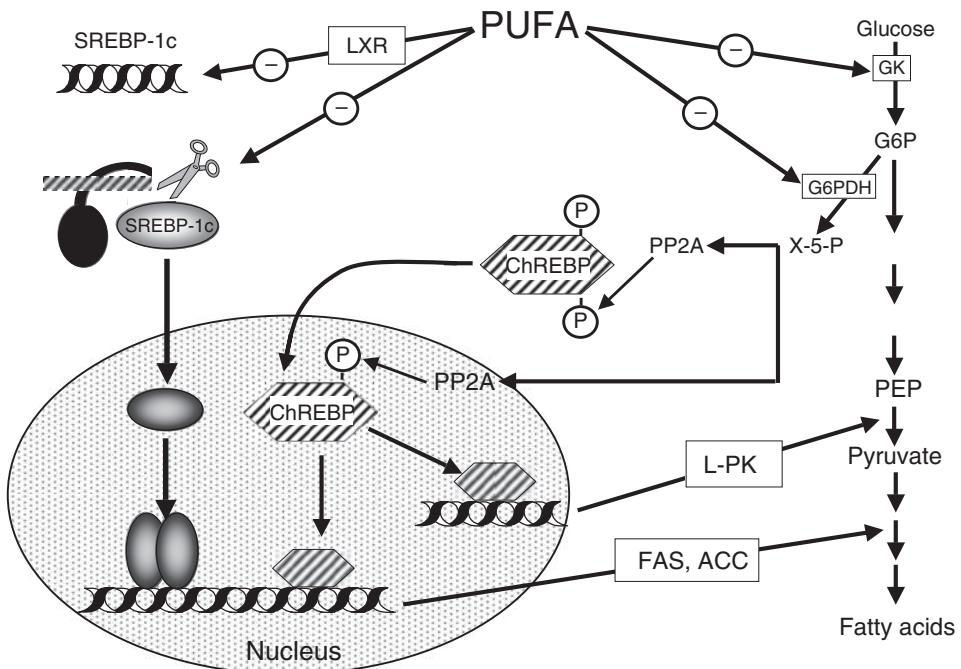
site of a single gene) and SREBP-2 encoded by another gene (Horton et al. 2002; Osborne 2000). SREBP-1c preferentially enhances transcription of genes involved in fatty acid, triglyceride, and phospholipids synthesis, whereas SREBP-1a and SREBP-2 activate genes involved in cholesterol synthesis (Horton et al. 2002; Osborne 2000). SREBP belongs to the basic helix-loop-helix-leucine zipper (bHLH-Zip) family of transcription factors, but differ from other bHLH-Zip proteins in that they are synthesized as inactive precursors bound to the endoplasmic reticulum (Horton et al. 2002; Osborne 2000). In order to reach the nucleus and to act as a transcription factor, the NH<sub>2</sub>-terminal domain of SREBPs must be cleaved proteolytically from the endoplasmic reticulum membrane (Horton et al. 2002; Osborne 2000). Briefly, four proteins play a major role in the maturation and translocation of SREBP. In the endoplasmic reticulum, SREBP binds to SREBP-cleavage-activating protein (SCAP) that interacts with a third protein, INSIG (insulin-induced gene). When intracellular sterol levels fall, SCAP escorts SREBP to the Golgi, whereas INSIG facilitates retention of SREBP in the endoplasmic reticulum (Yabe et al. 2002, 2003; Yang et al. 2002). In the Golgi, two proteases S1P and S2P (Site 1 and 2 protease) cleave inactive SREBP precursor and release mature transcription factor from the Golgi for its translocation to the nucleus. SREBP binds, as homodimers, to SRE (sterol responsive element) in the promoter region of many genes involved in lipid metabolism (Foufelle and Ferre 2002; Jump 2004).

As previously mentioned in the section “Peroxisome Proliferator-Activated Receptors,” PUFA inhibit the transcription of lipogenic genes (ATP citrate lyase, ACC, FAS, Spot 14) in the liver of PPAR $\alpha$ -null mice (Ren et al. 1997). Indeed, overexpression of mature SREBP-1c in transgenic mice liver (Yahagi et al. 1999) or in cultured hepatocytes (Materi et al. 1999) overrides the PUFA suppression of lipogenic gene expression, supporting a role for SREBP-1c in mediating the negative effects of PUFA. However, the inhibitory effects of PUFA through SREBP-1c are complex. At least two mechanisms have been described. First, it was reported that treatment of CHO cells with PUFA activated a sphingomyelinase, leading to the redistribution of cholesterol from the plasma membrane to the endoplasmic reticulum (Worgall et al. 2002). The increase in the level of cholesterol in endoplasmic reticulum membrane induces the inhibition of proteolytic process and decreases the nuclear SREBP abundance (Worgall et al. 2002). This mechanism probably represents the main mechanism by which PUFA suppress

the lipogenic gene expression (see below). Secondly, PUFA markedly reduce the hepatic SREBP-1c levels (Figure 8.4) through inhibition of its gene transcription (Ou et al. 2001; Xu et al. 1999b), an accelerated mRNA turnover (Xu et al. 2001), and finally by accelerating its proteosomal degradation (Botolin et al. 2006). While PUFA-induced SREBP-1c mRNA degradation is still unresolved, the effect of PUFA on SREBP-1 gene transcription is controversial. Originally attributed to an antagonistic effect of PUFA on LXR activity (see section “Liver X Receptors”), a strong activator of SREBP-1c gene transcription (DeBose-Boyd et al. 2001; Schultz et al. 2000), this has been recently questioned. For instance, in rat liver or isolated hepatocytes, it was shown that PUFA did not antagonize the oxysterol-induced LXR transcriptional control of target genes such as CYP7A or certain ATP-binding cassette genes (Pawar et al. 2003). Moreover, the PUFA-induced suppression of SREBP-1c promoter activity does not require LXR responsive element (LXRE; Deng et al. 2002). Thus, despite evidences for an inhibitory effect of PUFA on SREBP-1c gene expression, additional work is required to fully understand the molecular mechanisms involved in this regulation, at least in the liver.

#### CARBOHYDRATE RESPONSE ELEMENT-BINDING PROTEIN

As already mentioned (section “Peroxisome Proliferator-Activated Receptors”), L-PK gene transcription is markedly decreased by PUFA in a PPAR $\alpha$ -independent mechanism. The PUFA-mediated suppression of L-PK gene expression cannot be attributed to SREBP-1c since the L-PK promoter does not contain an SRE-binding site (Stoeckman and Towle 2002). Functional mapping analysis of L-PK promoter has shown that the PUFA response element contains a carbohydrate response element (ChoRE) binding site (Diaz-Guerra et al. 1993; Liimatta et al. 1994). Recently, ChREBP was shown to play a crucial role in the induction of glycolytic and lipogenic genes by glucose (Dentin et al. 2004; Iizuka et al. 2004) by its capacity to bind to ChoRE present in promoters of these genes (Ishii et al. 2004; Stoeckman et al. 2004; Yamashita et al. 2001). The fact that ChREBP DNA-binding activity on L-PK promoter is decreased in hepatic nuclear extracts from rats fed a high-fat diet suggests that ChREBP may be involved in PUFA-induced downregulation of glycolytic genes (Yamashita et al. 2001). At least two different mechanisms have been involved in the negative effects of PUFA on L-PK gene expression (Figure 8.4): (1) a decrease in



**Figure 8.4.** Schematic representation of PUFA-induced repression of glycolytic and lipogenic gene expression. PUFA inhibit, in an indirect manner, the transcription of genes encoding lipogenic (FAS, ACC) and glycolytic enzymes (L-PK, liver-type pyruvate kinase) secondarily to a reduction in nuclear abundance of stimulatory transcription factors such as SREBP-1c (sterol regulatory element-binding protein) or ChREBP (carbohydrate response element-binding protein). For SREBP-1c, this results mainly from a decrease in mRNA levels (via an inhibition of LXR-mediated gene transcription and mRNA levels) and in the cleavage of the precursor form of SREBP-1c. For ChREBP, this is due to a decrease in xylulose-5-phosphate (X-5-P) concentration (secondarily to a reduction in glycolytic flux via glucokinase (GK) and glucose-6-phosphate dehydrogenase (G6PDH) activities). In these conditions, ChREBP is not dephosphorylated by PP2A (protein phosphatase 2A) and thus not translocated in the nucleus.

ChREBP gene expression (Dentin et al. 2005; He et al. 2004; Kawaguchi et al. 2002); (2) a decrease in nuclear translocation of ChREBP (Kawaguchi et al. 2002). Indeed, the translocation of ChREBP from the cytosol to the nucleus is an important process to activate target genes in response to glucose (Uyeda et al. 2002). ChREBP is a phosphoprotein that contains at least three phosphorylation sites important for its activation (Kawaguchi et al. 2001). The dephosphorylation of Ser<sup>196</sup> requires the activation of glucose metabolism in which xylulose-5-phosphate (X-5-P), a metabolite of pentose phosphate pathway, activates a phosphatase (PP2A) that dephosphorylates ChREBP, leading to its migration in the nucleus ((Kabashima et al. 2003), Figure 8.4). As high-fat diets are known to reduce glycolytic (Dentin et al. 2005) and pentose phosphate pathways (Dentin et al. 2005; Stabile et al. 1996, 1998; Tomlinson et al. 1988), this would reduce the nuclear shut-

ting of ChREBP. Once inside the nucleus, dephosphorylation of Ser<sup>568</sup> is required for ChREBP DNA binding (Kawaguchi et al. 2002) (Figure 8.4). This dephosphorylation is ensured by X-5-P-induced nuclear PP2A (Kawaguchi et al. 2002) (Figure 8.4). Conversely, Kawaguchi et al. (2002) reported that fatty acids (whatever the chain length and degree of saturation) induced an increase in intracellular AMP concentration that activated AMP kinase and led to the phosphorylation of ChREBP on Ser<sup>568</sup> (Figure 8.4). However, these results are questionable since it was reported by several groups that dietary PUFA suppress lipogenic and glycolytic gene transcription without affecting hepatic ATP levels (Salati and Clarke 1986; Suchankova et al. 2005). A possible explanation for these discrepancies is that PUFA could modulate AMP kinase activity by governing the activity of phosphatases controlling its dephosphorylation (Suchankova et al. 2005). Such

kind of a regulation, that is through phosphorylation/dephosphorylation mechanisms, has been described for other transcription factors. Indeed, fatty acids and/or their metabolites can affect either directly or indirectly the activity of various kinases (PKB, PKC, IKK, etc.; Jump 2004) that in turn change the degree of phosphorylation and activity of transcription factors such as NF- $\kappa$ B (Gao et al. 2004) or nuclear receptors, especially PPAR (Burns and Vanden Heuvel 2007; Diradourian et al. 2005). For instance, it was shown that fatty acids activate PKC in a tissue-dependent manner (PKC $\theta$  in muscle and adipocyte (Gao et al. 2004; Le Marchand-Brustel et al. 2003), PKC $\epsilon$  in liver (Samuel et al. 2004)) that in turn induces other kinases such as I $\kappa$ B kinase (IKK) or c-Jun N-terminal kinase (JNK) that contribute to the development of insulin resistance. Indeed, activation of IKK in liver of high-fat diet mice has been clearly identified as a major determinant of insulin resistance through NF- $\kappa$ B-regulated genes (Arkan et al. 2005; Cai et al. 2005). Nuclear abundance of NF- $\kappa$ B transcription factor can be controlled by fatty acids through at least two different ways: (1) the modulation of Toll-like receptors activity; (2) a PPAR-mediated transrepression mechanism. These two aspects will be briefly described.

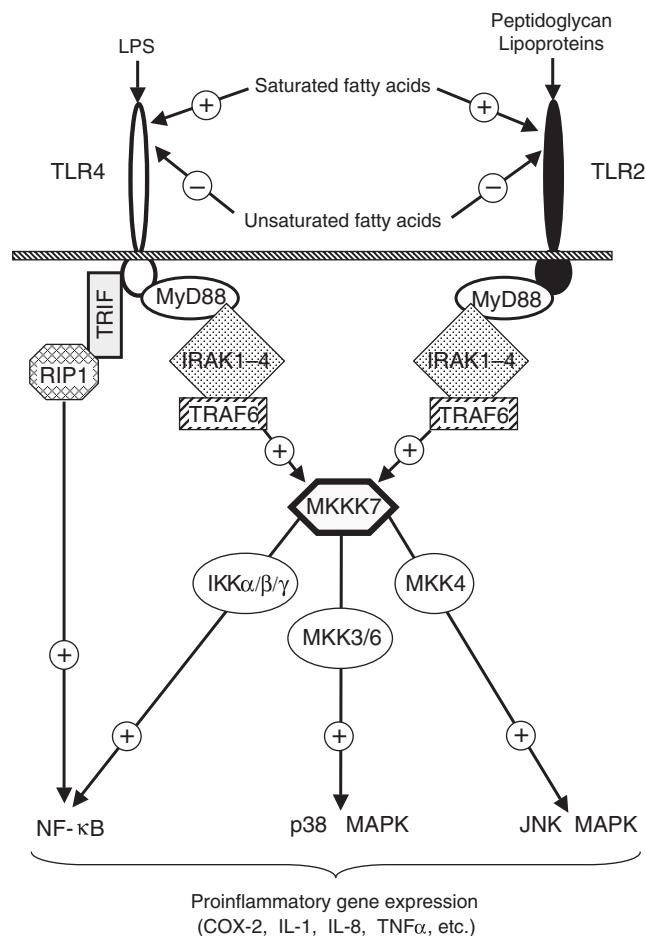
#### TOLL-LIKE RECEPTORS

Toll-like receptors (TLRs) are mammalian homologue of Toll proteins identified in *Drosophila* as mediators of immune response. Eleven TLRs have been identified in human and 13 in mouse genome (Bjorkbacka 2006; Takeda and Akira 2001). TLRs stimulated with specific agonists recruit adaptor molecules to activate downstream signaling pathways. Each TLR uses different combinations of adaptor proteins to provide the specific signaling, which will be briefly described for the two TLRs that are modulated by fatty acids, that is, TLR4 and TLR2 (Lee and Hwang 2006). In general, the activation of TLR4 triggers the activation of both MyD88-dependent (myeloid differentiation factor 88) and -independent signaling pathways, whereas TLR2 activation primarily induces the activation of MyD88-dependent pathway (Figure 8.5). MyD88 recruits IL-1 receptor-associated kinase 4 (IRAK-4) and induces its phosphorylation that in turn induces the phosphorylation and activation of IRAK-1 which associates with tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6). This leads to the activation of IKK $\alpha/\beta/\gamma$  complex, resulting in the nuclear translocation of NF- $\kappa$ B transcription factor or the activation of other upstream kinases such as

p38 and JNK MAP kinases (Zhang and Ghosh 2001) (Figure 8.5). MyD88-independent pathway is due to the recruitment of a TRIF adaptor molecule (Toll/IL-1R domain-containing adapter inducing interferon  $\gamma$ ) that interacts with RIP1 leading to the activation of NF- $\kappa$ B transcription factor (Lee and Hwang 2006) (Figure 8.5). Saturated fatty acids (mainly lauric C12:0 and palmitic C16:0 acids) induce the expression of proinflammatory gene products such as cyclooxygenase 2 (COX-2), inducible nitric oxide synthase (iNOS), TNF- $\alpha$ , or IL-1 $\alpha$  in macrophages (Lee et al. 2001) as a result of NF- $\kappa$ B transcription factor activation. This is due to the activation of MyD88-dependent and TRIF-dependent signaling pathways of TLR4 or TLR2 as demonstrated by using dominant negative mutant of various proteins involved in the cascade of NF- $\kappa$ B activation in macrophages (Lee et al. 2003). In contrast to the stimulatory effect of saturated fatty acids on TLR activation, various PUFA (EPA, DHA, or linoleic acid) suppressed NF- $\kappa$ B activation induced by TLR4 agonist, LPS (Lee et al. 2001b). Indeed, LPS-induced COX-2, IL-1, or TNF- $\alpha$  gene expression was markedly reduced in human peripheral monocytes or mononuclear cells by the consumption of meals containing fish oil, a major source of *n*-3 PUFA (Endres et al. 1993; Lee et al. 2003a). Interestingly, *n*-3 PUFA (EPA, DHA) are much more potent inhibitors of TLR4 and TLR2 activation as compared to *n*-6 (arachidonic and linoleic acids) or *n*-9 (oleic acid) unsaturated fatty acids. This may account for the reason *n*-3 PUFA are considered as more effective anti-inflammatory compounds than *n*-6 PUFA. Moreover, atherosclerosis in high-fat diet fed ApoE-deficient mice is markedly reduced in TLR4 or MyD88 knockout mice (Bjorkbacka et al. 2004; Michelsen et al. 2004), suggesting that TLR-mediated inflammatory gene expression could be involved in the development of atherosclerosis induced by lipid components.

#### PPAR-MEDIATED TRANSREPRESSION

Recent studies have demonstrated protective roles of PPARs in a number of inflammation-related diseases such as atherosclerosis, inflammatory bowel diseases, and liver fibrosis (Ricote and Glass 2007). The first evidence that PPARs played a role in inflammation was demonstrated in PPAR $\alpha^{-/-}$  mice that present prolonged inflammatory response to leukotrienes and arachidonic acid (Devchand et al. 1996). Similarly in hyperlipidemic and atherosclerotic patients, fenofibrate treatment (a PPAR $\alpha$  synthetic ligand) markedly reduces the plasma concentration of acute-phase proteins (fibrogen, C-reactive



**Figure 8.5.** Schematic representation of TLR4 and TLR2 signaling pathways in response to fatty acids. MyD88, myeloid differentiation factor 88; IRAK1–4, IL-1 receptor-associated kinase 1–4; TRIF, Toll/IL-1R domain-containing adapter-inducing interferon  $\gamma$ ; TRAF6, tumor necrosis factor receptor associated factor 6; MKKK7, MAP kinase kinase kinase 7; MAPK, MAP kinase; MKK3/6,4, MAP kinase kinase.

protein) and proinflammatory cytokines (IL-6, TNF- $\alpha$ ; Madej et al. 1998; Staels et al. 1998). In contrast to transcriptional activation, which involves the binding of PPAR to DNA-specific sequences of target genes (see section “Peroxisome Proliferator-Activated Receptors”), transrepression does not involve binding of the receptor to PPRE. Several molecular mechanisms have been described to account for transrepression activity of PPARs (Ricote and Glass 2007). Along various mechanisms, it has been shown that ligand-activated PPAR $\alpha$  binds directly NF- $\kappa$ B and AP-1 transcription factors via protein–protein interactions and prevents binding to their specific DNA response elements (Delerive et al. 1999). As PUFA are potent PPAR $\alpha$  activators (see section “Peroxisome Proliferator-Activated Receptors”), it is tempting to

speculate that in addition to the reduction in nuclear abundance of SREPB-1c, ChREBP, or TLR activity, this mechanism of transrepression would participate in the benefit effect of PUFA in reducing inflammation-related diseases such as atherosclerosis, diabetes, obesity, or metabolic syndrome.

Finally, another level of regulation of gene expression by fatty acid is the role played by fatty acid-binding proteins (FABP). At least nine different FABP have been characterized in mammals (Haunerland and Spener 2004). Among this great family of protein, it was shown that liver isotype (L-FABP) interact physically with PPAR $\alpha$  and therefore L-FABP is considered as a coactivator in PPAR $\alpha$ -mediated gene expression (Wolfrum et al. 2001). Similarly, E-FABP (mainly expressed in adipose tissue and

muscle) interacts with PPAR $\beta$  and A-FABP (mainly expressed in adipose tissue) with PPAR $\gamma$  (Tan et al. 2002), underlying the crucial role of these proteins as link between intracellular fatty acid level and the regulation of gene expression.

## CONCLUSIONS

All the experimental data presented in this chapter emphasize the major role of dietary fat as a source of signal molecules for the regulation of an extensive network of genes involved in hepatic fatty acid metabolism. They also underline the great diversity of fatty acid-sensor proteins, and the list of potential transcription factors involved in fatty acid-mediated gene expression is probably not closed. This chapter is focused on two main pathways by which fatty acids can control gene expression: (1) direct binding on nuclear receptors (PPAR, LXR, HNF-4, RXR, ROR, etc.); (2) an indirect action through changes in abundance or activity of transcription factors (SREBP, ChREBP, NF- $\kappa$ B, etc.). Knowledge of the mechanisms by which fatty acids control specific gene expression may provide insight into the development of new therapeutic strategies for a better management of whole-body lipid metabolism and the control of blood levels of triglycerides and cholesterol, and important risk factors involved in several chronic diseases like insulin resistance, obesity and diabetes, metabolic syndrome, atherosclerosis and coronary heart diseases, inflammation, cancer, etc.

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# 9

## Nonnutritient Functionality of Amino Acids

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### INTRODUCTION

The history of amino acids began in 1936 when Rose recognized an individual amino acid, threonine (Tanford and Reynolds 2001). The exploration of amino acids originated with the physiology of proteins to the physiology of amino acids and more recently has shifted to the functionality of amino acids (Cynober 2004). Although amino acids are widely known as the building blocks of protein, their functions in living organisms are vast as they can interact with the endocrine, neuronal, and immune systems to influence the balance between health and disease (Li et al. 2007). These systems, particularly in diseased states, affect the amino acid availability and may induce pathways to alter protein synthesis. The underlying mechanism of the regulation of the biological functions is partially due to amino acid control of gene expression. Presently, there are two well-documented pathways of amino acid control of gene regulation, mammalian target of rapamycin (mTOR) and amino acid response (AAR) pathways. This chapter reviews the importance of amino acid balance and the consequences of amino acid imbalance at the genetic level. The focus is mainly on the mammalian control of gene regulation in amino acid-depleted models at the transcriptional and posttranscriptional levels of gene regulation. Furthermore, health and disease implications through amino acid deficiency and supplementation are explored.

### AMINO ACID CLASSIFICATION

Amino acids are composed of an amino group, a carboxyl group, a hydrogen atom, and a unique side group (R) attached to an alpha carbon atom. It is the R group that dictates the properties of each amino

acid to impact protein structures and the functionality in the biological system. Amino acids that are classified as essential or indispensable are those that cannot be synthesized *de novo* by an organism at a sufficient rate to meet the amino acid requirement to maintain optimal growth (Rose 1957). Another group of amino acids, termed conditionally essential amino acids, are those that become essential under specific circumstances where amino acid synthesis is compromised or there is a greater demand of amino acids. For example, tyrosine becomes essential for those with the disease phenylketonuria because they must reduce the intake of phenylalanine, which is the precursor for tyrosine (Scriver et al. 1995). Furthermore, tumor cells utilize glutamine, arginine, and sulfur-containing amino acids for its growth, furthering tumor development and reducing amino acid availability to host cells (Lazo 1981). The availability of essential and some conditionally essential amino acids plays an important role in the control of gene regulation, which are further discussed in this chapter.

### AMINO ACID REQUIREMENT

The requirement of essential amino acids was presented by FAO/WHO/UNU in 1985 using nitrogen balance to determine the requirement for normal growth. The daily requirement of amino acids is variable depending on the age, gender, and the physiological state of an individual. The requirement values can be defined as the dietary intake of indispensable amino acids to sufficiently meet the irreversible losses in an initially healthy human at energy balance under conditions of moderate physical activity (Young and Tharakan 2004). Failure to intake or maintain the adequate concentration of essential

amino acids could pose health risks by starving cells of substrates for protein synthesis and also by initiating cell signaling pathways to reduce overall protein synthesis at the gene regulation level.

On the opposite spectrum of amino acid deficiency is amino acid toxicity. Studies reveal that overconsumption of various amino acids can have adverse health effects such as severe growth depression. Unfortunately, accurate numerical values of amino acid toxicity have not been generated for two reasons. First, the additional administration of a single amino acid versus several amino acids presents a different biological effect (Sauberlich 1961). An increase in a single amino acid alters the entire amino acid pool by competitively inhibiting the absorption of other amino acids that share a common transporter (Renwick 2004). Second, most toxicity studies originate from animal models where the data are extrapolated for human doses. One toxicologist reported that there is a 100-fold uncertainty factor based on toxicodynamics, toxicokinetics, and species differentiation (Renwick 2003). Therefore in amino acid supplementation studies, it is important to maintain an adequate dosage below toxicity levels.

## AMINO ACID METABOLISM

### AMINO ACID ABSORPTION

Amino acids are absorbed into the cell through various transporters. There are three common transporters, system A, system L, and system N. System A transporters are  $\text{Na}^+$  dependent and pH sensitive, and transport neutral amino acids (Neville et al. 1980; Shennan et al. 1997). Extracellular amino acid concentration, starvation, and hormones heavily influence system A transporter activity (Collarini and Oxender 1987). System L transporters transfer branched chain amino acids (BCAA) and aromatic amino acids. They are temperature sensitive and influenced by the concentration of the amino acids they transfer (Thompson et al. 1973). System N amino acids can uptake glutamine, asparagine, and histidine and are  $\text{Na}^+$  dependent (Collarini and Oxender 1987). These transporters are commonly found in the liver and its activity depends on protein synthesis. Some amino acid transporter genes have shown to be upregulated by amino acid deprivation, which could result in an increase in the intracellular concentration of their substrates (Franchi-Gazzola et al. 2006; Lopez et al. 2007). The mechanism of the feedback system between transporter expression and amino acid deprivation is yet to be well defined.

### AMINO ACID HOMEOSTASIS

A balance of metabolic inputs and outputs regulate the homeostatic balance of free amino acids. The amino acid inputs include protein breakdown, de novo synthesis (of nonessential amino acids), and dietary supply, whereas the outputs are protein synthesis and amino acid degradation. The balance of free amino acids is maintained after a protein-rich meal because the increase in circulating amino acids results in a series of cell signaling mechanism to initiate protein turnover (Proud 2002). In particular, essential amino acids initiate the translation process of muscle synthesis, whereas nonessential amino acids do not (Iresjo et al. 2005). The carbon skeleton of amino acids is conserved as glucose via gluconeogenesis or as fatty acids via fatty acid synthesis. There are typically two types of amino acids, glucogenic or ketogenic. There are 18 glucogenic amino acids that act as intermediates of the Krebs cycle in the form of pyruvate,  $\alpha$ -ketoglutarate, succinyl CoA, fumarate, and oxoacetate. On the other hand, 7 amino acids are ketogenic and are stored in the form of acetyl CoA and acetoacetate. In the rare case of high-energy demands or starvation, amino acids partake in energy production by being oxidized to form  $\text{CO}_2$  and  $\text{H}_2\text{O}$ . Nonessential amino acids can be synthesized via transamination of  $\alpha$ -keto acids followed by subsequent steps dependant on the amino acid. These amino acids follow the reverse pathway of amino acid metabolism. Essential amino acids may be produced; however, since mammals lack the enzyme for their synthesis, it is not synthesized at a sufficient rate to maintain organism growth.

### AMINO ACID RECOGNITION PATHWAYS

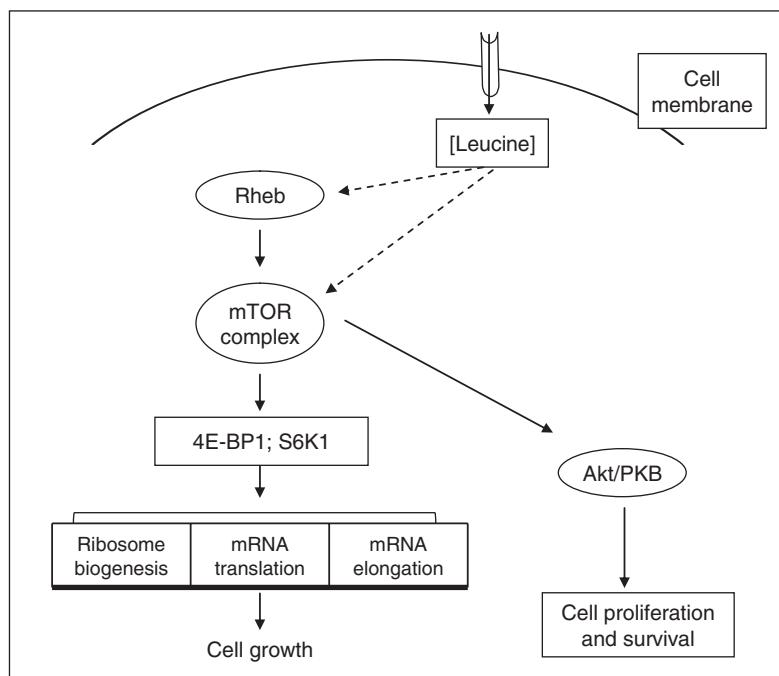
Amino acid metabolism is tightly regulated to provide adequate concentration for the survival of the organism. It is well known that amino acids are crucial in life as they are the substrate for protein synthesis, and more recently have been shown to interact at the genomic level to influence gene expression. The two well-documented mammalian pathways for detecting and responding to amino acid availability are the mTOR pathway and the AAR pathway (Kilberg et al. 2005). The mTOR pathway detects for excess of amino acids, especially leucine, and ultimately leads to an increase in protein synthesis and cell growth. The exact mechanism of detecting extracellular or intracellular amino acid concentration is unknown. On the other hand, AAR pathway recognizes amino acid deficiency and leads to a general reduction in

protein synthesis, with the exception of upregulation of selected genes. The two pathways influence protein synthesis in opposing manners, but whether their recognition pathways are directly linked is unknown (Kilberg et al. 2005).

#### MAMMALIAN TARGET OF RAPAMYCIN PATHWAY

The discovery of mTOR pathway was in association to the functionality of BCAA in the muscular system (Garlick and Grant 1998). BCAA are unique from other amino acids because they are predominantly metabolized in the skeletal muscle as to the liver and their metabolism can be enhanced by intense exercise by the activation of branched-chain  $\alpha$ -keto acid dehydrogenase complex (Kobayashi et al. 1999; Wagelmakers et al. 1989). Extensive studies in BCAA's ability to augment protein synthesis have revealed that leucine is the culprit of enhanced protein synthesis via interaction with the mechanism of mTOR where leucine initiated the translation and elongation of mRNA (Anthony et al. 2000, 2001; Fujita et al.

2007; Wang and Proud 2006). Supplementation of leucine is able to enhance signal transduction by activating Ras homologue enriched in brain (Rheb) and mTOR (Figure 9.1). Rheb is a GTPase that promotes protein synthesis by enhancing the phosphorylation of S6 kinase (S6K1) and eIF4E-binding protein 1 (4E-BP1). Furthermore, Rheb is able to bind directly to mTOR in an amino acid-dependent manner to enhance mTOR activity (Long et al. 2005). Leucine is also able to enhance mTOR independent of Rheb, although its direct mechanism is yet unknown. Studies have reported that high supplementation of leucine was able to enhance phosphorylation of S6K1 and 4E-BP1 (Anthony et al. 2000; Fujita et al. 2007). 4E-BP1 activation results in the release of eIF4E, the mRNA cap-binding protein, to bind to eukaryotic initiation factor (eIF) 4E, which acts as a scaffolding protein to bridge eIF4E-mRNA complex and the 40 S ribosomal subunit. On the other hand, phosphorylation of S6K1 allows for activation of various proteins for the elongation of mRNA translation. Therefore, mTOR phosphorylation of 4E-BP1 and S6K1 affects



**Figure 9.1.** Mechanism of leucine via the mammalian target of rapamycin (mTOR) signaling pathway in mRNA translation. Intracellular concentration of leucine is able to activate mTOR complex to directly phosphorylate eIF4E-binding protein 1 (4E-BP1) and ribosomal protein S6 kinase (S6K1). Subsequent steps lead to activation of mRNA translation and elongation. In addition, leucine-activated mTOR complex was able to phosphorylate protein kinase B (Akt/PKB) for cell proliferation and survival. Lines ending in arrowheads denote activation and dashed lines represent incompletely defined steps. Rheb, Ras homologue enriched in brain.

the initiation and elongation stages of mRNA translation. Leucine supplementation studies have also revealed an increase in protein kinase B (Akt/PKB). The phosphorylation of Akt/PKB leads to the inhibition of tuberous sclerosis complex that inhibits the activity of mTOR (Sarbassov et al. 2005); thus, it is able to indirectly activate mTOR.

BCAA, in particular leucine, have received tremendous attention from its ability to influence protein metabolism. It is only recently that the mechanism by which protein synthesis occurs has been investigated. Leucine is able to promote mRNA translation by activating the mTOR complex to further activate Akt/PKB, 4E-BP1, and S6K1. Implications from BCAA supplementation have been useful for studying muscle-wasting diseases, cancer, liver cirrhosis, exercise-induced fatigue, and immunosuppressant diseases.

#### AMINO ACID RESPONSE PATHWAY

AAR pathway detects and adjusts to amino acid deficiency. In general, it is the “essential” amino acid(s) to the particular cell that will trigger this pathway, not necessarily for the whole organism (Kilberg et al. 2005). The AAR pathway begins with an increase in uncharged tRNA due to a limitation in the extracellular supply of amino acids, which binds to and activates the general control nonderepressible protein 2 (GCN2) kinase (Sood et al. 2000; Zhang et al. 2002). The activated GCN2 kinase then phosphorylates translation initiation factor, eIF2 $\alpha$ , to cause a general suppression of protein synthesis (Kimball 2002). Nonetheless, the mammalian activating transcription factor (ATF) 4, also known as the “master regulator” transcription factor in mammals, gene expression is upregulated (Scheuner et al. 2001). The activation of eIF-2 $\alpha$  and the short upstream open reading frames of the ATF4 mRNA are able to control the translational regulation of ATF4 (Lu et al. 2004). The full extent of ATF4’s role in mammalian gene regulation is yet to be discovered; however, it plays an essential role in the transcriptional control of many genes.

#### GENE REGULATION BY AMINO ACID STARVATION

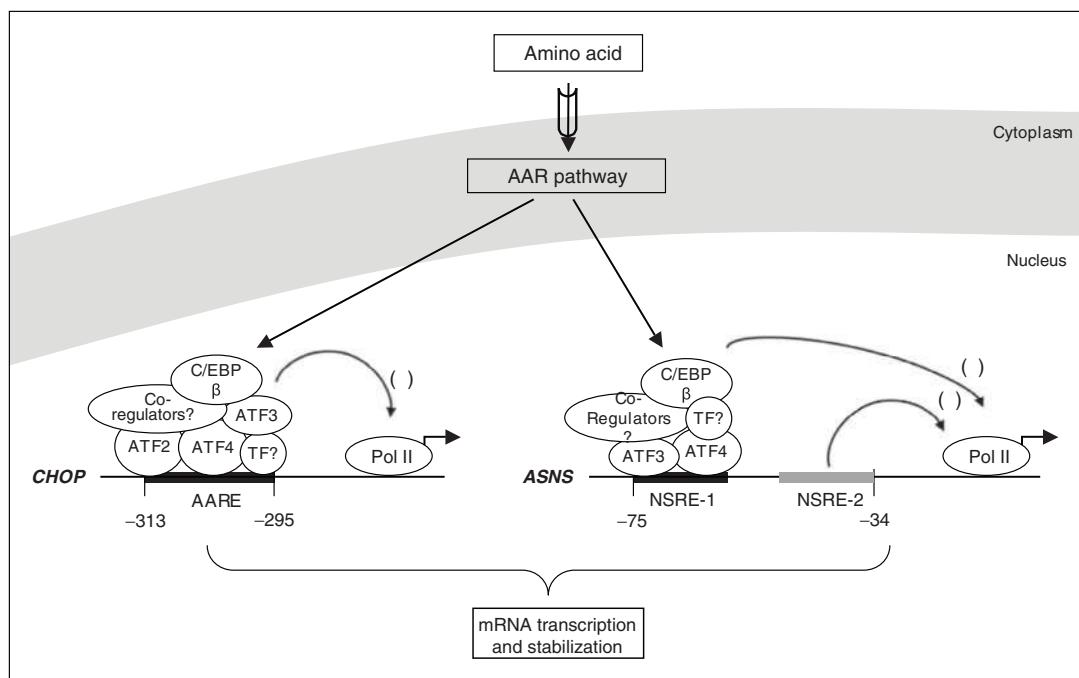
Amino acid starvation models have been used to investigate the changes that occur at the transcriptional and posttranscriptional level of gene regulation. The amino acid response pathway is stimulated upon amino acid starvation, which triggers a general

suppression of protein synthesis. However, selected genes have been documented to be upregulated by amino acid depletion. At the transcriptional level, amino acid starvation promoted the gene expression of CCAAT/enhancer binding protein (C/EBP) homologous protein (*CHOP*), asparagine synthetase (*ASNS*), and system A neutral amino acid transporter 2 (*SNAT2*), and their mechanisms have been well investigated. Furthermore, amino acid starvations induce posttranscriptional regulation of gene expression, as found in cationic amino acid transporter-1 (*Cat1*) and mTOR pathway. Amino acids likely have a myriad control of gene expression in mammals but the molecular mechanisms involved are presently not fully understood.

#### HUMAN CHOP GENE REGULATION

*CHOP* is known as a growth arrest-DNA damage-inducible 153 (GADD153) protein and its expression is enhanced by various stress stimuli, especially to endoplasmic reticulum stress (Zinszner et al. 1998). *CHOP* expression is associated with cell-cycle arrest and apoptosis during cellular stress. Recently, amino acid deprivation has been shown to induce *CHOP* mRNA and protein expression, both transcriptionally and posttranscriptionally (Abcouwer et al. 1999; Jousse et al. 1999). Protein degradation found in certain diseased conditions may follow AAR pathway for the loss of muscle mass and body weight.

In the work by A. Bruhat group, the location of amino acid response element (AARE) in the *CHOP* promoter was identified to be between -313 and -295 (Bruhat et al. 2000). The AARE provides a site that is essential for several individual amino acid regulation of *CHOP* expression (Figure 9.2). The AARE has the ability to bind ATF2, ATF3, and ATF4, C/EBP $\beta$ , and other coregulators and unknown transcription factors. The key transcription factors mandatory for amino acid responsiveness is ATF2 and ATF4 where the loss of their expression eradicated *CHOP* expression by amino acid deprivation (Averous et al. 2004; Bruhat et al. 2000). Following amino acid starvation, ATF2 and ATF4 are activated and become phosphorylated (Averous et al. 2004). Using chromatin immunoprecipitation, Bruhat et al. (2007) showed that phospho-ATF2 and ATF4 are associated with acetylation of histones H4 and H2B in a time-dependent manner. In addition, Bruhat et al. (2007) showed that ATF-4 binding and histone acetylation are two independent occurrences that are necessary for amino acid regulation of *CHOP*. Therefore, ATF-2 activation by amino acid starvation results in the modification of the chromatic structure to promote the



**Figure 9.2.** Scheme for the transcriptional activation of mammalian gene C/EBP homologous protein (*CHOP*) and asparagine synthetase (*ASNS*) by amino acid starvation on a *cis*-regulatory sequence in the amino acid response element (AARE) and nutrient-sensing response elements (NSRE) 1 and 2. Following amino acid starvation, amino acid response (AAR) pathway promotes activating transcription factors (ATF) 2, 3, and 4, unknown transcription factor (TF?), CCAAT/enhancer binding protein (C/EBP), and other coregulators to bind to AARE for the transcriptional activation of *CHOP* and *ASNS*. P, phosphate; Pol II, RNA polymerase II.

transcription of *CHOP*. On the other hand, ATF4 interacts with a cofactor p300/CBP-associated factor (PCAF) to increase the histone acetylation of *CHOP* (Cherasse et al. 2007). A recent *in vitro* study demonstrated that Jun dimerization protein-2 (JDP2) acted as a repressor of the *CHOP* gene where amino acid abundance revealed JDP2 bound to the AARE of the *CHOP* gene. However, upon amino acid starvation, the binding of JDP2 decreased (Cherasse et al. 2008).

#### HUMAN ASPARAGINE SYNTHETASE GENE REGULATION

*ASNS* catalyzes the biosynthesis of asparagine and glutamate from aspartate and glutamine (Richards and Schuster 1998). The human *ASNS* gene is transcriptionally regulated by amino acid starvation via AAR pathway and endoplasmic reticulum stress response pathway. In the promoter region of *ASNS*, nutrition-sensing response elements (NSRE)-1 and -2 are present between nucleotides -70 to -34 that are highly sensitive to amino acid starva-

tion (Barbosa-Tessmann et al. 2000). NSRE-1 (5'-TGATGAAAC-3', nucleotides -68 to -60) and NSRE-2 (5'-GTTACA-3', nucleotides -48 to -43) are both required for activation of the human *ASNS* gene following the AAR pathway by amino acid deprivation (Barbosa-Tessmann et al. 2000). NSRE-1 and -2 function and bind very similar to AARE of *CHOP*; however, since *ASNS* transcription is sensitive to both amino acid and glucose deprivation, it is termed NSRE rather than AARE. The transcription factors ATF4, C/EBP $\beta$ , and ATF3-full length (ATF3-FL) are involved with NSRE-1 to increase the expression of *ASNS* during amino acid starvation. Chen et al. (2004) showed the temporal sequence of transcription factors and their role in *ASNS* regulation. During phase I of amino acid deprived regulation of *ASNS* gene (0–4 h), there was an increase in binding of ATF4 and constitutively low binding of C/EBP $\beta$ . Consequently, there is an increased acetylation of histones H3 and H4, promoting the binding of various transcription factors, coactivators, and RNA polymerase II (Pol II). However, during phase II of amino

acid deprivation (4–24 h), ATF3-FL binds to NSRE-1 antagonistically (Fairley et al. 2002) to suppress, but not completely reverse, ASNS gene expression. The binding of C/EBP $\beta$  and ATF3 $\Delta$  Zp3 to NSRE-1 is also significantly increased during this phase. The end result is still an increased transcription of *ASNS* gene but at a reduced rate than during phase I. Therefore, the increased binding of ATF3-FL and C/EBP $\beta$  results in a suppression of ATF4-dependent upregulation of *ASNS* gene.

The *cis*-acting elements required for the induction of *CHOP* and *ASNS* are shared because the nucleotide sequence on *CHOP* AARE and *ASNS* NSRE-1 are similar, only differing by a few nucleotides (Bruhat and Fafournoux 2001). However, *CHOP* AARE can function independently whereas *ASNS* NSRE-1 functions weakly without the presence of NSRE-2 (Bruhat et al. 2002). The binding proteins of NSRE-2 are presently unknown but it is required for the amino acid-deprived regulation of *ASNS* gene control.

#### AMINO ACID REGULATION OF TRANSPORTERS

There are several transmembrane amino acid transporters to assist its entrance into cells. It has been previously reported that the expression of some amino acid transporters such as *Cat1*, *SNAT2*, and cystine/glutamate transporter (xCT) are regulated by amino acid availability (Lopez et al. 2007). Thus, the availability of the transporter substrate is able to regulate the expression of its transporter.

The amino acid regulation of *Cat1* has been well documented where control mechanisms have been found in transcription: mRNA degradation and translation level (Hatzoglou et al. 2004). *Cat1* is a system Y $^{+}$  transporter for arginine and lysine and the AARE has been found in the first exon. Following an amino acid deficient diet, the  $\alpha$  subunit of eIF2 becomes phosphorylated (Jefferson and Kimball 2001). The phosphorylation of eIF2 decreases the translation of most mRNAs by inhibiting the delivery of the initiator methionyl-tRNA (Met-tRNA) to the initiation complex; however, it can cause increased translation of certain mRNAs, such as ATF4 (Lu et al. 2004), ATF3 (Jiang et al. 2004), and C/EBP $\beta$ . ATF4 has been found to generally activate the expression of *Cat1* to where ATF3 was shown to suppress *Cat1*. Thus, *Cat1* transcription can be regulated at the transcriptional level via binding of transcriptional factors as well as phosphorylation of eIF2 (Lopez et al. 2007). The phosphorylation of eIF2 is also able to control the translation of *Cat1* mRNA via internal ribosome entry site (IRES). IRES can be activated

by translation of a small open reading frame within the *Cat1* mRNA ladder, which causes a conformation change to form the active IRES (Yaman et al. 2003). The transcriptional and translational control of *Cat1* is similar to *CHOP* and *ASNS* but has overlapping binding proteins.

More recently, other amino acid transporters have received attention regarding amino acid deprivation control of its gene regulation. For example, *SNAT2* mRNA level and activity during amino acid starvation requires phosphorylation of eIF2 $\alpha$  and internal ribosome entry site-mediated translation (Gaccioli et al. 2006). Although the mechanisms are similar, they are not identical. The location of the AARE differ; for example, *SNAT2* AARE is found in the intron (Palii et al. 2004), xCT AARE is in the promoter region (Sato et al. 2004), and *Cat1* is found in the exon (Lopez et al. 2007). In a state of cellular stress by amino acid deprivation, most protein synthesis is suppressed. However, through the AARE found in selected genes, they may provide the cells with an attempt to replenish the amino acid concentration by increasing the expression of amino acid transporters.

#### HEALTH IMPACT OF AMINO ACIDS

Amino acids are intricately involved in many metabolic pathways as substrates and regulators. Their concentrations have been measured frequently from blood, urine, and amniotic fluid to obtain nutritional and biochemical information of various disease conditions (Noguchi et al. 2006). An imbalance or abnormalities in amino acid concentrations have been associated with physiologic conditions, such as liver failure, renal failure, cancer, diabetes, muscle dysfunction, and aminoacidemia (Cynober 2004). The amino acid imbalance found in diseased conditions may further contribute to the disease pathology by inducing the stress response in cells to suppress protein synthesis.

There have been attempts to use amino acid concentrations as a diagnostic tool to understand their profile for certain diseased states. For example, the Fischer's ratio measures the ratio between BCAA and aromatic amino acids and is established as a diagnostic marker to monitor the development of liver fibrosis (Kano et al. 1991). Reduced Fischer's ratio indicates worsened liver damage via elevated aromatic amino acids (Shiota et al. 1984). In cancer patients, the overall plasma-free amino acid profile is decreased due to hypermetabolic state required for cancer growth (Lai et al. 2005). Depending on the

location of the cancer, the plasma-free amino acid profile varies. In muscle dysfunction, BCAA play a major role by making up the bulk of muscle protein. BCAA become reduced during extended exercise and muscle diseases due to an increase in protein breakdown in the skeletal muscles. Studies have shown that BCAA supplementation promotes protein synthesis and inhibits protein degradation as well as being the building block via the mTOR pathway (Bolster et al. 2004). Amino acid profiling becomes important in other diseases such as diabetes, renal disease, aminoacidemia, and others. However, the underlying mechanism of action of amino acid impact in disease and health is just beginning to be uncovered. The amino acids and their metabolites likely initiate a series of cell signaling and gene-regulating pathways to augment the amino acid balance in disease pathogenesis.

### AMINO ACIDS AND IMMUNE FUNCTION

The immune system plays an important role in providing protection to the organism from infectious diseases and through wound healing. The immune system can also play a role in disease pathogenesis of autoimmune diseases and cancer. New emerging studies have shown that dietary protein or amino acid imbalance can impair the function of the immune system by (1) regulating the activation of immune cells; (2) producing antibodies, cytokines, and other cytotoxic substances; and (3) cellular redox state and gene expression (Li et al. 2007). In a state of disease, malnutrition and altered amino acid metabolism may contribute to the imbalance of free amino acids, which can impair the function of the immune system. For example, insufficient dietary intake of BCAA reduced the lymphocyte-mediated lysis of tumor cells by 80–90% (Jose and Good 1973) and increased susceptibility to *Salmonella typhimurium* in mice and reduced antibody production (Petro and Bhattacharjee 1981). BCAA availability is crucial for the synthesis of cytokines and antibodies because their absence completely halts protein, DNA, and RNA synthesis (Dauphinais and Waithe 1977; Waithe et al. 1975). Leucine in particular has the greatest impact on immune function (Konashi et al. 2000) likely due to its regulatory function via the mTOR pathway (Meijer and Dubbelhuis 2004). Furthermore, cysteine depletion, through the synthesis of glutathione (GSH), was able to regulate the cellular signaling pathways in response to H<sub>2</sub>O<sub>2</sub> immunological challenge by suppressing NF- $\kappa$ B transcription activation and DNA methylation (Fratelli et al. 2005). In addition, asparagine played an important role in regulating

T-lymphocyte functions. In mice, a diet deficient in L-asparagine revealed T-lymphocyte cell-cycle arrest in the G<sub>0</sub>–G<sub>1</sub> phase, contributing to a weak immune system (Rodriguez et al. 2007). Lastly, autoimmune inflammatory diseases can benefit from immunosuppressive amino acids such as tryptophan. Tryptophan and its catabolites provide local immunosuppressive environments to control T-cell homeostasis and self-tolerance by downregulating the mRNA expression of T helper I cytokines (Platten et al. 2005). There is emerging evidence of amino acids and amino acid metabolites playing a role at the genetic level of the immune system. The cell signaling mechanisms have begun to be exposed but the impact at the genetic level remains mostly unknown. Future studies addressing the genetic regulation of amino acids in the immune system may play an important role in dictating the state of health and disease.

Glutamine is the most abundant free amino acid in the body and is known to play a regulatory role at the gene and protein level in several cell-specific processes including metabolism (e.g., oxidative fuel, gluconeogenic precursor, and lipogenic precursor), cell integrity (survival, cell proliferation), protein synthesis and degradation, redox potential, respiratory burst, insulin resistance, insulin secretion, and extracellular matrix synthesis. Glutamine has been shown to regulate the expression of many genes related to metabolism, signal transduction, cell defense, and repair and to activate intracellular signaling pathways (Curi et al. 2005). Glutamine is a precursor for the synthesis of glutathione and stimulates the formation of heat-shock proteins. Moreover, there are suggestions that glutamine plays a crucial role in osmotic regulation of cell volume and causes phosphorylation of proteins, both of which may stimulate intracellular protein synthesis (Roth 2007). Glutamine depletion in the cultivation medium decreases the mitogen-inducible proliferation of lymphocytes, possibly by arresting the cells in the G<sub>0</sub>–G<sub>1</sub> phase of the cell cycle. Glutamine depletion in lymphocytes prevents the formation of signals necessary for late activation. In monocytes glutamine deprivation downregulates surface antigens responsible for antigen preservation and phagocytosis. One of the important functions of the gut is to prevent migration of bacteria and/or toxins from the gut lumen into the systemic circulation. Moreover, glutamine exerts a trophic effect on the intestinal mucosa and decreases the intestinal permeability, and thus may prevent the translocation of bacteria (Roth et al. 1996).

Nitric oxide (NO) is a signaling molecule that plays a key role in the pathogenesis of inflammation. It gives an anti-inflammatory effect under

normal physiological conditions. On the other hand, NO is considered as a proinflammatory mediator that induces inflammation due to over production in abnormal situations. NO is believed to induce vasodilatation in cardiovascular system, and furthermore, it involves in immune responses by cytokine-activated macrophages, which release NO in high concentrations. In addition, NO is a potent neurotransmitter at the neuron synapses and contributes to the regulation of apoptosis. NO is involved in the pathogenesis of inflammatory disorders of the joint, gut, and lungs. Therefore, NO inhibitors represent important therapeutic advance in the management of inflammatory diseases. Selective NO biosynthesis inhibitors and synthetic arginine analogues are proved to be used for the treatment of NO-induced inflammation (Sharma et al. 2007). Recent findings indicate that increased metabolism of L-arginine by myeloid cells can result in the impairment of lymphocyte responses to antigen during immune responses and tumor growth. Two enzymes that compete for L-arginine as a substrate—arginase and nitric oxide synthase—are crucial components of this lymphocyte-suppression pathway, and the metabolic products of these enzymes are important moderators of T-cell function (Bronte and Zanovello 2005).

Arginine, often found in immunonutrition regimens, is an important modulator of immune system activation. However, the mechanism of how arginine may be beneficial in immunonutrition is poorly understood. The metabolism of arginine is determined by the expression of the arginine-metabolizing enzymes, inducible nitric oxide synthase and two arginase isoforms (arginase I and II). Inducible nitric oxide synthase is induced by T helper I cytokines (interleukin (IL)-1, tumor necrosis factor, and gamma-interferon), while arginases are induced by T helper II cytokines and other immune regulators such as IL-4, -10, and -13, transforming growth factor-beta, and prostaglandin E2. Endotoxin induces inducible nitric oxide synthase and arginases I and II. Arginase plays an important role in the production of ornithine, a precursor of proline and polyamines, both of which are necessary for cellular proliferation and wound healing. Arginase also induces nitric oxide synthase activity by competing for arginine availability in the extracellular environment, and producing polyamines, which may modulate macrophage activation. Through limitation of arginine availability in the extracellular environment, arginases also potentially regulate other “arginine-dependent” immune functions such as T-lymphocyte activation, although this hypothesis remains to be proven. The availability of arginine during critical illness may be regulated by

arginase activity. Thus, arginase expression appears to be essential in the regulation of the cellular immune response and the inflammatory process during critical illness (Bansal and Ochoa 2003).

There is emerging evidence of amino acids and amino acid metabolites playing a role at the genetic level of the immune system. The cell signaling mechanisms have begun to be exposed but the impact at the genetic level remains mostly unknown. Future studies addressing the genetic regulation of amino acids in the immune system may play an important role in dictating the state of health and disease.

#### AMINO ACIDS AND OXIDATIVE STRESS

Alleviation of oxidative stress in the human biological system has been the focus of many health studies because oxidative stress is associated with disease progression of cardiovascular, central fatigue, and neurodegenerative diseases and also in prevention of aging. Amino acids may reduce oxidative stress by acting directly as free radical scavengers and indirectly through antioxidant metabolites and by preventing amino acid starvation-induced oxidative stress (Elsier et al. 2004). Although many amino acids possess antioxidant properties, key amino acids and their mechanism of actions will be explored.

Sulfur amino acids (methionine, cysteine, and taurine) hold very significant places among amino acids in controlling oxidative status. The two mechanisms of how sulfur amino acids reduce oxidative stress lie in the synthesis of intracellular antioxidants and in the methionine sulfoxide reductase antioxidant system. First, GSH is a tripeptide (L-glutamyl-L-cysteinyl-glycine) that acts as the most important intracellular antioxidant of the body (Oz et al. 2007). Its synthesis is limited by the cellular uptake of L-cysteine, and therefore, cysteine availability plays a crucial role in GSH synthesis (Shoveller et al. 2005). GSH functions as a direct scavenger of reactive oxygen species (ROS) and can prevent deleterious effects, such as lipid and protein oxidation and DNA strand-break damage and affect metabolic processes (Metayer et al. 2008). Protein and lipid oxidation may lead to cellular death by altering cellular protein and lipid metabolism and function (Petropoulos and Friguet 2005). Furthermore, GSH is able to influence the transcription process. GSH peroxidase catalyzes the GSH-dependent reduction of hydrogen peroxide while oxidizing GSH to GSH dimer (GSSG). The GSH/GSSG ratio and the concentration of intracellular GSH determines the redox status of the cell, which then regulates metabolic pathways by activating inhibiting enzymes and cellular genetic

processes. For instance, the DNA-binding capacity of PAX transcription factor family may be controlled by GSH/GSSG ratio through DNA-binding domain that contains two cysteine residues (Cao et al. 2005). Second, methionine acts to reduce oxidative stress by acting as a catalytic antioxidant through the methionine sulfoxide reductase (MSR) system (see Weissbach et al. 2005 for reviews). The MSR system utilizes methionine residues to act as scavengers of ROS. Mice lacking the *MsrA* gene were more sensitive to ROS, whereas transgenic flies overexpressing the *MsrA* gene had increased resistance to ROS (Metayer et al. 2008; Moskovitz et al. 1995). The MSR system involving methionine is the only method whereby protein damage via oxidative damage can be repaired (Metayer et al. 2008). Therefore, sulfur amino acids are able to reduce oxidant status by acting directly as a ROS scavenger and indirectly by altering cellular signaling and gene expression.

There are other amino acids that possess antioxidant properties and have the capability to reduce oxidant status. Histidine functions as an antioxidant in two distinct ways: (1) by intervening with metal ion redox reactions that produce hydroxyl radicals and (2) by direct interaction of a singlet oxygen and the histidine imidazole ring (Wade and Rucker 1998). The imidazole ring of histidine has shown to be the culprit of antioxidant activity of several biologically important dipeptides, such as carnosine (Guittot et al. 2005). Histidine and histidine-containing molecules have been widely applied in cardiovascular, neurodegenerative, gastrointestinal, and respiratory systems as an antioxidant and an anti-inflammatory agent (Wade and Rucker 1998). Furthermore, tryptophan metabolites, such as 5-hydroxytryptophan, 3-hydroxykynurenone, and melatonin, have shown to express antioxidant activities (Christen et al. 1990). Melatonin can regulate oxidative stress by regulating antioxidant enzyme activity, such as GSH peroxidase, superoxide dismutases, and catalase, and regulating the expression of these enzymes (Rodriguez et al. 2004). It has been shown that histidine and lysine inhibit oxidative stress-induced IL-8 production in intestinal epithelial cells (Caco-2 and HT-29).

Specific amino acids such as histidine exerted as inactivator of transcription factor, nuclear factor-kappaB (NF- $\kappa$ B), which is a regulatory element for IL-8 gene expression (Son et al. 2005). The protective effects of amino acids against H<sub>2</sub>O<sub>2</sub>-induced oxidative stress were investigated in an in vitro assay using human intestinal epithelial cells. The inhibition of H<sub>2</sub>O<sub>2</sub>-induced IL-8 secretion from Caco-2 cells was observed by pretreatment with cystine, va-

line, isoleucine, leucine, tryptophan, histidine, lysine, and alanine. Cystine enhanced glutathione (GSH) biosynthesis enzyme activity and increased cellular GSH levels. BCAA elevated activities of GSH S-transferase (GST) and catalase. Tryptophan, histidine, and lysine caused increases in GST activity. Alanine enhanced GSH reductase activity (Katayama and Mine 2007).

The abilities of certain amino acids to reduce oxidative stress has been well documented; however, it is only recently that their mechanisms of action and genomic interaction have been investigated. As future studies further probe gene interaction of amino acids and oxidative stress, the application of amino acids to target certain diseases may become more evident.

## CONCLUSIONS

Amino acids play a vital role in the synthesis of protein by acting as a substrate, and more recently have been discovered to regulate this process at the genetic level. The experimental data supporting the amino acid regulation of mammalian gene expression has been closely investigated mainly in the last decade and have provided valuable information. In this chapter, two main pathways are discussed: the mTOR pathway and the AAR pathway. The exact mechanism of how the cells sense the amino acid concentration is presently unknown; however, during amino acid starvation, high supplementation of leucine is able to have drastic alterations in protein synthesis. It is mainly essential amino acids in the cells that have a profound effect on genomic regulation. Amino acid deprivation leads to a general suppression in protein synthesis; however, certain genes such as *CHOP*, *ASNS*, *C/EBP*, and *ATF4* are upregulated. Many amino acid studies have reported health benefits during diseased states, such as cancer, inflammatory disorders, diabetes, gastrointestinal disorders, and muscular wasting diseases (Cynober 2004). Understanding the mechanism of amino acid control of genes, both singly and in unison, may provide its involvement in disease progression and prevention.

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# 10

## Functional Bioactive Proteins and Peptides in Nutrigenomics

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### BACKGROUND

#### DEFINITION

Bioactive peptides are peptide sequences present in the intact protein that under normal circumstances do not have biological properties, but when they are released as peptides *in vitro* (Korhonen and Pihlanto 2006) or *in vivo*, they exert biologically active properties. *In vitro* release can be achieved through the use of heat, acid or base, or proteolytic enzymes. *In vivo* release involves protein cleavage by various digestive enzymes in the gastrointestinal tract. Regardless of how the peptides are produced, it is of utmost importance to address how the peptides arrive at their site of action and if they are still in a biologically active state when they do so.

#### GASTROINTESTINAL PEPTIDE DIGESTION, ABSORPTION, AND TRANSPORT

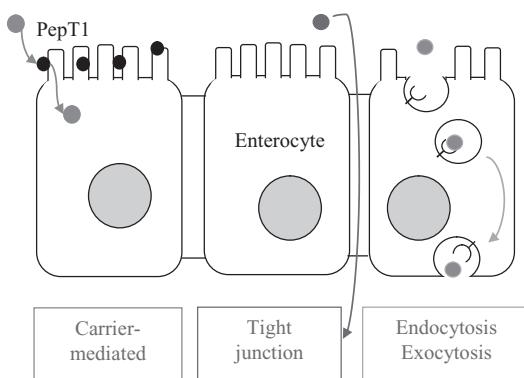
Bioactive peptides can be a natural breakdown product of proteins ingested, digested in the gastrointestinal tract, and absorbed by intestinal epithelial cells. Upon ingestion, the stomach's pepsin hydrolyses proteins into large oligopeptides, which then get cleaved into short di- or tripeptides and free amino acids by trypsin and chymotrypsin in the small intestine. These di- or tripeptides can be further digested by brush border membrane oligopeptidases and, if transported into the cell, by intracellular peptidases (Aito-Inoue et al. 2007; Ganapathy et al. 1994). Although this was the traditionally accepted pattern of digestion and absorption of protein, it is now known that not all peptides are fully broken down into amino acids. In fact, many dietary oligopeptides are transported intact across the apical side of the brush border membrane, past the basolateral side, and into the

bloodstream. As shown in Figure 10.1, there are three major transport routes that can result in intact peptide: (1) PepT1 carrier-mediated transport, (2) tight junction paracellular diffusion, and (3) endocytosis–exocytosis.

The main features of each peptide transport route are summarized in Table 10.1. PepT1 is an  $H^+$ -coupled peptide transport protein responsible for the absorption of small peptides after digestion of dietary protein in the small intestine (Daniel 2004). It specifically transports di- and tripeptides from the apical side of the epithelial membrane into the cell. Once the peptide is inside the cell, it can be cleaved by cytoplasmic peptidases to amino acids and then transported by amino acid transporters across the basolateral membrane (Ganapathy et al. 1994). Alternatively, depending on the peptide structure and composition, it can escape intracellular hydrolysis and be transported intact (Saito and Inui 1993). Paracellular transport involves the passive transport of oligopeptides via the pores formed by tight junctions (Tsukita et al. 2001). This is typically a nondegradative route whereby peptides remain intact. Oligopeptides that have affinity with the apical cell membrane can be transported via endocytosis and exocytosis (Shen et al. 1992). However, within the vesicle, many peptides are hydrolyzed into amino acids. Only the PepT1 carrier-mediated and the tight junction paracellular transport routes allow for the transport of intact peptides into the bloodstream.

### PRODUCTION OF BIOACTIVE PEPTIDES

The production of bioactive peptides by the body's digestion process is limited by the substrate



**Figure 10.1.** Intestinal transport routes for the transport of intact peptides.

specificities of pepsin, trypsin, and chymotrypsin. Pepsin cleaves after the N-terminal of aromatic amino acids such as phenylalanine and tyrosine (Fruton 1970), trypsin at the carboxyl side of lysine and arginine (Brown and Wold 1973), and chymotrypsin at the carboxyl side of tyrosine, tryptophan, and phenylalanine (Sweeny and Walker 1993). Since consumed proteins are usually in a food mixture of varying buffering capacities and pH, and there are a variety of brush border membranes and intracellular peptidases that can hydrolyze peptides even further, there may be inconsistencies in the amount and types of bioactive peptides released. To maximize control over the quantity and quality, and to discover or verify biological activities, peptides can be made *in vitro* using similar digestive enzymes and tested *in vitro* cell culture systems or *in vivo* animal models.

However, the more compelling research involves the production of novel bioactive peptides. This can be achieved by the use of heat, acid, or base hydrolysis, or by using proteolytic microorganisms or their proteolytic enzymes. Microbial enzymes are particularly interesting since they possess different substrate specificities than that of digestive enzymes; hence, new peptides can be produced which (depending on the amino acid sequence) may be able to escape cleavage by gastrointestinal enzymes and arrive intact at epithelial cells, travel in the bloodstream, and arrive at the target site.

In many cases, the use of proteolytic enzymes in the digestion of a protein results in a wide array of peptides with varying lengths and sequences. Although only a few peptides may exert bioactive properties, these peptides may be targeted for further production and concentration once they have been separated by HPLC and identified as possessing useful properties in cell cultures and animal trials. Figure 10.2 shows an overview of the steps involved in the fractionation of food-derived bioactive peptides. Membrane-based separation techniques as unit operations for general fractionation/purification process are powerful tools for peptide separation (Mine 2007). Membrane-based separation can be used as a preliminary step for the removal of enzyme and nonhydrolyzed proteins as well as for further fractionation of the peptide mixture.

## BIOACTIVE PEPTIDES

There is increasing commercial interest in the production of bioactive peptides from various sources such as egg, milk, cereal, and fish proteins. Bioactive

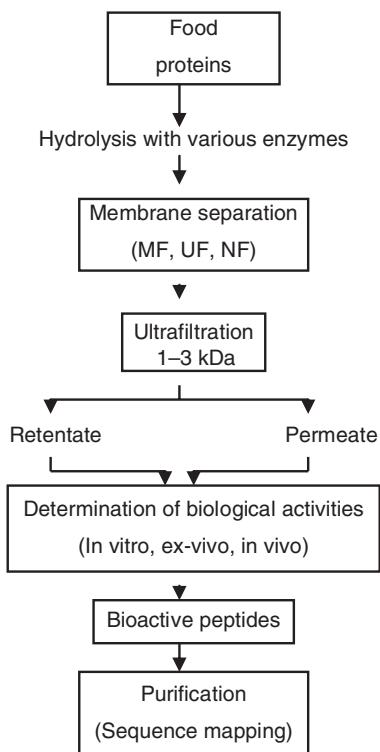
**Table 10.1.** Summary of the features associated with the peptide transport routes

	Carrier mediated	Paracellular	Transcytosis (endocytosis–exocytosis)
Type of transport	Active	Passive	Active
Molecules involved	PepT1 transporter <sup>a</sup>	Tight junction proteins	Epithelial cell membrane surface
Peptides transported	Di- and tripeptides only	Oligopeptides <sup>b</sup>	Basic and hydrophobic peptides
State of peptide at bloodstream	Some fully intact; others cleaved into amino acids	Intact	Very few intact; over 90% hydrolyzed into amino acids <sup>c</sup>

<sup>a</sup> Meredith and Boyd (1995).

<sup>b</sup> Pappenheimer et al. (1994).

<sup>c</sup> Heyman and Desjeux (1992).



**Figure 10.2.** Processing steps for production and purification of bioactive peptides from food proteins by enzymatic hydrolysis.

peptides from egg proteins can be obtained by in vitro hydrolysis using appropriate enzymes. For example, a number of peptides produced by enzymatic hydrolysis of egg white and yolk proteins have been shown to have various biological activities with high potential for pharmaceutical and nutraceutical applications (Kovacs-Nolan et al. 2005). Peptides and amino acids were first thought of as breakdown products of proteins and contributed to the body's energy supply and growth. After the discovery of the PepT1 intestinal peptide transporter and further knowledge of paracellular transport, it is now appreciated that some peptides can be absorbed intact and further transported to sites of need for use in energy metabolism or biological modulation via the alteration of gene expression. In fact, there is increased pharmaceutical research in developing cell-penetrating peptides that are able to deliver peptides, proteins, or viral vectors into the cytoplasm or nucleus to influence gene regulation (Jarver and Langel 2004). Although these particular peptides are synthetic, they highlight the fact that peptides can influence

biological systems, and understanding their mode of action is pertinent to unraveling their biological activities.

#### MECHANISMS OF ACTION OF BIOACTIVE PEPTIDES

The mechanisms of action of many bioactive peptides are not fully elucidated; however, their properties are broadly determined by the physiological effect they have on the body. Bioactive peptides can be broadly categorized into two categories: (1) peptides that exert their effects by direct physical interaction with another molecule and (2) peptides that interfere with gene expression.

#### Peptides that Directly Interfere with Other Molecules

Peptides in the first category include those with antihypertensive, antimicrobial, and mineral-sequestering properties. Antihypertensive peptides lower blood pressure within the body through inhibition of an enzyme or enzymes associated with blood pressure control, for example, angiotensin-converting enzyme (ACE) or endothelin-converting enzyme (Murray and FitzGerald 2007; Okitsu et al. 1995). ACE inhibition involves direct interaction of the peptide with noncatalytic binding sites in the enzyme (Murray and FitzGerald 2007). Antimicrobial peptides, in general, interfere with bacterial cell wall component to bring about cell death (Epand and Vogel 1999). Peptides with mineral-sequestering activities bind and solubilize ions, which can lead to increased bioavailability and intestinal absorption. A good example of this is casein phosphopeptides (CPPs) that sequester and solubilize  $\text{Ca}^{2+}$  ions (Lee et al. 1983) and phosvitin phosphopeptides that increase calcium and iron uptake (Jiang and Mine 2000, 2001).

#### Mechanisms for Peptide Effects on Gene Expression

Bioactive peptides that alter gene expression can do so by (1) epigenetic modification of the proteins that attach to the DNA, (2) alteration of the cell's primary signaling ligand to indirectly influence transcription factor activity, and (3) interference with cell signaling and gene expression via the direct binding of peptide ligand to receptor. Although there are a few bioactive peptides that can be classified into these categories, the goal of this chapter is not to provide a list of these

peptides, but rather to highlight specific peptides and discuss their effect on gene expression in relation to the mechanisms mentioned.

#### **Epigenetic modification**

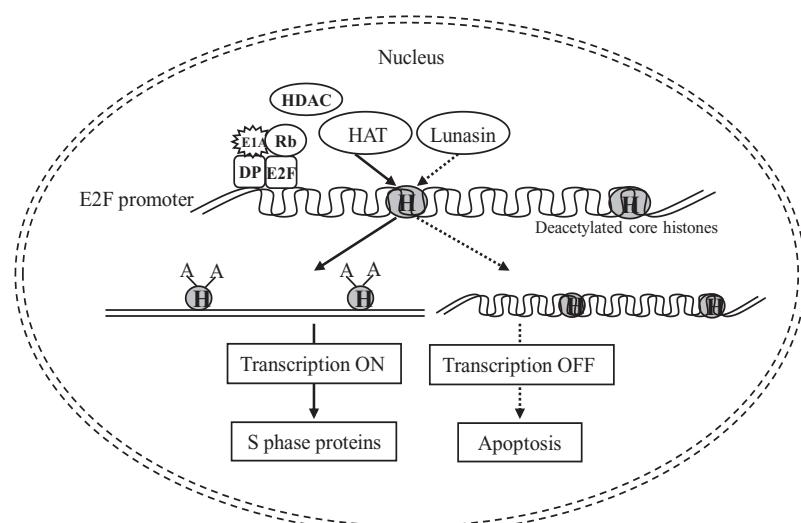
Epigenetic modification involves the alteration of histones in the chromatin of active or potentially active genes. Modifications such as acetylation, ubiquination, phosphorylation, and methylation of the histones determine whether specific regions of the chromatin are active or inactive (Jaskelioff and Peterson 2003; Khorasanizadeh 2004). Acetylation of histones H3 and H4 by histone acetyl transferase (HAT) enzymes results in opening of the chromatin structure, thereby allowing transcription to proceed (Brown et al. 2000; Carrozza et al. 2003).

Lunasin is a unique 43 amino acid soybean peptide, whose carboxyl end contains nine Asp (D) residues, an Arg-Gly-Asp cell adhesion motif, and a helix with structural homology to a conserved region of chromatin-binding proteins (Galvez 2001). It is this last property that prompted researchers to look at its potential role in suppressing carcinogenesis by arresting cell division and inducing apoptosis. Lunasin selectively kills cells that are being transformed or newly transformed by binding specifically to deacetylated histone substrates exposed by the transformation event and by inhibiting histone acetylation catalyzed by HATs (de Lumen 2005; Lam et al. 2003). This disturbance to histone acetylation–deacetylation is perceived as abnormal by the cell and results in cell death. Normal and established cancer

cell lines are not affected (Lam et al. 2003), making it a cancer-preventive rather than a cancer-curative agent.

As depicted in Figure 10.3, in a nonmutagenic cell, the retinoblastoma tumor suppressor protein, Rb, interacts with the E2F promoter (E2F/DP complex) and recruits histone deacetylase (HDAC). HDAC ensures core histones are in a hypoacetylated state so that they can interact with transcription factors that repress genes involved in carcinogenesis. Viral oncoproteins such as E1A disrupts the interaction between Rb and HDAC and displaces HDAC (Brehm et al. 1998; Magnaghi-Jaulin et al. 1998). When lunasin is present, it competes with HAT for the binding of the deacetylated core histones. If lunasin binds, the core histones remain deacetylated, transcription is turned off, triggering the cell to recognize this event as abnormal, and apoptosis ensues. If HAT binds, core histones are acetylated and the E2F transcription factors activate transcription and subsequent progression from the G1 to S phase of cell division (Lam et al. 2003). In normal cells, the Rb–HDAC complex keeps the core histones in the E2F deacetylated promoter and lunasin is physically unable to bind. In cells with established cancer, HAT is already bound to and has acetylated the core histones, leaving no opportunity for lunasin to act.

The efficacy of lunasin in suppressing carcinogenesis caused by chemical carcinogens and viral oncogenes has been proven in both *in vitro* cell culture and rodent models (de Lumen 2005; Galvez et al. 2001; Jeong et al. 2002; Lam et al. 2003). When



**Figure 10.3.** Epigenetic regulation of H3 and H4 histones by lunasin.

lunasin is administered orally, it is able to survive the gastrointestinal tract in sufficient amounts to maintain bioactivity, and in rats, it was found in an intact form in the blood and liver during long-term feeding (Jeong et al. 2002). Lunasin internalizes into cells within minutes of exogenous application and localizes in the nucleus after 18 h (de Lumen 2005). This bioactive soy peptide has great potential as a novel cancer chemopreventive agent and is quite unique in its role in regulating gene expression.

#### ***Indirect influence on transcription factor activity***

The binding of a ligand to an extracellular or intracellular receptor in a target cell triggers an initiating signal that activates transcription factors and other molecules necessary in the transcription process. Transcription factors then bind DNA at specific sites and activate gene transcription. Some ligands such as hormones or sterols are influenced by dietary composition; hence, their levels are not static. The ingestion of soy bioactive peptides can influence the hormonal balance and cholesterol concentration such that the amount of ligand (hormone, sterol) binding to the receptor is reduced and some genes are preferentially activated instead of others (Song et al. 2007).

Insulin and glucagon are two hormones with opposing activities that function in the maintenance of glucose and lipid homeostasis. When blood glucose levels are high, insulin promotes glucose uptake, glycogen storage, and increased fatty acid synthesis. However, when blood glucose levels are low, the pancreas releases glucagon, which influences the conversion of glycogen to glucose and the breakdown of amino acids and fatty acids into glucose. Both hormones modulate the expression levels of genes involved in cholesterol synthesis or uptake as well as fatty acid synthesis (Torres et al. 2006). These genes are primarily under the transcriptional control of the sterol regulatory element-binding proteins (SREBPs) (Brown and Goldstein 1997).

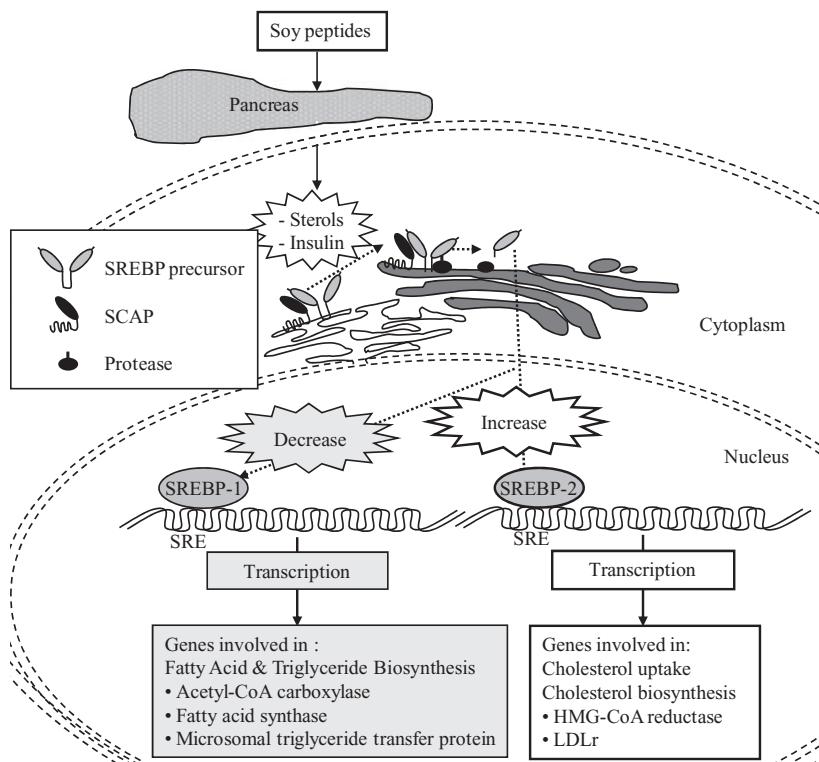
SREBPs are present in the endoplasmic reticulum (ER) in the form of a precursor protein. When cytoplasmic levels of sterols are low or insulin is present, another protein called the SREBP cleavage-activating protein (SCAP) escorts the SREBP precursor to the Golgi (Sakai et al. 1997). It is here that proteases cleave the SREBP precursor to an active form (Sakai et al. 1996; Wang et al. 1994) that is able to translocate to the nucleus and bind the sterol

response elements (SRE) in the promoter regions of genes involved in cholesterol, fatty acid, and triglyceride synthesis (Shimano 2001).

Of the three SREBP isoforms, SREBP-1a and -1c regulate lipogenic genes, while SREBP-2 more specifically regulates cholesterologenic genes (Horton et al. 1998; Shimano et al. 1999). SREBP-1c and -2 are subject to distinct forms of transcriptional regulation, whereas SREBP-1a is constitutively expressed at low levels in the liver and most tissues (Shimomura et al. 1997). Insulin tends to promote SREBP-1c expression and activation of fatty acid biosynthetic genes, but glucagon represses its expression. When (chole)sterol concentrations are low, SREBP-2 expression and activity increase.

As depicted in Figure 10.4, it is clear that diet influences the ratio of insulin to glucagon and the related expression of metabolic genes. In fact, studies have shown that rats fed a soy protein diet versus a casein diet had much lower short-term insulin concentrations (Ascencio et al. 2004; Tovar et al. 2002a), and over the long term had significantly higher glucagon concentrations (Torres et al. 2006). SREBP-1 expression was repressed and accordingly there was reduced expression of lipogenic genes. In addition to lowered insulin concentrations, soy ingestion results in decreased serum and hepatic cholesterol concentrations (Tovar et al. 2002b). When the liver senses lower cholesterol levels, SREBP-2 increases the expression of genes related to cholesterol uptake and biosynthesis, in particular, hydroxymethyl glutaryl-CoA reductase (HMG-CoA reductase) and the low-density lipoprotein receptor (LDLr) (Ascencio et al. 2004). In rats fed a soy diet, SREBP-2 expression was 119% higher than those fed a casein diet (Tovar et al. 2005). Soy is able to modulate the insulin/glucagon ratio may be because of its higher arginine/lysine ratio compared to animal proteins, since in some cases, a higher arginine/lysine ratio is correlated with higher glucagon levels (Radcliffe and Czajka-Narins 2000; Sanchez et al. 1988). The decrease in serum and hepatic cholesterol levels can be explained by an increase in LDLr (which is under SREBP-2 transcriptional control) as well as an increase in bile acid secretion (Potter 1995).

It is important to note that although many *in vivo* studies have used soy proteins, upon digestion and absorption in the gastrointestinal tract, many of these proteins are most likely digested to peptides. Of two primary soybean proteins,  $\beta$ -conglycinin and glycinin,  $\beta$ -conglycinin and its peptides have been found to upregulate LDLr activity (Lovati et al. 2000) and its oligopeptides have been found in the bloodstream (Matoba et al. 2001).



**Figure 10.4.** Indirect regulation of sterol regulatory element-binding proteins (SREBP) transcription factor by the ingestion of soy peptides.

#### **Regulation of cell signaling and transcription via the direct binding of peptide ligand to receptor**

In many cases, the binding of a specific ligand to a cell surface receptor initiates a series of cell signaling mechanisms that culminates in the activation of transcription factors, cofactors, and other proteins important in the transcription and expression of genes. The biological activity that a peptide exerts on a cell can be observed by a change in protein type and concentrations. After a peptide is administered to a cell culture or a host animal, supernatant, blood, or tissues are harvested for further analysis of biomarkers. These biomarkers pinpoint the biological activity that is affected but gives limited data regarding the mechanism of action. There are few peptides, however, in which further investigation has led to an increased understanding of how the genes underlying the activity are being regulated.

#### **Immunomodulation**

**Casein phosphopeptides** Casein phosphopeptides (CPPs) are phosphorylated peptide fragments resulting from the proteolysis of caseins by the action of

digestive enzymes. One commercially available CPP, CPP-III, consists of about 90% CPPs such as bovine  $\alpha$ s2-casein (1–32) and  $\beta$ -casein (1–28) and about 10% other casein peptides. CPP's role as a bioactive peptide lies in its immunostimulatory activity.

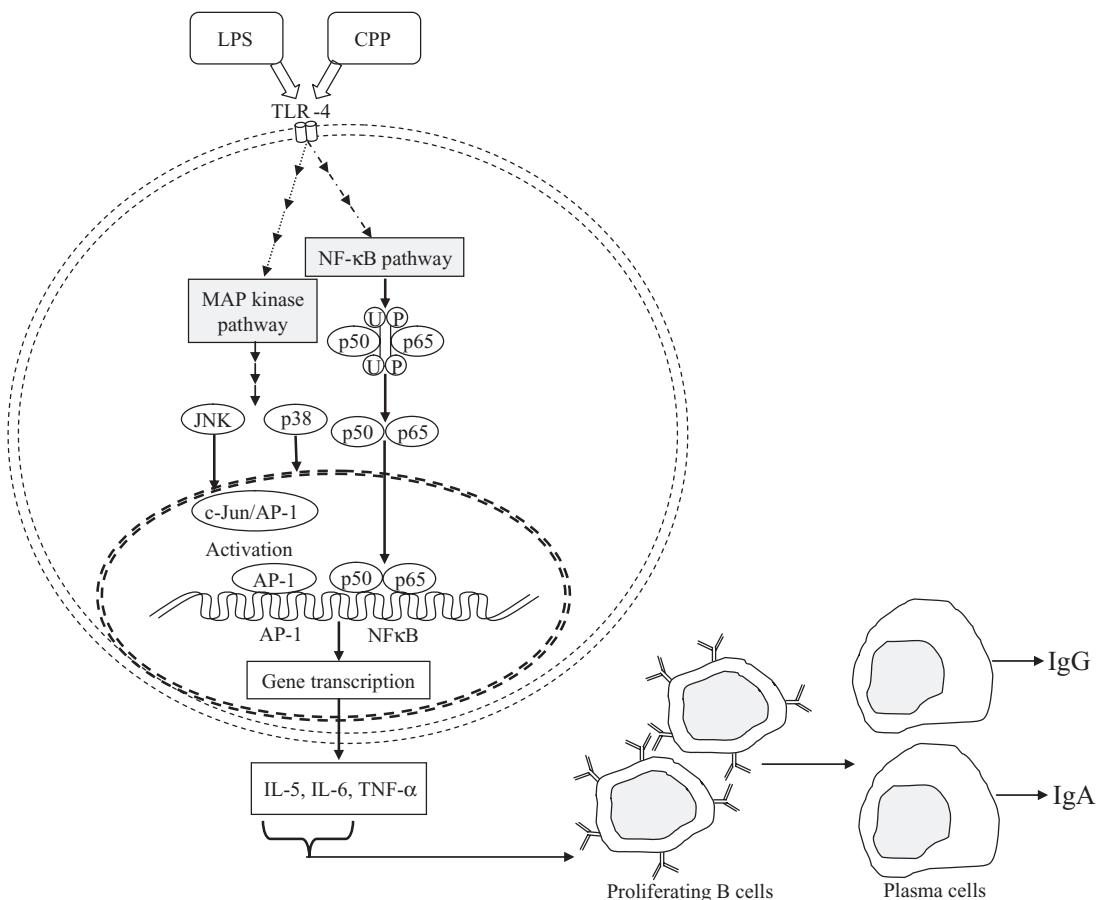
In vivo and ex vivo cell cultures and animal trials have shown that the administrations of CPP, CPP-III, or its peptide fragments have resulted in enhanced interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF- $\alpha$ ) mRNA (Kawahara and Otani 2004); increased production of IL-5 and IL-6 proteins (Otani 2001); significant stimulation of immunoglobulins in particular IgA and IgG (Hata et al. 1998; Kitamura and Otani 2002; Kitamura et al. 2002; Otani et al. 2000a, b); and enhanced proliferation of T cells, B cells, and monocyte cells (Kawahara et al. 2004). The increase in IgA concentrations is particularly interesting since these peptides may be able to enhance gut mucosal immunity. To support this further, in the presence of immune system stimulants such as bacterial peptidoglycan or lipopolysaccharide (LPS), the peptides' influence on IL-6 mRNA (Kawahara and Otani 2004), IL-5 and IL-6

proteins, and IgA is even more pronounced (Otani et al. 2003).

Several clues point to the peptides' mode of action. Otani et al. (2001) found CPP fragments containing the sequence SerP-X-SerP increased lymphocyte proliferation and immunoglobulin, in particular IgA production. The immunostimulatory activity of CPP-III is hardly influenced by peptic and pancreatic digestion, whereas it is significantly reduced by phosphatase action (Hata et al. 1999). In addition, the proliferation and IL-6 expression of CD19<sup>+</sup> spleen cells are deduced to occur via the interaction of the  $\beta$ -casein (1–28) peptides with Toll-like receptor 4 (TLR4) (Tobita et al. 2006).

The phosphoserine residue in the specific sequence of SerP-X-SerP appears to exert the most influence on biological activity and may form part of the ligand that physically interacts with TLR4. Since these peptides trigger the activation of naïve B CD19<sup>+</sup> lym-

phocytes without prior sensitization and result in enhanced proliferation, they act as mitogens, but without negative pyrogenic effects. Bacterial LPS commonly interacts with TLR4 to mediate cell signaling and enhanced expression of cytokines such as IL-6, IL-10, and TNF- $\alpha$  (Monodane et al. 2001; Schippers et al. 2005). Therefore, it is not unexpected to have an increase in IL-6 production in the presence of CPPs. In turn, IL-6 induces both proliferation and IgA production of B cells (Beagley et al. 1989). The increase in IL-5 discussed earlier is also important, since this cytokine is a growth factor for B cells and promotes their proliferation. Both IL-5 and IL-6 are necessary for the production of IgA (Yan et al. 1997). Figure 10.5 illustrates possible signal transduction mechanisms that bring about the immunostimulatory effects observed. Upon CPP interaction with TLR4, the B cell is activated and cell signaling pathways such as the mitogen-activated protein kinase (MAPK) and



**Figure 10.5.** Direct interaction of CPP with TLR4 on B cells. Not all cytokines or entire pathways are shown.

the nuclear factor kappa B (NF- $\kappa$ B) pathway can result in the expression of IL-5, IL-6, and TNF- $\alpha$  genes (Peng 2005). IL-5 and IL-6 interact with the B cell to promote proliferation and differentiation into plasma cells that release IgG or IgA (Kindt et al. 2007).

**Egg yolk digests** Some bioactive peptides that result from the enzymatic digest of food proteins can exert immunostimulatory properties on gut-associated lymphocytes (Low et al. 2003). In vivo studies using egg yolk protein digests have exhibited anti-infection activities in chickens by reducing the frequency of colonization of food-borne pathogens and preventing these organisms from colonizing the intestinal tract (Kassaify and Mine 2004a, b). It was found that the fractions conveyed an antiadhesive effect against the food-borne pathogens instead of a direct antimicrobial activity (Kassaify et al. 2005). The egg yolk protein digests induced an immune response with an increase in IgA+ cells, while orchestrating the Th1/Th2 response. The effect of the egg yolk digests on the small intestinal epithelial cells resulted in an increase in IL-6 secretion and this may have played an important role in preventing bacterial infection (Nelson et al. 2007).

**Carnosine** Carnosine ( $\beta$ -Ala-His), a dipeptide contained in skeletal muscles, is well known for its role as an antioxidant but its immunomodulatory role, especially as an potential anti-inflammatory agent, is emerging. This dipeptide is capable of reducing the inflammatory chemokine IL-8, in Caco-2 cells exposed to either hydrogen peroxide or TNF- $\alpha$  (Shimizu 2004; Son et al. 2008) by utilizing PepT1 to gain internal access and influence IL-8 suppression (Shimizu 2004). In most cases, mRNA expression is reflective of protein concentration; however, carnosine's reduction of IL-8 protein levels was not reflected in the IL-8 mRNA expression levels. In fact, the IL-8 mRNA levels were similar to positive hydrogen peroxide controls, but secretion levels were significantly inhibited. Since IL-8 inhibition by other histidine-containing dipeptides such as Gly-His, Ala-His, and anserine ( $\beta$ -Ala-1-methyl-His) resulted in IL-8 protein reduction following decreased IL-8 mRNA expression, carnosine may have influenced the posttranslational IL-8 expression. In the presence of hydrogen peroxide, the main regulatory initiation factor involved in the binding of mRNA to ribosome, eIF4E, is highly phosphorylated. However, pretreatment with carnosine inhibited this phosphorylation as well as phosphorylation of Akt, ERK1/2, and p38 MAPK. These results support carnosine's

role as a unique anti-inflammatory peptide (Son et al. 2008).

**Lactoferrin** Lactoferrin is an iron-binding glycoprotein that can be found in secretions such as breast milk, in epithelial secretions, and in the secondary granules of neutrophils. Lactoferrin is an iron-chelator and an antimicrobial agent, and exhibits a wide range of effects on cell growth and differentiation, embryonic development, myelopoiesis, endothelial cell adhesion, cytokine and chemokine production, immune system regulation, and immunomodulatory function (Severin and Wenshui 2005). Its recent role in promoting bone growth (Naot et al. 2005) is of great interest since it is able to provide novel nonpharmacological treatment options for patients suffering with osteoporosis.

Naot et al. (2005) found that at physiological concentrations, lactoferrin stimulates the proliferation and differentiation of primary osteoblasts and also acts as a survival factor-inhibiting apoptosis induced by serum withdrawal. Lactoferrin also inhibits osteoclastogenesis and in vivo studies measuring the hemicalvaria of adult mice results in significant increases in bone growth. Grey et al. (2006) found that lactoferrin's mechanism of action is via binding to the LDLr-related proteins-1 (LRP1) that play a role as an endocytic and signaling receptor (Herz et al. 2000; Li et al. 2001). Confocal laser scanning microscopy confirmed the presence of endocytosed lactoferrin in the osteoblastic cell cytoplasm. Lactoferrin is able to activate the p42/44 MAPK, which results in the mitogenic effect; however, this was found to be independent of endocytosis. When Cornish et al. (2006) examined the degree of glycosylation, iron-binding, and the structure-activity relationships of lactoferrin, they found that it is able to maintain its osteogenic activity in deglycosylated, holo, and apo forms, and with various small fragments of the molecule. This suggests that it is probable that lactoferrin signals through more than one membrane-bound receptor using diverse cell signaling pathways (Cornish et al. 2006).

**Val-Pro-Pro and Ile-Pro-Pro** Proteolytic hydrolysates of milk casein contain the bioactive tripeptides Val-Pro-Pro (VPP) and Ile-Pro-Pro (IPP). These peptides are known as ACE inhibitors that have been shown to improve vascular endothelial function (Hirota et al. 2007; Mizuno et al. 2005). In addition to these major functions, they have also been shown to play a role in bone formation and mineralization.

Narva et al. (2004, 2007) demonstrated that *Lactobacillus helveticus* fermented milk with IPP and VPP increased osteoblastic bone formation in vitro and in vivo using ovariectomized rats. A more in-depth examination of IPP, VPP, and another tripeptide Leu-Lys-Pro (LKP) on osteoblast proliferation and gene expression was conducted in UMR-106 osteosarcoma cells, human marrow-derived mesenchymal stem cells (hMSC), and osteoblasts differentiated from hMSC (Huttunen et al. 2007). IPP, in particular, showed the most promising results, increasing UMR-106 cell and hMSC proliferation and upregulating a wide range of cell differentiating, cell growth, and cell transcription genes including PThrP, cAMP response element-binding protein-5 (CREB-5), and bone morphogenetic proteins-5 (BMP-5). A long-term IPP treatment on bone formation and mineralization using hMSC-differentiated osteoblasts showed that IPP increases matrix formation-related osteoglycin expression and matrix mineralization and reduces apoptosis-related caspase-8 expression (Huttunen et al. 2008). In addition, the gene expression ratio of RANKL/osteoprotegerin was reduced, indicating decreased bone resorption. The influence of IPP is intriguing and is a great example of the role of bioactive peptides in promoting bone health.

**Trypsin inhibitors** The biological activities of a Kunitz trypsin inhibitor (KTI) and a Bowman–Birk trypsin inhibitor (BBI) recently attracted great interests as potent anti-inflammatory agents and as dietary supplements in the treatment of cancer metastasis. Soybean KTI and BBI inhibitors administered intraperitoneally and orally to mice given bacterial LPS-induced lethality were found to affect survival rates (Kobayashi et al. 2005a). In particular, KTI significantly reduced the LPS-induced lethality; decreased the LPS-induced expression of pro-inflammatory cytokines such as TNF- $\alpha$ , IL-1 $\beta$ , and IL-6; and inhibited LPS-activated ERK1/2, JNK, and p38 MAPK pathways in peritoneal macrophages. Similarly, in gingival fibroblasts, soybean KTI was able to abrogate LPS-induced upregulation of TNF- $\alpha$  mRNA and protein expression as well as reduce the induction of IL-6 and IL-1 $\beta$  proteins (Kobayashi et al. 2005b). The interference with TNF- $\alpha$  mRNA correlates with reduced activation of ERK1/2 and p38 MAPK, and interestingly, KTI pretreatment results in reduced NF- $\kappa$ B activity. These results support KTI's role as a novel anti-inflammatory agent.

The effect of oral administration of soy trypsin inhibitors was also examined in a mouse model of lung cancer and of peritoneal disseminated metastasis using human ovarian cancer cells, HRA (Kobayashi

et al. 2004). The prometastasis signaling in ovarian cancer cells can be triggered when transforming growth factor- $\beta$  (TGF- $\beta$ ) activates Src kinase, which in turn activates ERK-phosphatidylinositol 3-kinase (PI3)/Akt, the downstream targets of Src, and upregulates urokinase-type plasminogen activator (uPA) (Hirashima et al. 2003). The KTI-supplemented diet inhibited the formation of lung metastasis, but the antitumor effect was not direct. Rather, in the metastasis model, KTI reduced uPA protein expression, MAPK activation, and PI3 kinase proteins in the metastasis-promoting agonists: granulocyte colony-stimulating factor (G-CSF) and TGF- $\beta$ . KTI 4 may be a beneficial nutraceutical for ovarian cancer patients, since it reduces tumor burden by inhibiting MAPK and PI3 kinase phosphorylation and hence reduces the expression of uPA.

#### *Antioxidative stress activity*

Oxidative stress is a condition within cells whereby an increased level of free radicals results in a highly oxidized environment. Free radicals are highly reactive molecules with one or more unpaired electrons in their outer orbit; in most cases, this reactive molecule is oxygen and is referred to as reactive oxygen species (ROS). ROS represent the most important class of radical species generated in living systems (Miller et al. 1996) and are divided into free oxygen radicals and nonradical ROS. Free oxygen radicals include superoxide ( $O_2^-$ ), hydroxyl ( $\bullet OH$ ), nitric oxide ( $\bullet NO$ ), alkoxy- ( $RO\bullet$ ), or peroxy- ( $ROO\bullet$ ) radicals. Nonradical ROS include hydrogen peroxide ( $H_2O_2$ ), organic hydroperoxides ( $ROOH$ ) and hypochlorite ( $HOCl$ ). It has been estimated that the average person has approximately 10,000–20,000 free radicals in each cell each day (Valko et al. 2004). ROS are produced by neutrophils and macrophages during inflammation and are by-products of mitochondria-catalyzed electron transport reactions and other mechanisms (Valko et al. 2006). Many environmental factors are also implicated in the generation of ROS, including exposure to alcohol, cigarette smoke, atmosphere pollutants, UV light, toxins, and overexercise (Bunker 1992). In addition, free radicals and lipid hydroperoxides may either preexist in the diet or arise from polyunsaturated fatty acids (Aw 1998). Recent studies (Droge 2002) have revealed that ROS play an important role in several aspects of intracellular signaling regulation. Since some ROS are membrane permeable, they do not bind to a specific receptor, but instead act as signaling molecules by oxidizing the thiol moiety of sulphydryl-containing proteins involved in

signal transduction pathways. ROS can affect tyrosine kinases (Dalton et al. 1999), membrane receptors, and transcription factors (Arrigo 1999; Morel and Barouki 1999) and can stimulate the MAPK cell signaling pathways (Ogura and Kitamura 1998).

Recently, there has been considerable interest in the search for natural antioxidants. These include antioxidants that go beyond the quenching of ROS, and also upregulate the expression of proteins and enzymes involved in reducing oxidative stress. An abundance of antioxidants have been discovered from plant sources; however, egg yolk phosphopeptides have also been reported to have excellent antioxidative stress properties.

**Phosvitin oligophosphopeptides** Hen egg yolk phosvitin is a highly phosphorylated protein with a molecular weight of 35,000 Da that comprises 10% phosphorus and 6.5% carbohydrates (Taborsky and Mok 1967). It contains 123 serine (Ser) residues accounting for 57.5% of the total amino acid residues, and most of these Ser are monoesterified with phosphate. Jiang and Mine (2000) prepared phosvitin oligophosphopeptides (PPPs) with molecular weights of 1,000–3,000 Da from egg yolk phosvitin by partial alkaline dephosphorylation followed by trypic hydrolysis. PPPs with 35% phosphate retention enhanced calcium- and iron-binding ability and inhibited the formation of a phosphate precipitate, suggesting that PPPs can be looked upon as a potentially new nutraceutical that increases calcium and iron uptake in the intestinal tract (Feng and Mine 2006; Jiang and Mine 2001). It was further demonstrated that PPPs had free radical scavenging and antioxidant activities against lipid peroxidation (Xu et al. 2007).

Many studies strongly indicate that a variety of dietary antioxidants exhibit protective effects against oxidative injury. The protective effects of hen egg yolk PPPs against H<sub>2</sub>O<sub>2</sub>-induced oxidative stress were evaluated in vitro using human intestinal Caco-2 cells. Pretreatment of cells with PPP3 (a highly effective PPPs fraction) but not phosvitin resulted in an inhibition of H<sub>2</sub>O<sub>2</sub>-induced IL-8 secretion and decreased malondialdehyde levels. Not only was lipid peroxidation by-products reduced, but there was also an increase in intracellular glutathione levels, a significant increase in  $\gamma$ -glutamylcysteine synthetase ( $\gamma$ -GCS) activity and the expression of  $\gamma$ -GCS heavy subunit mRNA. In addition, intracellular glutathione reductase (GR), glutathione S-transferase (GST), and catalase activities were elevated by PPP3. As depicted in Figure 10.6, these data indicate that oligophosphopeptides from hen egg

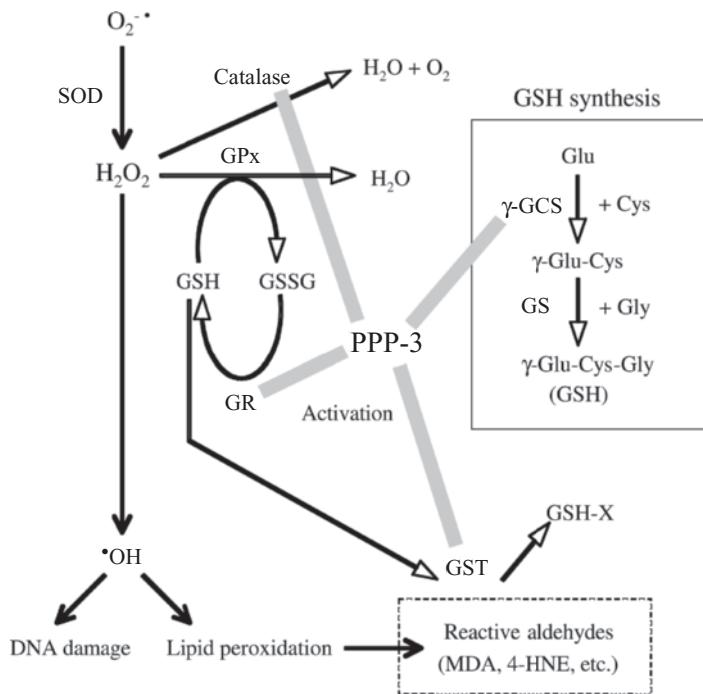
yolk phosvitin can upregulate cellular glutathione biosynthesis-associated enzyme activities as well as antioxidative stress activities, providing an effective defense against oxidative stress in human intestinal epithelial cells (Katayama et al. 2006; Katayama et al. 2007; Mine and Katayama 2008). It is probable that PPPs stimulate the transcription of antioxidant and detoxification defense systems such as  $\gamma$ -GCS, GST, GR, and GPx through antioxidant response elements (AREs) and do so through the activation of MAPK proteins (ERK, JNK, and p38), which subsequently phosphorylates the transcription factor, Nrf2; however; further studies are required to confirm this.

#### *Satiety (Antibesity)*

Leptin is a hormone secreted by adipocytes that plays a pivotal role in regulating food intake, energy expenditure, and neuroendocrine function. AMP-activated protein kinase (AMPK) is a key regulator of cellular energy balance and of the effects of leptin on food intake and fatty acid oxidation (Minokoshi et al. 2002). Obesity is usually associated with resistance to the effects of leptin on food intake and body weight. As such, both leptin and the AMPK pathways are of utmost importance for regulating appetite and ensuring overall energy balance.

**Black soy peptides** More recently, black soy peptides (BSPs) were investigated for its antibesity effects and its effects on signaling satiety (Jang et al. 2008). After feeding obese rats high-fat diets with an isoflavone-free peptide mixture, these rats gained less body weight than those without BSP. Since BSP-fed leptin-deficient ob/ob mice also ate less and had decreased body weight gain, leptin-like signaling may be involved. Indeed, BSP activated Janus kinase 2 (JAK2)-dependent STAT3 and increased the level of hypothalamic STAT3 phosphorylation in ob/ob mice. Since the leptin-mediated STAT3 phosphorylation pathway is of utmost importance in signaling satiety, BSP is activating leptin-like signaling to suppress food intake. In addition, BSP also phosphorylated AMPK and acetyl-CoA carboxylase of C2C12 myocytes, further complimenting the antibesity effects.

**Milk and soy peptides** Bioactive peptides from milk proteins can be derived from in vivo digestion or from enzymatic breakdown during starter culture fermentation. Dairy products have been known to reduce short-term food intake and contribute to a feeling of satiety (Aziz and Anderson 2007). The mechanism of action of casein and soy proteins and their hydrolyzates was recently determined (Pupovac and Anderson 2002). These peptides were found



**Figure 10.6.** Hypothetical mechanism of protective effect of PPP-3 against  $\text{H}_2\text{O}_2$ -induced oxidative stress. PPP-3 enhances the activation of GR and  $\gamma$ -GCS, key enzymes in GSH biosynthesis, and thereby maintains a high level of GSH in the cells. PPP-3 also increases catalase and GST activities, so that  $\text{H}_2\text{O}_2$  and reactive aldehydes derived from lipid peroxidation are detoxified. GS and 4-HNE denote GSH synthetase and 4-hydroxy-2-nonenal, respectively.

to independently activate opioid and cholecystokinin (CCK) receptors and contribute to satiety signaling. Nishi et al. (2003a) have also noted that soybean  $\beta$ -conglycinin hydrolyzates reduces food intake and gastric emptying by acting directly on the small intestinal mucosa to stimulate CCK release. In a follow-up study, it was found that the beta 51–63 fragment in  $\beta$ -conglycinin was responsible for binding the mucosa and releasing CCK, thereby reducing appetite (Nishi et al. 2003b).

## CLOSING REMARKS

Many researchers have reported that food proteins and their peptides expressed a variety of functions in the body, including a reduction of blood pressure, antimicrobial activity, antioxidative, anti-inflammatory, antisatiety, anticancer, antiobesity, antiallergy, modulation of immune cell functions, and regulation of nerve functions. The aim of this chapter was to highlight specific examples of the role bioactive peptides play in modulating and influencing gene expression. Understanding the behavior of dietary proteins and

peptides in the intestines is important for designing functional foods with physiological functions. With the advent of new proteomic and genomic techniques, the mechanisms underlying many of the biological properties of food-derived peptides may be soon revealed.

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# 11

## Antiobesity Effect of Allenic Carotenoid, Fucoxanthin

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### INTRODUCTION

Carotenoids represent a large group of isoprenoid structures with many different structural characteristics and biological activities. To date, a wide range of carotenoids have been isolated, identified, and quantified from the extracts of fruits and vegetables commonly consumed in the world. They are the most important pigments among those occurring in the nature that are responsible for various colors of different fruits, vegetables, and plant parts. The number of naturally occurring carotenoids reported continues to rise and has now reached more than 700. Animals including humans are incapable of synthesizing carotenoids and many are colored by carotenoids derived through their diets. More than 40 carotenoids may be available from the diet and absorbed, metabolized, or utilized by the human body.

The best-known biological function of carotenoids is their established role as provitamin A. Carotenoids such as  $\alpha$ - and  $\beta$ -carotene and  $\beta$ -cryptoxanthin can be converted to retinoic acid. Retinoic acid in their all-*trans* or 9-*cis* configuration is highly potent because of the activities of the retinoic acid receptors (RAR) and the retinoid-X receptors (RXR). By activation of these nuclear receptors, retinoic acids can influence the transcription of various retinoid-response genes (De Luca 1991). In addition, dietary carotenoids, including nonprovitamin A carotenoids, are considered to play a role in the prevention of common chronic diseases such as cardiovascular disease, age-related macular degeneration, and cancers (Cooper et al. 1999a, 1999b). Further, epidemiological studies established a positive correlation between carotenoid consumption and a reduced risk of cancer (Riboli and Norat 2003; Willett 2001).

Reactive oxygen species (ROS) and oxidative damage to biomolecules have been widely postulated to be involved in the cause and progression of several chronic diseases, including cancer and cardiovascular diseases. Carotenoids have been implicated as important dietary nutrients having antioxidant potential, being involved in the scavenging ROS, singlet molecular oxygen, and peroxy radicals generated in the process of peroxidation (Edge et al. 1997). The antioxidant properties of carotenoids have been suggested as being the main mechanism by which they afford their beneficial health effects (Giovannucci 1999; Hadley et al. 2002; Tapiero et al. 2004). Much of the research has focused on the potential role of the carotenoids as dietary antioxidants (Evans and Halliwell 2001; Halliwell 1996). There is little doubt that, under the right conditions, carotenoids can provide cells, tissues, and other structures such as lipoproteins with a degree of antioxidant protection (Astley et al. 2004; Collins 2001; Porrini and Riso 2000).

However, the true significance of the antioxidant capacity of carotenoids both under normal physiological conditions and conditions of oxidative stress remains unclear. Some might argue that the initial focus of carotenoids research on their role as antioxidants has set back progress in research into other biological activities of these compounds. While it remains entirely possible that some of their biological effects result from changes in cellular redox status through redox-sensitive cell-signaling pathways (Jackson et al. 2002), other mechanisms of action that are independent of their antioxidant properties are also likely to be important. The science behind the effects of dietary carotenoids on human health is complex, and there are unlikely to be simple and straightforward answers to the outstanding questions.

While modulation of transcriptional activity by carotenoids have been reviewed in their anticancer effect (Bertram and Vine 2005; Molnár et al. 2006; Palozza et al. 2004; Sharoni et al. 2003), in most cases, their underlying mechanisms of other action remain uncertain. Certain effects have been observed with provitamin A carotenoids that are not elicited by vitamin A itself (Rühl 2007). Equally, the non-provitamin A carotenoids are also capable of altering patterns of gene and protein expressions and cell function with a specific and important nutritional and biofunctional impact on the body (Chew and Park 2004). The nutritional functions of carotenoids depend on their chemical structures that differ depending on the length of the polyene, nature of the end group, and various substituents they contain. The specific regulation of a carotenoid on a particular biomolecule will be responsible for the characteristic physiological effect of the carotenoid.

In this chapter, we mainly describe the nutrigenomic study on the antiobesity effect of allenic carotenoids from seaweed and vegetables with special reference to its regulations on relative gene and protein expressions.

## CAROTENOID STRUCTURE

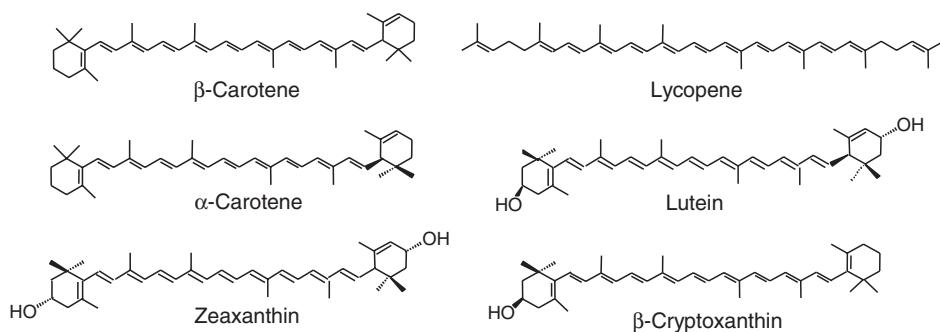
### GENERAL

Carotenoids are a class of hydrocarbons (carotenes) and their oxygenated derivatives (xanthophylls). They consist of eight isoprenoid ( $C_5H_8$ ) units joined in such a manner that the arrangement of isoprenoid units is reversed at the center of the molecule in such a way that the two central methyl groups are in a 1,6-position relationship and the remaining nonterminal methyl groups are in a 1,5-position relationship. All carotenoids may be formally derived from the acyclic  $C_{40}$  structure, having a long central chain

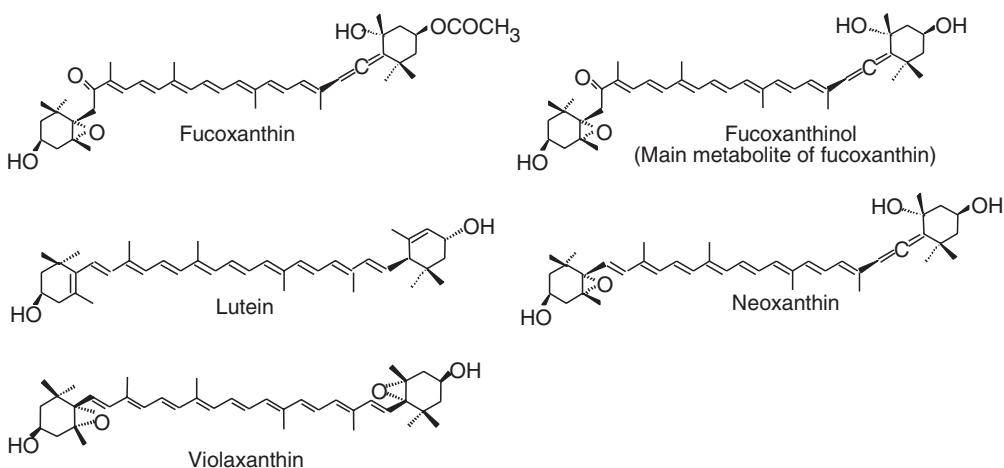
of conjugated double bonds. The extended system of conjugated double bonds in the carotenoid backbone is largely responsible for their color and antioxidant properties.

Typical  $C_{40}$  carotenoids carry cyclic  $\beta$ -ionone end groups that can be substituted by oxo, hydroxyl, and epoxy groups at different positions. The chemical structures of the six carotenoids found in the highest concentrations in human blood,  $\beta$ -carotene, lycopene,  $\alpha$ -carotene, lutein, zeaxanthin, and  $\beta$ -cryptoxanthin, are shown in Figure 11.1 (Basu et al. 2001). Owing to its two unsubstituted  $\beta$ -ionone rings at the ends of the isoprenoid chain,  $\beta$ -carotene is the carotenoid with the highest provitamin A activity, while other carotenoids such as  $\alpha$ -carotene,  $\gamma$ -carotene, and  $\beta$ -cryptoxanthin have lower provitamin A activities. On the other hand, the interest in the absorption and function of other carotenoids arose from epidemiological data, supporting the protective effects of carotenoid-rich vegetables and fruits against many degenerative diseases, including cardiovascular diseases, age-related macular degeneration, and some types of cancer.

Among the 700 known carotenoids in nature, only about 20 have been reported in human plasma and tissue (Tapiero et al. 2004). Lycopene is the most predominant carotenoid in human plasma and has a half-life of about 2–3 days. Lycopene is a nonprovitamin A carotenoid and one of the major carotenoids in Western diets, accounting for more than 50% of the carotenoids in human serum (Gerster 1997). However, carotenoids levels in foods vary; in individuals in Western societies, levels of  $\alpha$ - and  $\beta$ -carotene and lycopene are higher, while those of carotenoids such as lutein and zeaxanthin are much lower (Rühl 2007). Other than  $\beta$ -carotene and lycopene the absorption of other major carotenoids is not well known. The bioavailability of carotenoids is extremely variable, being influenced by many dietary and



**Figure 11.1.** Structure of main carotenoids found in human blood.



**Figure 11.2.** Structure of major carotenoids found in natural products.

physiological factors (Rao and Rao 2007). Recent studies have established a link between increased intake of carotenoids from plant sources with reduced incidences of chronic diseases. Although this positive link between decrease in a risk of cancer and higher intake and serum concentrations of  $\beta$ -carotene and lycopene is well studied, physiological characteristics of other kinds of carotenoids have received relatively less attention.

## ALLENIC CAROTENOIDS

The total production of carotenoids in nature has been estimated at 100 million tones per year (Britton 1995; Che Man and Tan 2003). The four major carotenoids occurring in nature are fucoxanthin, lutein, violaxanthin, and neoxanthin (Che Man and Tan 2003; Matsuno 2001) (Figure 11.2). Among them, lutein has been thoroughly reviewed with respect to its biological functions and possible health benefits (Seddon 2007). However, there have been a few studies on the physiological effects or beneficial applications of other kinds of carotenoids.

Fucoxanthin and neoxanthin (Figure 11.2) are the major carotenoids present in chloroplasts of brown seaweeds and higher plants, respectively. In dark green vegetables such as spinach,  $\beta$ -carotene, lutein, violaxanthin, and neoxanthin are the four most abundant carotenoids. Fucoxanthin is the most abundant of all carotenoids accounting for >10% of the estimated total natural production of carotenoids (Matsuno 2001). Seaweeds are a part of the staple diet in Japan, Korea, and China apart from being used as delicacies in some of the Western world. Seaweeds

have been used since ancient times as food, fodder fertilizer, and as sources of medicinal drugs. Today, seaweeds are the raw material for industrial production of agar, carrageenan, and alginates, but they continue to be widely consumed as food in Asian countries. They are nutritionally valuable, in both fresh as well as dried forms, as ingredients in a wide variety of prepared foods (Wong and Cheung 2000).

# INHIBITORY EFFECT OF ALLENIC CAROTENOID ON CANCER CELLS

Fucoxanthin and neoxanthin have a unique structure including an unusual allenic bond and 5,6-monoepoxide in its molecule (Figure 11.2). Of approximately 700 naturally occurring carotenoids, about 43 carotenoids contain the allene group. The principal allenic carotenoids are fucoxanthin in brown seaweeds and neoxanthin in higher plants (Dembitsky and Maoka 2007). Fucoxanthin has been reported to be very effective in inducing apoptosis in human leukemia cells (Hosokawa et al. 1999; Kotake-Nara et al. 2005) and colon cancer cells (Das et al. 2005; Hosokawa et al. 2004).

The strong inhibitory effect of fucoxanthin on the growth of cancer cells has been also confirmed using human prostate cancer cells (Kotake-Nara et al. 2001). In their study, the effect of 15 kinds of carotenoids (phytoene, phytofluene, lycopene,  $\beta$ -carotene,  $\beta$ -cryptoxanthin,  $\alpha$ -carotene, canthaxanthin, astaxanthin, capsanthin, lutein, zeaxanthin, violaxanthin, neoxanthin, and fucoxanthin) present in foodstuffs on the growth of the human prostate cell cancer lines (PC-3, DU 145 and LNCap) was

evaluated. Among the carotenoids evaluated, they reported neoxanthin and fucoxanthin to cause a remarkable reduction in the growth of prostate cancer cells.

DNA fragmentation revealed that these two carotenoids apparently reduced the cell viability by inducing apoptosis. Although other acyclic carotenoids such as phytofluene,  $\beta$ -carotene and lycopene also significantly reduced cell viability, the effect was lower than that of neoxanthin and fucoxanthin. Further, other carotenoids did not affect the growth of the prostate cancer cells. The higher activity of fucoxanthin and neoxanthin will be due to their characteristic chemical structure including allenic bond and other polar groups. The specific activity of allenic carotenoids has been more remarkably found in their antiobesity effect.

### ANTIOBESITY EFFECT OF FUCOXANTHIN

#### OBESITY AND DISEASE

The rise in the prevalence of obesity is now recognized as a worldwide problem, with ominous implications for public health and health-related costs. It may be the second most important preventable cause of death, exceeded only by cigarette smoking. Obesity is a potent risk factor for type-2 diabetes, hypertension, and dyslipidemia, comorbidities that markedly increase the risk of cardiovascular disease. Obesity is a multifunctional condition affected by the combined effects of genes, environment, and their interactions. Other important parameters considered to explain increase in obesity are food availability, increased dietary fat content, greater energy density of foods, and decreased physical activity. Thus, a large number of studies have investigated the role of food components for the prevention of obesity.

Adipocytes have an important role in energy homeostasis. Adipose tissue stores energy in the form of lipid and releases fatty acids in response to nutritional signals or energy insufficiency (Spiegelman and Flier 1996). Further, adipocytes have endocrine functions by secreting hormones and factors that regulate physiological functions, such as immune response, insulin sensitivity, and food intake (Frühbeck et al. 2001; Gregoire 2001). Excessive fat accumulation in the body and white adipose tissue (WAT) causes obesity and results in the increase of the risk for many serious diseases, including type-2 diabetes, hypertension, and heart disease. The regulation of adipose differentiation has been focused for the prevention of lifestyle-related diseases (Kopelman

2000; Visscher 2001), because adipose tissue development is closely related to adipocyte differentiation.

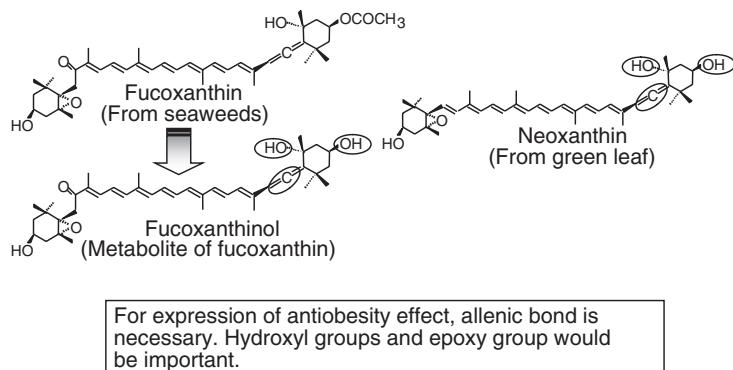
#### EFFECT OF ALLENIC CAROTENOID ON LIPID ACCUMULATION IN ADIPOSE CELLS

The biological functions of carotenoids are primarily determined by their chemical properties, and are therefore dependent on the general structure of the polyene chromophore, its substituents and end groups. When various carotenoids were screened for potential suppression effects on adipocyte differentiation (Maeda et al. 2006; Okada et al. 2008), only fucoxanthin, fucoxanthinol, and neoxanthin (Figure 11.2) showed an encouraging suppressive effect on the differentiation of 3T3-L1 adipose cells, while other carotenoids did not show such an effect. The above-mentioned three kinds of carotenoids significantly inhibited intercellular lipid accumulation during adipocyte differentiation of 3T3-L1 cells and significantly decreased glycerol-3-phosphate, an indicator of adipocyte differentiation, as compared with the control cells.

Studies of the uptake and metabolism of fucoxanthin in 3T3-L1 cells indicated that fucoxanthin added into the culture medium was incorporated in cells and further converted to fucoxanthinol by deacetylation within 24 h (Maeda et al. 2006). The levels of fucoxanthinol, but not fucoxanthin, increased in a time-dependent manner. In addition, the carotenoid accumulation in 3T3-L1 cells was greater following treatment with fucoxanthinol than after treatment with fucoxanthin. It has been reported that orally administrated fucoxanthin is detected as fucoxanthinol and amarouciaxanthin A in the blood and liver (Asai et al. 2004; Sugawara et al. 2002). Fucoxanthin is easily converted into fucoxanthinol in human intestinal cells and in mice (Sugawara et al. 2002), suggesting that the active form of fucoxanthin in biological system would be fucoxanthinol.

Interestingly, neoxanthin is very similar in structure to fucoxanthinol, which has been suggested to be the biologically active form of fucoxanthin (Figure 11.2). The only structural difference between neoxanthin and fucoxanthinol is the existence of a keto substituent at the end of the polyene chromophore of fucoxanthinol. Thus, it was hypothesized that the specific structure that both carotenoids contain is somewhat responsible for the suppressive effect on the adipocyte differentiation (Figure 11.3).

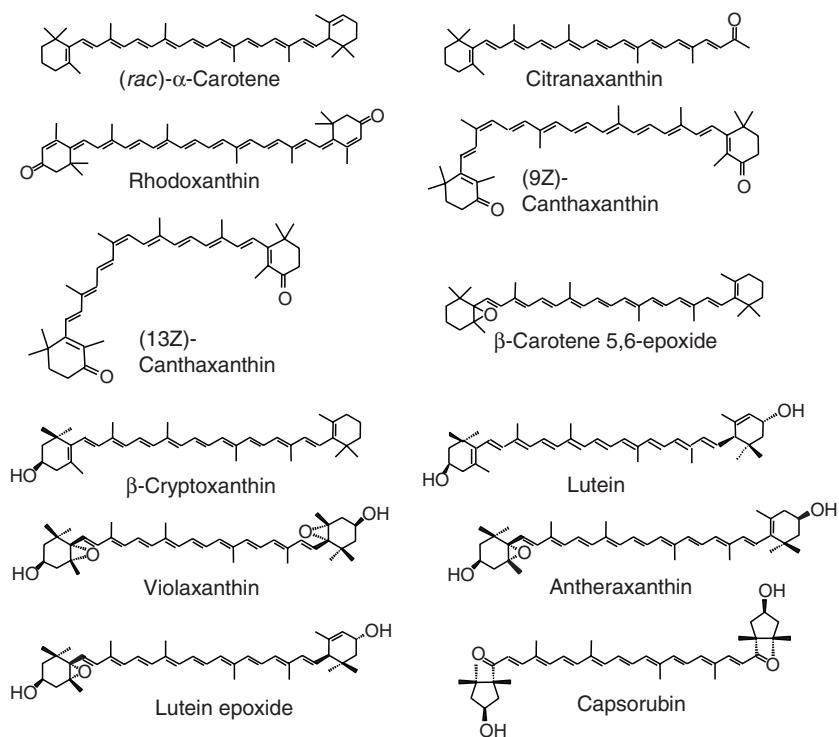
To test this theory, the effects of neoxanthin and additional 12 kinds of carotenoids (Figure 11.4) on adipose cell differentiation were analyzed (Okada et al. 2008). The result clearly indicated that only



**Figure 11.3.** Structure of carotenoids with no suppressive effect on the differentiation of 3T3-L1 adipose cell.

neoxanthin did show suppressive effects on lipid accumulation, glycerol-3-phosphate dehydrogenase (GPDH) activity, and aP2 expression in the 3T3-L1 differentiation. However, treatment with (rac)- $\alpha$ -carotene, carotenoids with keto group (citraxanthin, rhodoxanthin, canthaxanthin), and an epoxy group ( $\beta$ -carotene 5,6-epoxide) did not result in apparent changes in the level of GPDH activity. The same was true for hydroxyl carotenoid ( $\beta$ -cryptoxanthin, lutein), epoxy-hydroxy carotenoids

(violaxanthin, antheraxanthin, lutein epoxide), and keto-hydroxy carotenoids (capsorubin). These findings provide further evidence for the theory that suppressive effects on adipocyte differentiation in carotenoids are related to structural properties, where allenic bond is essential for the expression of the activity; however, carotenoids with an epoxy group, a keto group, or a keto and a hydroxyl group as part of the end group are not active without allenic bond.



**Figure 11.4.** Relationship between chemical structure of carotenoids and their antioesity effect.

When 3T3-L1 cells were treated with fucoxanthin, fucoxanthinol, and neoxanthin, PPAR $\gamma$ , a regulator of adipogenic gene expression, was downregulated by these carotenoids in a dose-dependent manner (Maeda et al. 2006; Okada et al. 2008). These results suggest that fucoxanthin and fucoxanthinol inhibit the adipocyte differentiation of 3T3-L1 cells through downregulation of PPAR $\gamma$ . PPAR $\gamma$  has an important role in the early stages of 3T3-L1 cell differentiation (Gregoire et al. 1998; Tontonoz et al. 1994), because it is a nuclear transcription factor that regulates adipogenic gene expression (Grimaldi 2001). Regulation of PPAR $\gamma$  would be one of the expected mechanisms underlying the antiobesity effect of these dietary carotenoids.

When the adipocyte differentiation cascade occurs within a cell, C/EBP $\beta$  and C/EBP $\delta$  expressions are initiated at the onset of differentiation, whereas regulation of C/EBP $\alpha$  production takes place in the later phase (Rosen et al. 2000). Subsequently, C/EBP $\alpha$  becomes the predominant family member in mature adipocytes, functioning in the activation and maintenance of adipocytes, along with concurrent reduction/disappearance of C/EBP $\beta$ . It has been reported that C/EBP $\beta$  was expressed immediately (within 2–4 h) after differentiation of 3T3-L1 preadipocytes, reached maximal levels within 4 h, and began to disappear 2 days after initiation of differentiation (Lane et al. 1999).

In addition, it has been well documented that C/EBP $\beta$  is a transcriptional activator of PPAR $\gamma$  (Elberg et al. 2000; Rosen et al. 2002; Wu et al. 1996) and that C/EBP $\alpha$  and PPAR $\gamma$  promote a level of adipogenesis and sustain each other's expression during maturation of adipocytes. C/EBP $\alpha$  and PPAR $\gamma$  contribute to adipogenesis by participating in the control of genes involved in lipogenesis, insulin sensitivity, and other pathways (Elberg et al. 2000; Wu et al. 1999). Neoxanthin treatment could have interfered with events occurring downstream from the C/EBP $\beta$ -induced transcriptional cascade and caused significant inhibition of C/EBP $\alpha$  and PPAR $\gamma$  mRNAs expression in a dose-dependent manner (Okada et al. 2008).

#### IN VIVO STUDY OF ANTOBESITY EFFECT OF FUcoxanthin

Fucoxanthin is the most characteristic pigment of brown seaweeds and little of other carotenoids is found in the brown seaweed lipids (Czeczuga and Taylor 1987), while neoxanthin is found in dark green leaves of vegetables together with other carotenoids such as  $\beta$ -carotene, lutein, and violaxanthin. Fucox-

anthin can relatively be easily prepared from brown seaweeds as pure form or crude seaweed lipid form containing more than 10% fucoxanthin (Hosokawa et al. 1999).

In the first in vivo study on antiobesity effect of seaweed carotenoid, fucoxanthin, lipids were separated from edible seaweed, Wakame (*Undaria pinnatifida*), which contained 10% fucoxanthin (Maeda et al. 2005). Fucoxanthin (0.2%) intake significantly reduced the weight of abdominal WAT (comprising perirenal and epididymal abdominal adipose tissues) of both rats and mice. Furthermore, body weight of mice fed fucoxanthin containing *Undaria* lipids was significantly ( $P < 0.05$ ) lower than that of control, although there was no significant difference in the mean daily intake of diet between both the groups. In order to confirm the active component of *Undaria* lipids, fucoxanthin-rich fraction and *Undaria* glycolipids fraction were administered to obese KK-A $y$  mice. The WAT weight of fucoxanthin-rich fraction-fed mice was significantly lower than that of control mice. However, there was no difference in WAT weight of mice fed *Undaria* glycolipids and control diet. This result indicates that fucoxanthin is an active component for antiobesity effect of *Undaria* lipids.

The antiobesity effect of fucoxanthin was also found in purified sample (Maeda et al. 2007a,b). By feeding with 0.2% fucoxanthin, body weight gain was significantly reduced compared with that of the control mice ( $P < 0.05$ ), although there was no difference in the amount of food intake. This reduction was consistent with the decrease in the weight of uterine, mesentery, perirenal, and retroperitoneal. Weight of WAT normalized by body weight in the mice fed 0.2% fucoxanthin was significantly lower than in control group. Further, the brown adipose tissue (BAT) weight normalized by body weight, which is related to energy expenditure, was increased in the mice fed 0.1 and 0.2% fucoxanthin compared with the control group. Other tissue weights were not affected by fucoxanthin.

#### UCP1 AS IMPORTANT MOLECULAR TARGET FOR ANTOBESITY

Many functional foods have been shown to alter energy metabolism of fat partitioning by influencing the substrate utilization or thermogenesis (Kovacs and Mela 2006; St-Onge 2005). These may not influence the absorption of the nutrients, but they act postabsorptively and increase the oxidation rate. Nutritional antiobesity products commonly focus on limiting fat digestion and/or absorption (i.e., structured lipids, nondigestible fats, lipase inhibitors, fat absorbers)

or enhancing fat catabolism. Newer products do not focus on limiting fat digestion and absorption but on edible oils that have an impact on fat metabolism through inherent differences in their natural digestion and absorption. Such products include diacylglycerol (DAG)-rich oils (Flickinger 2006; Flickinger and Matsuo 2003) and medium-chain triacylglycerol (MCT)-rich oils (Che Man and Manaf 2006). MCTs have been utilized extensively in special clinical settings such as fat malabsorption and treatment for burn patients for several decades.

Although regulation of fat digestion and/or absorption and stimulation of lipolysis and reduction of lipid synthesis in liver will be effective for antiobesity, the most important target has been recognized to be direct action on adipocytes. Especially, a great deal of interest has been focused on adaptive thermogenesis by uncoupling protein (UCP) families (UCP1, UCP2, and UCP3) as a physiological defense against obesity, hyperlipidemia, and diabetes (Dulloo and Samec 2001; Ježek 2002). UCPs are found in BAT (UCP1, UCP2, and UCP3), WAT (UCP2), skeletal muscle (UCP2 and UCP3), and brain (UCP4 and UCP5) (Dalgard and Pedersen 2001; Ježek 2002).

UCP2 and UCP3 are members of the mitochondrial anion carrier superfamily with high homology to UCP1, a well-characterized UCP playing a key role in facultative thermogenesis in rodents. Interest in UCPs increased with the discovery of proteins similar to UCP1, including UCP2 and UCP3. These proteins are expressed in tissues besides BAT and, thus, are candidates to influence energy efficiency and expenditure (Fleury et al. 1997; Ježek 2002). Since metabolic rate, metabolic efficiency, and obesity are integrated properties of the whole animal, researchers have produced mice lacking UCP2 (Arsenijevic et al. 2000) and UCP3 (Gong et al. 2000; Vidal-Puig et al. 2000). However, despite of UCP2 or UCP3, no consistent phenotypic abnormality was observed in the knockout mice. They were not obese and had normal thermogenesis. These results suggest that UCP2 and UCP3 are not a major determinant of metabolic rate in normal condition but, rather, have other functions (Arsenijevic et al. 2000; Harper et al. 2001; Ježek 2002; Lowell and Spiegelman 2000). Apart from UCP2 and UCP3, it is certain that UCP1 can potentially reduce excess abdominal fat (Nedergaard et al. 2001).

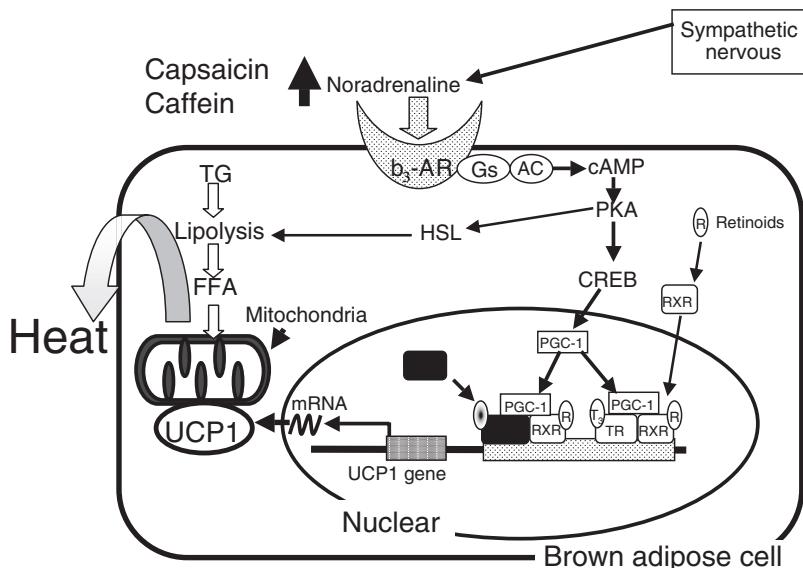
#### ADAPTIVE THERMOGENESIS IN BAT

Involvement of BAT in cold-induced thermogenesis is well established. Cold-induced nonshivering thermogenesis has been defined as an increase in

metabolism as a response to cold exposure in the absence of measurable shivering. The principal organ responsible for nonshivering thermogenesis in the rat is BAT, which adaptively increases its capacity for heat production in the course of cold acclimation, a process known as recruitment. This adaptive thermogenesis also plays an important role in energy balance, by dissipating excess energy intake as heat to resist body weight gain. The recruitment process of BAT involves the concerted activation of cell proliferation and cell differentiation, and thus involves changing the expression of numerous genes. These genes are under active study in several investigations in the realm of thermogenesis function, regulation, and the relationship with obesity.

Further, data from rodents have also demonstrated the role of BAT in diet-induced thermogenesis (Rothwell and Stock 1979; Smith and Horwitz 2005). Adaptive thermogenesis in BAT is mainly mediated by UCP1, an inner mitochondrial membrane protein that can catalyze the reentry of protons into the mitochondrial matrix, thus bypassing ATP synthase, uncoupling oxidative phosphorylation, and releasing chemical energy as heat. UCP1 is exclusively expressed in BAT, where the gene expression is increased by cold, adrenergic stimulation,  $\beta_3$ -agonists, retinoids, and thyroid hormone (Silva and Rabelo 1997). Thermogenic activity of BAT is dependent on UCP1 expression level controlled by the sympathetic nervous system via noradrenaline (Argyropoulos and Harper 2002; Del Mar Gonzalez-Barroso et al. 2000; Mozo et al. 2005; Nedergaard et al. 2001) (Figure 11.5). As a consequence of noradrenaline binding to the adipocyte plasma membrane, protein kinase (PKA) is expressed, and then cyclic AMP response element-binding protein (CREB) and hormone-sensitive lipase (HSL) are expressed. HSL stimulates lipolysis and the free fatty acids that are liberated serve as the substrate in BAT thermogenesis (Mozo et al. 2005).

Bioactive ingredients of dietary origin show a propensity to stimulate energy expenditure by influencing subtle cellular and metabolic processes linked with energy dissipation. There is immense interest in these naturally occurring substances in view of their potential application in body weight reduction. Natural ingredients have received particular attention as alternatives to conventional weight management strategies with limited long-term effectiveness. One such natural-product-derived ingredient is tea. Green tea extract is reported to increase energy expenditure and fat oxidation in humans (Dulloo et al. 1999). The tea extract contains caffeine and catechin. Epigallocatechin gallate, a main tea catechin, promotes fat



**Figure 11.5.** Molecular pathway for upregulation of UCP1 in brown adipose cell.

oxidation and decreases fat synthesis, but does not activate  $\beta_3$ -adrenergic receptor ( $\beta_3$ -AP) (Klaus et al. 2005). Antioesity activity of green tea extract will be attributed to effects of both UCP1 upregulation by caffeine (Figure 11.5) and lipid metabolism control by catechin. Capsaicin (Figure 11.5), the major pungent principal of red pepper, also upregulates UCP1 in BAT by release of catecholamine such as norepinephrine (Kawada et al. 1986, 1991; Masuda et al. 2003; Watanabe et al. 1994).

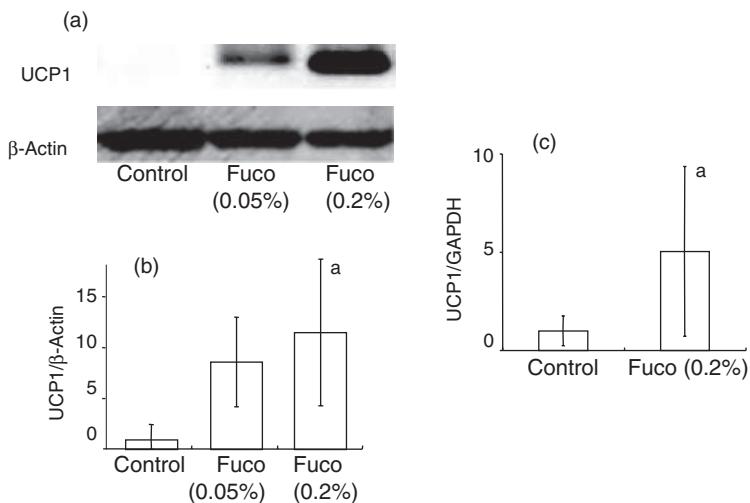
#### ANTIOBESITY EFFECT OF SEAWEED CAROTENOID, FUcoxanthin

There is no doubt that UCP1 is a key molecule for antioesity. UCP1 expression is known as a significant component of whole-body energy expenditure and its dysfunction contributes to the development of obesity. However, adult humans have very little BAT and most of the fat is stored in WAT. Even though BAT is scanty in adult humans, it is true that BAT serves as a good model for the study of energy expenditure regulation; moreover, due to the high thermogenic capacity of BAT (500 W/kg), even very small quantities such as those found in adult humans may influence heat production.

Considered as breakthrough discoveries for an ideal therapy of obesity, regulation of UCP expression in tissues other than BAT by food constituent would be also important. UCP1, usually expressed

only in BAT, has been also found in WAT of mice overexpressing *Foxc2*, a winged helix gene, with a change in steady-state levels of several WAT and BAT derived mRNAs (Cederberg et al. 2001). This result suggests the possibility of UCP1 expression in WAT, which would be an increasingly attractive target for the development of antioesity therapies. As the key molecular components become defined, screening for food constituent that increase energy dissipation is becoming a more attainable goal. From this viewpoint, the antioesity effect of edible seaweed carotenoid, fucoxanthin, is very interesting, as its activity depends on the protein and gene expressions of UCP1 in WAT (Maeda et al. 2005).

In the study on antioesity effect of seaweed carotenoid, BAT weight of mice fed 0.2% fucoxanthin was significantly greater than that in control mice (Maeda et al. 2005). However, there was no significant difference in UCP1 expression among the different dietary groups. Thus, the decrease in abdominal fat pad weight found in 0.2% fucoxanthin-fed mice could not be explained only by energy expenditure in BAT mitochondria by UCP1. As shown in Figure 11.6, UCP1 expression was found in WAT of 0.2% fucoxanthin-fed mice, although there was little expression in that of control mice. Expression of UCP1 mRNA was also found in WAT of 0.2% fucoxanthin-fed mice, but little expression in that of control. The finding that fucoxanthin induces both protein and mRNA expressions of UCP1 in WAT will give a clue



**Figure 11.6.** UCP1 expressions in white adipose tissue (WAT) of mice fed fucoxanthin. (a) Western blot analysis of UCP1. (b) UCP1 protein expression. (c) UCP1 mRNA expression. a: Significant difference from control ( $P < 0.05$ ). The dietary fats for mice were 13% soybean oil (control), 12.5% soybean oil + 0.5% *Undaria* lipids (fucoxanthin (Fuco): 0.05%), 11% soybean oil + 2% *Undaria* lipids (Fuco: 0.2%). (Adapted from Maeda et al. 2005.)

for new dietary antioesity therapy. An enormous amount of data has been collected on thermogenesis in BAT through UCP1 expression. However, there had been little information on UCP1 expression in WAT induced by a dietary component until the above report had appeared. An excessive accumulation of fat in WAT induces some diseases such as type-2 diabetes. Direct heat production by fat oxidation in WAT, therefore, will reduce risk of these diseases in humans.

Various studies have shown that the white and brown preadipocytes differentiate in vitro in characteristic white and brown adipocytes, respectively (Ailhaud et al. 1992; Kopecký et al. 1990; Rehnmark et al. 1990). Multilocular fat cells, expressing UCP1 and rich in mitochondria, have been observed for the first time in WAT (Young et al. 1984). The emergence of these so-called ectopic brown adipocytes in the WAT was found to be induced by cold acclimation rats (Cousin et al. 1996) and mice (Guerra et al. 1998; Loncar 1991). Selective  $\beta_3$ -AR such as CL 316243 induced the emergence of brown adipocytes in WAT depots of mice (Collins et al. 1997; Ghorbani and Himms-Hagen 1997; Guerra et al. 1998; Nagase et al. 1996). Further, transgenic overexpression of the human  $\beta_1$ -AR in the WAT of mice also induced the appearance of abundant brown adipocytes in this tissue (Soloveva et al. 1997). Although molecular pathway for the UCP1 expression in mice fed fucox-

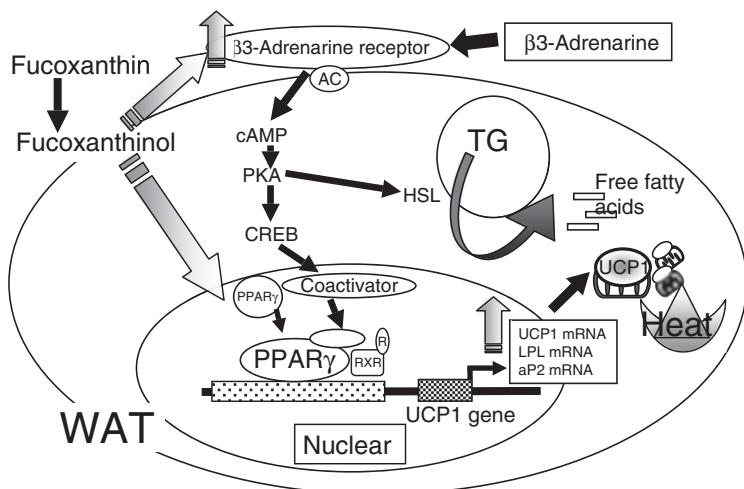
anthin is still unknown, antioesity effect of fucoxanthin with UCP1 expression in WAT may be related to the upregulation of  $\beta_3$ -AR in the WAT (Figure 11.7). Investigations are needed to evaluate the mechanism from this viewpoint.

## OTHER NOVEL PHYSIOLOGICAL EFFECTS OF FUCOXANTHIN

### ANTIDIABETIC EFFECT OF FUCOXANTHIN

KK- $A^y$  mice, a good model of obesity and type-2 diabetes, develop obesity and show hyperleptinemia and hyperinsulinemia along with insulin resistance. When the mice fed 0.1 and 0.2% fucoxanthin, significant reduction was found in blood glucose level and in insulin concentration with a significant decrease in abdominal WAT (Maeda et al. 2007b) (Figure 11.8). Water intake, which is related with elevated blood glucose level in diabetic mice (Lee and Bressler 1981), was also decreased by feeding fucoxanthin diets (Maeda et al. 2007b).

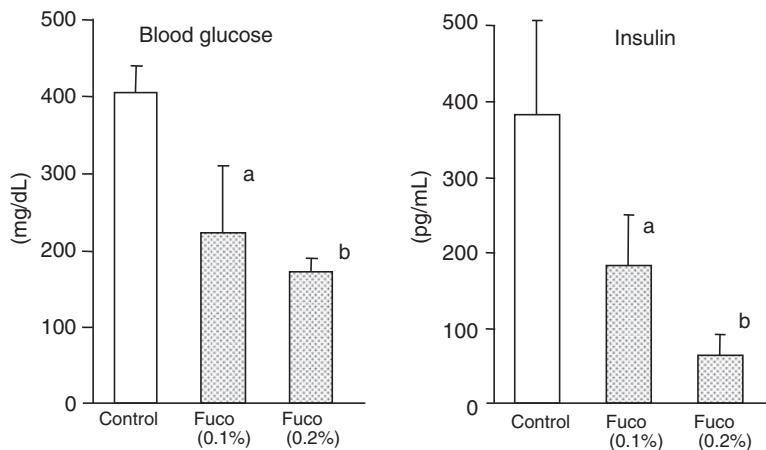
Excess fat accumulation in abdominal WAT found in KK- $A^y$  mice causes a disturbance in cytokine secretion in adipose tissue and is involved in the pathogenesis of type-2 diabetes, cardiovascular, and hypertension (Kadowaki et al. 2006; Matsuzawa 2006). TNF- $\alpha$  (Hotamisligil et al. 1993, 1995) and leptin (Masuzaki et al. 1999) secretion is elevated through



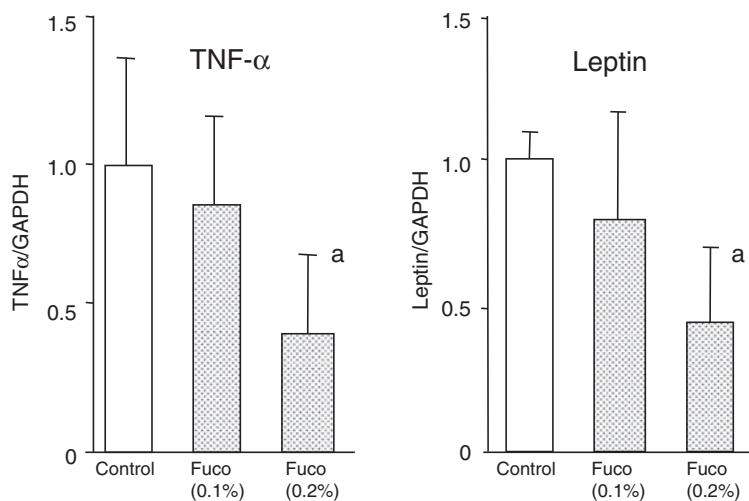
**Figure 11.7.** Possible molecular mechanism for UCP1 expression in white adipose tissue (WAT) of animals fed fucoxanthin.

the accumulation of fat in the adipocytes and causes insulin resistance in obese animal models. Insulin resistance in peripheral tissue is one of the major pathogenic characters of type-2 diabetes. The significant improvement in blood glucose and plasma insulin level of KK- $A^y$  mice by fucoxanthin intake (Figure 11.8) was strongly correlated with the regulatory effect of fucoxanthin on these adipokines mRNA in WAT (Maeda et al. 2007b) (Figure 11.9).

In general, leptin controls body weight and fat weight by regulating the food intake and energy expenditure (Halaas et al. 1995; Pelleymounter et al. 1995). However, KK- $A^y$  mice are known to have elevated plasma leptin levels and exhibit hyperleptinemia (Masuzaki et al. 1999). Thus, the lower leptin level (Figure 11.9) in KK- $A^y$  mice fed fucoxanthin may reflect the size of WAT, because leptin is mainly produced in adipocyte (Frederich et al. 1995).



**Figure 11.8.** Blood glucose and serum insulin levels in KK- $A^y$  mice. KK- $A^y$  mice were fed control or experimental diets for 25 days. Values are expressed as means  $\pm$  standard division (S.D.) g WAT per 100 g body weight ( $n = 7$ ). a and b, Significant difference from control (a,  $P < 0.05$ ; b,  $P < 0.01$ ). The dietary fats for mice were 1.35% soybean oil (control), 1.34% soybean oil + 0.1% fucoxanthin (Fuco), and 0.2% Fuco. (Adapted from Maeda et al. 2007b.)



**Figure 11.9.** TNF- $\alpha$  and leptin mRNA expression in white adipose tissue (WAT) of KK- $A^{\gamma}$  mice fed fucoxanthin. KK- $A^{\gamma}$  mice were fed control or experimental diets for 25 days. The expression levels of adipokine mRNA were measured by quantitative RT-PCR and expressed relative to control. Values are expressed as means  $\pm$  S.D. g WAT per 100 g body weight ( $n = 7$ ). a, Significant difference from control ( $P < 0.01$ ). The dietary fats for mice were 1.35% soybean oil (control), 1.34% soybean oil + 0.1% fucoxanthin (Fuco), and 0.2% Fuco. (Adapted from Maeda et al. 2007b.)

Significant decrease in TNF- $\alpha$  mRNA expression in WAT of KK- $A^{\gamma}$  mice fed fucoxanthin (Figure 11.9) implies that fucoxanthin improves insulin resistance and decreases blood glucose level, at least in part, through downregulation of TNF- $\alpha$  mRNA. However, downregulation of TNF- $\alpha$  mRNA was not significant in the WAT of mice fed 0.1% fucoxanthin, even though the blood glucose and plasma insulin levels were significantly decreased. Therefore, the reduction of blood glucose and insulin levels of KK- $A^{\gamma}$  mice by fucoxanthin seems to be induced by another signaling pathway.

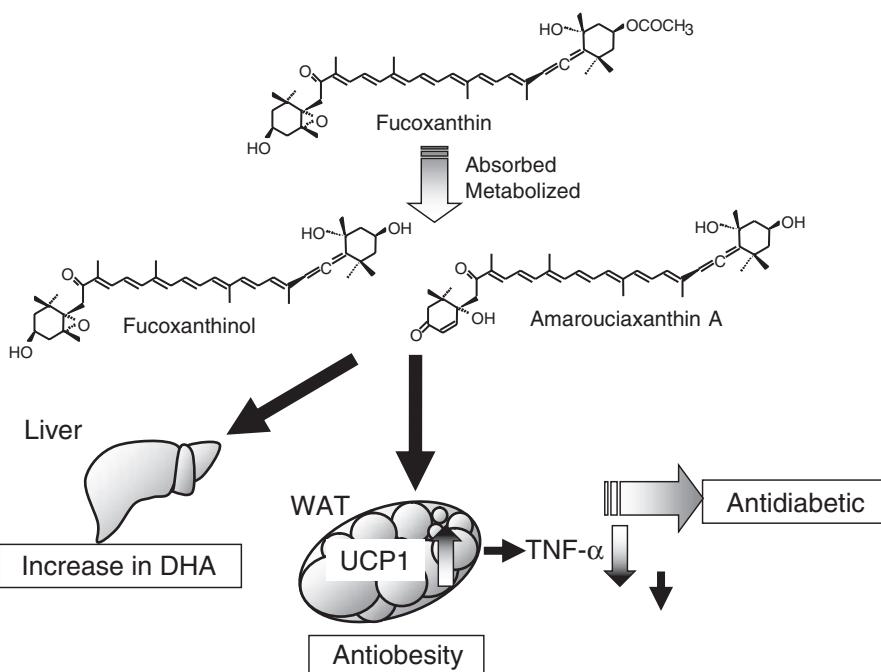
#### DHA INCREASE IN LIVER LIPIDS OF ANIMALS FED FUCOXANTHIN

Another characteristic change in animals fed fucoxanthin is the increase in the proportion of docosahexaenoic acid (DHA) in liver lipids (Tsukui et al. 2007). The feeding of fucoxanthinol, the main metabolite of fucoxanthin, also induced DHA content in the liver lipids. When mice were fed with fucoxanthin- and fucoxanthinol-containing diets with the same fatty acid composition for 4 weeks, food intake, liver weights, and the total amount of liver lipids did not differ among groups (Tsukui et al. 2007). However, the total amount of hepatic DHA in mice fed 0.1 and 0.2% purified fucoxanthin diets increased 1.7 and 1.9 times higher than that of the control group,

respectively. In addition, 22:5  $n$ -3 increased in mice fed 0.2% fucoxanthin diet. Hepatic 20:4  $n$ -6 also increased with the fucoxanthin diets, although this increase was not significant. A significant increase in hepatic DHA was found in mice fed diets containing 0.2% purified fucoxanthinol. Furthermore, an increase in 20:4  $n$ -6 was observed in mice fed fucoxanthinol diets.

Fucoxanthin is metabolized to fucoxanthinol and then to amarouciaxanthin A in mice (Asai et al. 2004). Therefore, fucoxanthinol and amarouciaxanthin A, but not fucoxanthin, are suggested to be the key substances to enhance the amount of DHA in the liver of KK- $A^{\gamma}$  mice. DHA can be biosynthesized through desaturation and elongation reaction steps beginning with  $\alpha$ -linolenic acid in the liver. The increase in hepatic DHA found in the animals fed fucoxanthin might be due to the upregulation of enzymatic activities related to the bioconversion of  $\alpha$ -linolenic acid to DHA.

$n$ -3 Polyunsaturated fatty acids such as EPA and DHA have been reported to have pivotal roles in a number of physiological functions including cardioprotection activities, the reduction of triacylglycerol and cholesterol, as well as anti-inflammatory and anticancer effects. The effects of EPA and DHA on lipid metabolism could be strongly correlated with the antioesity effect of marine lipids. Therefore, the novel effects of fucoxanthin in increasing the total DHA in



**Figure 11.10.** Health beneficial effects of seaweed carotenoid, fucoxanthin.

the liver of rodents may be indirectly related to the health beneficial effects of fucoxanthin.

As described in this chapter, dietary fucoxanthin reduces the risk of metabolic disorders by affecting two or more molecular targets as shown in Figure 11.10.

## CONCLUSION

Obesity and insulin resistance or type-2 diabetes are pathologies with rapidly growing prevalence in most of the world. Although much remains to be learned until we have a complete understanding of these complex systems, remarkable progress in the understanding of the mechanisms controlling energy balance has been achieved in the last decade. A few molecular targets offer the most hope for antiobesity and antidiabetic therapeutics. These promising targets have been quite well validated biologically (in animals) and are known to be reachable with small molecules. Carotenoids are part of this category. For fucoxanthin mentioned in this chapter, the key to success would be induction of UCP1 in WAT and downregulation of adipokines such as TNF- $\alpha$ . The regulatory effect of fucoxanthin on PPAR $\gamma$  and  $\beta_3$ -AR in WAT would be correlated its antiobesity effect and antidiabetic effect.

Furthermore, the relationship between carotenoid structure and suppressive effect on the differentiation of 3T3-L1 adipose cells shows that carotenoids containing an allene bond and an additional hydroxyl substituent on the side group may show the characteristic antiobesity activity. This suggestion that carotenoid health effects are likely linked to structural characteristics may help in the discovery of additional carotenoids that possess health-promoting properties. Along these lines, it will be of great interest to assess the health beneficial effects of other naturally occurring carotenoids that possess the specific structures, including allene bond, acetylene bond, and polar substituents on the adjacent end group.

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# 12

## Control of Systemic Inflammation and Chronic Diseases—The Use of Turmeric and Curcuminoids

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### ABSTRACT

The world suffers an epidemic of both critical illness (CI) and chronic diseases (ChDs), and both groups of diseases increase from year to year, and have done so for several decades. It is strongly associated to the modern, so-called Western, lifestyle: stress, lack of exercise, abuse of tobacco and alcohol, and the transition from natural unprocessed foods to processed, calorie-condensed, and heat-treated foods. There is a strong association between reduced intake of plant fibers and plant antioxidants and increased consumption of industrially produced and processed products especially dairy, refined sugars, starch products and ChDs. Heating up foods such as milk (pasteurization) and production and storage of milk powder produce large amounts of advanced glycation end products (AGEs) and advanced lipoxidation end products (ALEs), known as potent inducers of inflammation.

Numerous plant-derived, but also microbe-derived, substances, often referred to as chemopreventive agents, have documented anti-inflammatory effects and are believed to reduce speed of aging and prevent degenerative malfunctions of organs and also development of acute and chronic diseases. Among these are various curcuminoids, active ingredients in turmeric curry foods, and thousands more of hitherto little or totally unexplored substances. This chapter focuses on documented experimental and clinical effects of supplementation of turmeric, various curcuminoids, and pure curcumin. Regrettably, only a few clinical studies in humans have been performed in contrast to an abundance of studies in experimental animals.

### AN EPIDEMIC OF CHRONIC DISEASES AND CRITICAL ILLNESS

Modern medicine has to a large extent failed in its ambition to control both acute and chronic diseases. The world suffers an epidemic of chronic diseases of a dimension never seen before, and these diseases are like a prairie fire also spreading to the so-called developing countries. As an example, there are more cases of diabetes reported in China (24 million) and India (44 million) than in the United States (17 million), and the increase in incidence is faster in these countries than in Western societies. Today, chronic diseases—for example, diseases such as cardiovascular and neurodegenerative conditions, diabetes, stroke, cancers, and chronic respiratory diseases—constitute 46% of the global disease burden and 59% of the global deaths; each year approximately 35 million individuals die in conditions related to chronic diseases, and the numbers are fast increasing and have done so for several years (World Health Organization 2003).

Also acute diseases, often referred to as medical and surgical emergencies—myocardial infarction, stroke and severe pancreatitis, or diseases/complications following advanced medical and surgical treatments such as organ and stem cell transplantation and other large operations—have an unacceptably high morbidity and mortality. Sepsis, the most common medical and surgical complication, is estimated to annually affect as many as 751,000 individuals only in the United States (Angus et al. 2001; Arias and Smith 2003) and cause death of approximately 215,000 patients/year (29%) (Angus

et al. 2001), making sepsis the tenth most common cause of death in this country. It is especially alarming that both morbidity and mortality in critical illness (CI), and sepsis, is fast increasing worldwide and has done so for several decades. With a documented 1.5% rate of increase per year, the incidence is forecasted to double within the coming 50 to 60 years.

### LIFESTYLE ASSOCIATED DISEASES

Accumulating evidence supports the association of ChDs to modern lifestyle, stress, lack of exercise, and abuse of tobacco and alcohol, and most important, the transition from natural unprocessed foods to processed, calorie-condensed and heat-treated foods are contributing to this development. The strong association between ChD and reduced intake of plant fibers and plant antioxidants, and increased consumption of industrially produced and processed dairy products, refined sugars, and starch products is well documented. The per capita consumption of refined sugar has increased from about 0.5 kg/person/year in 1850 to almost 50 kg/person/year in the year 2000 and the per cow milk production from 2 to 50 liters/day. Dairy products, especially milk (mostly from pregnant cows), are rich in proinflammatory molecules: hormones such as estrogens (Howie and Shultz 1985; Malekinejad et al. 2006) and growth factors such as IGF-1 (Holmes et al. 2002). Consumption of bovine milk has also been shown to release inflammatory mediators, increase intestinal permeability, and induce leakage of larger molecules such as albumin and hyaluronan into the body (Bengtsson et al. 1996). Heating up milk (pasteurization), and especially production and storage of milk powder, produces large amounts of advanced glycation end products (AGEs) and advanced lipoxidation end products (ALEs) (Baptista and Carvalho 2004), known as potent inducers of inflammation. This information is especially important as many foods such as ice cream, industrially produced enteral nutrition solutions, and baby formulas are based on milk powder. Such formulas are reported to increase inflammation and induce microbial intestinal translocation (Deitch et al. 2002; Mosenthal 2002; Xu et al. 1998). Bread, especially when from gluten-containing grains, is rich in molecules with documented proinflammatory effects, and bread crusts are often used experimentally to induce inflammation. See further Bengmark (2004, 2006a, 2007).

### PLANT-DERIVED PROTECTION

Common to those suffering from ChD as well as CI is that they suffer an increased degree of systemic inflammation. We are increasingly aware that plant-derived substances, often referred to as chemopreventive agents, have an important role to play in control of inflammation. These substances are generally inexpensive, easy available, and have no or limited toxicity. Among the numerous chemopreventive agents are a whole series of phenolic and other compounds believed to reduce the speed of aging and prevent degenerative malfunctions of organs, among them various curcumenoids found in turmeric curry foods and thousands of other hitherto little or not at all explored substances.

Curcumin and many other plant-derived substances are increasingly regarded as shields against disease (Bengmark 2006b). Curcumin is the most explored of a family of the so-called active chemopreventive substances in the spice turmeric, collectively referred to as curmenoids. The health-promoting effects of turmeric are widely recognized as the spice has been used for centuries, especially in Indian Ayurveda medicine, to treat a wide variety of disorders such as pains and colics, rheumatism, skin diseases, intestinal worms, diarrhea, intermittent fevers, hepatic disorders, urinary problems, dyspepsia, intestinal conditions such as colitis and constipation, amenorrhea, and inflammatory conditions in general. However, it is only in the most recent years that the interest has exploded, much in parallel to the availability of molecular biological techniques, but also due to increasing concern for severe side effects of synthetic cyclooxygenase-2 (COX-2) inhibitors that the pharmaceutical industry is marketing. Most of the curcumin studies reported in the literature are experimental and few clinical studies are this far presented.

### TURMERIC—APPROVED AS FOOD ADDITIVE

Curcumin (1,7-bis(4-hydroxy-3-methoxyphenol)-1,6-heptadiene-3,5-dione) a polyphenol, richly available in turmeric, is received from dried rhizomes of the perennial herb *Curcuma longa* Linn, a member of the ginger family. Turmeric is since long known to be an excellent food preservative and is approved as such in most Western countries. It is mainly produced in Asian and South American countries. Only in India about 500,000 metric tonnes are produced each year, of which about half is exported. The content of curcumin in turmeric is

usually 4–5%. The molecule of curcumin resembles ubiquinols and other polyphenols known to possess strong antioxidant activities. Its bioavailability on oral supplementation is relatively low, but can be improved by dissolution in ambivalent solvents (glycerol, ethanol, DMSO). (Sharma et al. 2001). It is also reported to be dramatically elevated by co-ingestion of piperine (a component of pepper), as demonstrated both in experimental animals and humans (Shoba et al. 1998). Several studies have demonstrated that curcumin is atoxic, also in very high doses Bravani Shankar et al. 1980; Shainani-Wu 2003). Treatment of humans for 3 months with 8,000 mg curcumin per day lead to no side effects (Shainani-Wu 2003). It is estimated that adult Indians consume daily 80–200 mg curcumin per day (Grant and Schneider 2000). A common therapeutic dose is 400–600 mg curcumin three times daily, corresponding to up to 60 g fresh turmeric root or about 15 g turmeric powder.

## INFLAMMATION—CENTRAL TO DEVELOPMENT AND PREVENTION OF DISEASE

The process of inflammation is well known. Activated monocytes and macrophages release proinflammatory cytokines such as tumor necrosis factor alpha (TNF $\alpha$ ) and interleukin-1 (IL-1), which induce inflammation in the tissues. Also important for the development of inflammation is the production by macrophages and neutrophils of prostaglandins, thromboxanes, and leukotrienes, collectively known as eicosanoids, which are mediators of inflammation synthesized through enzymatic degradation by COX-2 or lipoxygenase (LOX) of arachidonic acid (AA). COX-2 is induced by physical and mental stress, and a variety of inflammatory stimuli, including endotoxins, cytokines, growth factors, tumor promoters, and COX-2, catalyze the synthesis by mononuclear phagocytes, endothelial cells, polymorphonuclear leukocytes, and platelets of series-2 prostaglandins (e.g., PGE2, PGF2 $\alpha$ , PGI2, PGD2) and thromboxanes (e.g., TXA2, TXB2). PGE2 is a well-known promoter of production both of IL-10, a potent immunosuppressive cytokine, produced especially by lymphocytes and macrophages, and suppressor of IL-12 (Stolina et al. 2000).

Nuclear factor-kappa B (NF- $\kappa$ B) plays a critical role for induction of several signal transduction pathways involved in inflammatory diseases (Bernes and Karin 1997) such as asthma, arthritis and various

cancers (Amit and Ben-Neriah 2003). Activation of NF- $\kappa$ B is linked with apoptotic cell death, either promoting or inhibiting apoptosis, depending on cell type and condition. The expression of several genes such as COX-2, matrix metalloproteinase-9 (MMP-9), inducible nitric oxide synthase (iNOS), TNF, IL-8, eotaxin, various cell surface adhesion molecules, and antiapoptotic proteins are regulated by NF- $\kappa$ B (Pahl 1999). COX-2 is inducible and barely detectable under normal physiological conditions, but is rapidly, but transiently, induced as an early response to proinflammatory mediators and mitogenic stimuli including cytokines, endotoxins, growth factors, oncogenes and phorbol esters. iNOS, activated by NF- $\kappa$ B is another enzyme that plays a pivotal role in mediating inflammation, especially as it acts in synergy COX-2.

Curcumin is not only an inexpensive atoxic and potent COX-2 and iNOS inhibitor (Surh et al. 2001), but also a potent inducer of heat shock proteins (Hsps) and a cytoprotector (Chang 2001; Dunsmore et al. 2001). Curcumin inhibits not only COX-2, but also LOXs and leukotrienes such as LTB4 and 5HETE (Wallace 2002), especially when bound to phosphatidylcholine micelles (Begun et al. 1999). Curcumin is also reported to inhibit cytochrome P450 isoenzymes and thereby activation of carcinogens (Thapliyal and Maru 2001). Curcumin has the ability to intercept and neutralize potent prooxidants and carcinogens, both ROS (superoxide, peroxyl, hydroxyl radicals) and NOS (nitric oxide, peroxynitrite) (Jovanovic et al. 2001). It is also a potent inhibitor of TGF- $\beta$  and fibrogenesis (Gaedeke et al. 2004), which is one of the reasons why it can be expected to have positive effects in diseases such as kidney fibrosis, lung fibrosis, liver cirrhosis, and Crohn's disease and prevent formation of tissue adhesions (Srinivasan and Libbus 2004). Curcumin is suggested to be especially effective in Th1-mediated immune diseases, as it effectively inhibits Th1 cytokine profile in CD4 $^+$  T cells by activation of IL-12 (Kang et al. 1999).

Furthermore, curcumin is also known to:

- Inhibit the release of AA through hydrolysis of membrane phospholipids (Hong et al. 2004).
- Inhibit the induction of COX-2 mRNA and protein expression (Zhang et al. 1999).
- Inhibit extracellular signal-regulated kinase (ERK) activity (Chun et al. 2003).
- Inhibit 5-hydroxyeicosatetraenoic acid (5-HETE) production in human neutrophils (Flynn et al. 1986).

- Inhibit the so-called Janus kinase (JAK)—STAT signaling cascade (Kim et al. 2005a).
- Inhibit the production of superoxide and nitric oxide by inflammatory cells. (Bhaumik et al. 2000; Brouet and Ohshima 1995).
- Moderately increase the number of T-and B-cells without altering the numbers of phagocytic macrophages (Gautam et al. 2007).
- Increase the phagocytic activity of macrophages (Antony et al. 1999; Li and Liu 2005).
- Increase the numbers of B-cells in the small intestinal mucosa (Churchill et al. 2000).
- Suppress surface expression of costimulatory molecules CD80 and CD86 and major histocompatibility complex (MHC) II, but not MHC class I (Kim et al. 2005a).
- Impair the production by dendritic cells of IL-12, IL-1, IL-6, and TNF $\alpha$  (Kim et al. 2005a).
- Inhibit the activation of mitogen-activated protein kinase (MAPK) and nuclear translocation of nuclear factor-beta (NF- $\beta$ ) (Gautam et al. 2007; Kim et al. 2005a).
- Reduce accumulation in the body of proinflammatory molecules such as AGEs and ALEs (Sajithlal et al. 1998).
- Induce apoptosis of various tumor cells by a variety of mechanisms: decreasing cellular levels of antiapoptotic Bcl-2, Bcl-xL, and cIAP proteins, increasing levels of proapoptotic Bax, inhibiting constitutively active JAK—STAT pathways, activating MAPK and PI3 k/PKB and Fas receptor/caspase-8 pathway independent of p53. See further Gautam et al. (2007).
- Induce heme oxygenase-1 (HO-1), a redox-sensitive inducible protein that provides protection against various forms of stress (Balogun et al. 2003).

See also Jagetia and Aggarwal (2007) for further information.

Many medicinal herbs and pharmaceutical drugs are therapeutic at one dose and toxic at another, and interactions between herbs and drugs, even if structurally unrelated, may increase or decrease the pharmacological and toxicological effects of either component (Fugh-Berman 2002; Grotan et al. 2000). It is suggested that curcumin may increase the bioavailability of vitamins such as vitamin E and decrease blood levels of cholesterol, as in experimental studies curcumin will significantly raise the concentration of  $\alpha$ -tocopherol in tissues such as lung and decrease plasma cholesterol (Kamal-Eldin et al. 2000). Polyphenols, isothiocyanates such as curcumin, and flavonoids such as resveratrol are all made accessible

for absorption into the intestinal epithelial cells and the rest of the body by digestion/fermentation in the intestine by microbial flora (Shapiro et al. 1998).

## CURCUMIN IN ACUTE AND CHRONIC DISEASES

### AGING

Oxidative stress is believed to play a major role in the aging process and in pathogenesis of diseases most commonly responsible for morbidity and mortality in older age. Dietary factors influence considerably both disease processes and longevity by modifying oxidative stress. Bala et al. (2006) investigated the influence of chronically administered curcumin on normal aging-related parameters—lipid peroxidation, lipofuscin concentration and intraneuronal lipofuscin accumulation—and on activities of a series of other factors—superoxide dismutase (SOD), glutathione peroxidase (GPx), and Na $^+$ , K $^+$ -adenosine triphosphatase (Na $^+$ , K $^+$ -ATPase) in different brain regions (cerebral cortex, hippocampus, cerebellum and medulla) in 6- and 24-month-old rats. Chronic curcumin supply to both 6- and 24-month-old rats resulted in significant decreases in lipid peroxide and lipofuscin content in the brain regions, and was accompanied by significant increases in activities of SOD, GPx and Na $^+$ , K $^+$ -ATPase in various brain regions. In a rat study, supply of tetrahydrocurcumin, a biotransformed metabolite of curcumin, was demonstrated to increase average life span by 12% ( $P < 0.01$ ) and average life expectancy after 24 months of age by 126% (Kitani et al. 2004). However, no human study is this far reported.

### ALLERGY

Curcumin has a potential therapeutic value for control of allergic responses to exposure to allergens. Intragastric treatment of latex-sensitized mice with curcumin demonstrated a diminished Th2 response and a concurrent reduction in lung inflammation (Kurup et al. 2007). In addition, in curcumin-treated mice eosinophilia was markedly reduced, costimulatory molecule expression (CD80, CD86, and OX40L) on antigen-presenting cells decreased, and expression of MMP-9, OAT, and TSLP genes attenuated. Another recent study suggests that that the hydroxy groups of curcumin play a significant role in exerting both antioxidative and antiallergic activities, and that most of the compounds develop antiallergic activities through mechanisms related to antioxidative activities, but some most likely also through antioxidation unrelated mechanisms. A significant

decrease in histamine release from rat basophilic leukemia cells, RBL-2 H3, was observed when cells were cultivated with curcumin or tetrahydrocurcumin (Suzuki et al. 2005). No human study in allergy is this far reported.

### ARTHRITIS

Treatment *in vitro* of chondrocytes with curcumin is shown to suppress IL-1 $\beta$ -induced NF- $\kappa$ B activation via inhibition of I $\kappa$ B $\alpha$  phosphorylation, I $\kappa$ B $\alpha$  degradation, p65 phosphorylation, p65 nuclear translocation and inhibition of upstream protein kinase B Akt, events which correlate well with downregulation of NF- $\kappa$ B targets including COX-2 and MMP-9 (Shakibaie et al. 2007). IL-18 is a novel proinflammatory cytokine that has been suggested to play a pathogenic key role in a number of autoimmune diseases such as inflammatory bowel diseases (IBD), psoriasis, and rheumatoid arthritis (RA) (McInnes et al. 2000). Vascular endothelial growth factor (VEGF) is deeply involved in angiogenesis in rheumatoid synoviocytes and IL-18 will dose-dependently increase both production of VEGF IL-18 and VEGF levels of sera and synovial fluids of RA patients. These factors were found to be significantly higher in RA than in osteoarthritis A patients. Curcumin did dose-dependently abrogate the effect of IL-18 on VEGF production (Cho et al. 2006). A recent *in vitro* study compared the potential anti-inflammatory effects of curcumin and quercetin. Both agents inhibited neutrophil activation, synoviocyte proliferation, and angiogenesis (Jackson et al. 2006). In addition, curcumin also strongly inhibited collagenase and stromelysin expression, effects not obtained by quercetin.

In 1980, Deodhar and colleagues had already performed a clinical study in which 18 RA patients were treated with curcumin and comparisons were made with phenylbutazone. Improvements in morning stiffness, walking time, and joint swelling were observed after 2 weeks of curcumin supplementation (1,200 mg/day), and reported to be equal to those induced by phenylbutazone therapy (300 mg/day) (Deodhar et al. 1980). Another now classical study did also conclude that five days of oral curcumin supplementation (1,200 mg/day) is equally effective as phenylbutazone to reduce postsurgical edema, tenderness, and pain (Satoskar et al. 1986). Most interesting are recent observations that curcumin has the ability to potentiate the effects of pharmaceutical COX-2 inhibitors such as celecoxib (Lev-Ari et al. 2006a). Such combinations might enable to use pharmaceutical drugs at much lower and safer concentra-

tions, especially when used for longer periods and in conditions such as osteoarthritis and other rheumatological disorders.

### ATHEROSCLEROSIS

Curcumin has a strong capacity to prevent lipid peroxidation, stabilize cellular membranes, inhibit proliferation of vascular smooth muscle cells, and inhibit platelet aggregation, all important ingredients in the pathogenesis of arteriosclerosis. Curcumin is also found to be the most effective, when the ability of butylated hydroxy anisole, curcumin, quercetin, and capsaicin to inhibit the initiation and propagation phases of low-density lipoprotein (LDL) oxidation was compared (Naidu and Thippeswamy 2002). Supply of not only curcumin, but also capsaicin and garlic (allecin), to rats fed a cholesterol-rich diet prevented both increases in membrane cholesterol and fragility of the erythrocytes (Kempaiah and Srinivasan 2002). Significant prevention of early atherosclerotic lesions in thoracic and abdominal aorta in rabbits fed an atherogenic diet for 30 days was observed, accompanied by significant increases in plasma concentrations of coenzyme Q, retinol, and  $\alpha$ -tocopherol and reductions in LDL-conjugated dienes and TBARS (thiobarbituric acid-reactive substances, an expression of ongoing oxidation) (Quiles et al. 2002).

Curcumin is also shown to protect the myocardium *per se* against ischemic insults. A single oral dose of curcumin (15 mg/kg), administered 30 min before and/or after the onset of isoprenaline-induced ischemia in rats not only prevented decrease in levels of xanthine oxidase, superoxide anion, lipid peroxides, and myeloperoxidase (MPO) and increase in levels of SOD, catalase (CAT), GPx, glutathione-S-transferase (GST) activities, but also reduced myocardial damage as documented by histopathology and electron microscopy (Manikandan et al. 2004). It is especially observed in *in vitro* studies that treatment with curcumin will produce a pronounced induction of the defensive protein HO-1, which will, when added to Celsior preservation solution, significantly prevent storage-induced damage of atrial myoblasts (Abuarqoub et al. 2007).

Studies on mice have also demonstrated that oral administration of curcumin will suppress aortic wall degeneration and prevent development of abdominal aortic aneurysms. Curcumin preserves medial elastin fibers and reduces aortic wall expression of cytokines, chemokines, and proteinases, known to mediate aneurysmal degeneration (Parodi et al. 2006). Recent studies on isolated porcine coronary arteries also demonstrate in a concentration-dependent

manner a considerable relaxant effect of curcumin via mechanisms involving NO, cGMP, and adrenergic  $\beta$ -receptor, but not by prostaglandins (Xu et al. 2007). Studies on porcine coronary arteries also demonstrate that curcumin effectively reverses homocysteine-induced endothelial dysfunction (Ramaswami et al. 2004). Curcumin, in doing so, blocks the homocysteine-induced superoxide anion production and downregulation of eNOS. However, no human study is this far reported.

## CANCER

Genomic approaches to cancer prevention and treatment are becoming increasingly important. In addition to characterizing potential mechanisms of cancer prevention, significant issues for future research are identification and selection of specific dietary bioactive food components, and especially identifying individuals with special nutrient requirements for optimal cancer protection.

Dietary bioactive food components that interact with the immune response have a considerable potential to reduce the risk of cancer. Numerous substances identified in fruits and vegetables have the ability to modulate the effects of deregulated cell cycle checkpoints and contribute to prevention of cancer. Not only curcumin, but numerous other plant-origin agents, possess this potential, among them apigenin (celery, parsley), epigallocatechin-3-gallate (green tea), resveratrol (red grape, peanuts, and berries), genistein (soybean), and silymarin (milk thistle). There is also accumulating evidence that cancer prevention can be achieved by some probiotic bacteria alone or in combination with prebiotic fibers, known to have a similarly strong effect on the immune system as plant antioxidants (see further Ferguson and Philpott 2007).

Curcumin has been tried in various animal models in order to achieve dietary prevention of development and spreading of cancer. Injection of human mammary cancer cells (MDA-MB-231) into the mammary fat pad of nude mice leads to the formation of tumors and distant metastases in lungs, brain, and lymph nodes. This spreading was to a great extent prevented by curcumin treatment: 68% of curcumin-treated in contrast to only 17% of untreated animals showed no or very few lung metastases (Bachmeier et al. 2007). Curcumin has in experimental models also demonstrated the ability to inhibit intrahepatic metastases (Ohadshi et al. 2003).

Curcumin seems to suppress several steps in tumorigenesis: cellular transformation, proliferation, invasion, angiogenesis, and metastasis. However, this

far most of the mechanisms are not fully understood. NF- $\kappa$ B is most likely playing a central role, as several genes, known to mediate these processes, are known to be regulated by NF- $\kappa$ B.

Different analogs of curcumin present in turmeric (curcumeneoids) exhibit variable anti-inflammatory and antiproliferative activities, which, however, do not entirely correlate with their ability to modulate the ROS status (Sandur et al. 2007). A comparison of the ability of curcumin and 20 curcumin analogues to suppress TNF-induced NF- $\kappa$ B activation demonstrated that the strongest effects are obtained by curcumin in itself, achieved by inhibition of NF- $\kappa$ B-regulated gene expression and inhibition of I $\kappa$ B kinase (IKK) and Akt activation (Aggarwal et al. 2006b). Later studies by the same group, but also others, demonstrate that other mechanisms are also involved such as:

- curcumin-induced downregulation of expression of cyclin E correlating with decreased proliferation of human prostate and breast cancer cells (Aggarwal et al. 2007).
- curcumin-induced enhancement of expression of tumor cyclin-dependent kinase (CDK) inhibitors p21 and p27 and tumor suppressor protein p53 (Aggarwal et al. 2007).
- curcumin-induced suppression of STAT3 activation, a mechanism linked with chemoresistance and radioresistance (Aggarwal et al. 2006a; Chakravarti et al. 2006). Curcumin is also known to inhibit JAK2, Src, Erb2, and EGFR, all known to be involved in STAT3 activation (see further Aggarwal et al. 2006b).
- curcumin-induced inhibition of both COX and LOX pathways of eicosanoid metabolism. Curcumin is reported to inhibit 12-folded block proliferation of human breast cancer cells (MCF-7 ADRs) in cell cultures (Hammamieh et al. 2007).

Curcumin has been reported to augment cytotoxic effects of both chemotherapy and radiation therapy (Aggarwal et al. 2005; Hour et al. 2002). There is also some evidence that subtoxic concentration of curcumin might promote apoptosis by ligands such as TNF-related apoptosis-inducing ligand (TRAIL). Prostate cancer cells, for example, are generally resistant to induction of apoptosis by anticancer agents and death ligands. However, in recent years, it has been demonstrated that a combination of subtoxic concentrations of curcumin and TRAIL induces apoptosis of prostate cancer cell lines, mainly through inhibition of NF- $\kappa$ B and activation of extrinsic and intrinsic pathways of

apoptosis (Deeb et al. 2005, 2007). When in an orthotopic murine model the effects of curcumin on two ovarian cancer cell lines (SKOV3ip1, HeyA8) were studied, curcumin alone did induce 49% ( $P = 0.08$ ) and 55% ( $P = 0.01$ ), respectively, reductions in mean tumor growth, an effect that was further increased by combining curcumin with the chemotherapeutic drug docetaxel, and demonstrating 96% ( $P < 0.001$ ) and 77% reductions, respectively (Lin et al. 2007). Also in mice with multidrug-resistant HeyA8-MDR tumors, treatment with curcumin alone and in combination with docetaxel resulted in significant reductions in tumor growth, 47% and 58%, respectively ( $P = 0.05$ ). SKOV3ip1 and HeyA8 tumors treated with curcumin alone or in combination with docetaxel demonstrated not only decreased proliferation ( $P < 0.001$ ), but also reduced microvessel density ( $P < 0.001$ ) and increased tumor cell apoptosis ( $P < 0.05$ ). The growth of induced colorectal cancer, measured as average number of aberrant crypt foci (ACF), was  $64.2 \pm 3$  in the control group,  $39 \pm 5$  in the curcumin-treated group,  $47 \pm 10$  and in celecoxib-treated group, but only  $24.5 \pm 6$  in the group that had received both agents (Shpitz et al. 2006). Another nude mice study undertaken with four different head and neck squamous cell carcinoma (HNSCC) cell lines documented that topical application as a curcumin paste is superior even to intratumoral injection of curcumin (LoTempio et al. 2005). Curcumin induces apoptosis in vitro in almost all cell lines: breast cancer (Xia et al. 2007; Zhang et al. 2007), head and neck cancer (Chakravarti et al. 2006; LoTempio et al. 2005), hepatocellular cancer (Labbozzetta et al. 2006), laryngeal cancer (Mitra et al. 2006), leukemia (Liao et al. 2008; López-Lázaro et al. 2007), lung cancer (Lee et al. 2005; Lev-Ari et al. 2006b), myeloma (Bharti et al. 2003, 2004), melanoma (Marín et al. 2007; Siwak et al. 2005), neuroblastoma (Liontas and Yeger 2004; Vanisree and Ramanan 2007), oral cancer (Atsumi et al. 2005; Sharma et al. 2006), osteosarcoma (Huang et al. 2005; Walters et al. 2008) pancreatic cancer (Lev-Ari et al. 2006b, 2007), and prostatic cancer (Deeb et al. 2007; Shankar and Srivastava 2007).

Although encouraging results have been obtained in *in vitro* and animal studies, only a small number of small clinical studies are thus far reported (see further Steward and Gescher 2008). A study intended as a phase I study reports histologic improvement of precancerous lesions in one out of two patients with resected bladder cancer, two out of seven patients of oral leucoplakia, one out of six patients of intestinal metaplasia of the stomach, and two out of six patients with Bowen's disease (Cheng et al. 2001). However,

the main purpose of the study was to document that curcumin is not toxic to humans when administered by mouth for 3 months in a dose of up to 8,000 mg/day.

## DIABETES

The oxidative stress observed in diabetic rats is clearly reduced significantly by curcumin administration. As a consequence of curcumin supply, nonenzymic antioxidants such as vitamin C, vitamin E, and glutathione are preserved at near normal levels and accumulation of lipid peroxidation products is significantly reduced.

Curcumin is also reported to prevent the accelerated accumulation of glycated collagen in diabetic animals. An interesting study reports significant prevention by curcumin of the extensive cross-linking of collagen in tendons and skin normally seen in diabetic animals (Sajithlal et al. 1998). Also interesting is the observation that curcumin contributes to control of hyperglycemia and also to some extent prevents islet cell death. In a streptozotocin-induced islet damage model, the *in vitro* islet viability and secreted insulin remained significantly higher after exposure to curcumin than in the controls. Furthermore, curcumin pretreatment significantly prevented streptozotocin-induced changes in isolated mouse islets such as DNA fragmentation, and reduced the concentrations of peroxynitrite, nitric oxide, and poly(ADP-ribose) polymerase-1 (Meghana et al. 2007). Curcumin administration also prevented the formation of the AGE-related malonyl dialdehyde in streptozotocin-treated islets. Oral administration of diabetic rats for 45 days with tetrahydrocurcumin at 80 mg/kg body weight significantly reduced blood glucose and increased plasma insulin levels parallel to significant increases in activities of SOD, CAT, GPx, GST, reduced glutathione, vitamin C, and vitamin E. Furthermore, significant decreases in TBARS and hydroperoxide formation in liver and kidney were observed, all suggesting a protective role of curcumin against lipid peroxidation-induced membrane damage, observations supported by observed improvements on histopathological examination of liver and kidney sections (Murugan and Pari 2006a).

Subsequent studies by the same group demonstrated that these changes are also accompanied by:

- reduced lipid peroxidation (TBARS and hydroperoxides) and reduced levels of lipids (cholesterol, triglycerides, free fatty acids, and phospholipids) in serum and tissues (Murugan and Pari 2006b).

- normalization of liver cholesterol, triglycerides, free fatty acids, phospholipids, HMG CoA reductase activity, and very low-density lipoprotein (VLDL), LDL, and high-density lipoprotein (HDL) cholesterol (Pari and Murugan 2007a).
- decreased levels of not only blood glucose, but also glycosylated hemoglobin and erythrocyte TBARS, and increased levels of plasma insulin, hemoglobin, erythrocyte antioxidants and activities of membrane bound enzymes, observations also accompanied by histopathological improvements (Murugan and Pari 2007a, Pari and Murugan 2007a).
- normalization of total protein, albumin, globulin, and albumin/globulin ratio, and near normalization of urea, uric acid, and creatinine (Murugan and Pari 2007b).
- decreased levels of brain lipid peroxidative markers: TBARS and hydroperoxides and increased brain activities of SOD, CAT, GPx, GST (Pari and Murugan 2007b).
- decreased to near normal tissue levels of hexose, hexosamine, and fucose (Pari and Murugan 2007c).
- reduced cross-linking of collagen (Pari and Murugan 2007d).
- Similar observations are made in alloxan-induced diabetes (Giltay et al. 1998). It is also observed that cryopreserved islets will be better preserved in the presence of curcumin (Kanitkar and Bhonde 2008).

Furthermore, it was observed in these studies of cryopreserved islets that curcumin increases the release of heat shock response proteins, Hsp70 and HO-1, which significantly contributes to a better preservation result.

No human clinical trials in diabetes with curcumin or turmeric are reported.

#### GASTRIC DISEASES

*Curcuma longa* is since long used commonly as a traditional remedy for gastritis and gastric ulcer. A recent study suggests that supply of 60 mg/kg body weight (bw) of curcumin is as effective as 20 mg/kg bw of omeprazole to restore suppressed MMP-2 gene transcription and translation and oxidative inactivation of basal MMP-2 and thereby prevent/reduce development of induced gastric ulcer in rats (Ganguly et al. 2006). Curcumin is shown not only to protect from formation of gastric ulcers but also to accelerate healing, mainly through attenuation of

MMP-9 activity (Swarnakar et al. 2005). Curcumin as well as turmeric have both the capacity to inhibit gastric acid secretion by blocking histamine receptors (Kim et al. 2005b). A potential use of turmeric or curcumin as alternative or complementary therapeutic agents against pathogenic processes initiated by *Helicobacter pylori* infection is supported by observations that curcumin has the capacity to inhibit *H. pylori*-induced NF- $\kappa$ B activation, subsequent release of IL-8, degradation of I $\kappa$ B- $\alpha$ , I $\kappa$ B kinases  $\alpha$  and  $\beta$  (IKK $\alpha$  and  $\beta$ ) activity, and NF- $\kappa$ B DNA binding (Foryst-Ludwig et al. 2004). When the in vitro effects of turmeric and curcumin against 19 different strains, including five cagA+ strains (cag A is the strain-specific *H. pylori* gene linked to premalignant and malignant lesions), were investigated and compared, both treatments were equally effective to significantly reduce the growth of all the *H. pylori* strains studied (Mahady et al. 2002).

#### HEPATIC DISEASES

Several studies have demonstrated the unique ability of turmeric and curcumin to preserve the integrity and function of liver cells. Studies have been undertaken with various models of acute toxic injuries to the liver, chronic supply of hepatotoxins and with liver perfusion and preservation. Curcumin has also been shown to protect the hepatocytes from oxidative injury, most likely and to a large extent through activation of HO-1. In a recent study acute hepatotoxicity was induced by oral administration of CCl<sub>4</sub> (4 g/kg) and curcumin supplemented orally (200 mg/kg), both before and 2 h after the CCl<sub>4</sub> administration. The CCl<sub>4</sub>-induced translocation of NF- $\kappa$ B to the nucleus, CCl<sub>4</sub>-induced NF- $\kappa$ B DNA-binding activity, and increases of TNF- $\alpha$  and IL-1 $\beta$  protein were blocked by curcumin, and most importantly, the destruction of hepatic tissues totally abolished (Reyes-Gordillo et al. 2007). Similar observations are also made in a model of endotoxin-induced hepatic dysfunction (Kaur et al. 2006). In another rat study, fulminant hepatic failure (FHF) was induced by two intraperitoneal injections of 300 mg/kg thioacetamide (TAA) at 24-h intervals. The experimental groups received intraperitoneally either a low dose (200 mg/kg/day) or a high dose (400 mg/kg/day) of curcumin, initiated 48 h prior to the first TAA injection. Curcumin significantly improved survival, minimized oxidative stress, reduced hepatocellular injury, hepatic necroinflammation, NF- $\kappa$ B binding and iNOS expression, and hepatic levels of TBARS (Shapiro et al. 2006). Furthermore, it inhibited nuclear binding of NF- $\kappa$ B and iNOS protein expression.

Biochemical parameters of liver injury, blood ammonia, and hepatic necroinflammation were significantly reduced in the low-dose curcumin group but were further reduced in the high-dose group ( $P < 0.05$  and  $P < 0.01$  respectively) (Shapiro et al. 2006). Curcumin induced, when cold preservation of human hepatocytes was applied, a significant elevation of HO-1 and exhibited a strong cytoprotection throughout the cold storage and rewarming (McNally et al. 2006). Injection of curcumin (50 mg/kg) into the portal system 30 min before applying hepatic warm ischemia/reperfusion (I/R) did significantly reduce the postperfusion increases in iNOS activity and content of malondialdehyde (MDA) in liver tissue and prevent the reductions in CAT and SOD activities (Shen et al. 2007). It also increased the expression of other Hsps such as Hsp70, reduced the rate of apoptosis, and, most importantly, significantly increased the survival. No human clinical trial is this far reported in liver disease.

### INFECTIOUS DISEASES

Sepsis is a leading cause of death. It affects each year about three quarters of a million North Americans and results in death of almost one quarter of a million. The challenge in CI is less infection than the exuberant inflammatory response (Taneja et al. 2004), often presented as a syndrome of prolonged systemic inflammation, frequently leading to a potentially lethal condition of irreversible organ system dysfunction. The development seems to occur especially in individual with a chronically dysfunctional innate immune system and sustained elevated inflammation. Apoptosis of circulating neutrophils in patients with clinical sepsis is through a mechanism that involves NF- $\kappa$ B activation profoundly suppressed, and also associated with reduced activity of cystein proteases (caspases-9 and -3) (Taneja et al. 2004). Suppression of this inflammation seems to reduce the inflammation and prevent development of infection and organ dysfunction. In a recent study, attempts were made to suppress inflammation by intravenous administration of curcumin for three days before sepsis was induced by the method called cecal ligation and puncture (CLP). The curcumin treatment did significantly attenuate tissue injury, reduce mortality, decrease the expression of TNF- $\alpha$ , downregulate peroxisome proliferator-activated receptor-gamma (PPAR $\gamma$ ) in organs like the liver and also prevent morphologic alterations in macrophages (Siddiqui et al. 2006). Most importantly, the same results were obtained even if curcumin was only administered after the onset of sepsis. These findings

are especially interesting as downregulation of PPAR in other models and by other tools has produced similar effects (Thiemermann 2006.) In animal models, curcumin is shown to prevent endotoxin-induced pulmonary sequestration of neutrophils via mechanisms such as induction of HO-1 and inhibition of endothelial ICAM-1 expression (Olszanecki et al. 2007). Curcumin will also attenuate endotoxin-induced coagulopathy and prevent disseminated intravascular coagulation (DIC) (Chen et al. 2007). These observations are of even greater interest as curcumin in itself demonstrates antibacterial (Di Mario et al 2007), antiviral (Kutluay et al. 2008), antifungal (Apisariyakul et al. 1995), antimalarial (Reddy et al. 2005), and antiprotozoal (Pérez-Arriaga et al. 2006) effects. No human clinical trial is this far reported except a study demonstrating great effects on scabies from topical treatment with a turmeric paste for 3–15 days in 814 patients (Charles and Charles 1992).

### INTESTINAL DISEASES

It is clear from what has been discussed above that curcumin to a large extent mediates its anti-inflammatory effects through inhibition of activation of NF- $\kappa$ B. This makes curcumin a promising candidate for treatment of IBD, alone or combined with other treatment modalities. Several successful experimental studies with curcumin in induced colitis are reported in recent years (for summary, see Camacho-Barquero et al. 2007). A recent study in experimental animals with trinitrobenzenesulfonic acid (TNBS)-induced colitis focussed on MAPKs such as the p38 and the c-Jun N-terminal kinase (JNK), known to regulate NF- $\kappa$ B activation and modulate the transcription of many genes involved in the inflammatory process. Oral supply of curcumin (50–100 mg/kg/day) not only dramatically reduced morphological signs of cell damage and stimulate the healing process, but also significantly reduced colonic levels of nitrites, colonic mucosa activity of MPO and TNF- $\alpha$ , and downregulated the expression of COX-2 and iNOS, and reduced activation of p38 MAPK (Camacho-Barquero et al. 2007). Few human studies have this far been performed. In an open study one capsule of pure curcumin (360 mg) was administered 3–4 times a day for 3 months to five patients with ulcerative proctitis and to five with Crohn's disease. All proctitis patients had improved reductions in disease activity & eliminate other concomitant medications could be done in four patients (Holt et al. 2005). Four of five Crohn's disease patients demonstrated reduced Crohn's disease activity index scores and lower erythrocyte sedimentation rates. The

ability of curcumin to prevent relapse was studied in a randomized, double-blind, multicenter trial in patients with quiescent ulcerative colitis (Hanai et al. 2006). Curcumin was administered for 6 months as 1 g after breakfast and 1 g after the evening meal. All patients received in addition to curcumin either sulfasalazine or mesalamine. Two of the 43 patients (4.65%) who received curcumin relapsed within 6 months, compared to 8 out of 39 patients (20.51%) in the placebo group ( $P = 0.040$ ). Some effects were also reported in clinical activity index ( $P = 0.038$ ) and especially in endoscopic index ( $P = 0.0001$ ) (Hanai et al. 2006).

#### NEURODEGENERATIVE DISEASES

Biochemical and physiologic stimuli: perturbation in redox status, accumulation and expression of misfolded proteins, altered glyc(osylation) and glucose deprivation, overloading of products of polyunsaturated fatty acid peroxidation, cholesterol oxidation and decomposition are among the factors that lead to the accumulation of unfolded or misfolded proteins in brain cells. Alzheimer's (AD), Parkinson's (PD), Huntington's diseases (HD), amyotrophic lateral sclerosis (ALS), and Friedreich's ataxia (FRDA) are all major neurological disorders, strongly associated with the production of abnormal proteins and, as such, belong to the so-called "protein conformational diseases" (Calabrese et al. 2006). Furthermore, a defect elimination/phagocytosis of amyloid-beta (A $\beta$ ) and clearance of A $\beta$  plaques by the innate immune cells, monocyte/macrophages, are reported to further contribute to the development of these neurodegenerative diseases (Zhang et al. 2006). Hsps and particularly HO-1 are also in this group of diseases identified to play a key role in cellular defense (Calabrese et al. 2003). Since it has been demonstrated that the expression of HO is closely related to amyloid precursor protein (APP), an increasing interest has focused on identifying dietary compounds that have the potential to inhibit, retard, or reverse the multistage pathophysiological events underlying these pathologies. Not only curcuminoids but also other antioxidants such as ferulic acid are known to be strong inducers of the heat shock response, which might provide exciting candidates for chemoprevention and treatment of these diseases (Calabrese et al. 2007). One small clinical pilot study was just concluded in AD patients. Unfortunately the treatment period was only 6 months and did not allow any definite conclusions to the clinical effects of curcumin treatment. However, the investigators showed that the treatment is safe and recommended that larger controlled studies are

undertaken (Baum et al. 2008). More pronounced effects of treatment will most likely be obtained if undertaken in a group of patients who are at risk but have not developed clinical signs of Alzheimer as yet. Panels of markers of inflammation should make it possible to identify such patients years before occurrence of clinical signs.

#### OCULAR DISEASES

Age-related cataractogenesis, for example, development of opacity of the eye lens, constitutes a significant health problem worldwide. Cataract is the leading cause of blindness worldwide, responsible for blindness of more than 20 million in the world. Nutritional deficiencies, especially lack of consumption of enough antioxidants, diabetes, excessive sunlight, smoking, and other environmental factors, are known to increase the risk of cataracts. Oxidative stress is regarded as the common mechanism behind cataractogenesis, and augmentation of the antioxidant defenses of the ocular lens has been shown to prevent or delay cataractogenesis. Curcumin feeding to experimental animals prevents the loss of alpha-crystallin chaperone activity and delays the progression and maturation of diabetic cataract (Kumar et al. 2005). Several other experimental studies report significant preventive effects of curcumin against cataracts when induced by various methods: naphthalene (Pandya et al. 2000), galactose (Raju et al. 2006, Suryanarayana et al. 2003), and selenium (Padmaja and Raju 2004).

In two uncontrolled studies, oral curcumin (1,125 mg/day) for 12 weeks to 22 months was found to improve chronic anterior uveitis, idiopathic inflammatory orbital pseudotumor, and other inflammatory conditions of the eye (Lal et al. 1999, 2000).

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#### ORAL CAVITY DISEASES

There are remarkable similarities in the pathogenesis of periodontal diseases and RA. Increased incidences of plaque, calculus, and gingival inflammation and increased prevalence and severity of destructive periodontal diseases are seen in most other chronic diseases. Periodontitis is clearly a sign of an increased systemic inflammatory burden, also manifested in signs such as elevation of C-reactive protein (CRP). Only one plant polyphenol, green tea polyphenol epigallocatechin gallate (EGCG) is so far tried and reported to reduce gingival inflammation and prevent periodontal diseases (Sakanaka and Okada 2004), but no human clinical study with curcumin is this far reported.

### PANCREATIC DISEASES

The effect of curcumin to reduce the damage to pancreas was studied in two different models: cerulein-induced and ethanol and cholecystokinin (CCK)-induced pancreatitis (Gukovska et al. 2003). Curcumin was administered intravenously in parallel with the induction of pancreatitis. A total of 200 mg/kg bw of curcumin was administered during a treatment period of six hours. Curcumin treatment significantly reduced histological injuries, the acinar cell vacuolization and neutrophil infiltration of the pancreatic tissue, the intrapancreatic activation of trypsin, the hyperamylasemia and hyperlipasemia, and the pancreatic activation of NF- $\kappa$ B, I $\kappa$ B degradation, activation of activator protein (AP)-1, and various inflammatory molecules such as IL-6, TNF- $\alpha$ , chemokine KC, iNOS and acidic ribosomal phosphoprotein. Curcumin did significantly stimulate pancreatic activation of caspase-3 in both models (Gukovska et al. 2003).

### RESPIRATORY DISEASES

Acute and chronic inflammatory lung diseases due to occupational and environmental exposures to mineral dusts, airborne pollutants, cigarette smoke, chemotherapy and radiotherapy are increasingly common. Curcumin offers a wide spectrum of therapeutic properties for these conditions. Curcumin is, as mentioned above, a potent inhibitor of TGF- $\beta$  and fibrogenesis (Gaedke et al. 2004), and suggested to have positive effects in various fibrotic diseases in kidneys, liver, intestine (Crohn's disease), pancreas, and in body cavities (prevention of fibrous adhesions) and on conditions with lung fibrosis, including cystic fibrosis (CF). CF is of special interest as it is especially linked to glutathione deficiency. The effect of curcumin against amiodarone-induced lung fibrosis was studied in rats. Significant inhibition of LDH activity, infiltration of neutrophils, eosinophils, and macrophages in lung tissue, LPS-stimulated TNF- $\alpha$  release, phorbol myristate acetate-stimulated superoxide generation, MPO activity, TGF- $\beta$ 1 activity, lung hydroxyproline content, and expression of type I collagen and c-Jun protein were observed when curcumin was supplemented in a dose of 200 mg/kg bw weight in parallel with intratracheal instillation of 6.25 mg/kg bw of amiodarone (Punithavathi et al. 2003). Curcumin exhibits structural similarities to isoflavonoid compounds that bind directly to the CFTR protein and alter its channel properties. Egan et al. (2004) observed that curcumin inhibits the calcium pump in endoplasmic reticulum, suggest-

ing that reducing the calcium levels might liberate the mutant CFTR and increase its odds of reaching the cell surface. A dramatic increase in survival rate and in normal cAMP-mediated chloride transport across nasal and gastrointestinal epithelia was observed when curcumin was supplemented to gene-targeted mice homozygous for the  $\Delta$ F508 (Illek et al. 2000). CF is characterized by at least three major biochemical deficits: diminished expression and activity of PPARs, increased PGE2 production, and elevated oxidative tissue injury. Curcumin should have the capacity to activate the PPAR anti-inflammatory pathway, typically underexpressed in CF, inhibit PGE2 synthesis, and protect against oxidative stress. Curcumin treatment in CF might function as an alternative until gene or other therapies aimed at restoring the CF transmembrane conductance function are realized (Emanuele et al. 2007). No human studies are, however, yet reported for CF. Significant reductions were observed in both airway constriction and airway hyperreactivity to histamine when the antiasthmatic effect of curcumin was tested in guinea pigs sensitized with ovalbumin (Ram et al. 2003).

### SKELETO-MUSCULAR DISEASES

Osteoporosis represents a major healthcare burden, affecting only in the United States approximately 10 million people aged over 50 years and with another 30 million or more at risk. Human and animal experiments indicate that proinflammatory cytokines such as IL-1, IL-6, and TNF $\alpha$  are primary mediators of osteoclastic bone resorption in aging individuals and in a variety of chronic diseases with accelerated bone loss (Mundy 2007). Increased production of proinflammatory cytokines is regularly associated with osteoclastic bone resorption both in chronic disease states and in individuals after estrogen withdrawal. The fact that the activation of NF- $\kappa$ B is, as discussed above, strongly linked with a large number of chronic diseases (see above) and that polyphenols like curcumin have the ability to inhibit this activation makes plant polyphenols, especially curcumin, an ideal candidate for prevention and treatment of incipient osteoporosis. However, the only studies with polyphenolic and other bioactive plant constituents, which have demonstrated preventive effects, have been performed with soy phytoestrogens and green tea polyphenols (Siddiqui et al. 2004).

Another obscure common disease, fibromyalgia, which has dramatically increased in the last decades, is also increasingly associated with increased systemic inflammation. Higher levels of IL-10, IL-8, and TNF- $\alpha$  are also reported in fibromyalgia patients

compared to healthy controls (Bazzichi et al. 2007). Although no studies with treatment with polyphenols have yet been undertaken, there are good reasons to assume that this category of patients could benefit from treatment with curcumenoids.

### **SKIN DISORDERS**

Curcumenoids have been recommended in a series of skin diseases from acne to psoriasis, but few studies have been undertaken. There exist many anecdotal reports of patients' successful treatment with curcumin, especially patients with psoriasis. A phase II, open-label trial was done with supplementation of 4.5 g/day of curcuminoids orally to patients with psoriasis (Kurd et al. 2008). Eight of 12 patients concluded a 16-week trial, with supplementation of 4.5 g/day of curcumin (3 pills of 500 mg, 3 times daily). Only a minority were reported to respond, but those who did respond achieved after 12 weeks excellent responses of 83% and 88% improvement in psoriasis area and severity index (PASI) scores. It is a general experience that polyphenol treatments often need to be continued for 6–12 months before results are seen. Unfortunately many patients do not have enough patience to wait that long. Large and long-lasting placebo-controlled studies are necessary before oral curcumin can be recommended as a treatment of psoriasis. However, most interesting animal experiments suggest that curcumin reduces aging effects on skin (Rattan and Ali 2007), reduces the skin destruction in irradiation (Okunieff et al. 2006) and burns (Singer et al. 2007), and promotes uneventful healing of skin (Panchatcharam et al. 2006).

### **TOBACCO/CIGARETTE SMOKE-INDUCED INJURIES**

Exposure to tobacco results in increased lipoxidation in the body and also dramatically increased activation and expression of NF- $\kappa$ B and its downstream target COX-2, and significant decreases in the levels of antioxidants such as ascorbic acid, vitamin E, reduced glutathione, GPx, SOD, and CAT. Animal studies demonstrate that such tobacco- and nicotine-induced changes are effectively counteracted by regular supply of turmeric and curcumin (Kalpana and Menon 2004; Thapliyal et al. 2004)

### **CONCLUSIVE REMARKS**

It is a great disappointment that only a few controlled clinical studies with curcumin or turmeric

have this far been performed and presented. It is a general experience that it is easier to get excellent results in young healthy animals with induced diseases than in humans with spontaneous diseases. Over the years numerous treatment modalities, successful in animals, have failed when tried in humans. One contributing factor most certainly is that humans under treatment usually continue with the unhealthy lifestyle that has led to the disease. Furthermore, treatments with natural products take a long time, often years, before results are seen. Modern day humans are often not prepared to spend that time waiting for clinical results. The whole idea of chemoprevention is that the plant-origin nutraceuticals are used regularly for the rest of the life.

However, the high incidence of side effects of pharmaceuticals has made an increasing number of individuals, especially among those with higher education, to turn to alternative and complementary compounds. Using medicinal plants and their active components remains an attractive approach for the prevention and treatment of various chronic diseases all based in impaired innate immunity and increased systemic inflammation. Food derivates have the advantage of being relatively nontoxic. Combination with other "nutraceuticals" such as other polyphenols and/or probiotic bacteria constitutes attractive but not explored treatment modalities. Preliminary observations suggest that such compounds have the ability to potentiate the effects of each other.

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# 13

## Alteration in Gene Expression and Proteomic Profiles by Soy Isoflavone

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### INTRODUCTION

With the development of microarray and proteomic technologies in recent years, nutrigenomics and nutriproteomics have received increased attention in the research field of basic and clinical nutrition (Maraman 2007; Trujillo et al. 2006). The emerging knowledge of nutrigenomics and nutriproteomics has strongly influenced research and practice in human nutrition. The term nutrigenomics refers to the effect of diet on gene expression profile, specifically the alteration of mRNA expression. However, the changes in mRNA concentration do not necessarily result in differences in the concentration and/or activity of the encoded protein. Therefore, nutriproteomic profiling is also an important strategy for investigating the diet–gene interaction to elucidate the true value of a diet. So far, a large number of reports regarding gene expression profiling of dietary compounds has been published; however, the proteomic profiling of dietary compounds has only been poorly investigated and reported because of the limitation of proteomic technique, which is beginning to be improved and is affordable.

Epidemiological studies have shown that a significant difference exists in the incidence of cancers, cardiovascular, and other diseases among different ethnic groups, and this difference has been believed to be partly attributed to dietary factors (varez-Leon et al. 2006). Soy isoflavones including genistein, daidzein, and glycinein, mainly derived from soybean have received much attention as dietary components having inhibitory effects on cancers, cardiovascular diseases, postmenopausal disorders, obesity, and diabetes. Soy isoflavones have been shown to inhibit carcinogenesis and cancer cell growth *in vivo*

and *in vitro* (Barnes 1995). It has also been found that soy isoflavones lower total cholesterol and low-density lipoprotein cholesterol, suggesting the effect of isoflavones on cardiovascular disease prevention (Jenkins et al. 2002). The Food and Drugs Administration has approved a health claim for soy-based food products for health benefit primarily based on epidemiological data, indicating that high soy consumption is associated with a lower risk of coronary artery disease (Food and Drug Administration 1999). Soy isoflavones also show beneficial role in obesity, diabetes, and osteoporosis in postmenopausal women (Allison et al. 2003; Bhathena and Velasquez 2002; Cotter and Cashman 2003). Therefore, it is important to investigate the molecular effects of isoflavone on the global gene and protein expression in order to elucidate the molecular mechanism of isoflavone action and design the molecular-based strategy of using isoflavone for the benefit of human health.

A number of gene expression profiles altered by soy isoflavone in different types of normal and cancer cells have been reported in recent years; however, few report proteomic profiling altered by isoflavone, although proteomic approaches are increasingly employed in nutritional research (Zeisel 2007). It has been found that soy isoflavone regulates the expression of genes that are related to estrogen regulation, organ differentiation, and fat and bone metabolism in normal cells (Ise et al. 2005; Pie et al. 2006). Soy isoflavone also inhibits the growth of cancer cells through the modulation of genes that control cell proliferation, cell cycle, apoptosis, oncogenesis, transcription regulation, and cell signal transduction system (Chen et al. 2001; Li and Sarkar 2002a, b; Takahashi et al. 2006). In this chapter, we provide current

evidence on the molecular effects of soy isoflavone as documented by nutrigenomic and nutriproteomic researches.

## STRUCTURE, METABOLISM, AND FUNCTION OF SOY ISOFLAVONE

### MOLECULAR STRUCTURE OF ISOFLAVONES

Isoflavones are a subclass of the flavonoids and are much more narrowly distributed in soybeans. The isoflavones including genistein and daidzein have been found in relatively high concentration in soybeans and most soy-protein products, while much lower amounts of glycitein are present in soybeans (Song et al. 1998). Precursors of daidzein and genistein, formononetin and biochanin A, respectively, are mainly found in clover (Tsao et al. 2006). The basic structural feature of isoflavone compounds is the

flavone nucleus, which is composed of two benzene rings (A and B) linked through a heterocyclic pyrane ring (Figure 13.1) (Messina 1999).

### METABOLISM OF SOY ISOFLAVONES

Soy isoflavones are present in foods mostly as glycosidic conjugates, which are more water soluble and require enzymatic cleavage of the sugar moiety by mammalian or microbial glucosidases before absorption. It has been found that in humans genistein is metabolized to dihydrogenistein and 6-hydroxy-*O*-desmethylangolensin while daidzein is metabolized to isoflavonoids, equol, dihydrodaidzein, and *O*-desmethylangolensin. Using various measurement techniques, isoflavones and their metabolites have been detected in human fluids such as plasma, prostatic fluid, and urine (Adlercreutz et al. 1993; Hedlund et al. 2006; Knight and Eden 1996). The

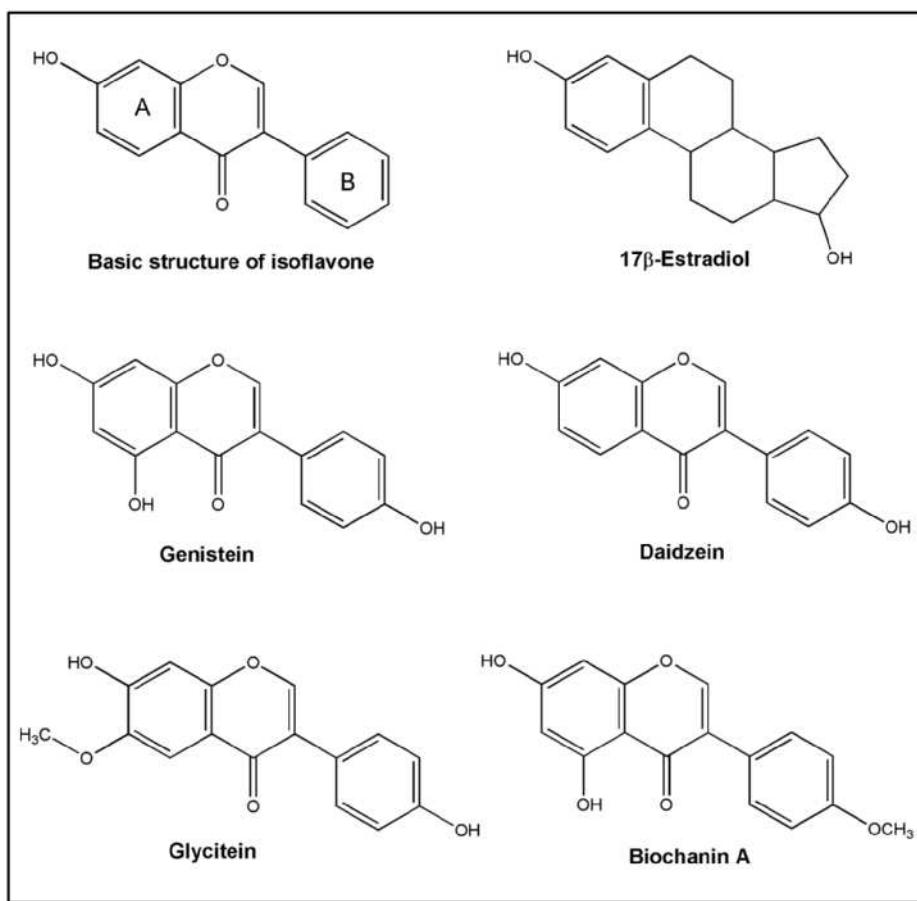


Figure 13.1. Molecular structures of isoflavones.

physiologic concentration of isoflavones in urine and plasma varies in different populations with different amounts of soy food intake. It has been found that the plasma level of genistein in people having a soy-rich diet was  $1.4 \pm 0.7$  to  $4.09 \pm 0.94 \mu\text{M}$  after metabolism and excretion (King and Bursill 1998; Xu et al. 2000). Genistein is relatively hydrophobic and expected to be taken up by cells and, thus, it may exert its effects on human health.

### FUNCTION OF ISOFLAVONES

Soy isoflavones have a close similarity in structure to estrogens (Figure 13.1), and have been known as phytoestrogens. Because of the structural similarity, both genistein and daidzein can bind to estrogen receptors (ERs); however, the binding affinities of genistein and daidzein for ERs are different, although they have very similar structures (Kuiper et al. 1998). After binding to ER, genistein might exert estrogen agonistic or antagonistic effect depending on its concentrations. Genistein at concentrations  $\leq 1 \mu\text{M}$  appears to act as an agonist and stimulates the growth of estrogen-dependent breast cancer cells when cultured in estrogen-free conditions, while concentrations  $\geq 5 \mu\text{M}$  inhibit  $17\beta$ -estradiol-stimulated cell proliferation (Martin et al. 1978).

In addition to the estrogen agonist and antagonist effect, soy isoflavones also have many important biochemical effects that are believed to mediate some of the health benefits, especially anticancer effect of soy isoflavone. Genistein is a known inhibitor of protein-tyrosine kinase and inhibits cell growth by downregulation of protein-tyrosine-kinase-mediated signaling pathway (Akiyama et al. 1987; Ullrich and Schlessinger 1990). Studies from our laboratory have shown that genistein is also a serine kinase inhibitor such as inhibition of Akt (Li and Sarkar 2002c). Genistein also inhibits topoisomerase I and II,  $5\alpha$ -reductase, and protein histidine kinase activity (Evans et al. 1995; Huang et al. 1992; Okura et al. 1988), all of which may contribute to antiproliferative or proapoptotic effects of genistein. In addition, isoflavone also regulates lipid and bone metabolism, suggesting its beneficial effect on cardiovascular diseases and postmenopausal disorders.

It has been found that soy isoflavones have antioxidant effect and also protect cells against reactive oxygen species by scavenging free radicals, inhibiting the expression of stress-response-related genes and the progression of carcinogenesis (Ruiz-Larrea et al. 1997; Zhou and Lee 1998). Among many stress-response genes and gene regulatory factors, NF- $\kappa$ B is known as a critical molecule-regulating

stress-response. We and others have found that the antioxidant effect of isoflavone is mediated through the inhibition of NF- $\kappa$ B pathway (Baxa and Yoshimura 2003; Davis et al. 1999; Davis et al. 2001). These effects of isoflavone are also involved in the induction of apoptotic processes in isoflavone-treated cells.

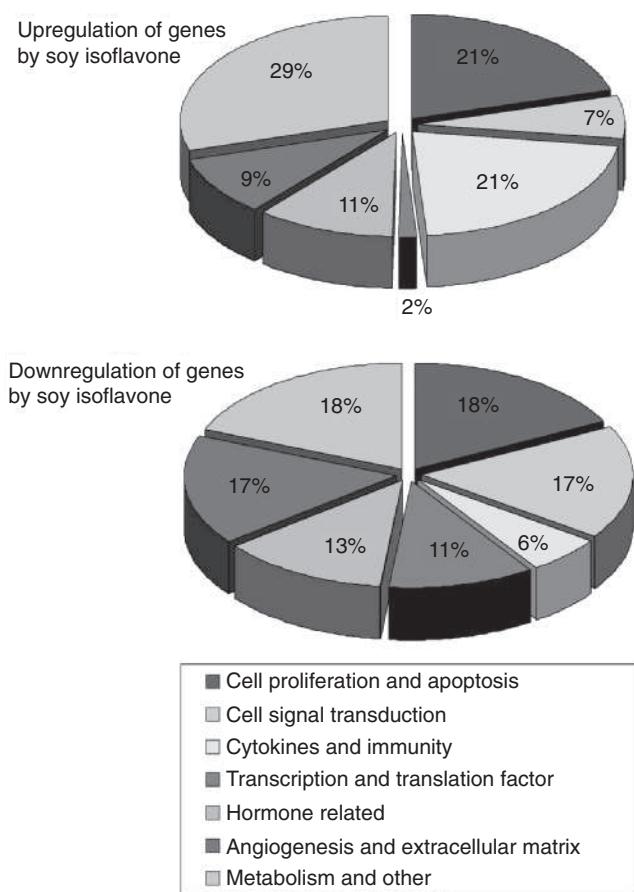
In addition to the NF- $\kappa$ B pathway, isoflavone also regulates other cell signal transduction pathways including Akt signaling pathway that plays important roles in cell survival and apoptotic death (Brunet et al. 1999; Li et al. 2002c). We found that inhibition of NF- $\kappa$ B activation by isoflavone was mediated via the inhibition of Akt signaling pathway (Li et al. 2002c). By inhibiting Akt and NF- $\kappa$ B pathways, isoflavone inhibits cell growth and induces apoptotic cell death. Importantly, isoflavone genistein has also been found to inhibit angiogenesis, cancer cell invasion, and metastasis (Fotsis et al. 1995; Li et al. 1999b, 2002a). Animal experiments also demonstrated that isoflavones inhibited bone metastasis of human breast cancer cells in a nude mouse model, and metastasis of androgen-sensitive human prostate cancers in mice (Iwasaki et al. 2002; Zhou et al. 2002). Gene expression and proteomic profiling analysis has revealed that soy isoflavones regulate a large number of genes related to different cellular physiological functions (Figure 13.2), suggesting the pleiotropic effects of soy isoflavone on human cells.

### ALTERATION OF GENE AND PROTEIN EXPRESSION PROFILES BY ISOFLAVONE IN NONCANCER CELLS

Several gene expression profilings have been conducted in noncancer cells treated with soy isoflavone. It has been reported that soy isoflavone regulates the expression of genes that are related to hormone regulation, differentiation, organogenesis, lipid and bone metabolism, immunity, cell signal transduction, transcription, and cell growth in noncancer cells (Table 13.1).

### REGULATION OF THE GENES RELATED TO HORMONE REGULATION, REPRODUCTIVE ORGANOGENESIS, DIFFERENTIATION, AND BONE METABOLISM

To investigate the effect of isoflavone on hormone regulation, mice were exposed to isoflavone genistein (1 mg/mouse/day) and tested for hormone-related genes using microarray analysis. The data from



**Figure 13.2.** Soy isoflavones regulate a large number of genes related to different cellular physiological functions.

microarray revealed that ER $\alpha$  and androgen receptor (AR) were downregulated in the testes of genistein-treated mice (Adachi et al. 2004), suggesting the effects of genistein on the regulation of hormone-related genes. In another comparison study, cDNA microarray and two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) analysis have been used to compare the different expression profiles of TM4 testicular cells treated with daidzein or 17 $\beta$ -estradiol (Adachi et al. 2005). The results from cDNA microarray and hierarchical clustering showed that daidzein treatment induced a different effect on gene expression compared to 17 $\beta$ -estradiol treatment. 2D-PAGE and hierarchical clustering of protein spot ratios also showed the difference in pattern of expression between daidzein and 17 $\beta$ -estradiol, consistent with microarray data (Adachi et al. 2005). Further analysis of the protein spot using mass spectrometry will elucidate the different proteomic profiles altered by daidzein and 17 $\beta$ -estradiol.

It has been known that exposure to some compounds with estrogenic activity during fetal development could alter development of reproductive organs, leading to abnormal function and disease either after birth or during adulthood. Microarray technology has been used to determine the transcriptional profile influenced by exposure to 17 $\alpha$ -estradiol or genistein during organogenesis and development in uterus and ovaries of rat (Naciff et al. 2002). A dose-dependent analysis of the transcript profile revealed a common set of genes whose expression is significantly and reproducibly modified in the same way by 17 $\alpha$ -estradiol and genistein (Table 13.1). However, each compound also induces changes in the expression of other transcripts that are not in common with the others, indicating that not all compounds with estrogenic activity act alike. Data from cDNA microarray analysis for examining the effects of genistein in the developing rat uterus indicated that genistein altered the expression of 6–8 times more genes

**Table 13.1.** Alteration of gene expression by isoflavone in noncancer cells.

Gene	Expression
<i>The genes related to cytokines and immunity</i>	
Histocompatibility 2, class II, locus Mb2	Up
Immunoglobulin lambda chain, variable 1	Up
Toll-like receptor 3	Up
Synuclein, alpha interacting protein (Synphilin)	Up
Chemokine receptor 5	Up
Interleukin 2 receptor, alpha chain (CD25)	Up
Immunoglobulin heavy chain 4 (serum IgG1)	Up
Immunoglobulin joining chain	Up
Immunoglobulin kappa chain variable 8	Up
Small chemokine (C-C motif) ligand 11	Up
Chemokine (C-C motif) ligand 8	Up
Complement component 3	Up
Complement component 1, q subcomponent	Up
Histocompatibility 2, D region locus 1	Up
Thymus LIM protein	Up
Activated leukocyte cell adhesion molecule	Up
Cadherin 2	Up
Receptor (calcitonin) activity modifying protein 2	Up
CD36 antigen	Up
Immunoglobulin heavy chain (J558 family)	Up
Immunoglobulin kappa chain variable 8 (V8)	Up
Tumor necrosis factor receptor superfamily, 11a	Up
Peptidoglycan recognition protein	Up
Interferon-related developmental regulator 2	Up
Interferon regulatory factor 2 binding protein 1	Up
mRNA for interleukin 4 receptor	Up
CD4 antigen	Down
Chemokine (C-C motif) ligand 6	Down
Defensin beta 7	Down
Interleukin 2	Down
CD84 antigen	Down
Complement component 1, r subcomponent	Down
T-cell receptor gamma, variable 4	Down
Natural killer tumor recognition sequence	Down
Interleukin 6	Down
Interleukin 12b	Down
Tumor necrosis factor-alpha-induced protein 6	Down
Tumor necrosis factor (ligand) superfamily, member 10	Down
Interleukin 1, beta	Down
<i>The genes related to hormone regulation, growth factor and cell growth</i>	
Insulin-like growth factor binding protein 2 (36 kD)	Up
Fibroblast growth factor receptor 3 (achondroplasia)	Up
Epidermal growth factor receptor pathway substrate 15	Up
Transforming growth factor-beta receptor II (70–80 kD)	Up
Insulin-induced growth-response protein (CL-6) mRNA, complete cds	Up
Insulin-like growth factor I mRNA	Up
Insulin-like growth factor I (IGF-I) mRNA, complete cds	Up
CDK7	Down

*(Continues)*

**Table 13.1. (Continued)**

ER $\alpha$ (M38651)	Down
ER $\beta$ (U81451)	Down
AR (M37890)	Down
<i>The genes related to kinase and cell signaling</i>	
Catenin (cadherin-associated protein)-alpha-like 1	Up
Signal transducer and activator of transcription 1	Up
Tyrosine protein kinase inhibitor alpha	Up
MAPK 10	Up
Ribosomal protein S6 kinase, 90 kD, polypeptide 4	Up
Protein tyrosine kinase 7	Down
Protein tyrosine phosphatase, receptor type, alpha polypeptide	Down
c-src tyrosine kinase	Down
<i>The genes related to cell size, extracellular and bone matrix</i>	
Potassium voltage-gated channel, subfamily G, member 2	Up
Chloride channel, nucleotide-sensitive 1A	Up
BMP 4	Up
mRNA for collagen alpha 1 type II, partial CDS	Up
Neural cell adhesion molecule BIG-1 protein (BIG-1) mRNA	Down
Laminin-gamma 2 (mouse)	Down
Laminin (W49392)	Down
Laminin, beta 1	Down
A disintegrin and metalloproteinase domain 9 (meltrin gamma)	Down
Integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)	Down
Integrin, alpha 3 (antigen CD49C-alpha 3 subunit of VLA-3 receptor)	Down
Integrin-alpha 5	Down
Integrin-beta 5	Down
Collagen, type III, alpha 1	Down
A disintegrin and metalloproteinase domain 9 (meltrin gamma)	Down
VE-cadherin	Down
Gap junction protein alpha 1 (connexin 43)	Down
Multimerin	Down
MMP 13 (collagenase 3)	Down
<i>The genes related to transcription factor, metabolism, and others</i>	
Sorting nexin 1	Up
Gamma-aminobutyric acid A receptor-delta	Up
Folylpolyglutamate synthase	Up
Profilin	Up
Homeo box A4	Up
Interferon-stimulated transcription factor 3 gamma (48 kD)	Up
Bromodomain adjacent to zinc finger domain, 1A	Up
Olfactomedin-like 3	Up
Pinin, desmosome associated protein	Up
Tachykinin receptor 3	Up
PDZ and LIM domain 2 (mystique)	Up
Formin 2	Up
EPH receptor A1	Up
Annexin V	Up
Lamin A	Up
Ubiquitin conjugating enzyme 12	Down
Sphingosine-1-phosphate phosphatase 1	Down

*(Continues)*

**Table 13.1.** (Continued)

Espin	Down
ATP-binding cassette, subfamily A (ABC1), member 1	Down
POU domain, class 4, transcription factor 1	Down
Stanniocalcin 1	Down
Ubiquitin carboxyl-terminal esterase L1 (ubiquitin thiolesterase)	Down
Dedicator of cytokinesis 3	Down
ATPase, H <sup>+</sup> transporting, lysosomal V0 subunit a isoform 2	Down
Adducin 2 (beta)	Down
ESTs, highly similar to endothelial NO synthase	Down
Recoverin	Down

compared to 17 $\beta$ -estradiol, suggesting that the effect of genistein is not just estrogenic (Naciff et al. 2002).

Proteomic technology has been used to investigate the mechanisms of action of isoflavone genistein in relation to differentiation and protection against chemically induced mammary cancer (Rowell et al. 2005). Rats were injected subcutaneously with isoflavone genistein and the proteins from the mammary glands were subjected to 2D-PAGE and mass spectrometry. The results showed that GTP-cyclohydrolase 1 (GTP-CH1) and tyrosine hydroxylase were upregulated and vascular endothelial growth factor receptor 2 (VEGFR2) was downregulated in the mammary glands of rats treated with genistein in the prepubertal period. This result suggests that early prepubertal exposure to genistein upregulates GTP-CH1, resulting in cell differentiation and gland maturation. The developmental maturation leads to reduced VEGFR2, which renders the mature mammary gland less proliferative and less susceptible to cancer (Rowell et al. 2005).

In addition, microarray analysis has been used to investigate the global gene expression profile altered by soy isoflavone supplement in lymphocytes from postmenopausal women (Niculescu et al. 2007). Expression of a large number of genes was altered by isoflavone supplement. Isoflavone induced genes associated with cyclic adenosine 3',5'-monophosphate (cAMP) signaling and cell differentiation (Table 13.1). Isoflavone also decreased expression of genes related to cyclin-dependent kinase (CDK) activity and cell division (Table 13.1). These findings suggest that isoflavone could increase cell differentiation and decrease cell proliferation (Niculescu et al. 2007). cDNA microarray has also been used in the survey of gene expression after genistein treatment in the mammary tissue differentiation. The results indicated that genistein treatment affected interactions

between steroid hormone receptor-mediated signals and growth factor-driven cellular pathways (Wang et al. 2006).

It has been known that bone metabolism is regulated by estrogen. By using microarray studies, the effect of genistein on bone metabolism-related genes has been investigated in animal model (Pie et al. 2006). Ovariectomized mice were injected daily with intraperitoneal genistein (0.1, 0.5, 1.5, and 3.0 mg/day) for 4 weeks. The administration of genistein restored bone mineral density in ovariectomized mice. Serum osteocalcin in ovariectomized mice treated with genistein was 1.6-fold higher than that in untreated mice. Importantly, genistein modulated bone metabolism-related gene expression, including calcitropic receptor, cytokines, growth factors, and bone matrix proteins (Pie et al. 2006), suggesting its beneficial effect on bone health.

#### REGULATION OF THE GENES RELATED TO ANGIOGENESIS, ATHEROGENESIS, LIPID METABOLISM, AND IMMUNITY

Endothelial cell plays an important role in the process of angiogenesis. Genistein is known to inhibit angiogenesis. To investigate the molecular effects of genistein on angiogenesis, a microarray gene expression-profiling study was performed to test the targeted genes of genistein in human umbilical vein endothelial cells (HUVECs) (Piao et al. 2006). The results showed that isoflavone genistein altered the genes that control cell proliferation, adhesion, transcription, translation, metabolism, cytoskeleton, apoptosis, and kinases in endothelial cells. Isoflavone genistein also downregulated the mRNA and protein expression of cell adhesion-related genes including VE-cadherin, gap junction protein alpha 1 (connexin 43), integrin alpha V, and multimerin

in endothelial cells (Piao et al. 2006), suggesting its inhibitory effects on the formation of blood vessels.

Endothelial cells also play a critical role in atherosclerosis. Epidemiological studies suggest that soy consumption contributes to the prevention of atherosclerosis. To identify proteins or pathways by which soy isoflavone might exert its protective activities on atherosclerosis, a proteomic analysis has been performed to investigate the response of HUVECs that were exposed to the pro-atherosclerotic stressors homocysteine or oxidized low-density lipoprotein (Ox-LDL) with or without isoflavone genistein treatment (Fuchs et al. 2005). Proteins from HUVECs were analyzed by 2D-PAGE and peptide mass fingerprints. The most interesting proteins that were potently decreased by homocysteine treatment were annexin V and lamin A, which are molecules for antiatherosclerosis. Importantly, genistein reversed the stressor-induced decrease of these antiatherosclerotic proteins. Ox-LDL treatment of HUVECs resulted in an increase in ubiquitin-conjugating enzyme 12, a protein involved in foam cell formation; however, treatment with genistein reversed this effect (Fuchs et al. 2005). The results from proteomic analysis suggest the protective effects of isoflavone on atherosclerosis.

It has been found that soy isoflavone genistein could also target adipose tissue by eliciting differential physiological effects depending on dietary intake. Gene microarray analysis identified factors in fat metabolism and obesity-related phenotypes that were differentially regulated by low and high doses of genistein, demonstrating its adipogenic and antiadipogenic actions. The lower dose induced the phospholipase A2 group 7 and the phospholipid transfer protein genes; however, the higher dose inhibited them (Penza et al. 2006), providing the molecular evidences for the adipogenic and antiadipogenic actions of genistein.

It has been known that genistein could induce thymic atrophy in mice and decrease both humoral and cell-mediated immunity. Microarray analysis of the effects of genistein on neonatal thymus indicated that genistein affected genes involved in the cell cycle, transcription, thymic development, and immune function (Table 13.1) (Cooke et al. 2006), suggesting its effects of genistein on immunity. These results are consistent with the genistein-induced inhibition of NF- $\kappa$ B, one of the important mediators of immune function. Therefore, as Dr Barnes indicated, isoflavones are much more than merely weak estrogens or tyrosine kinase inhibitors (Barnes 2004).

### ALTERATION OF GENE AND PROTEIN EXPRESSION PROFILES BY ISOFLAVONE IN CANCER CELLS

The gene expression and proteomic profiles altered by isoflavones has been investigated in several cancer cells including breast, prostate, bladder, endometrial, and pancreatic cancer (Bai et al. 2004; Chen et al. 2001, 2003; Lavigne et al. 2007; Li et al. 2002b; Takahashi et al. 2006). Profiling analysis has revealed that soy isoflavone regulates the expression of genes that are critical for the control of cell proliferation, apoptosis, cell signal transduction, transcription, translation, angiogenesis, invasion, and metastasis (Table 13.2). The data from these profiling analyses are considered very important for further designing molecular mechanism-based strategies for testing the beneficial role of isoflavone as cancer preventive and/or therapeutic agent.

### REGULATION OF THE GENES RELATED TO CELL PROLIFERATION AND APOPTOSIS

To better understand the precise molecular mechanisms by which genistein exerts its effects on cancer cells, we have utilized microarray technique to interrogate 12,558 known genes to determine the gene expression profiles of PC-3, LNCaP, and C4-2B prostate cancer cells treated with isoflavone genistein. We found that genistein regulated the expression of genes that are critically involved in the regulation of cell proliferation, cell cycle, apoptosis, cell signaling transduction, angiogenesis, tumor cell invasion, and metastasis (Li et al. 2002b). From the gene expression profiles of prostate cancer cells exposed to genistein, we found that genistein inhibited the expression of some genes (such as cyclin B, cyclin A, cdc25A, TGF- $\beta$ , ki67, and pescadillo), which are involved in the regulation of cell cycle. Some genes (e.g., p57<sup>KIP2</sup>, cyclin G2, growth arrest and DNA-damage-inducible protein, elafin) related to the control of cell proliferation were found to be upregulated (Table 13.2). Our results suggest that genistein may inhibit the cell growth through regulating the expression of these important genes related to cell proliferation.

Several studies have compared the effects of genistein at lower or higher concentrations on cancer cells using microarray profiling studies. Lavigne et al. (2007) have found that genistein altered the expression of genes involved in a wide range of pathways including estrogen- and p53-mediated pathways in breast cancer cells. At lower concentration (1  $\mu$ M), genistein elicited an expression pattern

**Table 13.2.** Alteration of gene expression by isoflavone in cancer cells.

Gene	Expression
<i>The genes related to cell cycle, cell growth, and apoptosis</i>	
Cdk-inhibitor p57KIP2 (KIP2) mRNA	Up
Cyclin G2 mRNA	Up
Growth arrest & DNA-damage-inducible	Up
Elafin gene	Up
CDC28 protein kinase regulatory subunit 2	Up
CDC28 protein kinase regulatory subunit 1B	Up
Chromatin licensing and DNA replication factor 1	Up
CCAAT/enhancer binding protein (C/EBP)-gamma	Up
Cyclin G1	Up
Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	Up
Jun D proto-oncogene	Up
Jun oncogene	Up
Stearoyl-CoA desaturase (delta-9-desaturase)	Up
7-dehydrocholesterol reductase	Up
M-phase phosphoprotein 9	Up
Homo sapiens programmed cell death 4 (PDCD4)	Up
Tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)	Up
Tumor necrosis factor receptor superfamily, osteoprotegerin	Up
BTG family, member 2	Down
Cyclin-dependent kinase (CDC2-like) 10	Down
Junction plakoglobin	Down
SMC5 structural maintenance of chromosomes 5-like 1	Down
Nuclear mitotic apparatus protein 1	Down
Parathymosin	Down
Cyclin B mRNA	Down
mRNA for cyclin A	Down
cdc25A mRNA	Down
cdc25B mRNA	Down
cdc25C mRNA	Down
Pescadillo mRNA	Down
mki67a mRNA	Down
mRNA for cytochrome b5	Down
TGF-beta-2 mRNA, complete cds	Down
B-cell CLL/lymphoma 3	Down
BCL2-associated athanogene	Down
BCL2-interacting killer (apoptosis-inducing)	Down
Tumor necrosis factor receptor superfamily, member 1A	Down
BCL2-like 1	Down
TNF receptor-associated factor 5	Down
Tumor necrosis factor (ligand) superfamily, member 10	Down
BCL2-antagonist of cell death	Down
Fibroblast growth factor 12	Down
Nuclear receptor coactivator 2	Down
Estrogen receptor 1	Down
Nuclear receptor coactivator 3	Down
Myeloid/lymphoid or mixed-lineage leukemia 2	Down
Apoptosis inhibitor (survivin)	Down
Topoisomerase (DNA) II	Down

(Continued)

**Table 13.2.** (Continued)

CDC6 (cell division cycle 6, <i>S. cerevisiae</i> )	Down
TNF-induced protein	Down
v-maf fibrosarcoma oncogene homolog	Down
<i>Hormone-responsive genes</i>	
Replication factor C (activator 1) 4, 37 kDa	Up
Amphiregulin (schwannoma-derived growth factor)	Up
Nuclear receptor interacting protein 1	Up
Topoisomerase (DNA) II alpha 170 kDa	Up
Seven in absentia homolog 2 ( <i>Drosophila</i> )	Up
Trefoil factor 1	Up
Insulin-like growth factor binding protein 4	Up
Chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)	Up
v-myb myeloblastosis viral oncogene homolog (avian)-like 1	Up
GREB1	Up
Retinoic acid induced 3	Up
Syntaxin binding protein 1	Up
Clusterin	Up
ER $\alpha$	Down
Serum response factor (SRF)	Down
Chromosome 11 open reading frame 8	Down
ATP-binding cassette, sub-family C (CFTR/MRP), member 5	Down
KIAA0922	Down
Rho family GTPase 3	Down
Chromobox homolog 6	Down
Breast carcinoma amplified sequence 1	Down
Glucosamine (UDP- <i>N</i> -acetyl)-2-epimerase/ <i>N</i> -acetylmannosamine kinase	Down
Polo-like kinase 2 ( <i>Drosophila</i> )	Down
Kynureinase (L-kynureanine hydrolase)	Down
Interleukin 1 receptor, type I	Down
Aryl-hydrocarbon receptor nuclear translocator 2	Down
N-myc downstream regulated gene 1	Down
Immediate early response 3	Down
Myosin IB	Down
Cathepsin H	Down
Dual specificity phosphatase 4	Down
Ephrin-A1	Down
Myoglobin	Down
v-erb-b2 erythroblastic leukemia viral oncogene homolog 2	Down
Selenium binding protein 1	Down
BCL2-interacting killer (apoptosis-inducing)	Down
Lipopolysaccharide-induced TNF factor	Down
SMAD family member 3	Down
B-cell linker	Down
Histone cluster 2, H2aa3	Down
Chromosome 10 open reading frame 116	Down
<i>The genes related to kinase and cell signaling</i>	
Protein kinase inhibitor (human, cell line)	Up
Protein kinase, Y-linked	Up
Thymidine kinase 1 (TK1)	Up
Serine/threonine protein kinase	Down

(Continued)

**Table 13.2.** (Continued)

NF- $\kappa$ B	Down
Defender against cell death 1 (DAD-1)	Down
PIM-2	Down
cdc2-related protein kinase mRNA	Down
Serine/threonine kinase (BTAK) mRNA	Down
mRNA for Ribosomal protein kinase B	Down
Stress-activated protein kinase 4 mRNA	Down
Receptor tyrosine kinase (DTK) mRNA	Down
RAB11B, member RAS oncogene family	Down
Pleckstrin homology, Sec7, and coiled/coil domains 3	Down
Ran GTPase activating protein 1	Down
Plexin B1	Down
Growth factor receptor-bound protein 2	Down
ADP-ribosylation factor 1	Down
Pleckstrin homology, Sec7, and coiled/coil domains 1	Down
MYC-associated zinc finger protein	Down
Mitogen-activated protein kinase kinase 3	Down
Nuclear receptor-binding protein	Down
Guanine nucleotide-binding protein beta polypeptide 2	Down
Mitogen-activated protein kinase 6	Down
Signal sequence receptor-a	Down
Ca/calmodulin dependent protein kinase kinase 2, beta	Down
Neuronal immediate early gene, 2	Down
IQ motif GTPase activation protein 2	Down
Inositol polyphosphate 4-phosphatase, type II	Down
NK3 transcriptional factor related	Down
Prostate epithelium-specific Ets transcriptional factor	Down
EGFR	Down
Akt2	Down
CYP1B1	Down
NELL2	Down
Ste-20-related kinase	Down
EphB3 receptor	Down
Protein kinase C-alpha mRNA, partial 3	Down
<i>The genes related to stress response, DNA repair and methylation</i>	
Homocysteine-induced endoplasmic reticulum ubiquitin-like domain member1	Up
Heat shock protein HSPA2 gene	Up
Heat shock protein 105	Up
Glutathione reductase	Down
Cytochrome b-245, alpha polypeptide	Down
P450 (cytochrome) oxidoreductase	Down
DNA (cytosine-5-)-methyltransferase 1	Down
Bcl-2-related ovarian killer protein (Bok)	Down
<i>The genes related to transcription and translation</i>	
Dual-specific phosphatase 1	Up
Nucleosome assembly protein 1-like 4	Up
Tumor necrosis factor receptor superfamily, member10b	Down
Interleukin 14	Down
Type I transmembrane protein Fn14	Down
Exostoses (multiple)-like 3	Down

(Continued)

**Table 13.2.** (Continued)

Gp25L2 protein	Down
translation initiation factor eIF-2 gamma	Down
Nuclear factor NF90 mRNA	Down
Translation initiation factor 5 (eIF5) mRNA	Down
Eukaryotic protein synthesis initiation factor	Down
Thioredoxin mRNA	Down
Forkhead box J1	Down
General transcription factor IIF, polypeptide 1, 74 kDa	Down
Chromobox homolog 6	Down
Eukaryotic translation initiation factor 5A	Down
Chromatin assembly factor 1, subunit A (p150)	Down
PRP8 pre-mRNA processing factor 8 homolog (yeast)	Down
TEA domain family member 3	Down
Nucleosome assembly protein 1-like 4	Down
Eukaryotic translation elongation factor 2	Down
Trf (TATA binding-protein-related factor)	Down
Tubby-like protein 3	Down
Small nuclear ribonucleoprotein polypeptide A	Down
Lysophosphatidic acid G-protein-coupled receptor, 4	Down
18s and 28s rRNA	Down
Transforming growth factor, beta 2	Down
<i>The genes related to migration, invasion, and angiogenesis</i>	
Connective tissue activation peptide III mRNA	Up
Tissue factor pathway inhibitor 2 (TFPI-2)	Up
Activating transcription factor 3 (ATF3)	Up
DNA (cytosine-5-)methyltransferase 1 (DNMT1)	Up
MT-1 matrix metalloproteinase cytoplasmic tail-binding protein-1 (MTCBP-1)	Up
Connective tissue growth factor	Up
E-cadherin	Up
Vascular endothelial growth factor gene	Down
Urokinase plasminogen activator	Down
Urokinase plasminogen activator receptor	Down
Protease M mRNA	Down
Type IV collagenase mRNA	Down
Bone-derived growth factor (BPGF-1) mRNA	Down
Lysophosphatidic acid receptor mRNA	Down
Thrombospondin-1 gene, controversial	Down
mRNA for thrombospondin, controversial	Down
Proteinase-activated receptor-2 mRNA	Down
Aminopeptidase N/CD13 mRNA	Down
Ankyrin 1, erythrocytic	Down
CD44 antigen	Down
Phosphodiesterase 7A	Down
Laminin, gamma 1 (formerly LAMB2)	Down
Integrin, alpha 3 (antigen CD49C, alpha 3 subunit of VLA-3 receptor)	Down
Integrin, beta 4	Down
Fibronectin 1	Down
A disintegrin and metalloproteinase domain 9	Down
mRNA for membrane-type matrix metalloproteinase 1	Down
Chemokine (C-X-C motif) ligand 12 (CXCL12)	Down

(Continued)

**Table 13.2.** (Continued)

Homo sapiens matrix metalloproteinase 2	Down
Homo sapiens matrix metalloproteinase 14	Down
Homo sapiens matrix metalloproteinase 11	Down
Homo sapiens matrix metalloproteinase 9	Down
Homo sapiens matrix metalloproteinase 7	Down
Homo sapiens matrix metalloproteinase 13	Down
<i>The genes related to structures and cholesterol synthesis</i>	
ATP synthase, mitochondrial F1 complex, epsilon sub	Up
Mitochondrial ribosomal protein S25	Up
Carbonyl reductase 1	Up
Procollagen, alpha-2 type IV	Up
Collagen, type XVIII, alpha 1	Down
Lamin	Down
Solute carrier family 12, member 4	Down
Microtubule-associated protein 4	Down
Tubulin beta polypeptide (TUBB)	Down
Potassium channel tetramerisation domain containing 2	Down
Mitochondrial ribosomal protein L49	Down
SEC61A1 protein transport protein	Down
KDEL endoplasmic reticulum protein retention receptor 1	Down
Mevalonate (diphospho) decarboxylase	Down
Transmembrane 4 superfamily member 1 (Tm4sf1)	Down
Keratin 8-like 2	Down
Stromal antigen 3	Down
Spindlin	Down
Keratin, hair, acidic, 4	Down
<i>Metabolism, protease, and other</i>	
Cockayne syndrome 1 (classical)	Up
Dopa decarboxylase	Up
Butyrylcholinesterase	Up
Phosphoinositide-3-kinase, regulatory subunit	Up
DnaJ (Hsp40) homolog, subfamily B	Up
Isopentenyl-diphosphate delta isomerase	Up
Stearoyl-CoA desaturase	Up
UDP-glucose dehydrogenase	Up
SEC24-related gene family, member D	Up
Elastase 3B, pancreatic (Ela3b)	Up
Pancreatic lipase related protein 1 (Pnliprp1)	Up
Trypsin 2 (Try2)	Up
Elastase 2 (Ela2)	Up
Amylase 2, pancreatic (Amy2)	Up
Phosphate cytidylyltransferase 1, choline, alpha isoform (Pcyt1a)	Up
Glucokinase activity, related sequence 2 (Gk-rs2)	Up
Chymotrypsin-like (Ctrl)	Up
Solute carrier family 2 (facilitated glucose transporter)	Up
Farnesyl diphosphate farnesyl transferase 1 (Fdft1)	Up
Ribonuclease, Rnase A family, 1 (pancreatic) (Rnase1)	Up
CD36 antigen (Cd36)	Up
Sorbitol dehydrogenase precursor	Down
Lysozyme (Lyzs)	Down

(Continued)

**Table 13.2.** (Continued)

Cleft lip and palate-associated transmembrane protein 1	Down
Cleft lip and palate-associated transmembrane protein 1	Down
Dopachrome tautomerase (tyrosine-related protein 2)	Down
Dentatorubral-pallidoluysian atrophy (atrophin-1)	Down
Nuclear pore complex interacting protein	Down
Autoantigen	Down
Papillary renal cell carcinoma (translocation-associated)	Down
Glutamate-ammonia ligase (glutamine synthase)	Down
Aldolase B, fructose-bisphosphate	Down
Transketolase (Wernicke-Korsakoff syndrome)	Down
NICE-3 protein	Down
Nuclear receptor subfamily 4, group A, member 1	Down
Ubiquitin specific protease 20	Down
<i>N</i> -ethylmaleimide-sensitive factor attachment protein	Down
Ataxin 2-like	Down
Vitelliform macular dystrophy 2	Down
Steroidogenic acute regulatory protein	Down
Aldehyde dehydrogenase 1, member A3	Down
24-dehydrocholesterol reductase	Down
Long-chain polyunsaturated FA elongation	Down
Sorbitol dehydrogenase	Down
Kallikrein 2, prostatic	Down
Kallikrein 3, prostate specific antigen	Down
Transmembrane protease, serine 2	Down
Transmembrane, prostate androgen induced RNA	Down
Disabled homolog 2 (DOC 2)	Down
Recombination activation gene 1 (RAG-1)	Down
UDP- <i>N</i> -acetylglucosamine pyrophosphorylase	Down

suggestive of increased mitogenic activity, consistent with the proliferative response to the lower concentration of genistein observed in cultured MCF-7 cells. However, genistein at 25  $\mu$ M effected gene expression that likely contributes to increased apoptosis and decreased proliferation, also consistent with the results from cell culture (Lavigne et al. 2007). A similar result has been observed in genistein-treated endometrial cancer cells (Konstantakopoulos et al. 2006). Genistein at low concentration induced multiple changes in the expression of genes that are implicated in oncogenesis. In contrast, treatment with higher concentration of genistein predominantly inhibited the expression of genes related to cell proliferation (Konstantakopoulos et al. 2006). To investigate the effect of isoflavone as a phytoestrogen, a customized cDNA microarray including 172 estrogen responsive genes was utilized and the expression profile altered by isoflavone was accessed and compared with 17 $\beta$ -estradiol-altered expression profile. The results showed that genistein at

lower concentration functioned as a weak estrogen to regulate p53-related genes (CDKN1A, TP53I11, and CDC14), Akt2-related genes (PRKCD, BRCA1, TRIB3, and APPL), and MAPK-related genes (RSK and SH3BP5) in MCF-7 breast cancer cells (Ise et al. 2005).

Global gene expression profile analysis of LNCaP prostate cancer cells exposed to genistein, equol, and daidzein has been conducted and compared (Takahashi et al. 2006). The multidimensional scaling analyses of the expression patterns suggested that these compounds exerted differential effects on cell cycle-related genes in LNCaP cells, suggesting the different potency of isoflavones on cell proliferation. However, these three compounds also exerted similar effect on genes that control other important cellular pathways including androgen-responsive genes, IGF-1 pathway gene, and MAP kinase-related pathway (Takahashi et al. 2006).

From the gene expression profiles of PC-3 cells exposed to genistein, we also found that genistein

downregulated the expression of some genes (such as survivin and pescadillo) that are involved in the inhibition of apoptosis (Li et al. 2002b). Moreover, additional genes (GADD, elafin, etc.) related to promoting apoptotic cell death were found to be upregulated. Our results suggest that genistein could inhibit overall cell growth through regulation of the expression of these important genes related to apoptosis. Other investigators also reported similar results showing that apoptosis inhibitor survivin was downregulated in genistein-treated LNCaP prostate cancer cells (Suzuki et al. 2002). They also found that DNA topoisomerase II, cell division cycle 6 (CDC6), and mitogen-activated protein kinase 6 (MAPK 6) were downregulated. However, the expression level of glutathione peroxidase (GPx)-1 was significantly increased after genistein treatment, suggesting that GPx activation might be one of the important effects of genistein on the inhibition of prostate cancer cell growth (Suzuki et al. 2002).

#### REGULATION OF THE GENES RELATED TO CELL SIGNALING

To better understand the molecular mechanisms underlying the beneficial roles of genistein in the prevention of prostate cancer, cDNA microarray approach has been used to examine the effects of genistein at concentrations in the physiologic range (0, 1, 5, or 25  $\mu$ M) on global gene expression patterns in androgen-independent LNCaP prostate cancer cells (Takahashi et al. 2004). A concentration-dependent modulation of multiple cellular pathways, which are important in prostate carcinogenesis, has been observed in genistein-treated LNCaP cells. Interestingly, the AR-mediated pathways, in particular, appeared to be downregulated by genistein, suggesting a chemopreventive mechanism for genistein administered at physiologic levels (Takahashi et al. 2004).

The gene expression profiling of genistein-treated Panc-1 pancreatic cancer cells has also been performed. Results showed that genistein significantly upregulated egr-1 and IL-8, and downregulated EGFR, Akt-2, CYP1B1, NELL2, SCD, DNA ligase III, Rad, 18s and 28s rRNA, and others. The most obvious target of genistein is the EGFR signaling pathway, since EGFR, AKT2, CYP1B1, and NELL2 all are related to this pathway and were reduced, suggesting that genistein could inhibit pancreatic cancer cell growth by the inhibition of EGFR signaling pathway (Bai et al. 2004).

A microarray for screening 847 genes involved in cytokine signal transduction has been used to test the effect of genistein on pancreatic cancer cells. The

expression of genes regulating tyrosine phosphorylation signaling was found to be altered by genistein treatment. The most highly downregulated gene was the EphB3 receptor that is a tyrosine kinase, suggesting the effect of genistein as tyrosine kinase inhibitor (Farivar et al. 2003).

Using cDNA microarray technology, signaling pathway genes altered by genistein were also identified in MCF-7 cells (Chen et al. 2003). Genistein upregulated heat shock protein 105 (HSP105) mRNA and downregulated mRNA expression of serum response factor (SRF), ER $\alpha$ , disabled homolog 2 (DOC 2), and recombination activation gene 1 (RAG-1). Western blotting analysis showed that the expression of the ER $\alpha$  and SRF protein decreased significantly with genistein treatment, suggesting that the inhibitory effect of genistein on human breast cancer cells could be mediated by the alteration of ER-dependent pathways (Chen et al. 2003).

Another study for further investigating the molecular mechanisms of the antiproliferative effects of biochanin A, another isoflavone, on LNCaP cells and xenografts has been reported. By cDNA microarray and Western blot analysis, the authors found that biochanin A mainly downregulated the genes involved in signal transduction, cell adhesion, ubiquitination, transcription, and translation (Rice et al. 2002). In mice with LNCaP xenografts, biochanin A significantly reduced tumor size and incidence, suggesting that biochanin A could regulate genes in multiple pathways and inhibit tumor growth (Rice et al. 2002).

#### REGULATION OF THE GENES RELATED TO TUMOR ANGIOGENESIS, INVASION, AND METASTASIS

We and others have found that isoflavone genistein decreased the angiogenic and metastatic potential of cancers (Fotsis et al. 1995; Li et al. 1999a, b). Our laboratory has examined the inhibitory effect of genistein on tumor cell invasion and metastasis of MDA-MB-435 breast cancer cells transfected with *c-erbB-2*, which has been shown to promote secretion of metalloproteinases (MMPs) and subsequent metastasis in experimental models. We found significantly higher levels of MMP-2 and MMP-9 in the conditioned medium from 435 transfectants (Li et al. 1999b). These MMPs were significantly decreased in cells treated with genistein. In order to better understand the precise molecular mechanisms by which genistein exerts its antiangiogenic and antimetastatic effects on cancer cells, we have also utilized

microarray gene expression studies for determining the comprehensive gene expression profile altered by genistein treatment (Li et al. 2002a). Among the genes related to angiogenesis and metastasis, we found that genistein downregulated the expression of MMP-9, protease M, uPAR, VEGF, neuropilin, TSP, BPGF, LPA, TGF- $\beta$ 2, TSP-1, and PAR-2, and upregulated the expression of connective tissue growth factor and connective tissue activation peptide (Table 13.2). The alteration of protein expression of these genes by genistein has been confirmed by Western blot analysis. These results demonstrated that genistein inhibited the transcription and translation of genes critically involved in the control of tumor angiogenesis, invasion, and metastasis (Li et al. 2002a), suggesting the possible therapeutic role of genistein for metastatic prostate cancer.

We have also utilized SCID-hu prostate cancer bone metastasis model and used microarray technique to investigate the effect of genistein on bone metastasis and metastasis-related gene expression. We found that genistein regulated the expression of multiple genes involved in the control of cell proliferation, apoptosis, and metastasis both *in vitro* and *in vivo* (Li et al. 2004). The expression of various MMPs in PC-3 bone tumors was inhibited by genistein treatment, whereas osteoprotegerin (OPG) was upregulated, suggesting that genistein could inhibit prostate cancer bone metastasis by regulating metastasis-related genes (Li et al. 2004).

Using oligonucleotide microarrays, other investigators have also determined the metastasis-related gene expression profile altered by genistein in HCC1395 cells, a cell line derived from an early-stage primary breast cancer. They found that TFPI-2, ATF3, DNMT1, and MTCBP-1, which are known to inhibit invasion and metastasis, were upregulated by genistein treatment, and MMP-2, MMP-7, and CXCL12, which are known to promote invasion and metastasis, were downregulated (Lee et al. 2007). A downregulated transcription of uPA, uPAR, and various integrin subunits was also demonstrated in genistein-treated cells using microarray analysis (Skogseth et al. 2006).

Perineural invasion (PNI) is the major mechanism of prostate cancer spread outside the prostate. An increase in cell proliferation and a decrease in apoptosis were observed in human PNI cells and the PNI model. In PNI, cDNA microarray analysis also showed upregulation of several genes including NF- $\kappa$ B and its downstream targets, defender against cell death 1 and PIM-2, suggesting that these molecules are involved in PNI invasion. Interestingly, genistein treatment resulted in a decrease in NF- $\kappa$ B, PIM-2

and defender against cell death 1, and was associated with the induction of apoptosis, suggesting the inhibitory effect of genistein on prostate cancer cell PNI (Ayala et al. 2004).

#### **REGULATION OF THE GENES RELATED TO THE ENHANCEMENT OF CANCER THERAPY**

In animal radiation therapies, soy isoflavone has been found to exert protective effects on the liver of the animal, enhancing the efficacy of radiation therapy. To elucidate the molecular mechanisms by which soy isoflavone protect liver against radiation damage, Song et al. (2006) utilized a cDNA microarray to investigate the expression profiles of 4,096 known genes in the livers of irradiated-mice with or without soy isoflavone treatment. They found that 68 genes were upregulated and 28 genes were downregulated in mice treated with irradiation alone, whereas only 6 genes were downregulated and 35 genes were upregulated in mice treated with soy isoflavone. Some of the downregulated genes in the irradiated group, such as DNA repair and stress response genes that are markers of cellular damage after irradiation, were maintained at close to normal expression levels after soy isoflavone treatment, suggesting the protective effect of soy isoflavone on irradiation damage (Song et al. 2006).

More importantly, we have also conducted animal experiment, microarray analysis, and Western Blot analysis to investigate the effects of genistein on the enhancement of therapeutic efficacy of docetaxel, one of the commonly used chemotherapeutic agents for the treatment of prostate cancer (Li et al. 2006). We found that the expression of OPG was induced by genistein and inhibited by docetaxel, whereas MMP-9 was downregulated by genistein and upregulated by docetaxel. Moreover, genistein significantly downregulated the expression and secretion of receptor activator of NF- $\kappa$ B (RANK) ligand (RANKL) and inhibited osteoclast formation, suggesting that the observed potentiation of antitumor activity of docetaxel by genistein in the animal model could be mediated by the regulation of OPG/RANK/RANKL/MMP-9 signaling (Li et al. 2006).

#### **CONCLUSION AND PERSPECTIVE**

In conclusion, emerging studies using gene expression profiling have indicated that soy isoflavones regulate a large number of genes that control cell proliferation, differentiation, apoptosis, cell signal

transduction, transcription, and translation. Clinical trials and epidemiological data have demonstrated that high soy consumption is associated with a lower risk of coronary artery disease. More evidence from epidemiological and experimental studies has also shown the beneficial effects of soy isoflavone on reducing the risk of cancers. However, additional studies from *in vivo* preclinical model and human intervention trials using genomics and proteomics are urgently needed for elucidating the molecular mechanisms underlying the beneficial effects of soy isoflavones for prevention and/or treatment of human diseases, which could be useful for further rational design of clinical trials.

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## 14

# Green Tea Polyphenol-Modulated Genome Functions for Protective Health Benefits

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## INTRODUCTION

The perspective with which the world views green tea is now in the process of changing. When tea culture is introduced, the Japanese tea ceremony still ranks right up there. It has been said that the twenty-first century is an era of health-oriented society of many differing civilizations, cultures, and ethnic customs. Human beings have a long history of tea plant cultivation and tea drinking. Today, tea beverages originated from the leaves of tea plant (*Camellia sinensis*) have become the most widely consumed beverages in the world. With excellence in unique and attractive colors, flavor, taste, and numerous health benefits, tea has increased its acceptance universally by attracting attention of consumers from all over the world. Thus, it can be said that the spirit of tea is the spirit of life.

Over the last two decades, more than five thousand peer-reviewed articles and ten thousand news articles have evidenced enhanced health benefits of tea. At present, multiple evidences have proven the involvement of tea beverages in health preservations that directly linked to its polyphenol content. Quality and concentration of polyphenol in tea infusion is directly influenced by tea-processing conditions such as complete fermentation (black tea), semi-fermentation (oolong tea), and least fermentation (green tea). Among all, green tea has firmly established its powerful strength in reducing oxidative stress, suppressing cancer-related risks, cardiovascular disease, neuronal damage, hepatic disorders, and so on. Epidemiological and clinical studies have also proven that individuals consuming tea or many forms of tea polyphenols suffer lower incidence of

cancers and other lifestyle-related diseases such as diabetes, obesity, and cardiovascular disease. In a previous contribution, we have accumulated plenty of data and information about green tea polyphenols and their physiological and preventive or therapeutic effects on diseases (Yamamoto et al. 1997). However, the question still to be answered is, how green tea polyphenols exert their health benefit effects? Whether it is a simple site-specific activity or it alters a pathway that ultimately leads to altered activity of one or more secondary molecules required to maintain normal cell function or it enhances the meaningful roles of the molecules to maintain cell machinery systems have long been questioned. Recent advances in the techniques related to molecular and cellular biology have answered many speculations that have come up in multiple levels of clinical and epidemiological studies. Several recent reports have provided evidences that green tea polyphenols target at the molecular level and alter gene functions that ultimately translate into benefits of tea consumption. On the basis of available evidences, this chapter briefly reviews how green tea polyphenols modulate genome function and gene repair, protect genes, and exert the roles considered auspicious, which remained unknown a decade ago. This chapter also lists the latest evidences in accordance with the enhanced physiological functions. Beginning with a basic introduction to tea catechins followed by genes expression studies, this chapter provides a realistic broad view of green tea catechin (GTC)-modulated signal transduction pathways and altered gene expression of various proteins, peptides, and hormones that are responsible for human health.

## STRUCTURE AND CHEMICAL PROPERTIES OF GREEN TEA POLYPHENOLS

Usually, the manufacturing process of green tea includes heating green tea leaves to inactivate enzymes and drying the leaves to preserve the constituents. However, the polyphenol composition of tea leaves varies with variety, cultivation, climate, season, and age of the tea leaves. The characteristic tea polyphenols are known as catechins, and the major tea catechins are (−)epicatechin (EC), (−)epigallocatechin (EGC), (−)epicatechin gallate (ECG), (−)gallocatechin, (+)catechin, and (−)epigallocatechin gallate (EGCG) (see Figure 14.1). The structure of catechins has two asymmetric carbon atoms at C-2 and C-3 positions. Epigallocatechin and gallocatechin contain an additional phenolic hydroxyl group as compared to epicatechin and catechin, respectively. Catechin gallates, such as EGCG and ECG, are gallic acid ester of catechins EGC and EC, respectively. Catechins and its derivatives have two nucleophilic centers at C-6 and C-8 positions. They are highly reactive toward electrophilic counterparts, and show the properties like metal chelating, free radical scavenging, and nitrosation inhibition. There is also a wealth of evidence, which suggests

that the free radical scavenging potential of catechins relates directly to the chemical structure of each compound, namely, the hydroxyl groups at the C-5 and C-7 positions on the A ring, the catechol group (3,4-dihydroxyl groups) on the B ring, and a gallate group attached at the C-3 position of the C ring. The vicinal hydroxyl groups also make these compounds susceptible to air oxidation under neutral or alkaline pH. It is proposed that auto-oxidation of EGCG leads to the generation of superoxide and hydrogen peroxide and the formation of dimers such as theasinensins. It is well documented that the polyphenolic structure of tea catechin provides them a good donor of H-bonding, which facilitates them in forming complexes with many kinds of compounds having similar or unrelated structures including itself and others, such as peptide, protein, amino acid, caffeine, metal ions and polysaccharides, and also nucleic acids. It is widely considered that catechin's radical scavenging activity and the ability to form complexes with other compounds as mentioned above is closely related to its physiological function.

Over the past two decades, extensive scientific evidences have linked the physiological functions of GTCs with altered cell signaling pathway in *in-vitro* and *in-situ* bioassay systems. Such altered cell signaling factors are readily translated into the

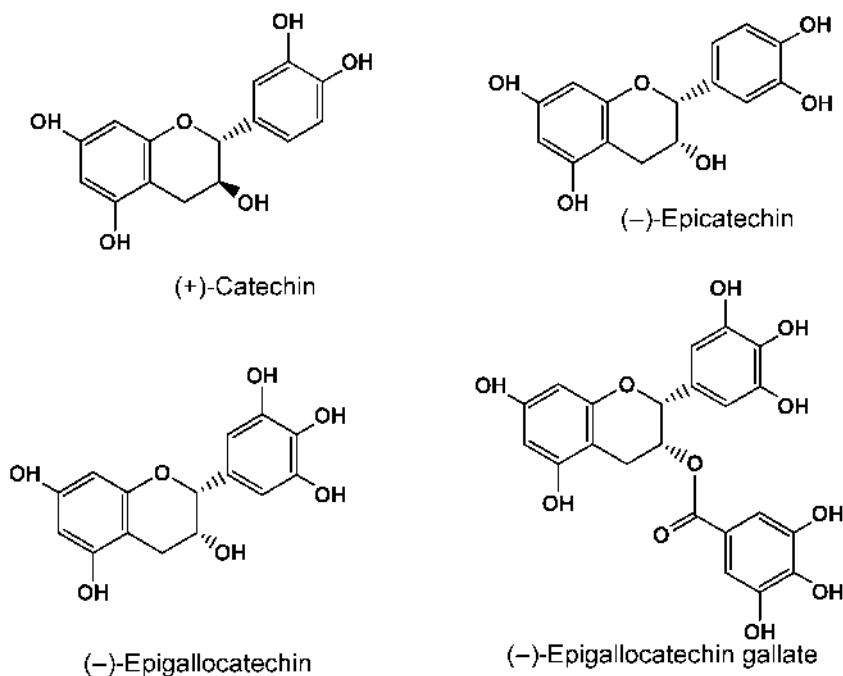


Figure 14.1. Molecular structures of four major green tea polyphenols.

modulated gene expressions in the multiple *in-vivo* models.

## FUNDAMENTALS OF GENE AND GENE EXPRESSIONS

A gene is defined as a functional unit of DNA, the host of all biological information of a cellular organism. DNA controls the formation of RNA that is required for the synthesis of polypeptides, which is a basic components of all cellular organisms performing diverse cellular functions in the synthesis of enzymes, receptors, proteins, transcription factors, signaling molecules, hormones, and others. Gene expression is a process by which the coded information located in the DNA is transcribed into a biological and physical functional genes product such as RNA or protein. Several steps, such as transcriptional, translational, or posttranslational modification, in the expression of a gene can be modulated, thereby changing the timing, location, and level of proteins. Gene expression provides full control over cellular structures and functions, the basis of cellular differentiation, morphogenesis, versatility, and adaptability of any organism.

Expression of a gene is primarily evaluated by the concentration of mRNA. Level of mRNA can be quantitatively measured in different ways. In Northern blotting, RNA extracted from a cellular or tissue extract is electrophoretically separated on an agarose gel. The procedure is suited for the detection of the specific RNA in a complex mixture of unrelated RNA species. In RT-PCR, an RNA molecule is reverse transcribed into complementary DNA followed by amplification of the DNA using polymerase chain reaction (PCR). This technique measures the abundance of mRNA in a cell lysate or tissue extract quantitatively and specifically. A DNA microarray usually contains thousands of DNA spots, which are fabricated on the plate by high-speed robotics. Each of the DNA spots, which have a probe with a known identity, is utilized to determine the complementary binding DNA on the basis of base-pair rules in unknown DNA samples. Thus, an experiment with a single DNA chip can provide massive information on parallel gene expression and gene discovery studies.

## PRIMARY TARGETS OF GREEN TEA POLYPHENOLS

Cell is the structural and functional unit of all living organisms. Therefore, it is obvious that physiological functions attributed to a drug or any functional

molecules such as hormone, neurotransmitter, and supplements are generated from the unique interaction between the cells and molecules. On the basis of many clinical and laboratory studies, it has been reported that GTCs, such as EGCG, exerts multiple biological functions. However, the question remains unanswered regarding the primary target of GTCs. There are drugs that specifically bind to a receptor molecule located on cell surface or within cytoplasm and regulate biochemical process such as ion conductance, protein phosphorylation, gene transcription, or enzyme activity. Molecules interacting with multiple receptor types are also reported. Physicochemical nature of tea catechins is diverse that enables these molecules to bind to multiple sites, located either on cell surface or inside the cells. The binding affinity may vary depending on the cell types or its physiology. Therefore, it has become rather difficult to conduct a thorough examination to determine the primary target of tea catechins. There are studies that have shown that catechins bind to laminin, a glycoprotein, and abrogate the effect of laminin on the morphology of some cancer cells (Bracke et al. 1987, 1991). Tachibana et al. (2004) have shown that EGCG binds to 67-kD laminin receptor (67LR), originally identified by Rao et al. (1983) as a link protein for anticancer activity of EGCG. Other research groups have also supported the involvement of 67-kD LR in EGCG-mediated anticancer effect (Shammas et al. 2006). In the study, INA6 myeloma cells transiently transfected with siRNA targeting LR1 are shown to abrogate EGCG-induced apoptosis in myeloma, as compared to nontransfected cells, wherein LR1 plays an important role in mediating EGCG activity in myeloma. More recently, EGCG-induced suppressive effect on the expression of the high-affinity IgE receptor (Fc epsilon RI) was found to correlate with the amount of 67LR. This observation, however, raised the possibility that the target of EGCG on cell membrane may involve other molecules in addition to 67LR (Fujimura et al. 2005). Although 67LR is known to be overexpressed on the cell surface of different forms of tumor cells, proteins similar in size exist on the surface of normal cells, such as epithelial cells, hepatocytes, neutrophils, macrophages, and neuronal cells that bind to laminin (Mecham 1991). Thus, it is postulated that EGCG may bind to 67LR and regulate the proliferation and differentiation of preadipocytes, although such a proposition requires further exploration.

In addition to 67LR, there is a wide speculation considering the involvement of other membrane or nuclear receptors that EGCG can bind to or alter activities of. It is widely known that androgens via their

cognate receptor may play a role in the development and progression of prostate cancer. Prostate cancer cell line, LNCaP, exposed to EGCG showed reduced expression of androgen receptor (AR) protein and mRNA by inhibiting expression, DNA binding, and transactivation of Sp1, an important regulatory component of AR gene expression (Ren et al. 2000). In human basophilic KU812 cells, IgE induced allergic response via Fc epsilon RI receptor. EGCG treatment to KU812 cells reduced both protein and mRNA expression by downregulating the expression of Fc epsilon RI  $\alpha$  and  $\gamma$  mRNA expression (Fujimura et al. 2001). Head and neck squamous cell carcinoma (HNSCC) cells often reported to overexpress the epidermal growth factor receptor (EGFR). HNSCC cells treated with EGCG showed reduced phosphorylation of the EGFR by inhibiting signal transducer and activator of transcription-3 (STAT3), and extracellular signal-regulated kinase (ERK) proteins with reduced c-fos and cyclin D1 promoter activity (Masuda et al. 2001). Similarly, EGCG treatment reduced the RAGE (receptor for advanced glycation end products) expression, an important element for cancer invasion in HLF cells (Takada et al. 2002). In addition, EGCG, either directly or indirectly, can suppress the activation or gene expression of most of the growth factor receptors, such as EGF, FGF, HER/neu, IGF-I, PDGF (Park et al. 2006b), and vascular endothelial growth factor (VEGF) receptors, in different cells, while it can stimulate the apoptotic receptor fatty acid synthase (FAS)/APO-1 in liver cancer cells.

Interactions of tea catechins with various peptides, proteins, enzymes, or lipopolysaccharides as well as transmitting signal from a cell surface receptor or intracellular components to alter genome function are widely accepted. However, until recently, when Kuzuhara et al. (2006) observed that nucleic acid extracted from catechins treated cells were colorful, there were no evidences showing GTCs had direct interaction with DNA or RNA molecules. The observation, however, motivated the research team in finding novel targets of GTC. In a study, the authors have used two methods, plasmon resonance assay (Biacore) and coldspray ionization-mass spectrometry, to investigate the nature of interactions. Applying EGCG (1.6, 3.3, 6.5, 13, 25, and 50  $\mu$ M) to poly(dT) 20-mer oligo ssDNA resulted in an initial increase in resonance along with a subsequent decrease in the resonance, indicating a direct association between catechins and DNA. Studying structure–function relationship of tea catechins binding to DNA oligomers, the authors have revealed that catechins having galloyl or catechol groups had significant ability to bind to DNA (Kuzuhara et al. 2006). However, the exact binding

sites and mode of interaction between catechins and DNA have not been revealed yet. Nevertheless, the results obtained in the study opened new dimensions in exploring novel target of drug and warrant further research to understand how the interactions between tea catechins and DNA would provide better health functions.

## TEA POLYPHENOL-MODULATED SIGNAL TRANSDUCTION PATHWAYS/GENE EXPRESSIONS

Signal transduction pathway refers to the pathways by which a cell responds to a change in environment. Signal transduction machineries are composed of many biochemical processes involving enzymes, cofactors, or other secondary messenger molecules that mediate sensing and processing of stimuli. Most signal transduction pathways occur inside the cells and are rapid. An ion channel signaling pathway usually takes a few milliseconds to generate responses. However, transmitting a signal mediated by protein or lipid cascades that ultimately leads into the altered gene function requires several hours or days. In most instances, a signal transduction involves binding of extracellular molecules or ligands to the receptors located on the cell surface and triggers events inside the cells. There are molecules that can diffuse through the cell membrane, bind to proteins, and directly interact with DNA to modulate gene function. This section of the chapter is focused on GTC modulation of cell signaling pathways affecting the expressions of gene in various disease states.

### CANCER

The term cancer is given to a disease or diseases that cause proliferation or divide the cells without any control, and allow cells to invade other cells. To date, over 100 different types of cancer have been identified, wherein most forms are thought to be linked with abnormal gene functions. The events that facilitate genetic changes to cause cancer and the nature of genetic changes are inconclusive and considered diverse. Epidemiological studies, the most direct way to assess the cancer incidence and its prevention, have revealed numerous data focusing why some group of people are more susceptible to cancer as compared to others. Several recent studies have proven the link between diet and cancer, showing that consumption of fruits and vegetables lowers the incidence of cancer in stomach, esophagus, lung, oral cavity and

pharynx, endometrium, pancreas, and colon (Steinmetz and Potter 1996).

Green tea is a rich source of polyphenols and contains powerful antioxidants. Anticancer properties of tea catechins have also drawn considerable attention from both scientific community as well as public. However, how GTCs suppress cancers and to what extent GTCs fixate the altered genetic events are the dominant issues that need proper clarifications. Thanks to the recent advance in knowledge on cellular and molecular basis of cancer etiology that have proven that GTCs target expression of several transcription factors, antiapoptotic proteins, proapoptotic proteins, protein kinases, cell cycle proteins, cell adhesion molecules, and COX-2, including growth factor signaling pathways.

### Nuclear Factor-kappa B Signal Transduction Pathway

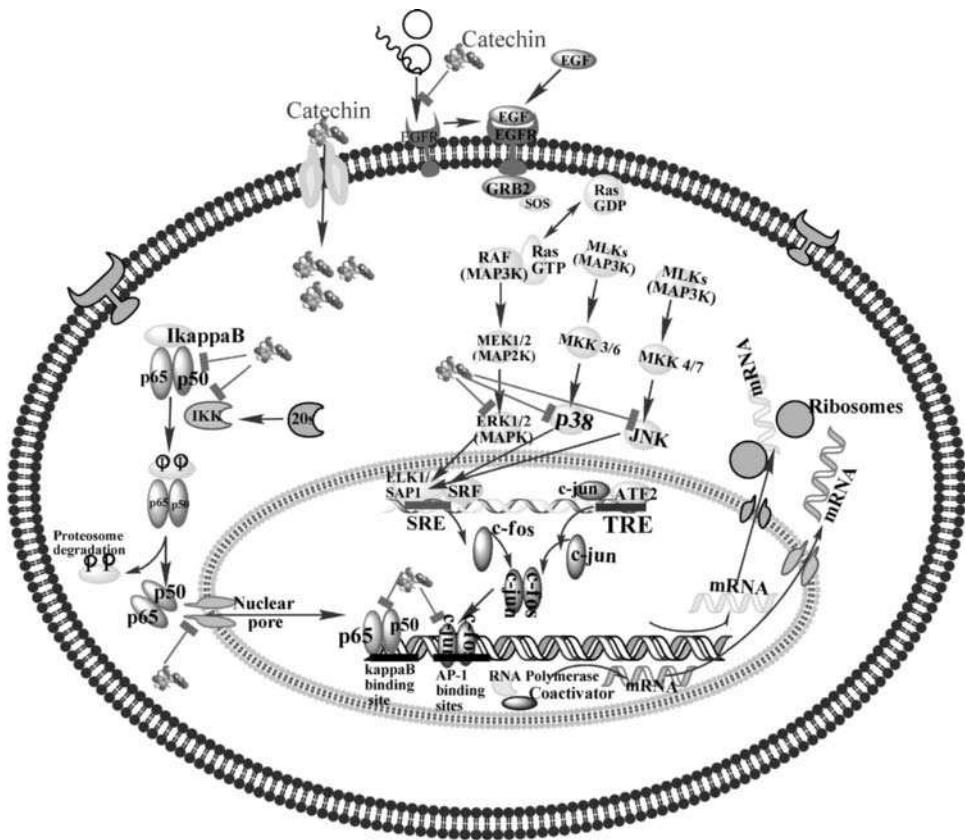
Transcription factor, nuclear factor-kappa B (NF- $\kappa$ B), also known as REL/NF- $\kappa$ B, is a group of closely related protein dimers. Under normal conditions, NF- $\kappa$ B resides in the cytoplasm bound to an inhibitory protein called inhibitory kappa B (I $\kappa$ B). There are, at least, six members identified so far in the family of mammalian NF- $\kappa$ B. They are NF- $\kappa$ B1 (also known as p50), NF- $\kappa$ B2 (p52), RELA (p65), RELB, c-REL, and v-REL. X-ray crystallography studies have revealed that the structures of p50–p50, p65–p65, p50–p65, c-Rel–c-Rel, and p50–p65–I $\kappa$ B complex readily bind to DNA. NF- $\kappa$ B inhibitory proteins (I $\kappa$ Bs) are also composed of several structurally related proteins such as, I $\kappa$ B $\alpha$ , I $\kappa$ B $\beta$ , I $\kappa$ B $\gamma$ , I $\kappa$ B $\epsilon$ , and Bcl-3. The most studied NF- $\kappa$ B–I $\kappa$ B interaction is that of I $\kappa$ B $\alpha$  with the NF- $\kappa$ B p50–p65 dimers. Various factors such as free radicals, inflammatory stimuli, cytokines, carcinogens, tumor promoters, endotoxins, gamma-radiation, ultraviolet (UV) light, and x-rays activate the dimers. This occurs primarily via the activation of a kinase called I $\kappa$ B kinase (IKK). The IKK has three structural domains: IKK- $\alpha$ , IKK- $\beta$ , and IKK- $\gamma$ . When activated, I $\kappa$ B is phosphorylated and released from NF- $\kappa$ B. After being activated, IKK phosphorylates two serine residues located on the I $\kappa$ B (Ser 36 and Ser 37 in human I $\kappa$ B $\alpha$ ) and degrades the inhibitory factor. Following separation from I $\kappa$ B, NF- $\kappa$ B translocates to the nucleus, where it binds to its distinct DNA sequence called the kappa B site and activates the transcriptions of a variety of genes that play critical roles in the development of many diseases or disease-like conditions such as cancer, inflammation, and autoimmune disorders. The misregulations of NF- $\kappa$ B, I $\kappa$ B, or IKK gene or in the

activity of their products have profound effect on cell survival or cell death. In tumors, NF- $\kappa$ B is active due to the mutation of either its own gene or of the genes that control the activity of NF- $\kappa$ B activity. There are also other factors that either are secreted from tumor cells or have entered from external sources that allow NF- $\kappa$ B to remain active. Thus, inhibition of one or more steps in NF- $\kappa$ B activation has become a target for the development of anticancer therapy.

Chemopreventive activity of many dietary factors have been shown to block one or more steps in the NF- $\kappa$ B signaling pathway such as the signals that activate the NF- $\kappa$ B signaling cascade, translocation of NF- $\kappa$ B into the nucleus, DNA binding of the dimers, or interactions with the basal transcriptional machinery. I $\kappa$ B kinase complex (IKK) has also been shown to mediate activation of the transcription factor NF- $\kappa$ B. GTC EGCG decreased NO generation and protein levels of iNOS with reduced expressions of iNOS mRNA in lipopolysaccharide (LPS)-stimulated macrophage by prevention of the binding of NF- $\kappa$ B to the iNOS promoter (Lin and Lin 1997). Tea catechins also inhibited I $\kappa$ B phosphorylation to prevent NF- $\kappa$ B activity and to inhibit NF- $\kappa$ B sequence DNA binding (Nomura et al. 2000; Yang et al. 2001). In Jurkat T cells, EGCG inhibits the chymotrypsin-like activity of the 20S proteasome, which targets a variety of proteins including p21, p53, Bax, p27kip1, and I $\kappa$ B $\alpha$  for degradation (Nam et al. 2001). In addition, catechins are also shown to inhibit translocation of NF- $\kappa$ B from cytosol to nucleolus and inhibits DNA binding of NF- $\kappa$ B at the MMP-1 gene transcription sites to inhibit the growth of lung carcinoma 95D cells (Yang et al. 2005). Numerous other studies have shown that GTCs induce suppressive effect on NF- $\kappa$ B by inhibiting (a) phosphorylation of I $\kappa$ B $\alpha$  and activation of IKK- $\alpha$  (Afaq et al. 2003; Beltz et al. 2006) or (b) nuclear translocation of NF- $\kappa$ B p65 translocation (Ahmed et al. 2006; Ahn et al. 2004), inhibiting NF- $\kappa$ B-DNA binding activity (Mackenzie and Oteiza 2006; Mackenzie et al. 2004), or due to loss of transactivation domains through caspase cleavage of NF- $\kappa$ B/p65 subunit (Gupta et al. 2004). In addition, EGCG suppresses expression of receptor activator of NF- $\kappa$ B ligand (RANKL) in *Staphylococcus aureus* infection in osteoblast-like NRG cells (Ishida et al. 2007).

### Inhibition of Mitogen-Activated Protein Kinase Pathways

In addition to NF- $\kappa$ B, mitogen-activated protein kinase (MAPK) signaling pathways have been found



**Figure 14.2.** MAPK signaling pathways involving activation of NF-κB and AP-1, and their modulation by green tea polyphenols.

to play important roles in many physiological processes, such as regulation of embryonic development, cell cycle progression, cellular differentiation, cell movement, and inflammation, as well as apoptosis. Therefore, the pathways have received significant attention as target pathways in preventing diseases such as cancer, cardiovascular diseases, and inflammation of various phytochemicals including green tea polyphenols.

The molecular events linking cell surface receptors to activation of MAPKs are *rather* complex (Figure 14.2). In the pathway, at least three different kinase modules have been found to participate: MAPK, MAP kinase kinase (MAPKK or MAP2K), and MAP kinase kinase kinase (MAPKKK or MAP3K). In the cascades, extracellular ligands stimulate EGFRs. Then, EGF binds to the EGFR, which becomes phosphorylated on tyrosine residue. Docking protein, GRB2, then binds to activate EGFR through its SH2 domain. Then, the domain SH3 of GRB2

binds to guanine nucleotide exchange factor SOS; thus, SOS is activated, which in turn converts Ras-GDP to Ras-GTP. The Ras-GTP interacts with and activates the serine/threonine protein kinase Raf-1, later called as MAP3K, which in turn phosphorylates and activates MEK1/2 (MAPKK) on two distinct serine residues. Activated MEK1/2 then phosphorylates ERK1 (p44MAPK) and ERK2 (p42MAPK) on both a tyrosine and a threonine residue. This pathway is also called as ERKs (also known as MAPKs). In addition to the ERK1/2 pathway, there are at least two other MAPK pathways that have been identified in mammalian cells. They are the JNK1/2/3 (also known as stress-activated protein kinases (SAPK)) and p38 $\alpha/\beta/\gamma$  pathways. Very recently, another pathway ERK5 has been recognized that is activated via stimuli and participates in cellular proliferation. In each cascade, an MAP3K activates an MAP2K, which in turn activates an MAPK (ERK, JNK, or p38).

There are numerous evidences that have shown that GTCs modulate MAPKs to prevent the pathological changes of various diseases, including cancer and atherosclerosis. In JB6, mouse epidermal cell line, EGCG, at the concentration range of 5–20  $\mu$ M, inhibited the PMA-induced p38 MAPK pathway (Dong et al. 1997). To investigate the molecular mechanism as to how EGCG, a major tea polyphenol, suppresses cancer, Chen et al. (1999) studied the effects of EGCG on ultraviolet B (UVB) radiation-induced *c-fos* gene expressions in a human keratinocyte cell line, HaCaT. EGCG inhibited UVB-induced transcriptional activation of the *c-fos* gene. EGCG, within the same dose range, also had an inhibitory effect on UVB-induced accumulation of the *c-fos* protein. Examining the status of upstream activators of the *c-fos* gene, they showed that EGCG significantly inhibited the activation of p38 MAPK but not *c-jun* NH<sub>2</sub>-terminal kinase or ERK protein activation. EGCG showed strong inhibition of tyrosine kinase and MAPK activities in transformed NIH-pATM ras fibroblasts without affecting the kinases in the normal cells (Wang and Bachrach 2002). When human epidermal keratinocyte cells were pretreated with EGCG, H<sub>2</sub>O<sub>2</sub>-induced phosphorylation of ERK1/2, JNK, and p38 was significantly inhibited (Katiyar et al. 2001). Thus, EGCG has the potential to inhibit oxidative stress-mediated phosphorylation of MAPK signaling pathways. The treatment of EGCG (10–40  $\mu$ M) to normal human epidermal keratinocyte before UVB exposure was shown to inhibit UVB-induced hydrogen peroxide production concomitant with the inhibition of UVB-induced phosphorylation of ERK1/2, JNK, and p38 proteins (Afaq et al. 2003). Maeda-Yamamoto et al. (2003) also reported that EGCG inhibited the phosphorylation of ERK1/2 and suppressed p38 MAPK activity in human fibrosarcoma HT1080 cells.

Although several studies have evidenced that the catechin-induced inhibition of MAPKs is preferentially linked with the suppression of cancer progression and inhibition of cardiovascular diseases, catechins are also shown to activate MAPKs in a number of pathophysiological processes. EGCG has been found to activate all three MAPKs (ERK, JNK, and p38) in a dose- and time-dependent manner in human hepatoma HepG2-C8 cells (Chen et al. 2000). It is also possible that activation of MAPK by low concentration of EGCG results in induction of antioxidant response element (ARE)-mediated gene expression, whereas higher concentration of EGCG causes activation of such MAPKs as JNK1, leading to apoptosis (Chen et al. 2000). In a study, Kong et al. (2000) had proposed that a low dose of EGCG may activate

the MAPK (ERK2, JNK1, p38), leading to expression of *c-Fos* and *c-Jun* genes. In the breast cancer cell line, T47D, catechins (containing approximately 53% of EGCG) phosphorylated JNK/SAPK and p38. The phosphorylated JNK/SAPK and p38 inhibited the phosphorylation of Cdc2 and regulated the expression of cyclin A, cyclin B1, and CDK proteins, thereby causing G2 arrest (Deguchi et al. 2002). In addition, the polyphenols triggered the phosphorylation of *c-Jun* (Ser63 and Ser73) and induced *c-Jun/c-Fos*, thereby increasing the DNA-binding activity of activator protein-1 (AP-1), as shown by an AP-1 luciferase reporter assay (Kim et al. 2005).

### Inhibition of AP-1 Signaling Pathway

Transcription factor AP-1 was originally identified by its binding to a DNA sequence in the SV40 enhancer. AP-1 is a protein complex that binds to a DNA via a leucine zipper that exists on it. The protein complex consists of either homo- or heteromeric dimers of the Jun (*c-Jun*, *JunB*, and *JunD*) or Fos (*c-Fos*, *FosB*, *Fra-1*, and *Fra-2*) family proteins. Fos cannot form homodimer but can ensue heteromeric dimer with Jun. These dimers bind to AP-1 recognition sites, and in humans, the sites have a nucleotide sequence of 5'-TGAG/CTCA-3', also known as TREs (phorbol 12-*O*-tetradecanoate-13-acetate (TPA) response elements). The resulting homomeric dimers such as Jun-Jun or heteromeric dimers such as Fos-Jun or Jun-Fra regulate the transcriptional activity after binding to the transcription sites of the numerous genes controlling cell proliferations, apoptosis, cell transformations, innate immune response, and are important for the pathogenesis of cancer, inflammation, and cardiovascular disease (Angel and Karin 1991).

AP-1 is activated predominantly via the MAPK cascade pathway, wherein serum response element (SRE) is a major component that mediates the activation of *c-fos* gene transcription. However, in the activation process, SRE binds to SRF (serum response factor) and recruits the ternary complex factors Elk-1, Sap1, and Sap2. The MAPKs ERK1, ERK2, JNK, and p38 phosphorylate and activate Elk-1 to enhance an SRE-dependent *c-fos* gene expression. FRK, also known as *c-Fos*-regulating kinase, phosphorylates and activates *c-Fos*, which forms a heterodimer with *c-Jun* and binds to AP-1 sites of target genes (Deng and Karin 1994). JNK phosphorylates and activates both *c-Jun* and ATF-2, which form heterodimers. p38 MAPK also phosphorylates and activates ATF-2.

AP-1 promotes cell proliferation by activating cyclin D1 gene and inhibiting tumor suppressor genes

(TSGs), such as p53, p21cip1/waf1, and p16. In various model studies, AP-1 is shown to play critical roles in the transition of tumor cells from an epithelial to mesenchymal morphology, one of the early steps in tumor metastasis. On the other hand, when tumor promoter-induced AP-1 activity was blocked, neoplastic transformation was inhibited (Dong et al. 1994). AP-1 thus appears to be a key target for chemopreventive agents such as green tea polyphenols. Barthelman et al. (1998) showed that EGCG inhibited AP-1 activity in the epidermis of a transgenic mouse model and in nonmelanoma skin cancer cells exposed to UVB. By studying the mechanisms of action through which EGCG or theaflavins prevented carcinogenesis in JB6 (mouse epidermal cell line), Dong et al. have shown that EGCG, at a dose range of 5–20  $\mu$ M, inhibited tumor promoter (EGF- or TPA)-induced cell transformation accompanied by reduced AP-1 transactivation with subsequent inhibition of AP-1 DNA-binding activity. The inhibition of AP-1 activation occurs through the inhibition of a c-Jun NH<sub>2</sub>-terminal kinase (JNK)-dependent pathway, but not of ERK1- or ERK2-dependent pathways (Dong 2000; Dong et al. 1997). Similarly, Chung et al. (1999) also investigated how EGCG inhibits AP-1 activation in JB-6 cells transfected with a mutant H-ras gene. They have shown that EGCG inhibited phosphorylation and protein level of ERK, c-jun, and fra-1, the important component of AP-1. Because the ras genes are activated in many human cancers, the inhibition of the phosphorylation of c-Jun and ERKs are considered as chemopreventive targets for tea catechins. Inhibition of both JNK and ERK activation was found when EGCG inhibited PMA-induced AP-1 activation in human gastric cells (Kim et al. 2004).

Very recently, He et al. (2008) have shown that EGCG suppresses EGF-induced cell transformation in JB6 cells. They have shown that EGCG directly binds to GST-Fyn-SH2 domain of Fyn kinase to inhibit phosphorylation activity. This activity of EGCG is proposed to inhibit the activity/expression of p38 MAPK, ATF-2, and STAT1, resulting in decreased AP-1 DNA-binding ability (He et al. 2008), suggesting that the MAPK pathway may also govern AP-1 activation, which was observed in some earlier studies (Chen et al. 1999; Chung et al. 2001). A very recent study has shown that EGCG suppressed c-jun gene expression in ischemia/reperfusion (I/R) injury in intestinal epithelial cells (Giakoustidis et al. 2008), suggesting that green tea polyphenols may regulate c-jun expression and subsequent activation of AP-1 at the transcription level.

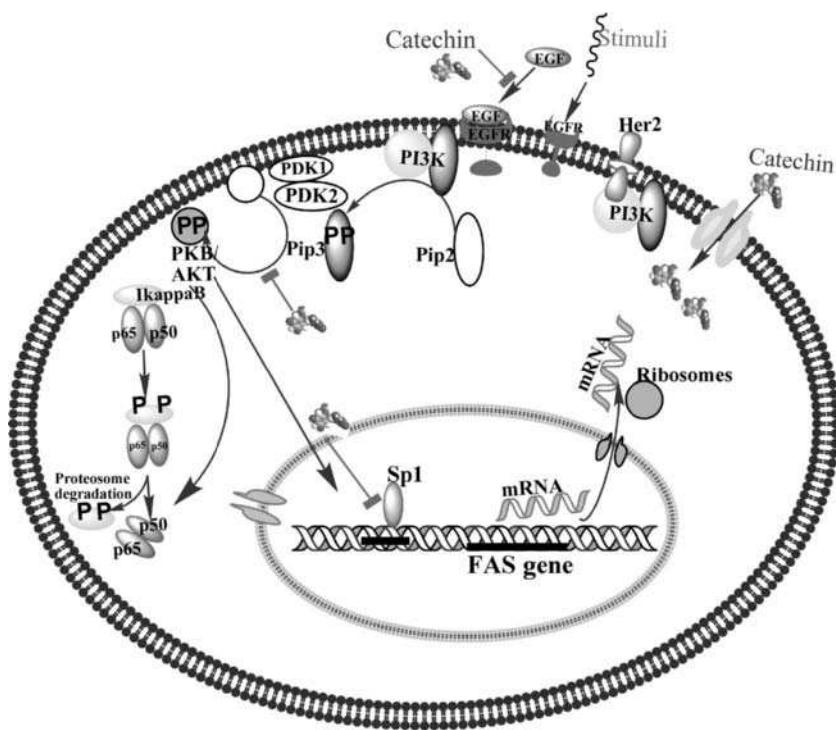
In contrast to these reports, EGCG has been shown to markedly increase AP-1 factor-associated

responses through a MAPK signaling mechanism in normal human epidermal keratinocytes, suggesting that the signaling mechanism of EGCG action could be markedly different in different cell types (Balasubramanian et al. 2002). EGCG treatment of HT-29 cells induced JNK activity with the increase in AP-1 luciferase activity in HT-29 cells exposed to TPA (Jeong et al. 2004). EGCG induced H<sub>2</sub>O<sub>2</sub> generation in HT-29 cells by increasing the activity of AP-1 transcription factor with the increased expression of pro-matrix metalloproteinase (MMP)-7 gene/protein (Kim et al. 2007). Taken together, it has become apparent that GTCs may have differential biological function on the AP-1 signal transduction pathway, depending on the state of cellular model and stage.

### Modulation of PKB/Akt Signaling

Protein kinase B or Akt (PKB/Akt) is a serine/threonine kinase, which in mammals comprises three highly homologous members known as PKB- $\alpha$  (Akt1), PKB- $\beta$  (Akt2), and PKB- $\gamma$  (Akt3). The Akt/PKB is a cellular homologue of the viral oncogene v-Akt and is activated in cells by diverse stimuli such as hormones, growth factors, and extracellular matrix components. Akt is located in the downstream of phosphoinositide 3-kinase (PI3K). PI3K generates phosphatidylinositol-3,4,5-trisphosphate (PIP(3)), which is essential for the translocation of Akt/PKB to plasmamembrane, where it is phosphorylated by PDK1 at Thr308 or by PDK2 at Ser473. Akt/PKB plays critical roles in mammalian cell survival by activating NF- $\kappa$ B signaling and by inhibiting apoptosis through inactivation of several proapoptotic factors such as Bad, fork-head transcription factors, and caspase-9. Akt has been shown to be activated in various cancers (Chang et al. 2003a; Clarke 2003). PKB/Akt activation can occur due to amplification of PKB/Akt genes or because of mutations in components of the signaling pathway that activates PKB/Akt. Thus, PKB/Akt has been considered as an attractive target for cancer prevention.

Several phytochemicals including genistein, indole-3-carbinol, and curcuminoids are known to suppress the Akt/PKB. Masuda et al. (2002) found that treatment with EGCG inhibited the constitutive activation of the Akt, EGFR, and STAT3 in both YCU-H891 head and neck squamous cell carcinoma and MDA-MB-231 breast carcinoma cell lines. Similarly, Tang et al. (2003) have shown that EGCG from green tea inhibits VEGF-induced angiogenesis *in-vitro* through suppression of VE-cadherin phosphorylation and inactivation of Akt. EGCG-induced



**Figure 14.3.** (–)Epigallocatechin gallate (EGCG)-induced suppression of Fas gene expression through modulation of the EGF receptor/PI3K/Akt/Sp-1 signal transduction pathway. In the pathway, EGCG inhibits the growth factor EGF binding to EGFR, blocks the activation of the PI3K/Akt signaling, and then reduces the DNA-binding capacity of nuclear transcription factor Sp-1 and finally lead to downregulation of Fas gene.

growth inhibition and proapoptotic signals in human breast cancer cell line (MCF-7) are linked with reduced activation of phospho-Akt and Akt kinase activity with the suppression of survivin promoter activity (Tang et al. 2007). Pan et al. (2007) found that treatment with EGCG inhibited activation of PKB/Akt, and expression of Fas gene in MCF-7 cells stimulated by heregulin-beta1 (HRG-beta1), suggesting that EGCG-induced Akt inactivation is regulated at the transcriptional level (see Figure 14.3). EGCG decreases the expression of PI3K, Akt gene in normal human bronchial epithelial cells (NHBE) exposed to cigarette smoke (Syed et al. 2007). A more recent study also evidenced that EGCG, at a concentration of 20  $\mu$ M, reduced N-cadherin mRNA expression while suppressing migration of bladder carcinoma cells by inhibiting Akt signaling in the bladder cells (Rieger-Christ et al. 2007). Another breakthrough research further evidenced that EGCG-induced inhibition of PI3K/Akt and MEK/ERK pathways acts synergistically and regulates transcriptional activation of FOXO gene when EGCG inhibits migration and capillary tube formation of

human umbilical vein endothelial cells (HUVEC), an antiangiogenic effect of EGCG (Shankar et al. 2008).

#### Modulation of Cell Survival/Death Genes

Apoptosis and necrosis are the two basic mode of cell death. Apoptotic cell death is precisely coordinated with multiple sets of molecular and cellular cascades, wherein at least 100 gene products either suppress or activate the effector molecules for modulating the process of self-destructions. Among those apoptosis-related genes, the members of Bcl-2 family genes are widely studied for their role in the apoptotic pathway, and depending on the mode of action, they are classified into two groups: cell death suppressor genes and cell death inducer gene. The cell death suppressor genes are Bcl-2, Bcl-xL, Bcl-w, Mcl-1, and A1. Cell death inducer genes are Bax, Bak, Mtd, and MH-3. In the last few years, several reports showed that activation of NF- $\kappa$ B promotes cell survival and proliferation by modulating several members of Bcl-2 family genes including Bcl-2 and Bcl-xL, in addition

to cIAP, survivin, TRAF1, and TRAF2 (Aggarwal 2004). Phytochemicals having the ability to inhibit NF- $\kappa$ B or AP-1 activation can significantly suppress cell proliferation and sensitize cells to apoptosis induction. Depending on the ability of the cell death suppressor gene to oppose the action of the inducer genes, fate of a cell, whether a cell lives or dies, is determined.

In many previous studies, low doses of GTCs, particularly EGCG, have been shown to be neuroprotective and suppress the expression of apoptosis inducer genes such as Bax, Bad, *GADD45*, and Fas ligands, with little or no impact on the expression of apoptosis suppressor genes. In a study by Weinreb et al. (2003b), a low micro molar concentration of EGCG was shown to abolish the expression of proapoptotic mRNAs in neuroblastoma SH-SY5Y cells, in which viability was challenged with a neurotoxin 6-hydroxydopamine (6-OHDA). In the study, mRNA was extracted from SH-SY5Y cells exposed to a low dose of EGCG and then to a high dose of 6-OHDA. After extracting mRNA followed by constructing cDNA and hybridization to a microarray membrane containing cDNA fragments of gene of proapoptosis and antiapoptosis, the authors have shown that EGCG treatment inhibits the expression of proapoptosis genes, and the results have been further supported by RT-PCR and protein profile analysis. Levites et al. (2002) have also obtained similar results in their study on molecular mechanism of neuroprotective effects of GTCs. They have shown that a low dose of EGCG prevented both the 6-OHDA-induced expression of several mRNAs, such as Bax, Bad, and Mdm2, and the decrease in Bcl-2, Bcl-w, and Bcl-xL, and also inhibited the expression proapoptotic genes such as Bax, Bad, and Mdm2. Other investigators have shown that the antiapoptotic action of EGCG regulates the expression of Bcl-2 family gene (Jung et al. 2007a, b). Several other studies also evidenced the antiapoptotic behaviors of GTCs in *in-vitro* and *in-vivo* models. Investigating molecular mechanisms in the EGCG-attenuated retinal damage caused by I/R, Zhang et al. (2007) showed that GTCs suppressed the I/R/oxidative-stress-induced expression of mRNAs of caspase-3, caspase-8, and GFAP, the markers of apoptosis, and inhibited the H<sub>2</sub>O<sub>2</sub>-induced apoptosis in ganglion cells. Hypoxia-induced damage to HepG2 cells is prevented by EGCG with subsequent decreased expression of proapoptotic genes, such as Bax and caspase-3 (Park et al. 2006a).

In contrast to the antiapoptotic behavior, GTCs are also known to exhibit proapoptotic features by increasing the expressions of proapoptotic genes, such as Bak and Bad. This feature of GTCs is usu-

ally observed when the effected cells are exposed to a comparatively higher dose of catechins. In a study, Weinreb et al. (2003b) showed that EGCG at a concentration of 50  $\mu$ M increased the expression of proapoptotic genes, such as Bax, *GADD45*, caspase-6, while decreasing the expression of antiapoptotic genes such as Bcl-2 and Bcl-xL in SH-SY5Y cells. Survivin gene that is a new member of IAP (inhibitors of apoptosis protein) family has been found to be overexpressed in majority of the human tumor cells including lung, breast, and colon. In a study, Tang et al. (2007) showed that EGCG treatment significantly decreased the survivin gene expression and induced apoptosis in human breast cancer cell line MCF-7 cells. Investigating the mechanism of action, the authors showed that EGCG inhibited survivin promoter activity by suppression of AKT pathway.

Antitumorigenic activity of several nonsteroidal anti-inflammatory drugs (NSAID), conjugated linoleic acid, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002), 3, 3'-diindolylmethane is shown to induce apoptosis and cell cycle arrest in colon cancer cells with increased expression of ATF3. Baek et al. (2004) for the first time have provided evidences that ECG, the third major catechins found in green tea, induces the ATF3 transcription factor, followed by NAG-1 induction at the transcriptional level in a p53-independent manner. Later, Cho et al. (2007a) have shown that ECG induces the expression of proapoptotic gene ATF3 at the transcription level. They found that both ECG and EGCG have increased ATF3 promoter activity by more than three folds and the sites lie between the -514 and -85 region of the ATF3 promoter. Using electrophoretic mobility shift assay and cotransfection techniques, the authors have shown EGR-1 involvement in ECG-induced ATF3 expression in HCT cells. Many cancer-preventive agents such as *N*-(4-hydroxyphenyl)retinamide (4HPR), curcumin, and thiazolidinedione induces apoptosis in cancer cells with increased expression of *GADD153* gene. Cancerous cells transfected with *GADD153* gene have shown to induce apoptosis without exposing to any stress-inducing factors, indicating that *GADD153* is directly involved in the regulation of apoptosis. Thus, for cancer-preventive agents, overexpression of *GADD153* gene is considered as a new molecular target for antitumor activity. In a study, Suganuma et al. (2006) have shown that a combination of EGCG and celecoxib strongly upregulated the expression of *GADD153* gene and activation of both ERK1/2 and p38 kinases, and synergistically induced apoptosis in PC-9 cancer cells. EGCG-induced upregulation of several proapoptotic genes such as Bax, Bid, and Bad

in MKN45 cells is also reported by Ran et al. (2007). Using a commercial green tea product polyphenon E (PPE) for the prevention of colon cancer in mice, Xiao et al. (2008) have identified that PPE-treated mice had increased apoptosis with decreased nuclear expression levels of  $\beta$ -catenin and cyclin D1. PPE treatment also suppressed the loss of RXR- $\alpha$  expression induced by carcinogenesis (Xiao et al. 2008).

### Miscellaneous Molecular Targets/Pathways

Cancer is generally known as a complex disease. It arises from a combination of changes that occur inside the cells involving modification of multiple macro- and micro-biomolecules. Biological effect of GTCs, particularly EGCG, also has multiple direct or indirect targets. Thus, molecular mechanism for the chemopreventive and anticancer effects of tea catechins are not only limited to the modulation of inhibition of MAPK, AP-1, NF- $\kappa$ B, binding of epidermal growth factor to its receptor, angiogenesis, and activation of apoptosis. There exist other targets for tea catechin's chemoprevention mechanisms.

#### **EGCG Inhibits BMP Signaling**

Bone morphogenic proteins (BMPs) are part of transforming growth factors-beta (TGF- $\beta$ ). These proteins have emerged as the key regulators of embryonic development including the induction of neuronal tissue, neurogenesis, and joint formation in the skeletal system. BMP signaling pathway has disparate role in tumorigenic progression and in the selection of cell fate. Recent research work on TGF- $\beta$ /MBP superfamily of growth factors reveals their role in tumor progression. Until recently, there has been no study focusing GTC's role on BMP signaling. Using microarray technology, Vittal et al. (2004) have shown that GTCs substantially modulate gene expression of BMP signaling pathway in H-ras transformed human bronchial epithelial 21BES cells. The cells when exposed to EGCG (25  $\mu$ M) showed downregulation of the type II receptor of BMP, as well as upregulation of its negative modulators such as dual specificity phosphatase 5 and 8, FK506- binding protein 5, and SMAD 7. Thus, EGCG-modulated BMP signaling/gene expression is providing new evidences in favor of its cancer chemoprevention activity.

#### **CYP1A Gene Expression**

The cytochrome P450 subfamily enzymes (CYP1A) are usually responsible for the metabolic activation of chemical carcinogens, such as polycyclic aromatic hydrocarbons (PAHs), 2,3,7,8-tetrachlorodibenzo-*p*-

dioxin (TCDD), and heterocyclic amines (McManus et al. 1990) to produce highly reactive intermediates that possess mutagenic and carcinogenic activity. The CYP1A enzymes are substrate inducible, and the induction of the CYP1A enzymes by PAHs or heterocyclic amines occurs at the level of transcription and is mediated by the cytosolic aryl hydrocarbon receptor (AhR). In the pathway, the ligand–receptor combination allows the receptor AhR to translocate to the nucleus, where it dimerizes with the AhR nuclear translocator protein (ARNT). AhR–ARNT heterodimers function as transcriptional activators by binding to consensus sequences termed dioxin-response elements (DRE), which are present in the 5'-flanking DNA of numerous genes (Denison and Whitlock 1995). Due to the importance of CYP1A in carcinogen activation, effect of dietary flavonoids on the AhR-mediated induction of CYP1A1 is being examined. In a study, Williams et al. (2000) exposed HepG2 cells, the 101L cells derived from HepG2 cells and primary hepatocytes to TCDD in the presence or absence of green tea extracts (GTEs) or individual catechins. The 101L cell was stably transfected with the human CYP1A1 promoter linked to the luciferase reporter gene. Then, the activation of CYP1A1 gene by classic CYP1A inducers was investigated. GTEs, at a concentration of 0.1 mg/mL, inhibited the activation of the CYP1A1-luciferase gene by an average of 50%. The study suggested that GTEs inhibited the transcription of a human CYP1A1 promoter-driven reporter gene induced by the AhR ligand TCDD in a concentration-dependent manner and inhibited the induced accumulation of both CYP1A1 and CYP1A2 mRNAs. GTEs blocked TCDD-induced binding of the AhR to DNA in HepG2 cells and *in-vitro* in isolated hepatic cytosol. In another study, polyphenon, a commercially available green tea extract, was shown to antagonize TCDD-induced binding of the AhR to DNA and inhibit subsequent transcription of human CYP1A1. In this study, Williams et al. (2003), however, proposed that a combination of two or more different catechins may be effective to modulate CYP1A gene expression because P100 or P100R (having different specifications) inhibited TCDD-induced CYP1A promoter-driven luciferase reporter activity and CYP1A gene expression similarly and more effectively as compared to that of a single catechin such as EGCG.

#### **Human Telomerase Reverse Transcriptase Gene Expression**

Various mechanisms have been shown to function in the transformation of normal cells to cancerous

cells. A major mechanism involves the activation of telomerase and subsequent maintenance of telomere in cancer cells. Telomerase activity in human cells is regulated through transcriptional control of telomerase catalytic subunit gene, human telomerase reverse transcriptase (hTERT). Elevated level of hTERT expression is found to be operative during carcinogenesis. Thus, molecules having suppressive effect on hTERT gene expression have drawn immense interest in the area of cancer research. EGCG exposed to breast cancer cells, MCF-7 cells, has been shown to suppress the hTERT expression by 40–55% (Mittal et al. 2004). Epigenetic alterations have been proposed in the downregulation of hTERT gene expression in MCF-7 cells because EGCG treatment resulted in a time-dependent decrease in hTERT promoter methylation and ablated histone H3 Lys9 acetylation (Berleth et al. 2008). EGCG-induced inhibition of hTERT gene expression is also linked to the antiproliferation mechanism in gastric cancer cells (Ran et al. 2005) and in oral squamous cell carcinomas (Hua et al. 2006). Lin et al. (2006) have investigated the efficacies of the suppressive effect of GTCs on the expression of hTERT gene. In the study, they have constructed a reporter system having active hTERT promoter activity. They have also shown that GTCs, EGCG and ECG, at a dose range of 20–40  $\mu$ M effectively suppressed hTERT gene expression in carcinoma cells, such as H1299, OECM-1, and SAS cells.

#### **RNA Polymerase III Transcription**

RNA polymerase III, also known as Pol III, transcribes many of the genes to synthesize ribosomal 5S rRNA, tRNA, and other small RNAs, thereby dictating the growth of cells. However, Pol III itself cannot identify its target promoter sites directly. Transcription factor IIIB (TFIIB) plays an essential role in the proper initiation of Pol III. Deregulation of TFIIB-mediated Pol III transcription is considered to be an important step in the development of tumor. This consideration is based on the fact that TFIIB is a molecular target of many tumor suppressor molecules such as ARF (Morton et al. 2007), p53, RB, the oncogene c-myc (Felton-Edkins et al. 2003b), and the MAPK ERK (Felton-Edkins et al. 2003a). Since Pol III activity in many cancer cells is overtranscribed, it is speculated that TFIIB-mediated Pol III transcription may be a target for chemopreventive agents such as GTCs. EGCG-induced RNA polymerase inhibition was first proposed to be due to competitive inhibition with the triphosphate substrate (GTP) (Shinozuka et al. 1988). Until recently, it remained unknown if

GTP inhibited a RNA polymerase at transcription level. In a recent study, Jacob et al. (2007) have shown a novel target of EGCG for its widely considered antitumor activity. The authors have shown that EGCG negatively regulates RNA pol III transcription in HeLa cells that resulted from the direct inhibition of expression of TFIIB subunits Brf1 and Brf2 by EGCG. In the study, the authors have also demonstrated that, in the case of U6 RNA pol III transcription, Brf2 expression is regulated at the transcriptional level by EGCG.

#### **Hypermethylation/Hypomethylation-Silenced Gene**

DNA methylation involves addition of methyl group to N5 of cytosine in DNA molecules. In humans, the process is carried out by a group of enzymes known as DNA methyltransferases (DNMTs). Although in every vertebrate, DNA undergoes methylation, and in humans, about 1% of total DNA bases undergo the process, a hypermethylation may cause adverse effect on DNA transcription mechanisms. For example, a hypermethylated DNA obstructs itself from binding to a transcription factor; in addition, methylated sites may bind to some proteins and by recruiting more proteins the sites become dormant, resulting in inactive chromatin termed as silent chromatin. Numerous TSGs have been found to be hypermethylated in cancer. For example, genes involved in cell cycle regulation (p16<sup>INK4a</sup>, p15<sup>INK4a</sup>, Rb, p14<sup>ARF</sup>), genes associated with DNA repair (BRCA1, MGMT) mechanisms, induction of apoptosis, drug resistance, angiogenesis, detoxification, or metastasis are described to be susceptible to hypermethylation in recent literatures. Since many TSGs undergo DNA methylation during carcinogenesis, there have been many attempts to reexpress these genes by inhibiting DNMTs.

Various food factors including green tea polyphenols have been found to reverse the effects of DNA hypermethylation. EGCG inhibits DNMT activity and affects DNA hypermethylation. EGCG-induced DNMT inhibition reactivates methylation-silenced genes in different types of cancerous cells such as human esophageal, colon, prostate, and mammary cancer cell lines. Esophageal cancer cells while treated with 5–50  $\mu$ M of EGCG for 12–144 h have showed reversal of hypermethylation, and this activity was associated with the demethylation of the CpG islands in the promoters as well as the reactivation of methylation-silenced genes such as *p16<sup>INK4a</sup>*, retinoic acid receptor  $\beta$ ,  $O^6$ -methylguanine methyltransferase, human

mutL homolog 1, and glutathione S-transferase-pi (Fang et al. 2003, 2007). Methylation-silenced genes in human colon cancer HT-29 cells, esophageal cancer KYSE 150 cells, and prostate cancer PC3 cells can also be reactivated by EGCG. Although hypermethylation of promoter of various TSGs causes their transcriptional silencing, hypomethylation of regulatory DNA sequences activates transcription of protooncogenes, retrotransposons, as well as genes encoding proteins involved in genomic instability and malignant cell metastasis (Luczak and Jagodzinski 2006). Mittal et al. (2003) have shown that EGCG induced a significant inhibition of UVB-induced DNA hypomethylation patterns in a photocarcinogenesis model. Thus, green tea polyphenol-induced hypomethylation and hypermethylation mechanisms are considered beneficial for the prevention of tumor suppression and carcinogenesis.

#### **Cyclooxygenase (COX)**

Cyclooxygenase is a heme-containing enzyme that mediates synthesis of biologically important molecules such as prostaglandins, prostacyclin, and thromboxane and plays an important role in inflammation and cancer progression. Thus, inhibition of COX synthesis provides relief from the symptoms of inflammation and anticancer support. The enzyme has two isoforms, COX1 and COX2. Several dietary components including galangin, luteolin, apigenin, 6-hydroxykaempferol, quercetagin, sasanqua, genistein, and wogonin have been shown to suppress COX-2. GTC EGCG inhibited COX-2 expression in keratinocytes induced by UV (Soriani et al. 1998). Similarly, phorbol ester-induced COX-2 expression in cultured human mammary epithelial cells was suppressed by EGCG (Kundu et al. 2003). While investigating molecular mechanism of COX-2 inhibition by green tea extract and EGCG, authors have shown that EGCG or catechins inhibited the catalytic activity of ERK and p38 MAPK, suggesting that these signal-transducing enzymes could be potential targets for previously reported antitumor promoting activity of EGCG. In addition, EGCG is also shown to suppress the phosphorylation of (i.e., activated) forms of EGFR, HER2, and HER3, with the decrease in the levels of both COX-2 protein and mRNA in colon cancer cells. EGCG inhibited the transcriptional activities of the COX-2, AP-1, and NF- $\kappa$ B promoters (Shimizu et al. 2005). EGCG also downregulated the ERK1/2 and Akt pathways in colon cancer cells. The effect of EGCG on COX-2 expression resulted in decreased COX-2 promoter

activity via inhibition of NF- $\kappa$ B activation (Peng et al. 2006a).

In contrast to the above observations, GTC EGCG is shown to induce COX-2 and mPGES-1 (microsomal prostaglandin E synthase 1) gene expression as well as PGE2 (prostaglandin E 2) biosynthesis in the lung alveolar type II pneumocytes, A549 cells (Moon et al. 2007). Increased activity of ERK1/2 MAPKs and early growth response gene 1 (EGR-1) was found as signaling mediators in mMMPGES-1 induction because by blocking the gene expression of EGR-1 with EGR-1 siRNA or ERK inhibitor, EGCG-induced mPGES-1 was suppressed. This effect of EGCG can be linked to the toxicological actions after human exposure to GTCs.

#### **Tumor-Suppressor p53**

p53, also known as protein 53 due its molecular mass of 53 kDa in SDS PAGE, is a tumor-suppressor and transcription factor. The human gene that encodes for p53 is termed as TP53. The protein p53 plays critical roles in many cellular processes such as cell signal transduction, DNA repair, genomic stability, cell cycle control, and apoptosis. In normal cells, p53 remains in an inactive form by binding to the protein MDM2; however, cancer-causing agents such as UV radiation, oncogenes, DNA-damaging drug activate the proteins. The critical event leading to the activation of p53 is the phosphorylation of its N-terminal domain. Two groups of kinases are mainly responsible for the activation of p53. One group consists of MAPK family (JNK1-3, ERK1-2, p38 MAPK) and responds to the stress like membrane damage, oxidative stress, osmotic shock, and heat shock. The second group contains ATR, ATM, Chk1, Chk2, DNA-PK, and CAK implicated in the genome integrity checkpoint. Once activated, the protein activates the transcription of downstream genes such as p21WAF1 and Bax to induce the apoptotic process, inhibiting the growth of cells with damaged DNA or cancer cells (el-Deiry et al. 1993; Vogelstein and Kinzler 1992). Cells bearing damaged TP53 gene usually show little responses to tumor suppression. In fact, more than 50% of human tumors contain a mutated TP50 gene. Obviously, the product of mutated TP50 gene, mutant p53, loses its ability to bind DNA effectively, and therefore the downstream protein p21 becomes unavailable to regulate cell division. Thus, cell division becomes uncontrolled, which results in tumor formation.

Green tea component EGCG inhibits cell proliferation and suppresses growth of various cancer models such as breast cancer cells (MCF-7 cells), liver

cancer cells (Hep G2), ovarian carcinoma cells, lung cancer cell line NCI-H460, and in sarcoma 180 cells by increasing the expression of p53. In an earlier study, EGCG treatment resulted in a dose-dependent increase in p53 in LNCaP cells (carrying wild-type p53), but not in DU145 cells (carrying mutant p53) (Gupta et al. 2000), suggesting that EGCG may target DNA to modulate the expression of p53. In fact, results obtained from another study, wherein EGCG-induced stabilization of p53 caused an upregulation of p53 transcriptional activity, resulted in the activation of its downstream targets such as p21WAF1 and Bax, and the induction of apoptosis (Hastak et al. 2003) strengthened the notion that EGCG targets and alters the molecular signaling to reduce the risk of cancer. Hastak et al. further took initiatives to explore the effect of EGCG on the transcription activity of p53 by taking a genetic approach. PC3 cells, wherein expression of wild-type p53 is inactivated using siRNA, showed resistance to EGCG, suggesting that catechins targets p53 gene to suppress the proliferation of cancer cells (Hastak et al. 2005). To investigate the molecular basis of EGCG resistant to the cells carrying nonfunctional p53, Amin et al. (2007), however, identified SHP-2 that protects cells from EGCG-mediated apoptosis.

Indeed, there are observations suggesting that cells lacking functional p53 may be susceptible to GTP-mediated apoptosis. For example, EGC suppressed growth of MCF-7 cells, which expresses wild-type p53 in a similar strength to MDA-MB-231 cells carrying mutated p53. In such a situation, GTP may target other than p53-related genes, for example, p73 (*Cyclin G1*, *Perp*, *Wig1*, and *Pig11*) and suppress the growth as proposed by Amin et al. (2007).

#### **Cell Cycle Checkpoints**

Cell cycle, also known as DNA cycle, is an ordered process composed of multiple events taking place in every eukaryotes and lead to production of daughter cells. Broadly, the events in cell cycle are divided into two phases such as interphase and mitotic phase. In interphase, cells grow, take nutrition, and synthesize DNA, RNA, and proteins. This phase of cell cycle is further divided into three phases, G1, S, and G2 phases. In mitotic (M) phase, cell splits into two daughter cells. Several proteins are known to regulate the timing of the events in the cell cycle. Defects in cell cycle regulations and controlling checkpoints resulted in genomic instability and a predisposition to cancer. Cyclins and cyclin-dependent kinases (CDKs) control the switches of major cell cycle events. Cyclins are regulatory sub-

unit and have no catalytic activity, and CDKs are also inactive without a cyclin. There are several cyclins and CDKs functioning in a cell. Cyclin D1, a component subunit of CDK4 and CDK6, is a rate-limiting factor in the progression of cells through the G1 phase. Improper function of cyclin D1 and CDKs, which mostly described due to the overexpression, is a reason of tumorigenesis. Dietary agents including curcumin, resveratrol, genistein, dietary isothiocyanates, apigenin, and silibinin suppress cancer cell proliferation, either by modulating expression of cyclins/CDKs or by blocking one or more steps in DNA cycle. Many cancers including breast, esophagus, head and neck, and prostate are shown to over-express cyclin D1. A 30- $\mu$ M EGCG is shown to block DNA cycle at G1 phase in MCF-7 cells (Liang et al. 1999). This effect of EGCG is shown to suppress cyclin D1, E, CDK2, and CDK4. EGCG also induces the expression of p21, which has inhibitory role on CDKs. Gupta et al. (2003) have investigated molecular mechanism of EGCG-induced G1 phase cell cycle arrest in human prostate carcinoma cells. In the study, EGCG treatment of LNCaP and DU145 cells resulted in dose- and time-dependent upregulation of WAF1/p21, KIP1/p27, INK4a/p16, and INK4c/p18; down-modulation of cyclin D1, cyclin E, CDK2, CDK4, and CDK6, but not of cyclin D2; increase in the binding of cyclin D1 toward WAF1/p21 and KIP1/p27, and decrease in the binding of cyclin E toward CDK2. Using a customized cDNA microarray and quantitative RT-PCR gene expression techniques, Weinreb et al. (2003a) have shown that a 50- $\mu$ M EGCG upregulated the expression of proapoptotic gene such as Bax, caspase-6, Fas ligand, and the cell cycle inhibitor *GADD45* genes, while decreasing antiapoptotic Bcl-2 and Bcl-xL in human neuroblastoma cells. EGCG also blocked cell cycle at G1 checkpoint in intestinal epithelial cells transfected with an inducible Ha-Ras(Val12) cDNA by downregulating the expression of cyclin D1 (Peng et al. 2006b). In addition, EGCG treatment resulted in cell cycle arrest at both G0/G1- and G2/M-phases in rat aortic SMCs (RASMCs), wherein the constitutive expression of NF- $\kappa$ B/p65 nuclear protein was lowered (Han et al. 2006).

#### **Tumor Necrosis Factor**

Tumor necrosis factor (TNF), also known as tumor necrosis factor alpha (TNF- $\alpha$ ), is a class of cytokine produced primarily by monocytes and macrophages. Initially, TNF- $\alpha$  was found to induce necrosis (cell death); however, later it was been shown to mediate tumor initiation, promotion, and metastasis. TNF- $\alpha$

is found to be a growth factor for most forms of tumors. The proinflammatory effect of TNF- $\alpha$  has also been extensively investigated due to its ability to activate NF- $\kappa$ B. TNF- $\alpha$ -induced induction of proinflammatory genes has been linked to many diseases. Almost all cell types, when exposed to TNF, activate NF- $\kappa$ B, leading to the expression of inflammatory genes, such as cyclooxygenase-2 (COX 2), lipoxygenase-2 (LOX-2), cell-adhesion molecules, inflammatory cytokines, chemokines, and inducible nitric oxide synthase (iNOS). Blocking the action of TNF or its transcription has been shown to be beneficial in reducing the inflammation in inflammatory diseases such as Crohn's disease and rheumatoid arthritis. Because of the critical role of TNF in mediating tumorigenesis, agents that can suppress TNF activity have the potential for therapy of TNF-linked diseases.

EGCG, the major green tea polyphenol, have been shown to downregulate the expression of TNF- $\alpha$  gene and TNF- $\alpha$  protein in different types of cancer cells and in the cells exposed to various proinflammatory stimuli. Oral gavage of green tea polyphenols to BALB/c mice (0.5 g green tea polyphenol/kg body weight) decreased LPS-induced serum TNF- $\alpha$  level by 80% of control (Yang et al. 1998). In the macrophage cell line, RAW264.7, EGCG decreased LPS-induced TNF- $\alpha$  production (50% inhibition at 100 mmol/L) and TNF- $\alpha$  mRNA (30–40% inhibition at 100 mmol/L) expression by inhibiting DNA-binding activity of NF- $\kappa$ B. Similarly, Okabe et al. have shown that green tea polyphenols ECG, EGCG, EGC, and EC inhibit release of TNF- $\alpha$  and TNF- $\alpha$  gene expression in KATO III cells, as well as okadaic acid-induced AP-1 and NF- $\kappa$ B activation. EGCG-mediated inhibition of AP-1 and NF- $\kappa$ B activation is shown to reduce expression of TNF- $\alpha$  gene in lung cancer cells (BALB/3T3) (Suganuma et al. 2000). This effect of green tea polyphenols was further evidenced in an animal experiment. In TNF- $\alpha$  transgenic mice, 1% green tea polyphenols reduced the expression of TNF- $\alpha$  gene and TNF- $\alpha$  protein. In addition, a recent study has shown that EGCG (100  $\mu$ M) attenuated PMA+A23187-induced NF- $\kappa$ B and ERK1/2 activation to inhibit PMA+A23187-induced TNF- $\alpha$ , IL-6, and IL-8 expression and production in mast cells (Shin et al. 2007).

GTC not only modulates the expression of TNF- $\alpha$  gene, but also alters the activity in TNF- $\alpha$ -modulated gene transcription of various cytokines. Chen et al. (2002) have shown EGCG inhibited TNF- $\alpha$ -mediated interleukin (IL)-8 gene promoter activity in A549 cells. In the study, A549 cells were tran-

siently transfected with an IL-8 promoter–luciferase reporter plasmid. EGCG treatment resulted in TNF- $\alpha$ -mediated activation of the IL-8 promoter in the cells.

#### **Signal Transducer and Activator of Transcription**

STAT (also called signal transduction and transcription) proteins govern cell proliferation, cell death, and cell differentiation at multiple regulatory points. Initially, these proteins were described as latent transcription factors, which had required phosphorylation for activation. In several recent studies, Janus kinase (JAK), G-protein-coupled receptors, or growth factor receptors (such as EGFR); platelet-derived growth factor receptors that have intrinsic tyrosine kinase activity; or intracellular nonreceptor tyrosine kinase recruitment have shown to play critical roles in the activation of these transcription factors. Being activated, these transcription factors bind to consensus DNA-recognition motif in the promoter region of cytokine-inducible genes and activate transcription of these genes. However, deregulation of the activation pathway is frequently observed in the multiple facets of tumor growth, spread of angiogenesis, and immunosuppression. At least seven different kinds of STAT proteins have been identified so far. Of them STAT3 and STAT5 have been identified to be implicated in myeloma, lymphomas, leukemia, and several solid tumors; thus, these two proteins have become targets for cancer therapy. These STAT proteins contribute to cell survival and growth by preventing apoptosis through increased expression of antiapoptotic proteins, such as Bcl-2 and Bcl-xL.

Several dietary components including green tea polyphenols have also been shown to suppress STAT activation in tumor cells. In HNSCC cells, treatment with EGCG has shown to suppress the phosphorylation of STAT3 (Masuda et al. 2001). In addition, EGCG-mediated suppression of interferon (IFN)- $\gamma$ -elicited STAT1 activation in human MDA-MB 231, HeLa, MCF7, MDA MB 468, HepG2, or HT-29 cell lines is shown to interfere with tyrosine phosphorylation (Magro et al. 2005; Menegazzi et al. 2001). Moreover, EGCG suppression of iNOS gene expression is mediated by downregulation of the DNA-binding activity of the transcription factor STAT1a. This downregulation of the STAT1a–DNA binding was shown to result from reduced tyrosine phosphorylation of the STAT1a protein (Tedeschi et al. 2002). Cytoprotective activity of EGCG on cardiac myocytes and the isolated rat heart was shown due to reduced expression of STAT1 proapoptotic target gene (Townsend et al. 2004). In a recent study,

mice bearing heterotopic tumors were given EGCG. Studying the level of VEGF protein level and activation of STAT3, the authors have found that EGCG reduced the activation of STAT3, but did not change the total STAT3 expression (Zhu et al. 2007).

### INFLAMMATION

Green tea polyphenols have been reported to have remarkable anti-inflammatory effects in many animal, cell culture, and epidemiological studies. Multiple lines of evidence support that NF- $\kappa$ B and AP-1 control the expression of genes encoding proinflammatory cytokines, chemokines, immune receptors, and adhesions molecules that play key role in inflammation-related injuries. Cigarette smoke is widely considered to be a powerful inducer of inflammatory responses resulting in disruption of major cellular pathways with transcriptional and genomic alterations, forcing the cells toward carcinogenesis. In a model study, Syed et al. (2007) have used NHBE cell to study the effect of GTC in preventing cigarette smoke condensate (CSC)-induced cell proliferation and found that EGCG (20–80  $\mu$ M) effectively suppressed the CSC-induced cell proliferation in NHBE cells. NHBE cells transfected with a luciferase reporter plasmid containing an NF- $\kappa$ B-inducible promoter sequence showed an increased reporter activity after CSC exposure that was specifically inhibited by EGCG pretreatment. Immunoblot analysis showed that pretreatment of NHBE cells with EGCG resulted in a significant downregulation of NF- $\kappa$ B-regulated proteins cyclin D1, MMP-9, IL-8, and iNOS. EGCG pretreatment further inhibited CSC-induced phosphorylation of ERK1/2, JNK, and p38 MAPKs and resulted in a decreased expression of PI3K, AKT, and mTOR signaling molecules.

In an animal study, Abboud et al. (2008) induced colitis in C57/BL6 mice by rectal administration of trinitrobenzenesulfonic acid (TNBS). After TNBS administration mice were treated with vehicle or EGCG (10 mg/kg) intraperitoneally, and vehicle-treated mice were found to experience colonic damage with hemorrhage, ulcers, and edema, with elevated plasma levels of TNF- $\alpha$ , IL-6, IL-10, and keratinocyte-derived chemokine. These events were paralleled by increased DNA binding of NF- $\kappa$ B and AP-1 in the colon of the vehicle-treated group. In contrast, the EGCG-treated mice experienced a very mild diarrhea and no weight loss. Tissue levels of myeloperoxidase were also significantly reduced as compared to vehicle-treated mice. These beneficial effects of EGCG were associated with a significant reduction of NF- $\kappa$ B and AP-1 activation.

Yu et al. (2005) evidenced that catechins attenuated acute urine retention and evoked proinflammatory response such as increased expression of NF- $\kappa$ B, AP-1, and ICAM-1 in the rat liver. IL-1 $\beta$  and TNF- $\alpha$  are two important proinflammatory cytokines that have been shown to orchestrate the inflammatory response through the activation of NF- $\kappa$ B and AP-1, with the subsequent induction of proinflammatory gene expression. In a model animal study (Wheeler et al. 2007), polymicrobial sepsis was induced in male Sprague-Dawley rats (hemodynamic study) and C57BL6 mice (mortality study) via cecal ligation and double puncture (CL2P). Rodents were treated with either EGCG (10 mg/kg intraperitoneally) or vehicle at 1 and 6 h after CL2P and every 12 h thereafter. In vehicle-treated rodents, CL2P was associated with profound hypotension and greater than 80% mortality rate. Epigallocatechin-3-gallate treatment significantly improved both the hypotension and survival. In vitro experiments further showed that EGCG inhibited activation of NF- $\kappa$ B and subsequent NOS2 gene expression in a primary culture of rat aortic smooth muscle cells. Epigallocatechin-3-gallate may therefore represent a potential nutritional supplement or pharmacologic agent in patients with sepsis. EGCG was also found to suppress the expression of IL-6, and in NF- $\kappa$ B ligand in *S. aureus*-infected osteoblast (Ishida et al. 2007).

### IL-8 Expression

IL-8 is a principle neutrophil chemoattractant; thus, interest in developing novel pharmacological inhibitors of IL-8 gene expression has become the interest of many recent research to combat inflammation-related disease. NF- $\kappa$ B has been shown to regulate IL-8 gene expression. A previous study has shown that EGCG inhibits 26S proteasome activity causing increase accumulation of I $\kappa$ B and inhibit NF- $\kappa$ B activation (Nam et al. 2001). In another study, Chen et al. have determined the effects of epigallocatechin-3-gallate (EGCG) on TNF- $\alpha$ -mediated expression of the IL-8 gene in A549 cells. Using ELISA and Northern blot analysis, the authors have shown that EGCG inhibited TNF- $\alpha$ -mediated IL-8 gene expression in a dose-response manner. Investigating the mechanism, the cells were transiently transfected with an IL-8 promoter-luciferase reporter plasmid. The authors found that EGCG inhibited TNF- $\alpha$ -mediated activation of the IL-8 promoter in cells. In addition, EGCG inhibited TNF- $\alpha$ -mediated activation of I $\kappa$ B kinase and subsequent activation of the I $\kappa$ B  $\alpha$ /NF- $\kappa$ B pathway, showing EGCG is a potent inhibitor of IL-8 gene expression *in-vitro* (Chen et al. 2002).

### **Toll-Like Receptor Signaling Pathway**

Toll-like receptors (TLRs) are defined as a class of receptors that recognize conserved molecules derived from pathogens once the molecules have crossed the physical barriers such as skin or intestinal tract, thereby activating immune cell response essential for host defense against invading microbial pathogens. Involvement of TLRs in the development of many inflammatory diseases including atherosclerosis, diabetes, rheumatoid arthritis, and cancer has been evidenced in many recent studies. Currently, at least 13 TLRs have been identified, and some of them have been shown to be activated in response to the endogenous agonists derived from nonpathogenic sources, such as heat shock protein 60 and 70, type III repeat extra domain A of fibronectin, taxol, and saturated fatty acids, RNAs, and lipopolysaccharides (LPSs).

*Helicobacter pylori*-induced TLR-4 activation is shown to be associated with increased glycosylation of the receptor molecules that help by moving out to the cell membrane after its translation to intensify its biological effects, such as inflammation, increased DNA breakdown, apoptosis of epithelial cells, and induction of cell cycle deregulation. Lee et al. (2004) have shown that EGCG pretreatment results in inhibiting the glycosylation of TLR-4 induced by *H. pylori* infection. This effect of EGCG was, however, shown to be associated with reduced activity in binding of NF- $\kappa$ B to DNA; thus, expression of inflammatory-related genes, including those for IL-8, IL-1beta, IFN- $\gamma$ , TNF- $\alpha$ , cyclooxygenase-2, and lipoxygenase, crucial for inflammation is prevented. While investigating the mechanism and elucidating the pathway involved in the process, it has shown that EGCG pretreatment attenuated the sequential activation of the ERK signaling pathway, NF- $\kappa$ B transcription, and IL-8 expression by *H. pylori* infection. To investigate the molecular target involved in downstream signaling of TLRs, Youn et al. (2006) have shown that EGCG inhibits both of MyD88-dependent and TRIF-dependent signaling pathways of TLRs, with subsequent modulation of inflammatory genes.

### **Monocyte Chemotactic Protein-1 Expression**

Monocyte chemotactic protein-1 (MCP-1), also known as CCL2, is one of the important chemokines that actively participate in the inflammation process. It regulates the migration and infiltration of monocytes/macrophages to the inflammation sites. High levels of MCP-1 have been detected in patients suffering from neuroinflammatory disease, including sclerosis, cerebral ischemia, and HIV-1 encephalitis.

MCP-1 mRNA expression has been detected in the endothelial cells, macrophages, and vascular smooth muscle cells (VSMCs) in atherosclerotic plaque (Seino et al. 1995). Various stimuli, such as LPS, IL-1, TNF- $\alpha$  platelet-derived growth factor, or IFN- $\gamma$  induce expression level of MCP-1 *in-vitro* in a wide variety of cells including monocytes, fibroblasts, vascular endothelial cells, and smooth muscle cells (Baggiolini et al. 1994). In a study, it has been shown that EGCG could suppress the PMA-induced MCP-1 expression in human endothelial ECV304 cells. Northern blot analysis using human MCP-1 cDNA revealed that EGCG significantly inhibited PMA-induced MCP-1 mRNA expression. Moreover, the suppression of MCP-1 expression has been described to occur at the transcriptional level, as shown by the transient transfection study using the MCP-1 promoter-reporter construct, which contained AP-1, NF- $\kappa$ B, and Sp1 sites for stimuli that can induce MCP-1 expression (Ueda et al. 1997). The possible mechanism involving an EGCG-induced anti-inflammatory effect by controlling MCP-1 expression is described to inhibit AP-1 and suppress p38 MAPK and NF- $\kappa$ B activation (Hong et al. 2007).

### **MMP-2 Expression**

MMP-2, also known as gelatinase A, plays an essential role in the pathogenesis of angiogenesis, inflammation, and fibrosis. Accordingly, elevated levels of MMP-2 gene expression have been detected after skin injury, liver fibrosis, and so on. In a model study, using MMP2/LacZ-reporter mice, Jansen et al. (2007) have shown that regulatory sequences -1686/+423 have driven injury-induced MMP-2 promoter activation. Many model studies have been conducted to evaluate the efficacy of food phytochemicals including green tea polyphenols on the modulation of MMP-2 gene expression. Initially, EGCG was found to inhibit MMP-2 (Annabi et al. 2002) protein expression by inhibiting the MT1-MMP-dependent proMMP-2 activation without affecting MMP-2 gene expression. In the study, the inhibitory effect of EGCG on MT1-MMP was linked with the downregulation of MT1 MMP gene transcription. Later, it was shown that EGCG modulates MMP-2 gene expression while it suppresses MMP-2 reactivity (El Bedoui et al. 2005). In a recent study (Zhen et al. 2007), EGCG treatment of CCl<sub>4</sub>-administered rats resulted in decreased hepatic level of MMP-2 activity. EGCG treatment significantly decreased the expression of MMP-2 protein and mRNA expression, as well as activation of MMP-2 in cultured rat HSCs. The mechanisms behind EGCG inhibition of MMP-2 gene expression

are rather unknown; however, several previous studies have demonstrated that MMP-2 expression requires the activation of NF- $\kappa$ B (Li et al. 2005). Since EGCG prevents the activation of NF- $\kappa$ B, its effect on MMP-2 expression is quite likely achieved through this inhibition.

#### ***Glucocorticoid-Induced Mouse Mammary Tumor Virus Gene Expression***

Glucocorticoids, because of the immunomodulating properties, are widely used to treat inflammation. The basic mechanism of the enhanced immunomodulating activity is based on interferences with the transcription factors. To study steroid-induced gene transcriptions, the gene mouse mammary tumor virus long terminal repeat (MMTV LTR) has been studied and used as a model system for the regulation of steroid-induced gene transcription (Archer et al. 1995).

In a model study, Abe et al. transfected luciferase reporter gene pBV2-MMTV-LUC into rat fibroblast 3Y1 cells under the transcriptional regulation of MMTV LTR gene. The cells when exposed to dexamethasone, a synthetic glucocorticoid, induce luciferase activity, which can be enhanced up to 180% by EGCG. The author proposed that GTPs might have therapeutic importance as it enhances glucocorticoid-induced gene transcriptions (Abe et al. 2001).

#### ***NSAID-Activated Gene Expression***

Nonsteroidal anti-inflammatory drugs (NSAID) have analgesic, antipyretic, and anti-inflammatory effects. Indomethacin, an NSAID, is known to relieve pain and inflammation associated with rheumatoid arthritis, osteoarthritis, ankylosing spondylitis, and gout. Baek et al. identified NAG-1 (also known as MIC-1, GDF-15, placental TGF- $\beta$ , and PLAB) gene from indomethacin-induced gene library. This gene represents a divergent member of the TGF- $\beta$  superfamily, although initially it was identified as a proapoptotic and anticarcinogenic.

NAG-1 gene is highly expressed in mature intestinal epithelial cells; on the contrary, a significantly reduced level is identified in human colorectal carcinoma samples and neoplastic intestinal polyps of Min mice (Kim et al. 2002). In addition, various previous reports have shown that NAG-1 is upregulated in human colorectal cancer cells by several NSAIDs (Baek et al. 2002b), as well as by dietary compounds such as resveratrol (Baek et al. 2002a), genistein (Wilson et al. 2003), and conjugated linoleic acids (CLAs) (Lee et al. 2006). In a recent report, Baek

et al. (2004) have shown that GTCs, EGCG, ECG, EGC, and EC, target NAG-1 gene and induce its expression with variable effects. EGCG at a higher concentration (100  $\mu$ M) have shown to cause oxidative damage of DNA followed by p53 protein induction and NAG-1 gene expression. However, for ECG, the authors have shown a novel mechanism, which is independent of p53 induction. In the report, it has been shown that ECG induces NAG-1 gene expression in cells lacking p53 activity and an NAG-1 promoter that lacks p53 binding site. Investigating the mechanism, the authors have further shown that transcription factor ATF3 mediates NAG-1 gene expression at the transcriptional level in ECG-induced NAG-1 expression in HCT116 cells. The authors also examined the sequences of ATF3 transcription factor binding sites through the 3.5-kb NAG-1 promoter sequences and have shown that ATF3 mediates NAG-1 expression through the putative ATF3 binding site located at -955 bp promoter region in pNAG1086/LUC promoter construct.

#### **DIABETES AND OBESITY**

Diabetes and obesity, the two common diseases often described to relate with lifestyle, have become epidemic with enormous global problems. Diabetes and obesity are closely linked and associated with each other. The reason why an individual develops diabetes/obesity has been extensively studied. In an individual, development of diabetes and obesity is characterized to elevated blood glucose level and increased number of adiposities, respectively. Both of the diseases are often described to be linked with genetic, endocrine, metabolic, neurological, pharmacological, environmental, and nutritional factors. It is widely considered that excessive consumption of food and less physical activity result in the development of obesity, which if not intervened properly may develop into diabetes within a short period. Many epidemiological and clinical studies have shown that consumption of healthy food may prevent the development of these diseases. Therefore, a better perception about healthy food, its active constituents, and how it prevents the development of diabetes and obesity would be of great benefit to individuals who are at risk of both diseases. Although numerous studies have shown that GTCs reduce body fat and regulate blood glucose level, little is known about the mechanism of action as compared to its anticancer activity. Such scientific studies are beginning to emerge that show GTC-modulated gene expression is linked with antiobesity and antidiabetic action.

### **PEFCK Gene Expression**

A major hallmark of diabetes is failure of insulin to inhibit hepatic glucose synthesis. Two rate-controlling gluconeogenic enzymes, namely phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) are considered to contribute to the increased hepatic glucose production. In several animal models of type II diabetes, it has been shown that PEPCK mRNA levels are increased two- to three folds over that observed in nondiabetic animals. Hormones like glucocorticoids and glucagons have shown to increase PEPCK gene transcription via their second messenger, cAMP. On the contrary, insulin dominantly suppresses PEPCK gene transcription. There are varieties of agents that have also shown to suppress PEPCK mRNA levels and exhibit insulin mimetic function. These include phorbol ester, vanadate, and various cytokines, such as TNF- $\alpha$ , IL-6, and IL-1. The G6Pase gene is also regulated in a manner similar to that of the PEPCK gene, and insulin represses this gene by a PI3K-dependent mechanism (Dickens et al. 1998). Thus, it is proposed that a suitable antidiabetic agent should have actions similar to insulin. In a study, Waltner-Law et al. (2002) have shown that GTC EGCG suppresses PEPCK and G6Pase gene expression in H4IIE cells. Using ribonuclease protection assay, the authors have shown that EGCG suppress PEPCK and G6Pase gene expression by a PI3K-dependent mechanism and control hepatic gluconeogenesis. Results obtained in the study suggest that prooxidant behavior of EGCG on the hepatoma cells may contribute to this activity, since *N*-acetyl cysteine (NAC) or superoxide dismutase (SOD) completely reversed the EGCG-mediated PEPCK and G6Pase gene suppression.

In another exciting study, Anton et al. have shown EGCG's ability to mimic insulin's inhibition of PEFCK gene expression. They have shown the extent of inhibition of PEFCK by EGCG is similar to that induced by insulin and IGF-1, since the effect of EGCG on PEPCK activity is completely inhibited by PtdIns 3-kinase inhibitors. Therefore, a common regulatory pathway involving PtdIns 3-kinase transcription factors was considered in the insulin-, IGF-1-, or EGCG-modulated PEFCK gene expression. At the end, the authors have proposed that EGCG-induced phosphorylation of FOXO1a transcription factor might play a critical role in the activation of PtdIns 3-kinase and then suppress PEPCK gene expression (Anton et al. 2007). In the experiment, human embryonic kidney cells (293 cells) were cultured in the presence or absence of EGCG, insulin, or IGF-1. Using specific antibodies that de-

tect phosphorylated FOXO1a proteins in cell lysates, the authors have shown that EGCG is effectively capable of mimicking action of insulin or IGF-1 on FOXO1 gene transcription factors. The authors have also shown that catechins having galloyl group, such as EGCG or GCG, are very effective in FOXO1 transcription factor phosphorylation in the cells. Investigating the involvement of any downstream effector molecules in EGCG-induced phosphorylation on FOXO1a transcription factors, the authors have shown that PKB/Akt or SGK catalyzed the phosphorylation reaction at Thr24, Ser256, and Ser319 residues, the latter residue then priming Ser322 and Ser325. In an extended experiment, EGCG and GCG were also found to induce FOXO1a phosphorylation hepatoma cell line HL1c, in which both EGCG and GCG repress PEPCK promoter activity. Consistent with the previous reports, the authors have shown that NAC and SOD, which scavenge ROS, can oppose the effectiveness of the catechins, and inhibit EGCG/GCG-induced FOXO1a phosphorylation. This effect of NAC or SOD is, however, in contrast to that of insulin or IGF-1, which are insensitive to the intervention.

### **Glucose Uptake/Transport**

Insulin is the principle hormone that regulates the uptake of glucose from blood to most cells. Therefore, deficiency in insulin synthesis or the insensitivity to its receptors has become the primary reason in most forms of diabetes mellitus. In most nonhepatic tissues insulin increases glucose uptake by increasing the number of glucose transporters (GLUTs). There are at least five different GLUT proteins, GLUT-1, 2, 3, 4, and 5, that exist in mammals. GLUT1 is found in most tissues, GLUT2 is found in liver and pancreatic B-cells, GLUT3 is distinct in the brain, and GLUT4 is found in heart, adipose tissue, and skeletal muscle. A distinct gene encodes each of the GLUT proteins. There are many other components existing in the insulin-signaling pathway that regulate the translocation of GLUT to the plasma membrane.

Any defect in the pathway may produce insulin resistance in animals or humans. There have been many reports showing green tea to reduce fasting plasma levels of glucose, triglyceride, free fatty acids, and insulin and increase the insulin-stimulated glucose uptake and GLUT4 proteins in mice fed a diet with high fructose (Wu et al. 2004). In a very recent study, Cao et al. (2007) investigated the effects of green tea extract on the relative expression levels of several member of glucose transporter family genes (Glut1/Slc2a1, Glut2/Slc2a2, Glut3/Slc2a3,

and Glut4/Slc2a4) and insulin signaling pathway genes (Ins1, Ins2, Insr, Irs1, Irs2, Akt1, Grb2, Igf1, Igf2, Igf1r, Igf2r, Gsk3b, Gys1, Pik3cb, Pik3r1, Shc1, and Sos1) in liver and muscle of rats fed a high-fructose diet known to induce insulin resistance and oxidative stress. They have shown that diet containing 1g/kg diet increased Glut1, Glut4, Gsk3b, and Irs2 mRNA levels by 110, 160, 30, and 60% in the liver, respectively, and increased Irs1 by 80% in the muscle. Diet containing green tea extract (2 g/kg) increased Glut4, Gsk3b, and Pik3cb mRNA levels by 90, 30, and 30% but decreased Shc1 by 60% in the liver and increased Glut2, Glut4, Shc1, and Sos1 by 80, 40, 60, and 50% in the muscle, respectively. The study have shown that green tea extract at 1 or 2 g/kg diet modulates gene expression in the glucose uptake and insulin signaling pathway in liver and muscle cells in rats fed a fructose-rich diet.

Several previous studies have shown that GTCs modulate the expression of various proteins or enzyme-related carbohydrate metabolism or transport across the brush borders in the intestinal tract. Green tea polyphenols have been shown to inhibit *in-vitro* enzymatic activity of many carbohydrate digestive enzymes, such as  $\alpha$ -glucosidase, gastric H<sup>+</sup>, K<sup>+</sup>-ATPase intestinal sucrose salivary  $\alpha$ -amylase. Kobayashi et al. (2000) have shown that green tea polyphenols, especially ECG and EGCG, inhibited intestinal glucose by inhibiting sodium-dependent GLUT (Na<sup>+</sup>-GLUT) of intestinal epithelial cells in a rat-everted sac. The effect was further confirmed in rabbit intestinal brush border system, wherein 1-mM ECG and EGCG inhibited glucose uptake by 53 and 35%, respectively. Using human intestinal epithelial Caco-2 cells and brush border membrane vesicles of rabbits small intestine, Shimizu et al. (2000) have shown that ECG inhibits glucose uptake by suppressing SGLT1 (sodium-dependent glucose transporter 1) expression. SGLT1 occurs in jejunum and transports glucose into blood through epithelial cells. Therefore, inhibition of SGLT1 expression is another strategy to prevent hyperglycemia, which causes diabetes.

#### **Adiponectin Gene Expression**

Adiponectin is a collagen-like protein specifically synthesized in adipose tissues and secreted into blood plasma. Adiponectin gene expression and plasma level of adiponectin are decreased in obese humans and in patients with type-2 diabetes with insulin resistance. In obese mice, administration of adiponectin resulted in total weight loss and reduced circulating free fatty acid by enhancing skeletal muscle fat oxidation (Fruebis et al. 2001). In diabetic mice,

adiponectin treatment reduced plasma glucose level by enhancing insulin sensitivity and suppressing hepatic glucose production. Understanding the mechanisms involved in the transcriptional regulation of adiponectin gene expression is still limited, but there are evidences showing multiple transcription factors, such as CCAAT enhancer-binding protein (C/EBP) $\alpha$ , Kruppel-like factor 7 (KLF7), liver receptor homolog-1, peroxisome proliferator-activated receptor- $\gamma$  (PPAR $\gamma$ ), and sterol regulatory element-binding protein-1c (SREBP-1c) regulate adiponectin gene expression. In many previous studies, GTCs have been shown to decrease blood glucose and insulin level, and increase glucose metabolism in adipocytes. A commercial tea infusion, sunphenon EGCG, enhances plasma adiponectin levels and decreases plasma triglyceride levels in nonobese diabetic rats (Shimada et al. 2007). In a recent study, Cho et al. (2007b) have shown that (−)catechins clearly enhance the expression of adiponectin and increase glucose uptake into 3T3-L1 adipocytes. In search of the molecular mechanism responsible for the inducible effect of (−)catechins on adiponectin expression, the authors have shown that (−)catechins markedly suppress the expression of Kruppel-like factor 7 (KLF7) protein, which has recently been reported to inhibit the expression of adiponectin and other adipogenesis-related genes, including leptin, PPAR $\gamma$ , C/EBP $\alpha$ , and aP2 in adipocytes. KLF7 is a transcription factor in adipocyte and plays an important role in the pathogenesis of type II diabetes. In the study, the authors have shown that (−)catechins increased insulin-dependent glucose uptake, and upregulated the expression of adipogenic marker genes, including PPAR $\gamma$ , C/EBP $\alpha$ , Fas, and stearoyl-CoA desaturase (SCD)-1. This report suggested that (−)catechin-induced upregulation of adiponectin might be associated with the suppression of KLF7 in 3T3-L1 cells.

#### **Lipogenic Gene Expression**

Lipogenesis encompasses the processes that are involved in the synthesis of fatty acid and subsequently in the synthesis of triglycerides. Various enzymes, such as acetyl-CoA carboxylase (ACC), FAS, glycerol-3-phosphate dehydrogenase (G3PDH), glucose-6-phosphate dehydrogenase (G6PDH), malic enzyme (ME), and SCD1, regulate lipogenesis pathways through which acetyl-CoA the key rate-limiting component is converted into triglycerides. Many previous studies have shown that GTCs modulate the activity of lipogenic enzymes by either inhibiting the activity of the enzymes or altering their synthesis at the transcription level.

ACC is a key enzyme that catalyzes the conversion of acetyl-CoA to malonyl-CoA, the precursor of fatty acid. The activity of ACC can be controlled at the transcription level as well as by modulators or posttranscription modifications. Human genome contains the gene for two different ACCs, such as ACACA(ACC-1) and ACACB(ACC-2). In a recent study, Wolfram et al. (2005) have shown that dietary supplementation of GTCs significantly reduced the ACC-1 mRNA expression in obese mice. In the same study, the authors have shown that dietary EGCG intervention reduced the mRNA expression of FAS and G3PDH, the two rate-limiting enzymes for fatty acid biosynthesis in epididymal adipose tissue. Reduced expressions of ME and G6PDH mRNA have also been observed in obese mice fed EGCG supplemented diet. EGCG-supplemented diet also decreased the level of SCD1 gene expression in obese rat. Although detail mechanisms involving the EGCG-modulated gene expression in lipogenesis is unknown, it is proposed that EGCG may recruit some downstream transcription factors that influence the transcription of the lipogenic gene in adipose tissue. This hypothesis is based on the fact that the EGCG induces suppression of tumorigenic Fas mRNA and protein expression in MCF-7 breast cells, which involves the downregulation of EGF receptor and its downstream Akt and Sp-1 proteins. Taken together, EGCG or green tea appears to reduce fatty acid and triglyceride synthesis by inhibiting enzyme synthesis at the transcription level.

Catechin administration to human is shown to increase fat oxidation and energy expenditure. This effect of tea catechins is also observed in obese mice fed with tea catechin (Murase et al. 2002). Feeding with tea catechin diet (0.1–0.5% w/w), obese mice C57BL/6J showed a significant reduction in body fat, visceral and liver fat accumulation, and the development of hyperinsulinemia and hyperleptinemia. Investigating gene expression in response to GTE-rich diet, the authors have extracted mRNA from the liver and found upregulation of two lipids  $\beta$ -oxidation enzymes acyl-CoA oxidase (ACO) and medium-chain acyl-CoA dehydrogenase (MCAD). In addition to this, downregulation of FAS mRNA was observed. Although the precise mechanism by which catechins modulate the synthesis of lipid metabolizing enzymes is unknown, but it has become apparent that PPARs regulate the transcription of several metabolizing enzymes, such as ACO and MCAD. It is proposed that GTC-induced NF- $\kappa$ B suppression may lead to upregulation of lipid catabolizing enzymes, because NF- $\kappa$ B was shown to negatively regulate the expression of PPAR $\alpha$  activity.

### **Resistin Gene Expression**

Resistin is a cysteine-rich polypeptide hormone secreted by adipose tissue. Resistin was discovered in 2001, and the name was given due to its action on insulin resistance in mice. Since its discovery, numerous actions of this hormone have been identified. The striking discovery related to the action of resistin is its link with obesity and type II diabetes. Rstn mRNA expression and protein level was found higher in obese and diabetic II subjects. Thus, controlling Rstn production and function especially by nutritional factors has become a strategy in the management of obesity or diabetes in these days. A recent study has shown that vitamin A reduced Rstn mRNA levels in white and brown adipocytes. GTCs have long been considered to deliver health benefits by reducing the mass of adipose tissue and blood glucose level. Until recently, there was no proof that GTCs modulate resistin gene expression. Using 3T3-L1 adipocytes, Liu et al. (2006) have shown that EGCG at 100  $\mu$ M suppressed Rstn mRNA levels by 50% after 3 h. Intracellular Rstn protein significantly decreased in the presence of 100  $\mu$ M EGCG 3h after treatment. Investigating molecular cascades involved in the EGCG-mediated resistin gene expression, the authors have shown that EGCG did not affect ERK1/2, phospho-JNK, phospho-p38, and phospho-Akt proteins but reduced the amounts of phospho-ERK1/2 proteins. To demonstrate the involvement of any cascades in the pathway, the authors have transiently overexpressed adipocytes with MEK1 and then examined the effect of EGCG on resistin gene expression, and at this point overexpression of either MEK1 or MEK1EE prevented EGCG-inhibited Rstn mRNA expression, suggesting that EGCG downregulates Rstn expression via a pathway that is dependent on the ERK pathway.

There is a wealth of literature that have evidenced that green tea-associated antiobesity effect is linked to increased thermogenesis in adipose tissue, increased lipid catabolism in liver, increased energy expenditure, and decreased respiratory quotient. Indeed, the details of the mechanism related to increased thermogenesis and fat oxidation is still unknown, but some emerging evidences suggest that GTCs, particularly EGCG, modulate expression of several key genes in lipid and glucose metabolism.

### **UCP Gene Expression**

Uncoupling proteins (UCP), UCP-1, UCP-2, and UCP-3, are considered to be involved in energy metabolism by facilitating oxidative phosphorylations and transferring ions across mitochondrial

membranes. Increased UCP gene expression enhances fat metabolism and regulates energy expenditure. Various stimuli such as cold exposure, prolong starvation (48 h), exposure of  $\beta$ -3-adrenergic agonist, and CL316, 243 have been shown to increase UCP gene expression. Klaus et al. (2005) have shown that GTC EGCG-supplemented diet (0.5 and 1.0%) resulted in the attenuation of body fat accumulation in obese rat. PCR amplification of mRNA isolated from rat liver have shown that EGCG induced UCP-2 gene expression in liver cells, and the effect is compatible with the increased energy metabolism in liver induced by EGCG as a consequence of increased fatty acid oxidation.

#### **SCD Gene Expression**

SCD is an enzyme that catalyzes the rate-limiting step in the biosynthesis of monounsaturated fatty acids. These unsaturated fatty acids are used as substrates in the synthesis of triglycerides, phospholipids, cholesterol, and so on. At least four isoforms of SCD have been identified. Mice lacking/mutant SCD1 gene show decreased lipid biosynthesis, reduced adiposity, and increased fatty acid oxidation. Therefore, this gene is considered as an important target in regulating lipidogenesis. Various dietary factors or drugs have shown to inhibit SCD1 gene expression. In a study, Klaus et al. (2005) have shown that EGCG treatment downregulates the SCD1 gene expression in liver tissue and white adipose tissue. In contrast to this observation, a recent study has reported that (–)catechins, if treated during adipocyte differentiation *in-vitro*, augmented lipid accumulation and increased the expression of SCD-1 with the increase in other adipocyte differentiation markers such as FAS, PPAR $\alpha$ , and C/EBP $\alpha$ . This contrasting report, particularly between *in-vivo* and *in-vitro* observations, may be due to the doses used, effected cells used, or any other unknown reasons.

#### **Leptin Gene Expression**

Leptin, a 16-kDa hormone protein, plays a key role in regulating energy intake and energy expenditure. Since leptin is synthesized in adipose tissues, EGCG-induced body weight loss may result in reduced expression of leptin gene as evidenced by Klaus et al. (2005). Recently, leptin-deficient mice are shown to gain weight due to the overexpression of GRP78 (glucose-regulate protein 78). GRP78 also known as immunoglobulin-binding protein promotes cancer cell proliferation, survival, metastasis, and resistance against a wide variety of anticancer drugs mainly due to its  $\text{Ca}^{2+}$ -binding and antiapoptotic properties.

Although EGCG was known to suppress the activity of GRP78 protein by directly interacting with the protein, it still remained to be known if EGCG had any role in suppressing the expression of gene that encodes GRP78 protein. A recent U.S. patent (20080075664), however, claimed that diet containing EGCG may inhibit the expression of GRP78 at the transcription level to suppress diabetes- and obesity-related disorders in obese mice, although the details of mechanism are still under exploration.

#### **CARDIOVASCULAR DISEASE**

Several previous epidemiological and clinical studies have shown that GTCs are effective in preventing cardiovascular disease. However, the studies related to GTC-modulated gene functions in preventing cardiovascular diseases are still at the primary level. There exist few studies that have shown that receptor-mediated signal transduction pathway modulated by GTCs may alter gene expression and/or alter the expression of many biomolecules that actively participate in the protection of cardiovascular system. While studying pathophysiological parameters of cardiovascular diseases, many previous studies have been attributed to superoxide generation with the occurrence of atherosclerosis and myocardial ischemia/reperfusion. Also, the antioxidant behavior of tea catechins has been focused to evaluate its beneficial effect related to cardiovascular diseases. Although GTCs have shown to be effective in scavenging different types of free radicals such as superoxide anions, singlet oxygen, nitric oxide, and peroxynitrite (Jovanovic and Simic 2000; Paquay et al. 2000), very few studies have revealed that GTCs enhance the activity of biological antioxidants that may protect oxidative damage of cardiovascular system. In cancer cells, NF- $\kappa$ B and AP-1, two redox-sensitive transcription factors, have been shown to be modulated by tea catechins. As discussed earlier, there is little evidence that shows that tea catechins also alter the expression of NF- $\kappa$ B and AP-1 in the cardiovascular system.

In a recent study, Aneja et al. (2004) have shown that intravenous administration of 10 g EGCG per kilogram of body weight of rat significantly reduced myocardial I/R injury. The beneficial effect of green tea was evidenced by reduced NF- $\kappa$ B and AP-1 DNA bindings. Suzuki et al. (2006) also supported the results that oral administration of tea catechins reduced NF- $\kappa$ B activity in murine cardiac transplants. Reduced activation of STAT1 and Fas receptor expression is also observed in EGCG-treated myocardial I/R injured rat (Townsend et al. 2004). Increased

levels of iNOS, which are responsible for the production of nitric oxide (also a free radical), are shown to be associated with cardiovascular abnormalities. Several previous studies have revealed that GTCs suppress the expression of iNOS gene. EGCG-induced induction of catalase in spontaneously hypertensive rat, an induction of heme oxygenase in endothelial cells, may also be considered to protect cardiovascular system from the lethal effect of ROS.

Abnormal VSMC proliferation and migration are involved in the pathogenesis of atherosclerosis, restenosis after angioplasty, and vein graft failure due to neointimal hyperplasia. A number of studies have revealed that GTCs are capable to inhibit SMC proliferation and migration in many *in-vitro* and *in-vivo* assay systems. Kim and Moon (2005) have shown that EGCG-induced SMC proliferation inhibition is partially linked to the increased expression of CDK inhibitor p21<sup>WAF1</sup> gene. Hofmann and Sonenshein (2003) also supported this observation; in addition, they have shown that a higher dose of EGCG (80 mg/mL) induces apoptosis mediated by the induction of p53 and activation of NF- $\kappa$ B and reduced induction of p21<sup>CIP1</sup>CKI. Tea catechin-mediated VSMC proliferation and migration are also linked to interference of platelets derived growth factor (PDGF)-induced mitogenic pathways. It is well established that enhanced activity of PDGF  $\beta$ -receptor and EGF receptor contribute to the development of atherosclerosis. Previous reports have shown that catechins with a galloyl group in the 3-position of the catechins structure selectively suppress PDGF-BB-induced stimulation of the PDGF-Rbeta-mediated signal transduction pathway in vascular SMC by inhibiting expression of several downstream signaling targets. In addition, EGCG was shown to inhibit PDGF-induced mRNA expression of c-fos and egr-1 gene in SMCs, thereby preventing atherosclerosis.

Basic fibroblast growth factor (bFGF), which is often described to play a role in SMC cell proliferation and migration, has been linked to the pathogenesis of atherosclerosis (Nugent and Iozzo 2000). In a study, Hwang et al. (2002) have shown that EGCG inhibits bFGF-stimulated cJun gene expression in rat VSMC and inhibits its proliferation and migration. EGCG-mediated interactions with MMP are also shown to be an important factor for inhibition of VSMC proliferation. In a study, Dell'agli et al. (2005) have reported that catechin-induced reduction in MMP-9 secretion can be correlated with decreased MMP-9 promoter activity and mRNA expression.

GTCs have also shown to modulate MAPKs to prevent the pathological changes in atherosclero-

sis. To inhibit serum-stimulated proliferate response in rabbit-cultured vascular smooth muscle, EGC at a concentration range of 10–100  $\mu$ M inhibited JNK1 activity and reduced the level of phosphorylated JNK1. In addition, EGC, at 10  $\mu$ M, significantly reduced c-jun mRNA expression with little or no effect on c-fos and c-myc mRNA (Lu et al. 1998). Similarly, EGCG inhibited PDGF-BB-induced activation of the 44-kDa and 42-kDa MAPK isoforms (p44(mapk)/p42(mapk)) in cultured VSMCs from rat aorta (Ahn et al. 1999). Treatment of VSMCs with 10 and 50  $\mu$ M EGCG resulted in an 80% and a complete inhibition of the PDGF-BB-induced activation of MAPK isoforms, respectively. EGCG showed inhibition of Ras activation and c-jun N-terminal kinase (JNK) activity without affecting protein kinase C expression in RAoSMCs stimulated by bFGF (Hwang et al. 2002). However, EGCG at a dose of 87.3  $\mu$ M significantly reduced expression of c-jun mRNA, suggesting that EGCG modulates gene function to alter the activity or expression level of MAPK, such as Ras/JNK, to inhibit the proliferation of RAoSMCs cells.

Angiotensin II (Ang II) implications in the pathogenesis of atherosclerosis are also well documented. Recent extensive investigations have proven that Ang II increases proliferation of VSMC due to the increase in phosphorylation of ERK 1/2, JNK1/2, or p38 MAPKs and mRNA expression of c-jun and c-fos. EGCG prevents Ang II-induced VSMC proliferation by inhibiting JNK activation and c-jun mRNA expression (Zheng et al. 2004). Won et al. (2006) have reported that EGCG inhibits VSMC proliferation via inhibition of Ang II-induced phosphorylation of ERK 1/2, JNK1/2, or p38 MAPK and the expression of c-jun or c-fos mRNA.

Chronic inflammation has also been considered to play an important role in pathogenesis of many diseases including atherosclerosis. Inflammation of vessel wall, stimulation of vascular endothelial cells, and increased adhesion of mononuclear cells are the initial events in inflammation-induced cardiovascular disorders. In addition to direct suppression of neutrophils and chemokine production, GTC EGCG has been shown to prevent cytokine (TNF- $\alpha$ )-induced VACM-1 (vascular cell adhesion molecules) gene expression in endothelial cells and reduced monocyte adhesion to the endothelial monolayer. ANG II is a vasoactive peptide and has been shown to implicate in the development of cardiovascular disease by promoting inflammation with the increase in production of ROS, inflammatory cytokines, and adhesion molecules (VCAM-1, ICAM-1). In a study, Chae et al. (2007) have reported that EGCG (10–50

$\mu\text{M}$ ) inhibited effect of ANG II-induced elevation of VCAM-1 and ICAM-1 mRNA expression in HUVEC. While studying the molecular cascades resulting in the inhibiting of VCAM-1 and UCAM-1 gene transcription, the authors have revealed that EGCG completely inhibited the ANG II-induced phosphorylation of ERK1/2, and p38 MAPK. A more recent study further provided an evidence revealing that EGCG inhibition of NF- $\kappa$ B and AP-1 transcription factor is involved in suppressing ANG II-induced early gene expression of ANP and BNP, and this activity of EGCG might prevent the ANG II-mediated progression of cardiac hypertrophy (Li et al. 2006).

It is widely considered that LPS-induced proinflammatory cytokines such as TNF- $\alpha$  play tremendous roles in atherosclerosis. In a study, Pan et al. (2000) have shown that EGCG reduced LPS-induced TNF- $\alpha$  production by blocking NF- $\kappa$ B activation via inhibition of  $I\kappa B$  activity. However, experimental data revealing tea catechin-modulated gene expression linked to prevention of cardiovascular disease are very limited. A recent study has shown that in HUVEC, only 65 of 12,500 genes were regulated more than 1.4-fold after treatment with 50- $\mu\text{M}$  EGCG, as determined by microarray analysis (Pfeffer et al. 2005).

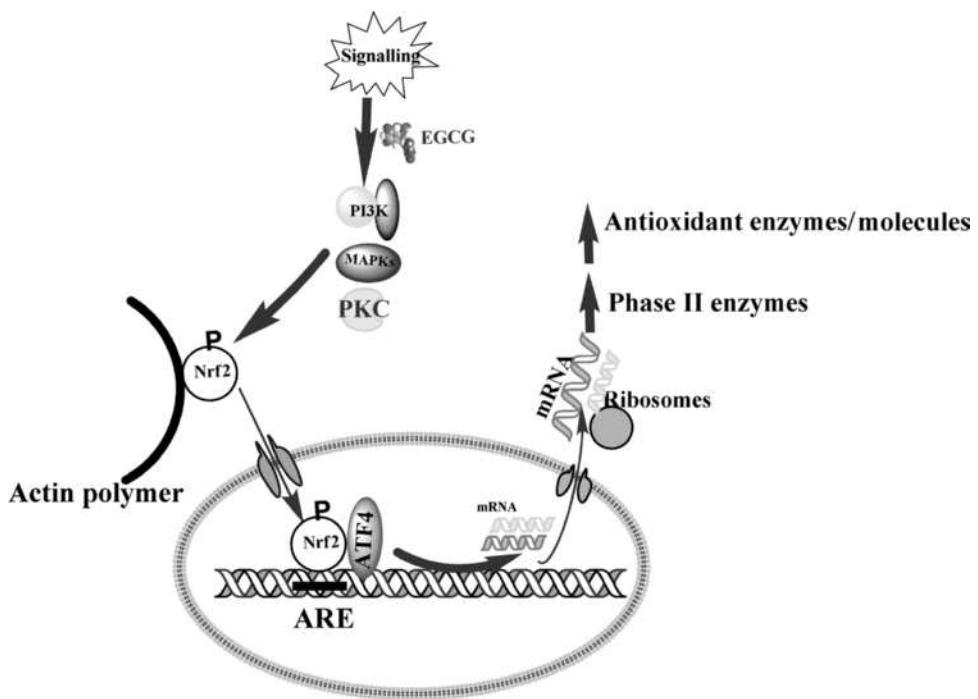
Controlling cell proliferation, cytokine production, and lipid metabolism within arterial wall has become the molecular targets to prevent atherosclerosis, in many recent strategies. Thus, the genes coding for LDLR, LXR $\alpha$ , CD36, PPARs ( $\alpha$ ,  $\gamma$ ), and c-myc are selected to assess the efficacy of potential phytochemical for treatment and prevention of cardiovascular disease. In a study, human mononuclear cells exposed to several phytochemicals found that GTP regulates the aforementioned genes at the transcription level. GTP downregulated PPAR $\gamma$ , CD-36, and LXR $\alpha$  and upregulated the expression of PPAR $\alpha$  (Kaul et al. 2005). This observation is quite pertinent as the tea polyphenol EGCG has been shown to decrease the expression, DNA-binding activity, and transactivation activity of Sp1 transcription factor (Ren et al. 2000).

### INTRACELLULAR ANTIOXIDATIVE MECHANISMS BY MODULATING MULTIPLE GENE EXPRESSIONS

Radical scavenging activity of green tea catechins has been extensively investigated. Various studies have also documented the role GTC on modu-

lating human's natural antioxidant defense system including detoxification process, thereby affording protection against free radicals, in addition to cytotoxic, genotoxic, or metabolic actions of environmental toxicants (see Figure 14.4). Water infusions or purified components extracted from *C. sinensis* enhances protection against TCA/DMBA-induced oxidative stress in rats by modulating the expression/activity of various antioxidant markers such as glutathione-S-transferase (GST), glutathione peroxidase (GPx), SOD, and catalase (CAT), reduced glutathione (GSH), glutathione reductase (GR), and lipid peroxidation (malondialdehyde, MDA) (Celik and Tuluce 2007; Saha and Das 2003). Similarly, catechins at a low concentration (0.1  $\mu\text{M}$ ) markedly increased SOD activity by increasing the expression of SOD gene in cultured rat brain astrocytes (RBA) (Chan et al. 2002). In addition, diet supplemented with GTC (10 mg GTC per ml) increased SOD, and catalase activity in wild-type Oregon-R-C (OR) but not in knocked out flies (Li et al. 2007). Increased expression of manganese superoxide dismutase (MnSOD) and copper/zinc superoxide dismutase (CuZnSOD) cells were also evidenced using Northern blot or RT-PCR analysis (Chow et al. 2002; Li et al. 2007). Thus, it is believed that catechins may modulate genes encoding antioxidant/detoxification enzymes at the transcription level. In fact, a common *cis*-element known as ARE exists on the promoter regions of many of these genes encoding the enzymes/proteins.

Chen et al. (2000) assessed the effect of GTCs on the induction of ARE reporter gene. They observed that (−)-epigallocatechin-3-gallate (EGCG) and (−)-epicatechin-3-gallate (ECG) potently induced ARE-mediated luciferase activity, at a lower concentration (25  $\mu\text{M}$  with EGCG), and the activity in the induction of ARE reporter gene appears to be structurally related to the 3-gallate group. Investigating the pathway involved in the ARE activation, they have proposed the involvement of MAPK, which is also expressed in ARE-mediated phase II gene expression. Under conditions where MAPKs are activated, GTP also activates ARE/EpRE (antioxidant/electrophile response element), with the induction of phase II genes such as NQO (Kong et al. 2001). One possible mechanism by which EGCG upregulates phase II enzymes may involve transcription factor Nrf2, because EGCG substantially modulates MAPKs and/or transcription factor Nrf2, and ARE-mediated luciferase reporter activity. In addition, a more recent study by Kweon et al. (2006) also suggests that EGCG activates Nrf2/ARE promoter to regulate the expression of heme oxygenase-1



**Figure 14.4.** A proposed mechanism of (-)epigallo catechin gallate (EGCG)-modulated intracellular antioxidant defense system.

(HO-1) in A549 cells showing resistance to apoptosis induced by EGCG.

It is quite interesting to note that catechins inhibit metabolic activation of carcinogens by suppressing the transcriptional activation of CYP enzymes. As discussed earlier, on the other hand, GTP induces the expression of several CYP enzymes in cells not exposed to any procarcinogens. A recent study revealed that GTE or EGCG significantly induced the expression CYP1A1 or CYP1A2 expression; however, EGCG significantly downregulated the expression of CYP1A1/CYP1A2 genes induced by promutagen benzo[a]pyrene (Netsch et al. 2006). EGCG activation of CYP3A4 promoter further evidences that GTP-induced activation of CYP enzymes is transcriptionally regulated (Kluth et al. 2007).

## REPAIR OF DNA DAMAGE

In humans, both the normal physiological activities and environmental factors such as smoke, UV radiation, x-rays and gamma rays, thermal factors, and mutagenic chemicals cause DNA damage and produce thousands of molecular lesions in a cell per

day. Many of these lesions cause structural damage to DNA and DNA mutation; thus, genome materials loose its normal transcription capacity to encode vital components required for cell function. Inherently, a cell poses a variety of superficial strategies that help repairing the damaged DNA and restoring the lost information encoded on genome materials. However, if a cell's inherent gene repair mechanism is challenged or the DNA damage exceeds the capacity of the cell to repair it, accumulated consequences may result in early senescence, apoptosis, cancer, angiogenesis, or premature aging.

As damaged DNA or impaired damaged DNA poses significant threat toward many of the life-threatening diseases, prevention of DNA damage or its early repair has become a strategy for the management of DNA lesion-related health disorders. Various previous studies have reported that green tea polyphenols inhibit DNA damage in *in-vitro* or in *in-vivo* model assay systems. Following an *in-vitro* assay protocol, Hayatsu et al. (1992) have shown that EGCG effectively repairs DNA breaks in a single-strand DNA caused by Glu-P-1 (NHOH). It is widely accepted that increased levels of biological ROS such as hydroxyl radical, superoxide, peroxy radical,

singlet oxygen, peroxy nitrite, and hydrogen peroxide can damage DNA, thereby altering the gene expression, cell growth, and cell differentiations. Previous studies have reported that GTCs also significantly reduced the formation of 8-oxodeoxyguanosine, a marker of oxidative stress-induced DNA damage. In addition, compounds in green tea have been found to inhibit the DNA strand breakage in rat hepatocytes after exposure to the tobacco-specific nitrosamine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). Leanderson et al. (1997) have shown that green tea polyphenols inhibit ROS such as cigarette smoke and  $H_2O_2$ -induced DNA damage in cultured human lung cells. The possible mechanism of this action of green tea polyphenols was considered to be the antioxidant effect, which restricts ROS to react and damage DNA. Investigating molecular mechanisms, Anderson et al. (2001) have shown that GTCs can undergo electron transfer (or hydrogen atom transfer) to ROS-damaged sites resulting in the chemical repair of DNA, thereby reducing both base damage and strand breaks. In another study, the same research group has shown that EGCG is effective in repairing oxidative stress-induced DNA damage among the four catechins, namely, EC, EGC, ECG, and EGCG.

Poly(ADP-ribose) polymerase (PARP) is a nuclear enzyme that also mediates the repair of DNA via the activation and recruitment of DNA repair enzymes. In a study, Huang et al. (2006) have shown that GTCs inhibited and significantly blocked DNA fragmentation and apoptosis of microglia cells by tBHP exposure. The authors have shown that catechins significantly enhance the expression of PARP required for repair of impaired DNA. GTCs have also been examined for its efficacies in UVB-induced DNA damage in mice. Meeran et al. (2006) have shown that a topical application of GTCs significantly reduced the formation of cyclobutane pyrimidine dimers (CPD), an indicator of DNA damage. Investigating mechanism of action, the authors have used IL-12-deficient mice and observed that the DNA damage or number of sunburn cells did not vary as compared to untreated control mice. At the end, the authors have proposed the involvement of IL-12 in UVB-induced DNA repair mechanisms. Alkylated DNA repair enzyme (alkB) is a DNA repair enzyme that releases replication block in alkylated DNA (Falnes et al. 2002). Thus, this enzyme has become a target for studying DNA repair mechanism in recent studies. Using Ha-ras gene transformed human bronchial epithelial 21BES cells and microarray technology, Vittal et al. (2004) have shown that GTC EGCG (25  $\mu$ M) enhances alkB gene expression in the 21BES cells. This study has also

evidenced that GTCs modulate genome expression in its DNA repair mechanism.

Further, *H. pylori*-induced gastric injury is shown to be associated with genome DNA fragmentation in epithelial cells. However, EGCG pretreatment significantly attenuated the damage as observed by comet assay (Lee et al. 2004). Concomitant with the changes observed in DNA damage, decreased expression of caspase-3 and reduced cleavage of PARP were observed in the cells pretreated with EGCG.

## ANTIVIRAL ACTION

Health benefit effect of tea catechins is not only limited to antioxidant activity, anticancer, anti-inflammation, or anticardiovascular activity, but also has been reported to have antiviral activity or antibacterial effect, in both *in-vivo* or *ex-vivo* assay systems. In recent reports, antiviral/antibacterial properties of tea catechins have been given tremendous importance because catechins are thought to be nonspecific against viral or bacterial growth; thus, microbes may never produce resistance against catechins.

There are some studies that have shown beneficial effects of tea catechins against the action of many viruses, including HIV-1. Several mechanisms including destabilizing viral particle, inhibiting viral attachment to the cell, blocking postadsorption entry into the cell, and binding CD4 molecules of anti-HIV effects of EGCG are commonly known. In a recent study, GTCs have been shown to have some inhibitory effects on the life cycle of HIV virus. It is also shown that transcription factor NF- $\kappa$ B is an important regulatory factor in the replication of HIV virus. In another report, Nakane and Ono (1990) have shown that GTCs inhibit RNA and DNA polymerases, and the action resulted in the inhibition of reverse transcription (RT) in cell-free chemical assays. Inhibition of RT by tea polyphenols in HIV-infected cells is also proposed by Yamaguchi et al. (2002). In the study, the authors have infected human monocyteoid cells (THP-1) with HIV virus and then stimulated with LPS. Following incubation with EGCG (1  $\mu$ M), the authors have extracted mRNA and found that EGCG treatment significantly reduced viral mRNA production in acute HIV-infected THP-1 cells. Interestingly, this effect of EGCG was not observed in HIV-infected T-lymphocytes. On the basis of the result obtained in the study, the authors have proposed that EGCG may target DNA replication cycle and downregulate HIV-1 gene promoter activity, which varies with the variation of target cells. Tillekeratne et al. (2002) have reported that systematic structural simplicity of epicatechin and

epigallocatechin can lead into inhibition of DNA strand transfer by inhibiting HIV-1 reverse transcriptase, which is critical for HIV-1 RT reproduction.

In addition to the anti-HIV effect, anti-EBV (Epstein–Barr virus) effect of EGCG is also extensively studied. EBV infection causes infectious mononucleosis and is shown to be associated with Burkitt's lymphoma, nasopharyngeal carcinoma, T-cell lymphoma, Hodgkin's disease, and posttransplant lymphoproliferative diseases. Chang et al. have used P3HR1, an EBV positive cell line, to study the mechanism of anti-EBV effect of EGCG. For proliferation, EBV must complete a lytic cycle. Using microarray, immunoblot, flow cytometry, and indirect immunofluorescence analyses, the authors have shown that EGCG inhibits the expression of EBV lytic proteins, including Rta, Zta, and EA-D, but does not affect the expression of EBNA-1. Moreover, DNA microarray and transient transfection analyses demonstrated that EGCG, rather than directly inhibiting EBV-encoded DNA polymerase and EBV DNA replication, blocks EBV lytic cycle by inhibiting the transcription of immediate-early genes, thus causing a block of EBV lytic cascade (Chang et al. 2003b).

## ANTICOLITIS AND ANTIMICROBIAL EFFECTS

This has long been known that green tea prevents food poisoning and has the ability to kill bacteria. Consumption of green tea is often recommended as a good treatment for diarrhea. Sato et al. (1998) assessed the efficacy of green tea against inflammation-related bowel disease in rat and reported that 0.05% catechins and 0.025%  $\alpha$ -tocopherol in diets significantly inhibited TNBS-induced rat colitis and colonic damage as compared to the control group. Since then, several researchers have reported that other tea polyphenols such as thearubigin and theaflavin digallate also inhibited TNBS-induced intestinal lesions in rats. In a study, Maity et al. (2003) have shown that pretreatment of mice with thearubigin (40 mg/kg/day, e.g., for 10 days) significantly ameliorated TNBS-induced intestinal lesions, diarrhea symptoms, and the disruption of colonic architecture in rats. This effect was associated with the reduced levels of NO and  $O_2^-$  ions. Consistent with these observations, NF- $\kappa$ B activation in colonic mucosa was suppressed in TNBS-treated mice by thearubigin.

In another study, Ukil et al. (2006) have found that pretreatment with TFDG (black tea polyphenol) markedly inhibited TNBS-induced increases in nuclear localization of NF- $\kappa$ B, cytosolic IKK activity,

and preserved I $\kappa$ B $\alpha$  in colon tissue. In a very recent study, the therapeutic efficacy of GTC (especially EGCG) was evaluated in experimental colitis, which was induced by rectal administration of TNBS in C57/BL6 mice (Abboud et al. 2008). After induction of colitis, vehicle-treated mice experienced bloody diarrhea and loss of body weight, colonic damage with hemorrhage, ulcers, and edema with elevated plasma levels of TNF- $\alpha$ , IL-6, IL-10, and keratinocyte-derived chemokine.

GTCs also exhibited inhibition activity against the mycobacterium survival in tuberculosis infection (Anand et al. 2006). It has been shown that tryptophan–aspartate containing coat protein (TACO) plays a crucial role in the arrest of mycobacterium maturation. RT-PCR and reporter assay technology revealed that EGCG had the capacity to downregulate TACO gene transcription within human macrophages through its ability to inhibit Sp1 transcription factor. The downregulation of TACO gene expression by EGCG was accompanied by inhibition of mycobacterium survival within macrophages as assessed through flow cytometry and colony counts.

A problem confronting antimicrobial action of drug is the ability of pathogen for mutation and drug resistance. It has long been known that GTCs have bacteriostatic and bactericidal activity against methicillin-resistant *S. aureus* (Hamilton-Miller and Shah 2000; Kono et al. 1994). However, such studies that investigated molecular targets of catechins in inhibiting the resistance are very few. *Bacillus subtilis* 168, a widely known pathogen, show resistance to high concentration of puromycin and lincomycin. These mutants also show resistance to several other drugs. The *lmrAB* genes encoding a transcriptional inhibitor and a drug efflux protein were found to be involved in this phenotype. DNase I footprinting analysis revealed that two transcriptional inhibitors, LmrA and YxaF, which are analogous to each other, bind specifically to LmrA/YxaF boxes, located in the promoter regions of the *lmrAB* operon for their repression. Hirooka et al. (2007) have shown that various flavonoids including catechins inhibit the LmrA binding to DNA and suppress resistance to the drug.

## CONCLUSION AND NOTES

Recent progress in our understanding of cellular and molecular mechanism of the occurrence of various diseases such as cancer, diabetes, cardiovascular diseases, obesity, and aging has made it easier to identify the molecular target of disease-preventive molecules including drug and supplements. In various instances, contemporary trends in the study

of cell signaling enabled us to link phytochemical-mediated disease-preventive mechanisms to the modulation of gene expression and cell signaling pathway. Since green tea is well known to exert various health benefit effects, molecular targets of tea catechins have been duly studied and linked to the modulation of genome function in the prevention of diseases such as cancer, diabetes, and obesity. Cancer-preventive mechanisms of tea catechins have associated with the inactivation of carcinogens, inhibition of cell proliferation, induction of apoptosis, and blockade of cell cycle progression. Multiple cell signaling pathways initiated by receptor molecules via activation of various kinases modulate gene expression involved in cancer prevention mechanism. In particular, inhibition of NF- $\kappa$ B and AP-1 transcription factor is shown to modulate the genome function. Results from multiple *in-vitro* and *in-vivo* studies also suggest the involvement of NF- $\kappa$ B and AP-1 in various other protective health benefits such as inhibition of inflammation.

Although the anticancer and anti-inflammation mechanisms of tea are closely related with the turnoff of genes regulating cell survival and stimulating cell proliferation and turn-on of the genes initiating cell death, other protective health benefits such as protection of neuronal cell death, stimulation of antioxidant defense system, protection of cardiovascular systems, and inhibition of I/R injury-related health disorders seem to be of the opposite effect. Thus, extreme care and precaution should be taken to explain protective health benefits of tea catechins. In fact, doses of tea catechins and pathophysiological condition of an individual are extremely important to determine the pathway that green tea polyphenols would utilize for its targets. More researches are still needed to elucidate the complete biological effect of tea catechins on humans.

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# 15

## Oat Avenanthramides: A Novel Antioxidant

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### INTRODUCTION

Reactive oxygen species (ROS) are generated ubiquitously in aerobic organisms (Halliwell and Gutteridge 1999). When these cytotoxic agents overwhelm endogenous antioxidant defense systems, serious oxidative stress and damage occur as reflected by the oxidative modification of macromolecules such as lipid, protein, and DNA (Ames et al. 1993). ROS generation has important implications in the etiology of numerous diseases and in aging (Finkel and Holbrook 2000; Harman 1956). Thus, it is critical that cells maintain optimal antioxidant defenses in order to reduce oxidative damage. Dietary supplementation and therapeutic use of antioxidants are emerging measures to prevent and treat oxidative stress-induced diseases (Meydani 2002; Yu 1994).

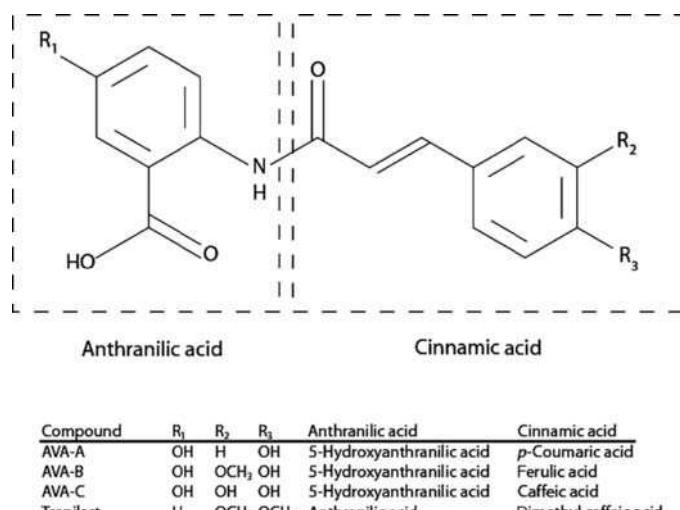
Nature offers an abundance of resources of antioxidants known as phytochemicals, most of which are present in plants, fruits, vegetables, and grains (Hertog 1996). While animals are equipped with both enzymatic and nonenzymatic antioxidants, plants rely more on the antioxidant phytochemicals to protect them from autoxidation of polyunsaturated fatty acids by natural irradiation and airborne oxidants. Tocols (such as tocopherols and tocotrienols), flavonoids (such as soy isoflavone, tea catechins, and anthocyanidines), carotenoids ( $\beta$ -carotene and other pigments), monophenolic acids (such as caffeic acid and ferulic acids), and polyphenolic acids (such as avenanthramides) are the most common phytochemicals. While much recognition has been made to fruits and vegetables as sources of phytochemicals, grains have been largely ignored despite the fact that they are a staple dietary component for most of the world's population (Peterson 2001).

Oat (*Avena sativa*), although consumed in considerably lower quantities worldwide than wheat and rice, has a highly edible quality and contains high nutritional value compared to other minor grains. Moreover, it is often consumed as a whole-grain cereal with intact bran that is rich in antioxidants. As early as 1937, Peters and Musher showed that adding oat flours to fats, margarine, and mayonnaise would increase the stability of fat and would also preserve bacon, potato chips, and nuts. These findings led to the practice of coating wrapping paper with oat flour. In the 1980s, oat extracts were widely included in lard as antioxidants. Over the past decade, the interest of restoring oat as a natural antioxidant additive in food has been increased (Berghofer et al. 1998; Collins 1989; Hammond 1983; Peterson 2001; White and Armstrong 1986).

Other than tocopherols, tocotrienols, and flavonoids, oat contains a unique group of approximately 40 different types of polyphenolic compounds called avenanthramides (AVA) that consist of an anthranilic acid derivative and a hydroxycinnamic acid derivative linked by an amide bond similar to those found in peptides (Figure 15.1).

These compounds may play the role of intrinsic antioxidants in oats. Of all the AVA that have been identified, three stand out due to their abundance in the grain and have been labeled as AVA-A, -B, and -C, which differ by a single moiety on the hydroxycinnamic acid ring (Figure 15.1).

Because of the high concentration of mono- and diunsaturated fatty acids and the presence of lipolytic enzymes, oat grain can be easily oxidized (Bryngelson et al. 2002). Heat treatment is used to preserve oat grains by reducing enzymatic rancidity; while many antioxidants are destroyed, AVA are relatively resistant to heat. The presence of AVA protects the



**Figure 15.1.** Structure of avenanthramide A, B, and C, and a synthetic pharmaceutical drug, Tranilast.

oat from becoming rancid through autoxidation and maintains the freshness of the grain. Most of the AVA are contained in the groats and hulls, where the combined AVA-A, -B, and -C concentration is 30–150 mg/kg, depending on the different cultivars and growth environment, but as high as 300 mg/kg AVA has been reported (Bratt et al. 2003; Emmons and Peterson 2001).

AVA dissolve readily in most organic solvents but are insoluble in water. However, at higher pH levels, they can be dissolved even in cold water (Collins 1989). AVA solutions are bright yellow to green in color. The anthranilic acid portions of AVA are highly susceptible to autoxidation induced by light and oxygen. This process results in the formation of orange, red, brown, and black coloring.

AVA can be synthesized by condensing the acyl chloride derivative of the protected hydroxycinnamic acid with the appropriate free anthranilic acid in the presence of pyridine (Peterson et al. 2002). Collins (1989) first synthesized AVA-A and -B, whereas AVA-C was synthesized by condensing caffeic acid with 5-hydroxyanthranilic acid using the peptide-coupling reagent dicyclohexylcarbodiimide (DCC) (Ishihara et al. 1998).

## ANTIOXIDANT FUNCTION IN VITRO

Phenols exert their antioxidant activity mainly by donating a hydrogen atom, thereby inhibiting the propagation of free radical chain reaction. Their antioxi-

dant potentials are dependent on the number and arrangement of the hydroxyl groups (Bratt et al. 2003). There are several methods used in the field to assess the antioxidant potential of a phytochemical in vitro (see Frankel and Meyer 2000, for a review). Two commonly used methods for AVA are 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, which measures reactivity toward DPPH (a hydrophilic system) (Brand-Williams et al. 1995) and linoleic acid peroxidation assay (Braughler and Pregenzer 1989).

The antioxidant potential of AVA was realized soon after the discovery of the compounds. Early assessment of AVA's antioxidant activity showed that AVA-B was more active than the hydroxycinnamic acid groups of AVA alone but less active than  $\alpha$ -tocopherol (vitamin E) (Dimberg et al. 1993). This activity was further investigated in the three major AVA fractions using the  $\beta$ -carotene bleaching assay (similar to the linoleic acid peroxidation assay) and DPPH assay. For both assays, AVA-C was found to have the greatest antioxidant activity, while AVA-A had the least and AVA-B was intermediate. This order matches with that of the hydroxycinnamic acid groups found in the AVA, that is, caffeic acid (AVA-C) is more active than ferulic acid (AVA-B), which in turn is more active than *p*-coumaric acid (AVA-A). AVA-C contains three hydroxyl groups, while AVA-A contains two, so AVA-C has more sites available for reducing oxidants. AVA-B contains two hydroxyl groups and one methoxy group, which may explain its intermediate activity. Furthermore, the Z isomer of AVA has been found to possess over 10 times the antiallergic activity of the naturally

occurring *E* isomer. AVA-E has also been patented as an *in vitro* lipoxygenase inhibitor (Wakabayashi et al. 1985).

AVA may be expected to function as more potent antioxidants than their constituent hydroxycinnamic acids because of resonance in the amide bond. However, in *in vitro* analyses, AVA and their corresponding hydroxycinnamic acids were equally active (Peterson 2001). The *in vitro* assays revealed that the ability to donate hydrogen atoms by the various antioxidants are dependent not on the nature of the attacking radicals, but rather on the environment of the assay (solvent nature). The observed kinetic effect is attributed to hydrogen bonding between antioxidant OH and NH groups and hydrogen bond-accepting solvents (Dimberg et al. 2001). These findings have great implications in understanding the behavior and efficacy of various phytochemicals when they are used for dietary supplements (see below).

It is interesting to note that many of the 40 different AVA-like compounds found in oat hulls and groats have previously been patented as pharmaceutical products with claims to have antiallergic, antihistaminic, and antiasthmatic activity (Devlin and Hargrave 1985). The best known is *N*-(3,4-dimethoxycinnamoyl)-anthranilic acid, a synthetic drug marketed with a trade name of Tranilast. Tranilast has been reported to inhibit the release of histamine from mast cells induced by antigen stimulation. Moreover, Tranilast inhibits the production and release of cytokines, prostaglandins, ROS, nitric oxide (NO), and cyclooxygenase (COX)-2 expression (Isaji et al. 1998). Another important effect of Tranilast is to prevent collagen deposition without affecting normal protein synthesis, possibly due to its inhibition of  $TGG_{\beta}$  production (Isaji et al. 1987). Recently, Tranilast was claimed to have an antiangiogenic effect due to its inhibition on the proliferation of VEGF (vascular endothelial growth factor)-induced chemotaxis. Although *N*-(3,4-dimethoxycinnamoyl)-anthranilic acid (i.e., monomethyl-AVA-E) represents only a small fraction of AVA found in natural oats, its intriguingly wide range of functions may provide some insight into the reason why AVA have demonstrated a wide range of biological functions in recent studies when they were supplemented *in vivo* (see below).

## BIOAVAILABILITY OF AVA

Bioavailability is a measurement of the extent that a therapeutically active compound reaches the systemic circulation and is available at the site of action (Shargel and Yu 1999). When a compound is admin-

istered orally, its bioavailability decreases (compared to intravenous injection which is defined as 100% bioavailable) due to incomplete absorption and/or first-pass metabolism by the liver and other organs before reaching the blood circulation. Thus, the *in vivo* efficacy of phytochemicals, including AVA, is largely dependent on their bioavailability.

To assess the bioavailability and antioxidant effects of AVA after an acute oral dose, Chen et al. (2004) fed a slurry containing 250 mg oat bran phenol-rich powder containing 40  $\mu$ mol of phenolics (including AVA-A, -B, and -C) to BioF1B strain golden Syrian hamsters by stomach gavage. This dose was based on the estimated daily phenolic intake for a 70-kg person and adjusted for the metabolism of the animals. Control hamsters received an equal volume of saline. Blood samples were collected at 0, 20, 40, 60, 80, and 120 min post gavage, and plasma was analyzed by high-performance liquid chromatography (HPLC) for the presence of phenolics. In addition to several other phenolic compounds in the mixture, AVA-A and -B were found to be bioavailable. Both detected AVA reached a peak concentration in the plasma at 40 min post gavage and returned to baseline concentration by 120 min. AVA-C was not detected in the plasma at any time point.

These results seem to conflict with those of Ji et al. (2003), who did not measure bioavailability of AVA-C but found that it had effects in tissues after chronic ingestion, suggesting that it might have been taken from the gastrointestinal tract. Two possible explanations exist for this discrepancy. First, AVA-C might have entered the circulation but was quickly modified, making it undetectable with the conventional method. Indeed, Chen et al. (2004) found two unidentified peaks on their chromatographs, one of which may correspond to a modified form of AVA-C. The compound with 30.95 min retention time showed a time course of plasma concentrations similar to those of AVA-A and -B. The other possibility is that there may be a species-specific difference in the pharmacokinetic profile of AVA, since one study was conducted in rats and the other in hamsters. Recent evidence supports this explanation as AVA-C, as well as -A and -B, was found to be bioavailable in rats (Koenig et al. 2007) and in humans (Chen et al. 2007).

In addition to assaying for bioavailability, Chen et al. (2004) examined the ability of the absorbed phenolic mixture present in the plasma to protect low-density lipoprotein (LDL) from  $Cu^{+}$ -induced oxidation. No protective effect was measured when the 40- and 60-min plasma samples were each added to the reaction mixture. However, when ascorbic

acid was added along with the 60-min plasma sample, a decrease in LDL oxidation that was greater than the effect of ascorbic acid alone was observed, suggesting a synergistic effect. The oxygen radical absorbance capacity (ORAC) assay confirmed that there was no antioxidant effect of the absorbed phenolics. The apparent lack of antioxidant effects may stem from the failure of the hamsters to take up AVA-C, the most potent antioxidant of the AVA. Indeed, when the raw oat phenolic mixture fed to the hamsters was used in the LDL oxidation assay, a dose-dependent protective effect as well as the synergistic effect with ascorbic acid was observed. These results suggest that hamsters may not absorb enough of the phenolics from the mixture to garner any antioxidant protection.

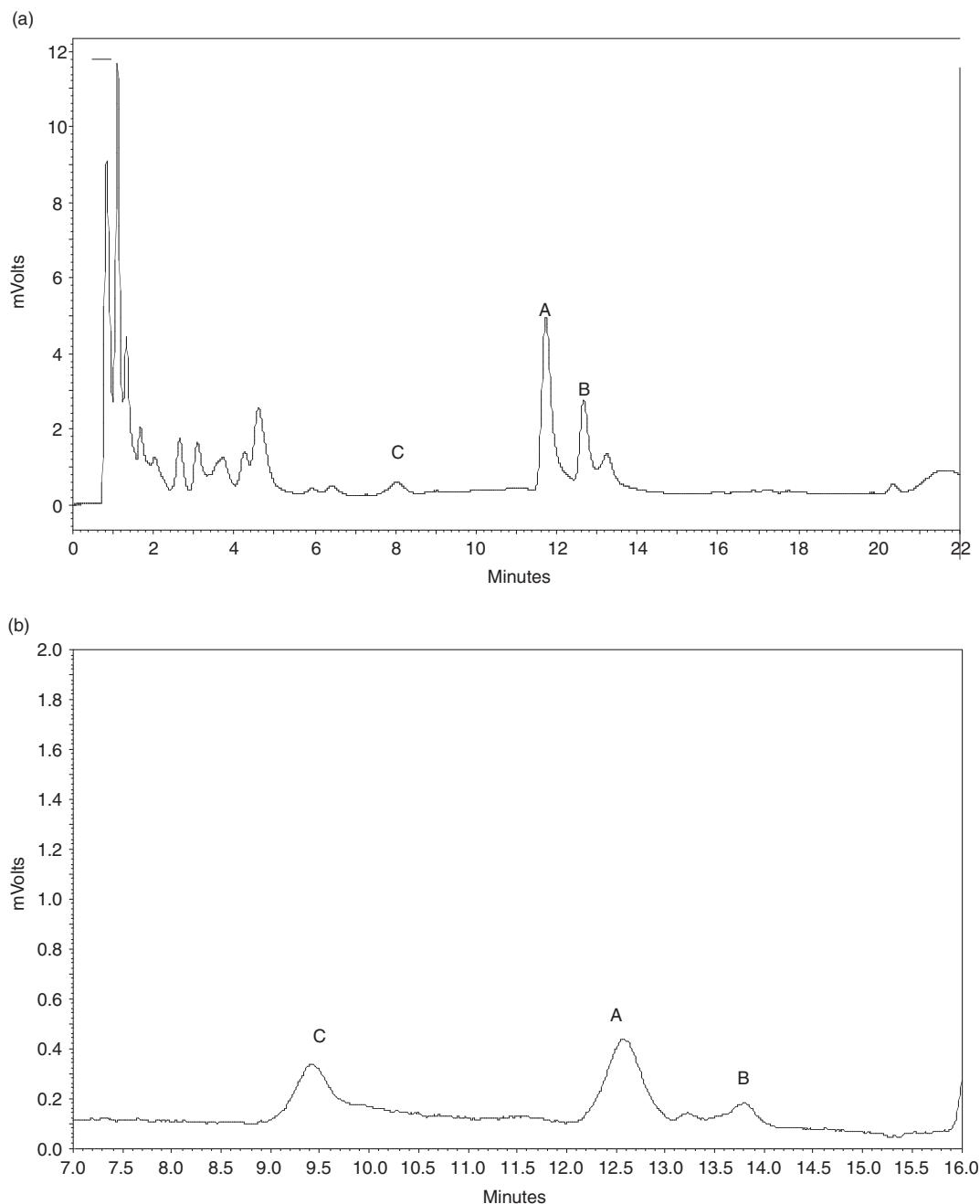
Recently we conducted a study to investigate the bioavailability of AVA-A, -B, and -C in rats by oral gavage followed by plasma and tissue measurement of their presence (Koenig et al. 2007). Rats were killed at 0 (no gavage), 1, 2, 4, or 12 h following gavage wherein plasma, liver, heart, and gastrocnemius (GAS), soleus, and deep (DVL) and superficial vastus lateralis (SVL) muscle samples were analyzed for AVA concentration with HPLC (Milbury 2001). All samples were analyzed under two conditions: with or without glucuronidase–sulfatase, which is known to cleave conjugated AVA, forming-free plasma products (Chen et al. 2004). Our results showed that all three AVA were present in the plasma with peak values occurring at 1 h post gavage (Figure 15.2).

AVA-A was the most prevalent at all time points, and it persisted up to 12 h after administration. The species detected with the highest concentration was conjugated AVA-A. AVA-B was found in the plasma only in the free form, and it was mostly eliminated at 12 h. AVA-C was detected in both free and conjugated forms at all time points except 12 h, when only the conjugated form persisted. Liver, heart, and GAS muscle each contained various AVA fractions following oral ingestion. Peak values tended to occur in the liver at 2 h, the time at which plasma AVA concentrations were beginning to decrease. Only conjugated AVA were detected in GAS tissue; that is, AVA were detected only in the assay with glucuronidase–sulfatase, suggesting an absorption preferential to the conjugated form. Only AVA-C was detected in heart tissue with increasing concentrations over time up to 12 h post gavage. Our data for the first time showed that AVA are not only available to the circulation, but also taken up by a variety of tissues. Presumably, the AVA affected the antioxidant capacity of these tissues and played a role in dealing with exercise-induced oxidative stress (see below).

Chen et al. (2007) recently studied AVA bioavailability in older human subjects with two oral doses of AVA-enriched mixture (AEM) extracted from the oat. Six subjects drank skim milk or milk containing 0.5 or 1.0 g AEM and crossed over with a 1-week washout period. Blood samples were collected 15, 30, and 45 min and 1, 2, 3, 5, and 10 h after AEM ingestion and were analyzed by HPLC for AVA concentrations. All three AVA in the AEM (AVA-A, -B, and -C) were detected in the plasma for both doses with peak concentrations occurring at 2.3, 1.75, and 2.15 h, respectively (compared to hamsters where the peak occurred at ~40 min). The elimination kinetics of plasma AVA followed first-order kinetics, whereas the bioavailability of AVA-A was 4-fold higher than that of AVA-B at the 0.5 g AEM dose. These data for the first time reported in humans revealed that AVA bioavailability can be 18- and 5-fold greater than in hamsters for AVA-A and -B, respectively. However, little is known about how much and when AVA could reach the tissue level in humans.

The confirmation of AVA bioavailability raised an interesting question, that is, whether and how AVA might affect the endogenous oxidant–antioxidant homeostasis. So far, there is only sparse data in this respect. Chen et al. (2007) examined plasma glutathione (GSH) concentration, lipid peroxidation (by measuring malondialdehyde, MDA), GSH peroxidase (GPX) activity, and LDL resistance to Cu<sup>+</sup>-induced oxidation as markers of antioxidant capacity in human subjects orally taking AEM. AEM at the dose of 1 g increased plasma GSH by 21% at 15 min with a return to baseline after a few hours. The ratio of GSH to oxidized glutathione (GSSG) did not change over the entire 10-h time course. Plasma GPX activity increased with oral consumption of either placebo or AEM but showed no treatment effect. MDA concentrations also were not different between treatment groups. Similarly, the copper-induced oxidation rate of LDL was not affected by oral AEM consumption.

Ji et al (2003) studied the effect of 50 days of dietary supplementation of a synthetic AVA-C (0.1 g/kg food) by measurement of ROS generation, antioxidant enzyme activities, and MDA content in the liver, heart, and several skeletal muscle fibers in rats at rest and after an acute bout of exercise. Basal rate of ROS production was not affected by AVA treatment in any of the tissues measured. However, AVA supplementation resulted in a significant increase in superoxide dismutase (SOD) activity in all tissues, an increased GPX activity in the heart, and no change in MDA levels in any of the tissues. The authors suggested that AVA-induced antioxidant enzyme activity might



**Figure 15.2.** A representative chromatogram of avenanthramides in rat samples of plasma (a) and liver (b). The rat was killed 1 h post an oral gavage containing 20 mg/kg body weight of AVA-A, -B, and -C. (Source: Koenig et al. 2007)

have offered enhanced antioxidant defense when rats were subjected to exercise-induced oxidative stress (see below). However, it is intriguing as to why orally supplemented AVA would increase basal SOD and GPX activities if it was made biologically available to tissues and served as an antioxidant.

## CARDIOVASCULAR AND IMMUNOLOGICAL PROTECTION

There has been both epidemiological and experimental data showing increasing oat consumption reduces the occurrence of cardiovascular diseases, but this cardiac protective effect was thought to be largely due to the high soluble fiber content in the grain (Anderson 1995; Katz et al. 2001). The finding that the inflammatory process plays an important role in the pathogenesis of atherosclerosis provided new insight into the prevention of this epidemic and raised new interest in studying oat antioxidants as a potential suppressor of atherosclerotic mechanisms.

Liu et al. (2004) were the first to investigate the effect of AEM on the markers of atherosclerosis—namely, monocyte adhesion, adhesion molecule expression, and proinflammatory cytokine production—in human aortic endothelial cells (HAEC). HAEC monolayers were incubated with various concentrations (4, 20, and 40  $\mu\text{g}/\text{ml}$ ) of AEM for 24 h. While AEM per se had no effect on the constitutive expression of any of the inflammatory markers, various concentrations of AEM resulted in reduced interleukin (IL)-1 $\beta$ -stimulated cell adhesion in a dose-response relationship. AEM also significantly inhibited IL-1 $\beta$ -stimulated expression of intracellular adhesion molecule-1 (ICAM-1), vascular adhesion molecule-1 (VCAM-1), and E-selectin on HAEC in a dose-dependent fashion. Furthermore, IL-1 $\beta$ -stimulated expression of cytokines such as IL-8, IL-6, and monocyte chemoattractant protein-1 (MCP-1) was decreased by AEM in a dose-dependent fashion. These results intimate that AVA can reduce the inflammatory response of endothelial cells to an atherogenic stimulus, namely IL-1 $\beta$ . The inhibitory effects of microgram levels of AVA used in this study were comparable to those of 17  $\mu\text{g}/\text{ml}$  of vitamin E, which was used as a positive control and is equivalent to a daily dose of 200 IU of vitamin E supplementation. Since upregulation of inflammatory cytokines, chemokines, and adhesion molecules by endothelial cells of affected blood vessels under IL-1, TNF (tumor necrosis factor)- $\alpha$ , and interferon is a critical step in the development of atherosclerosis, these findings may provide some important clues for the prevention of this disease.

The mechanism for the decreased cytokine production by AVA is not clear at present, but a study by Guo et al. (2008) examined the role of nuclear factor (NF- $\kappa$ B in the signal transduction of these proinflammatory cytokines in HAEC using an AVA-enriched oat extract (AVAO), a synthetic AVA-C, and the methyl ester of AVA-C ( $\text{CH}_3$ -AVA-C), which reportedly was 10 times more effective than AVA-C in some assays of antiatherogenic potential. NF $\kappa$ B is a dimeric transcription factor composed of members of the Rel family (Bauerle and Baltimore 1988; Meyer et al 1994). In mammals, these proteins include p50 (NF- $\kappa$ B1), p52 (NF- $\kappa$ B2), p65 (RelA), RelB, c-Rel, p105, and p100. NF- $\kappa$ B is activated by a variety of external stimulants, such as  $\text{H}_2\text{O}_2$ , TNF- $\alpha$ , IL-1, IL-6, lipopolysaccharide (LPS), and ionizing irradiation. These signals result in the phosphorylation and activation of  $\text{I}\kappa\text{B}$  kinase (IKK) that phosphorylates two critical serine residues and primes  $\text{I}\kappa\text{B}$  for ubiquitination and proteolytic degradation by the 26S proteasome.  $\text{I}\kappa\text{B}$  dissociation unleashes p50/p65 to dimerize and translocate into the nucleus and bind the  $\kappa$ B consensus sequence of the target genes.

AVAO, AVA-C, and  $\text{CH}_3$ -AVA-C were found to significantly and dose-dependently decrease mRNA expression and secretion of IL-6, IL-8, and MCP-1 by HAEC and to inhibit IL-1 $\beta$ - and TNF- $\alpha$ -stimulated NF $\kappa$ B activation (Guo et al. 2008). AVAO, AVA-C, and  $\text{CH}_3$ -AVA-C also inhibited the NF- $\kappa$ B-dependent reporter gene expression activated by TNFR-associated factor 2 and 6 (TRAF2 and TRAF6) and NF- $\kappa$ B-inducing kinase (NIK). It appeared that at least the  $\text{CH}_3$ -AVA-C effect was mediated by the decreased phosphorylation level of IKK and  $\text{I}\kappa\text{B}$  and the prevention of  $\text{I}\kappa\text{B}$  degradation due to an inhibition of proteasome activity.

Nie et al (2006a, b) investigated the effect of AVA on proliferation of vascular smooth muscle cells using an A10 rat embryonic aortic smooth muscle cell line model and provided additional insight into the mechanisms of AVA's inhibitory effect on atherosclerotic processes. Flow cytometry analysis revealed that 80  $\mu\text{M}$  AVA-C arrested the cell cycle in G1 phase as indicated by an increase in the number of cells in G1 phase and a decrease in the number of cells in S phase. The observed cell cycle arrest was associated with a decrease in the phosphorylation of retinoblastoma protein (pRb), the hyperphosphorylation of which marks the G1 to S transition (Sherr 1994). The inhibition of pRb phosphorylation with AVA-C was accompanied by a decrease in cyclin D1 expression and an increase in cyclin-dependent kinase (CDK) inhibitor p21cip1 expression.

In addition to these effects, AVA-C treatment caused a dose-dependent increase in p53 protein levels by stabilizing p53 and decreasing its half-life. The tumor suppressor p53 is a transcription factor for p21cip1, a CDK inhibitor, whose expression was upregulated with AVA-C treatment (Nie et al. 2006a). These results suggest that AVA-C arrests SMC cell cycle progression by increasing the level of p53, which upregulates p21cip1 expression, leading to inhibition of CDK and decreased phosphorylation of pRb.

In addition to its antiproliferative effect, AVA-C demonstrated an ability to increase NO production in SMC and HAEC (Nie et al. 2006b). NO is a vasodilator as well as an antiatherosclerotic agent as it can prevent platelet aggregation, leukocyte adhesion, SMC proliferation, and atherogenic gene expression. AVA-C treatment increased NO concentrations in both HAEC and SMC in a dose-dependent manner as measured by 4,5-diaminofluorescein (DAF-2) assay. Endothelial NO synthase (eNOS) is the enzyme that catalyzes NO production from arginine. Quantitative real-time polymerase chain reaction (PCR) analysis revealed that AVA-C treatment increased eNOS mRNA in a fashion that mirrored the increase in NO production. These results indicate that AVA-C may upregulate the transcription and translation of eNOS, which then increases NO production. These data indicate that increased NO bioavailability may be responsible for the inhibition of SMC proliferation. Zhou et al. (2004) have shown that NO can increase p53 levels; therefore, the increases in NO associated with AVA-C may cause the increase in p53 that could lead to SMC antiproliferation.

## ANTIOXIDANT PROTECTION DURING EXERCISE

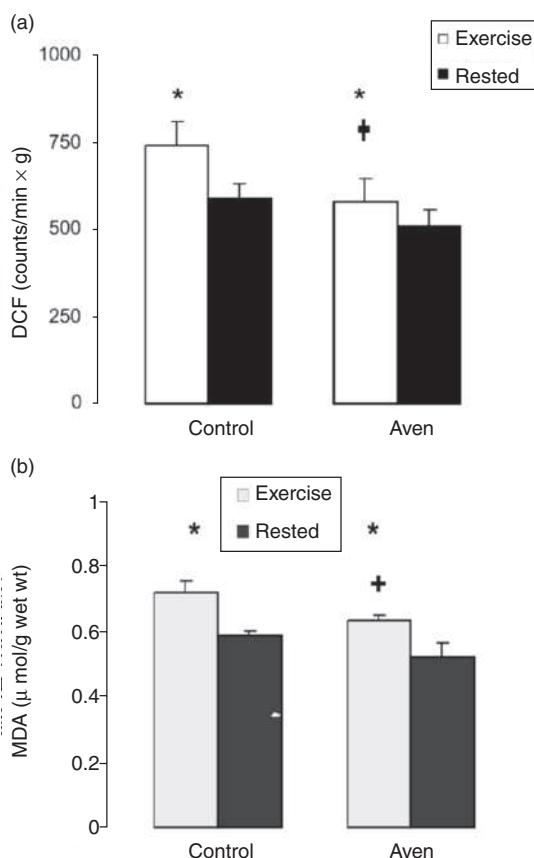
Physical activity is an important part of a healthy lifestyle as regular exercise decreases the occurrence of cardiovascular diseases, diabetes, and cancer; prevents degenerative disease; and improves quality of life during aging (Ji et al. 2004). Furthermore, exercise has been used as a therapeutic means in the rehabilitation programs for patients recovering from a wide range of diseases and surgical treatments such as myocardial infarction, transplantation, and cancer. Despite these clear benefits of participating in physical exercise, there is a concern that patients and aged individuals are more susceptible to some of the harmful effects of rigorous exercise as a result of increased exposure to ROS (Davies et al. 1982). Even healthy people are not exempted from the potentially cyto-

toxic effects of ROS during recreational exercise and sport events. Thus, increasing antioxidant protection is an important issue to almost the entire population. Since oats have demonstrated the potent antioxidant effects, as described above, AVA became an interesting and novel dietary antioxidant supplement for exercise studies.

There is a paucity of data regarding the efficacy of AVA in protecting against exercise-induced oxidative stress. Since pure AVA are available only through chemical synthesis, no human studies have been conducted to date to our knowledge. The following data are from animal studies primarily from our own work. In one study (Ji et al. 2003), female Sprague-Dawley rats ( $n = 48$ , age 6–7 week) were fed either an AIN-93-based control diet or the same diet containing 0.1 g/kg AVA-C for 50 days. Each group was further divided into two groups killed either at rest or after running on treadmill for 1 h at a speed of 22.5 m/min with 10% grade. AVA supplementation per se had no effect on ROS production measured by a 2',7'-dichlorofluorescein (DCF) assay method in most tissues; however, ROS level was decreased in soleus muscle (Figure 15.3a).

Exercise increased ROS production in the liver, DVL, and soleus, whereas AVA attenuated exercise-induced ROS in soleus but increased ROS in the heart. AVA-fed rats showed significantly higher SOD activity in the liver, kidney, DVL, and soleus muscle, but heart had lower SOD activity in the exercised rats (Ji et al. 2003). Interestingly, GPX activity was elevated in the heart compared to control rats. Lipid peroxidation measured by MDA content in the heart, liver, and DVL was increased as a result of the acute exercise bout, and AVA treatment decreased exercise-induced lipid peroxidation in the heart (Figure 15.3b). However, exercise rats had higher MDA levels in DVL muscle. These findings demonstrated for the first time that dietary supplementation of a synthetic AVA could attenuate exercise-induced ROS production and lipid peroxidation in selected tissues. The antioxidant effects of AVA seem to be related to its ability to influence tissue antioxidant enzyme systems such as SOD and GPX activities, but the mechanism is unknown. However, the combined effects of AVA and an acute bout of exercise to increase ROS production in the heart and MDA level in DVL muscle are difficult to explain at present.

Lengthening contraction (LC) of skeletal muscle has been shown to elicit delayed onset injury due to inflammatory response and ROS generation. Since AVA have demonstrated antioxidant effect and potential anti-inflammatory effect by inhibiting COX-2, prostaglandin, cytokine, and chemokine expressions,



**Figure 15.3.** Rate of dichlorofluorescein (DCF) formation in the soleus muscle (a) and malondialdehyde (MDA) in the heart (b) of rested and exercised rats fed an AIN-93-based control (C) diet or the same diet containing 0.1 g/kg AVEN-C for 50 days. Each bar represents mean  $\pm$  SEM ( $n = 12$ ). \*,  $P < 0.05$ , exercised versus rested. +,  $P < 0.05$ , aven versus controls. (Source: Ji et al. 2003)

a study has been conducted to investigate whether dietary supplementation of AVA would attenuate muscle oxidative damage induced by LC in rats (O'Moore et al. 2005).

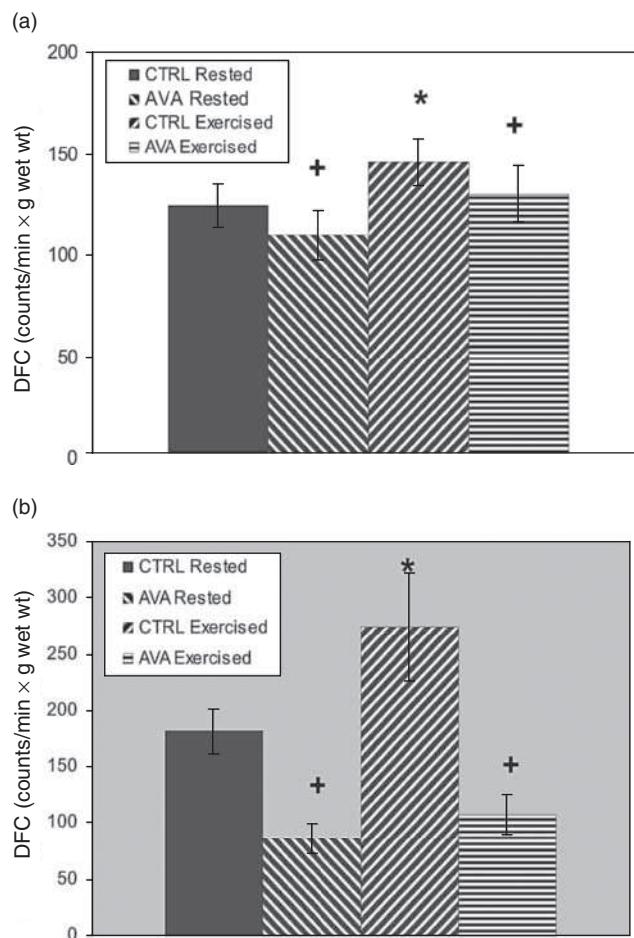
Female Sprague-Dawley rats (age 4 months) were randomly divided into two groups, receiving either an AIN-93-based purified diet containing 0.2 g/kg AVA-C ( $n = 20$ ) or a control diet ( $n = 18$ ) for 8 weeks. Each dietary group was further divided into rested and LC using a downhill treadmill running protocol at 25 m/min with -20% grade, for 2 h. Rats were killed 24 h after the LC treatment. The DVL, SVL, GAS and soleus muscle from both hind legs were quickly removed and homogenized. ROS production

from both mitochondrial and polymorphonuclear neutrophil (PMN) sources was assessed using different assay media. Several aspects have been learned from this study. (1) While oxidant production was significantly greater in all muscles tested comparing LC versus rested controls rats, regardless of the DCF fraction measured, oxidant production was not affected by AVA supplementation in DVL or GAS using either medium, but was decreased in SVL by 12% with mitochondrial respiration substrate (Figure 15.4a) and by 56% with a substrate for NADPH oxidase (Figure 15.4b). (2) LC significantly increased plasma lactate dehydrogenase (LDH) and creatine kinase (CK) activities, a clear sign of muscle damage. AVA treatment did not affect LDH or CK response to LC. GSH content and GSH:GSSG ratio in DVL were decreased with LC in control rats but were also unaffected with AVA supplementation. (3) AVA-supplemented rats had increased SOD activity in SVL muscle, but did not show substantial change in SOD activity in other tissues or altered activity of other antioxidant enzymes such as GPX and catalase in skeletal muscle.

Taken together, data from the above two studies indicate that AVA at the dose supplemented (0.1–0.2 mg/kg) via diet did not result in a large alteration of muscle redox status or exert major antioxidant effects as hypothesized. However, the effect on reducing ROS generation, especially those of PMN source, in selective muscle fibers suggests that AVA may have inhibitory effects on specific pathways that produce ROS. PMN produces ROS by increased expression of cytokine-induced neutrophil chemoattractant (CINC) and/or activated NADPH oxidase under the influence of cytokines (TNF- $\alpha$  and IL-1, 6). Many phytochemicals, such as quercetin and curcumin, are known to inhibit cytokine-induced ROS production and inflammation due to their inhibitory effect on NF- $\kappa$ B activation (Davis et al. 2007; Martinez-Florez et al. 2005). Thus, research on the potential role in the NF- $\kappa$ B signaling pathway may be an important area to understand the efficacy of AVA in protecting exercise-induced oxidative stress and injury. On the other hand, the effect of AVA on inducing SOD and other antioxidant enzymes is more difficult to explain and requires more investigation.

## CONCLUSION

AVA is a novel phytochemical that, though having been discovered for two decades, has not been well studied for its bioavailability and bioefficacy. Available data indicate that AVA can be quickly absorbed through the gastrointestinal tract and enter the blood circulation after oral ingestion, but different fractions



**Figure 15.4.** Rate of dichlorofluorescein (DCF) formation in the vastus lateralis muscle of rats at rest and after an acute bout of downhill running. Assay medium contained either 2 mM pyruvate and 2 mM malate (a) or 0.1 mM  $\text{FeCl}_3$ , 0.1 mM NADPH, and 1.7 mM ADP (b) to stimulate NADPH oxidase activity. \*,  $P < 0.05$ , exercised versus rested. +,  $P < 0.05$ , AVA versus controls. (Source: O'Moore et al. 2005)

of AVA appear to have different pharmacokinetic characteristics due to structural differences. Most of the in vitro research points to a strong antioxidant effect of the phytochemical; however, its antioxidant effects in vivo are less straightforward and somewhat controversial, suggesting that not all its bioefficacy is exerted through its antioxidant properties. On the other hand, there is strong evidence that AVA is a potent inhibitor of cell proliferation and inflammatory processes, especially in the endothelial cells and smooth muscle cells of blood vessels. These effects have been shown to be mediated by its inhibition of proinflammatory cytokine production and signaling. AVA has also been reported to modulate endogenous antioxidant defense such as increasing plasma GSH

level and upregulating tissue SOD activity, the mechanisms of which remain to be elucidated. Although oats have been recommended to be included as a source of dietary phytochemicals, the contribution of AVA to overall protection against atherosclerosis and other cardiovascular diseases still needs investigation.

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# 16

## Cancer-Preventive Effects and Molecular Actions of Anthocyanins

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### INTRODUCTION

Anthocyanins confer a wide range of colors with red, blue, purple, and violet in many fruits and vegetables, such as berries, red grapes, purple sweet potatoes, and red cabbages (Harborne and Grayer 1998; Mazza 1995). The contents of anthocyanins in colored fruits and berries vary a lot, from around 3.4 mg in reddish apples, 232 mg in strawberries, and 1064 mg in blackcurrants up to 3090 mg in blueberries per 100 g dried fruits (Kähkönen et al. 2001). In chemical structure, anthocyanin consists of an aglycone, the anthocyanidin, linked to a sugar moiety. The six most frequently found aglycones in fruits and berries are delphinidin, cyanidin, petunidin, pelargonidin, peonidin, and malvidin (Figure 16.1). These aglycones may be glycosylated or acylated by different sugars and acids in different positions, and become anthocyanin glycosides (Figure 16.1). The number and nature of the different attached sugar moieties are responsible for the high number of anthocyanins; to date, more than 400 anthocyanins have been found in plants (Harborne and Baxter 1999).

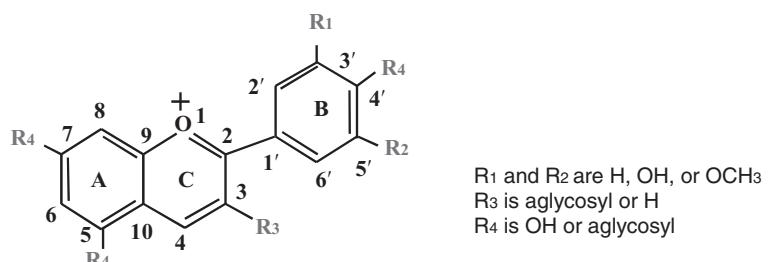
Daily intake of anthocyanins in humans differs depending on the nutrition customs. Mean dietary intake has been estimated to be 6–60 mg per day in Danish diet (Dragsted et al. 1997), 82 mg per day in Finland diet (Kähkönen et al. 2001), and 180–215 mg per day in the American diet (Kuhnau 1976; Manach et al. 2005), which are much higher than the intake (23 mg per day) of other flavonoids, including quercetin, kaempferol, myricetin, apigenin, and luteolin (Hertog et al. 1993). Moreover, an enhanced intake of anthocyanins is now increasing because extracts with higher anthocyanin contents from bilberry or elderberry are commercially available (Skibola et al. 2000). For example, servings of 200 g eggplant

or black grapes can provide up to 1.5 g anthocyanin and servings of 100 g berries up to 0.5 g. Thus, anthocyanins are now of great nutritional interest, especially in cancer chemoprevention.

Cancer chemoprevention, a relatively new and promising strategy to prevent cancer, is defined as the use of either natural or synthetic substances or their combination to block, reverse, or retard the process of carcinogenesis (Hong and Sporn 1977; Kelloff et al. 1997). Carcinogenesis can be categorized into three stages: initiation, promotion, and progression (Boutwell et al. 1982; Foulds 1965). These steps are made up of rate-limiting molecular events that occur along signal transduction pathways with attendant alteration of the expression of genes whose products are associated with transformation, inflammation, proliferation, apoptosis, and other biological processes.

Accumulating evidence from both population-based and laboratory studies indicate an inverse relationship between regular consumption of fruits and vegetables and the risk of cancer in general. Attention has recently been focused on nonnutritive phytochemicals present in plant-based diet as potential chemopreventive agents. It is now estimated that more than 1,000 different phytochemicals possess chemopreventive activities (Part and Pezzuto 2002). Most of these phytochemicals can target the cellular signaling transduction pathways and transcription factors, which further modulate downstream gene expression involved in cell transformation, inflammation, and proliferation (Surh 2003).

In this chapter, the cancer-preventive effects and molecular actions of anthocyanins are reviewed according to the accumulated data from the population-based survey, animal and culture cell investigations, and molecular mechanism studies.



Aglycone	R1	R2	Color	$\lambda_{\text{max}}^*$	Major source
Delphinidin	OH	OH	Bluish-red	526	Bilberry, blueberry
Petunidin	OCH <sub>3</sub>	OH	Bluish-red	526	Huckleberry
Cyanidin	OH	H	Orange-red	517	Popular
Pelargonidin	H	H	Orange	503	Strawberry
Peonidin	OCH <sub>3</sub>	H	Orange-red	517	Cranberry
Malvidin	OCH <sub>3</sub>	OCH <sub>3</sub>	Bluish-red	529	Grapes, blueberry

\* The  $\lambda_{\text{max}}$  values shown are those of the corresponding 3-glucoside anthocyanins at pH 3.

**Figure 16.1.** Structure and properties of anthocyanins.

## CANCER-PREVENTIVE EFFECTS

There is a population-based survey hinting the potential activity of anthocyanins on cancer prevention. The elderly individuals who consumed large amounts of strawberries had a lower odds ratio (0.3) for developing cancer at any site, compared to subjects who refrained from high berry consumption (Colditz et al. 1985). Other studies have suggested that the consumption of colored fruits and vegetables are associated with a reduced risk of human breast cancer (Adlercreutz 1998) and colorectal polyp recurrence (Almendingen et al. 2004). Intake of anthocyanin-containing foodstuffs could decrease the risk of coronary heart disease (Detre et al. 1986), and have been suspected to account, at least in part, for the “French paradox,” that is, the decreased risk of cardiovascular disease despite a high-fat diet in individuals living in France.

Several animal experiments revealed that anthocyanin-containing mixtures or anthocyanins could inhibit carcinogenesis (Table 16.1). The inhibitory effects of colonic carcinogenesis induced by 1,2-dimethyl hydrazine (DMH) and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) or azoxymethane (AOM) were observed in rats that were administrated with a dietary level of 5.0% anthocyanin-rich extract of purple corn or purple sweet potato or red cabbage for 36 weeks (Hagiwara et al. 2001, 2002), with a dietary level of 2.5%, 5.0%, and 10.0% lyophilized black raspberries for

33 weeks (Harris et al. 2001), or with a dietary level of 3.85 g/kg bilberry, chokeberry, or grape anthocyanins for 14 weeks (Lala et al. 2006). On the other hand, in *Apc*<sup>Min</sup> mice that received either a mixture of cyanidin glycosides at 800 mg/L or pure cyanidin at 200 mg/L and drinking water or freeze-dried tart cherries at 200 g/kg in diet, the number and volume of cecal adenoma were significantly reduced, compared to mice receiving control diet. However, number of colonic adenomas was not significantly influenced by these treatments (Kang et al. 2003). A significant reduction in small intestinal adenoma number was recently observed in *Apc*<sup>Min</sup> mice that received either an anthocyanin-containing blueberry extract or pure cyanidin-3-glucoside at 0.3% (w/w) in diet (Cooke et al. 2006).

The inhibitory effects of mammary carcinogenesis induced by 7,12-dimethylbenz[*a*]anthracene (DMBA) were observed in female rats that received Concord grape juice containing 15 different anthocyanins (12% in the extract) in drinking water at doses of approximately 10, 15, or 20 mg anthocyanins per rat per day. The incidence, multiplicity, and final weight of mammary tumors were reduced, compared to rats on drinking water without grape juice (Singletary et al. 2003).

Anthocyanins have also been shown to prevent skin carcinogenesis. In female CD-1 mice that received anthocyanin-containing pomegranate extract (2 mg per mouse) for 30 weeks, a delay in onset of skin carcinogenesis initiated by DMBA and

**Table 16.1.** Inhibitory effects of anthocyanin-containing mixtures and anthocyanins on animal tumorigenesis.

Tumorigenesis	Animal	Anthocyanin source	Administration	Carcinogen	Preventive outcome	Reference
Colonic adenoma	Rats	Purple corn color, purple sweet potato color; red cabbage color	5.0% in diet for 36 weeks	DMH–PhIP	Reduced the incidence and multiplicity of adenomas and adenocarcinomas, and number of aberrant crypt foci (21, 24, or 36%), and adenocarcinoma (28, 35, or 80%)	Hagiwara et al. (2001, 2002)
	Rats	Black raspberries	2.5, 5.0, or 10.0% in diet for 33 weeks	AOM	Reduced aberrant crypt foci (21, 24, or 36%), and adenocarcinoma (28, 35, or 80%)	Harris et al. (2001)
	Rats	Bilberry, chokeberry, grape	3.85 g/kg in diet for 14 weeks	AOM	Reduced number of aberrant crypt foci (67, 70, or 69%)	Lala et al. (2006)
	Rats	Concord grape juice	10, 15, or 20 mg per rat per day in drinking water for 21 weeks	DMBA	Reduced the incidence, multiplicity and weight of mammary tumors	Singletary et al. (2003)
	<i>Apc</i> <sup>Min</sup> mice	Tart cherry anthocyanins	200 g/kg in diet for 10 weeks	<i>Apc</i> <sup>Min</sup>	Reduced number and volume of cecal adenoma, but not colonic adenomas	Kang et al. (2003)
	<i>Apc</i> <sup>Min</sup> mice	Blueberry extract	0.3% in diet for 12 weeks	<i>Apc</i> <sup>Min</sup>	Reduced adenoma number (45%)	Cooke et al. (2006)
	<i>Apc</i> <sup>Min</sup> mice	Cyanidin	200 mg/L in drinking water for 10 weeks	<i>Apc</i> <sup>Min</sup>	Reduced number and volume of cecal adenoma, but not colonic adenomas	Kang et al. (2003)
	<i>Apc</i> <sup>Min</sup> mice	Cyanidin glycosides	800 mg/L in drinking water for 10 weeks	<i>Apc</i> <sup>Min</sup>	Reduced number and volume of cecal adenoma, but not colonic adenomas	Kang et al. (2003)
	<i>Apc</i> <sup>Min</sup> mice	Cyanidin-3-glucoside	0.3% in diet for 12 weeks	<i>Apc</i> <sup>Min</sup>	Reduced adenoma number (30%)	Cooke et al. (2006)
Mammary tumor	CD-1 mice	Pomegranate fruit extract	2 mg per mouse for 30 weeks	DMBA–TPA	Reduced incidence and body burden of skin tumors	Afaq et al. (2005)
Skin carcinogenesis	C57BL/6 mice	Cyanidin-3-glucoside	3.5 mM per mouse for 20 weeks	DMBA–TPA	Reduced number and size of papillomas (53%)	Ding et al. (2006)
Tumor cell metastasis	Nude mice	Cyanidin-3-glucoside	9.5 mg/kg, 3 times per week, injection for 14 days	A549 cells	Inhibited A549 cells xenograft growth (~50% inhibition), and tumor cell metastasis in abdominal, liver, kidney, pancreas, and perigastric lymph nodes	Ding et al. 2006

promoted by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) was observed, and the incidence and burden of skin tumors were decreased (Afaq et al. 2005). A recent study reported that cyanidin-3-glucoside derived from blackberry could reduce the number of papillomas in mouse skin tumors generated by DMBA–TPA treatment according to the two-stage mouse skin tumor model (Ding et al. 2006). Moreover, cyanidin-3-glucoside could inhibit tumor cell metastasis in mice. The cyanidin-3-glucoside-treated mice showed much less tumor involvement of the abdominal cavity, and fewer tumor nodules in the abdominal cavity and the mesenteric fat. There was no tumor detected in organ parenchyma in the cyanidin-3-glucoside-treated mice (Ding et al. 2006).

These data from animal model suggest that high intake of anthocyanins or anthocyanin-containing mixtures might have protective effects against tumorigenesis of colon, skin, and mammary glands.

## INHIBITORY EFFECTS ON THE GROWTH OR PROLIFERATION OF CANCER CELLS

Extensive studies have revealed that anthocyanins, anthocyanidins, and anthocyanin-rich extracts had inhibitory effects on the growth or proliferation of many cancer cells. These cell lines included human epidermoid carcinoma cells [A431 (Meiers et al. 2001)], human stomach carcinoma cells [AGS (Zhang et al. 2005)], human colon carcinoma cells [CoCa-2 (Lazze et al. 2004), HCT116 (Katsume et al. 2003; Lazze et al. 2004; Seeram et al. 2006; Zhang et al. 2005)], HT29 (Kang et al. 2003; Lala et al. 2006; Marko et al. 2004; Olsson et al. 2004; Zhao et al. 2004)], T-leukemia cells [Jurkat (Fimognari et al. 2004b)], human lung carcinoma cells [LXFL52 L (Meiers et al. 2001), NCIH460 (Zhang et al. 2005; Zhao et al. 2004)], human mammary cancer cells [MCF-7 (Olsson et al. 2004; Seeram et al. 2006; Zhang et al. 2005)], rat mammary adenocarcinoma cells [RBA (Koide et al. 1996)], human prostate cancer cells [LNCaP (Seeram et al. 2006)], human glioblastoma cells [SF-268 (Zhang et al. 2005)], human monocytic leukemia cells [U937 (Katsume et al. 2003)], human promyelocytic leukemia cells [HL-60 (Fimognari et al. 2004b; Hou et al. 2003, 2005a; Seeram et al. 2006)], human uterine carcinoma cells [HeLa S3 (Lazze et al. 2004)], and human oral cancer cells [CAL27 (Seeram et al. 2006), KB (Seeram et al. 2006)]. These data revealed that anthocyanins had a wide range of the inhibitory effects on various cancer cells. Studies on structure–

activity relationships suggest that (1) the inhibitory potency of anthocyanidins on cancer cell survival is greater than that of their glycosylated counterparts and (2) the orthodihydroxyphenyl structure on the B-ring of aglycone is important for the inhibitory action. Delphinidin and cyanidin with this structure showed higher growth-inhibitory activity, while pelargonidin, peonidin, and malvidin without such *ortho*-dihydroxyphenyl structure showed lower growth-inhibitory activity. Inconsistent with this inference is the finding that in some cell types the potency of malvidin was equivalent to, or greater than, that of delphinidin (Afaq et al. 2005; Ding et al. 2006; Meiers et al. 2001; Zhang et al. 2005). Anthocyanin-containing extracts of grapes, bilberries, or chokeberries at 25–75  $\mu$ g/ml inhibited the growth of human malignant HT29 colon cancer cells, but did not inhibit the growth of nontumorigenic colon cells [NCM460 (Lazze et al. 2004)]. They showed no inhibitory effects in normal human embryonic fibroblasts (NHF) (Zhang et al. 2005). Several reports showed that delphinidin, cyanidin, petunidin, delphinidin-3-sambubioside, cyanidin-3-glucoside, and bilberry extract could induce apoptosis in HL-60 cells (Hou et al. 2003, 2005a; Katsume et al. 2003), U937 cells (Katsume et al. 2003), Caco-2 cells (Lazze et al. 2004), lymphocytes (Fimognari et al. 2004a), Jurkat cells (Fimognari et al. 2004b), and HCT116 cells (Katsume et al. 2003). Thus, the cytotoxicity, antiproliferation, and apoptotic induction may be involved in the mechanisms of the growth-inhibitory effects of anthocyanins on cancer cells.

## MOLECULAR ACTIONS

### CELLULAR SIGNALING TRANSDUCTION PATHWAYS

#### Mitogen-Activated Protein Kinase

Mitogen-activated protein kinase (MAPK) pathway consists of a cascade in which an MAP3K activates an MAP2K that activates an MAPK. MAPK includes extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 kinase. ERK has been demonstrated to play a critical role in transmitting signals initiated by growth-inducing tumor promoters, including TPA, epidermal growth factor (EGF), and platelet-derived growth factor (PDGF) (Cowley et al. 1994; Minden et al. 1994). On the other hand, stress-related tumor promoters, such as UV irradiation and arsenic, potently activate JNK and p38 kinase (Bode and Dong 2000; Kallunki et al. 1994). Thus, MAPK pathway plays important roles on cell

growth and cell survival (Seger and Krebs 1995), and has been considered to be a target molecule for cancer prevention and therapy.

Anthocyanidins have been demonstrated to modulate the MAPK in several cell lines and animal models. In mouse epidermal JB6 cells, 15  $\mu$ M delphinidin, but not peonidin, blocked TPA-induced phosphorylation of ERK at early times (2 h) and JNK at later times (12 h) (Hou et al. 2004). Moreover, 15  $\mu$ M delphinidin suppressed the activation of JNK and ERK signaling cascades, which included the inhibition of phosphorylation of MAPK/ERK kinase (MEK), SAPK/ERK kinase 1 (SEK1), and c-Jun. The ability of delphinidin to suppress the MAPK signaling pathway linked to the inhibitory effects of TPA-induced cell transformation in mouse epidermal JB6 cell (Hou et al. 2004). Cyanidin-3-glucoside could block the activations of TPA-induced ERK, UVB-induced MAPK, and SEK1 in the same mouse epidermal JB6 cells (Ding et al. 2006), which led to the inhibition of transactivation of nuclear factor-kappa B (NF- $\kappa$ B) and activator protein-1 (AP-1), and to the inhibition of cyclooxygenase-2 (COX-2) and tumor necrosis factor (TNF)- $\alpha$  expression. In mouse macrophage cells (RAW264.7), 50  $\mu$ M delphinidin could attenuate the activation of MAPK induced by lipopolysaccharide (LPS), which led to the inhibition of COX-2 and inducible nitric oxide synthase (iNOS) expression, and sequential inflammation (Hou 2006; Hou et al. 2005b). In CD-1 mice model, anthocyanin-rich pomegranate fruit extracts inhibited TPA-induced phosphorylation of MAPK, which led to the inhibition of TPA-induced skin tumorigenesis (Ding et al. 2006).

### Growth Factor Receptors

Growth factor receptors on the cell surface are stimulated by growth factors and act as the primary result of activating cellular proliferation and/or differentiation. The potent cell proliferation signals, generated by various growth factor receptors such as EGF receptor (EGFR), insulin-like growth factor-1 (IGF-1) receptor, and vascular endothelial growth factor (VEGF) receptor, constitute the basis for receptor-driven tumorigenesis in the progression of several cancers (Hahn and Weinberg 2002). Abnormality of growth factor signaling pathways leads to increase in cell proliferation, suppression of apoptotic signals, and cell invasion.

Anthocyanidins such as delphinidin, cyanidin, and malvidin have been demonstrated to inhibit the tyrosine kinase activity of the EGFR isolated from A431 cells with IC<sub>50</sub> values of 18, 42, and 61

$\mu$ M, respectively (Meiers 2001). But, their glycosides such as cyanidin-3-D-galactoside and malvidin-3-D-glucoside did not affect the activity. Using the reporter gene assay for measuring EGF/MAPK-induced Elk-1 phosphorylation, the activation of the GAL4-Elk-1 fusion protein was suppressed by cyanidin and delphinidin at the same concentration ranges. The results suggest that cyanidin and delphinidin may be potent inhibitors for EGFR. The abilities of anthocyanidins to inhibit EGFR tyrosine kinase were reported to be in the order of delphinidin, cyanidin > pelargonidin > peonidin > malvidin (Meiers 2001), showing a positive correlation with the presence of hydroxyl functions in the ring B of aglycone.

### TRANSCRIPTIONAL FACTORS

#### Activator Protein-1

AP-1 is a dimeric protein typically composed of the products of the *jun* and *fos* oncogene families. AP-1 protein binds to the promoter regions on DNA that contain TPA-response elements (TREs) to activate the transcription of genes involved in cell proliferation (Angel and Karin 1991), transformation (Dong et al. 1994), and apoptosis (Sawai et al. 1995). A variety of stimuli, such as phorbol esters (Dong et al. 1994), UV radiation (Adler et al. 1996), growth factors (Lamb et al. 1997), and oxidative agents (Pinkus et al. 1996), can stimulate AP-1 activity. Enhanced AP-1 activity has been shown to be involved in the tumor promotion and progression of various types of cancers. In vivo data from mouse experiments have demonstrated that AP-1 activity was required for tumor promotion (Chen et al. 2001; Young et al. 1999).

In mouse epidermal JB cells, 5  $\mu$ M delphinidin (Hou et al. 2004) and 10  $\mu$ M cyanidin-3-glucoside (Ding et al. 2006) could inhibit transactivation of AP-1. These actions led to the inhibition of cell transformation induced by TPA or UVB.

In LPS-activated mouse macrophage cells (RAW264.7), 50  $\mu$ M delphinidin could attenuate phosphorylation of c-Jun, a partner of AP-1, which partially linked to the inhibition of COX-2 and iNOS gene expression induced by LPS (Hou 2006; Hou et al. 2005b).

#### Nuclear Factor-Kappa B

NF- $\kappa$ B is a family of closely related protein dimers that bind to a common sequence motif in DNA called the  $\kappa$ B site (Aggarwal 2004). NF- $\kappa$ B is one of the principal transcription factors mediated by free radicals, inflammatory stimuli, cytokines, carcinogens,

tumor promoters, endotoxins, radiation, UV light, and x-ray (Doyle and O'Neill 2006; Gloire et al. 2006). In most unstimulated conditions, NF- $\kappa$ B is sequestered in the cytosol and binds to inhibitory kappa B alpha (I $\kappa$ B) in an inactive state. When I $\kappa$ B is phosphorylated and degraded, NF- $\kappa$ B migrates to the nucleus, where it induces the expression of more than 200 genes that have been shown to induce cellular transformation, proliferation, invasion, metastasis, chemoresistance, radioresistance, inflammation, and to suppress apoptosis.

In mouse epidermal JB cells, cyanidin-3-glucoside suppressed NF- $\kappa$ B transcriptional activity induced by benzo[*a*]pyrene-7,8-diol-9,10-epoxide (BPDE) (Hecht et al. 2006), TPA- (Ding et al. 2006), or UVB (Ding et al. 2006), which led to the inhibition of cell transformation. In CD-1 mice model, anthocyanin-rich pomegranate fruit extracts inhibited TPA-induced phosphorylation of NF- $\kappa$ B and I $\kappa$ B alpha kinase (IKK $\alpha$ ), and degradation of I $\kappa$ B $\alpha$ , which led to the inhibition of TPA-induced skin tumorigenesis (Afaq et al. 2005).

#### DOWNSTREAM GENES

##### Cyclooxygenase-2

COX is a rate-limiting enzyme for synthesis of dienoic eicosanoids such as prostaglandin (PG) E<sub>2</sub>. COX exists in two isoforms (Funk et al. 1991; Hempel et al. 1994). COX-1 is expressed constitutively in many types of cells and is responsible for the production of prostaglandins under physiological conditions. COX-2 is induced by proinflammatory stimuli including mitogens, cytokines, and bacterial LPS in macrophages (Adler et al. 1996) and epithelial cells (Kelley et al. 1997; Mitchell et al. 1994), and is involved in many inflammatory processes and overexpressed in various carcinomas. Thus, COX-2 is considered to play a key role in inflammation and tumorigenesis (Hla et al. 1993; Mestre et al. 1999). Identification of COX-2 inhibitor is suggested to be a promising approach to protect against inflammation and tumorigenesis.

Several reports have showed that anthocyanidins, anthocyanins, and certain anthocyanin-rich extracts could inhibit COX-2 expression or/and activities. Pretreatment with 50  $\mu$ M delphinidin or 1 mg/ml blueberry anthocyanins inhibited the expression of both COX-2 mRNA and protein induced by LPS in mouse RAW264.7 cells (Hou et al. 2005b). In mouse epidermal JB cells, 20  $\mu$ M cyanidin-3-glucoside could attenuate COX-2 expression induced by TPA or UVB, which led to the inhibition of cell transforma-

tion (Ding et al. 2006). In cell-free system, cyanidin decreased COX-2 activities by 74% (Seeram et al. 2003).

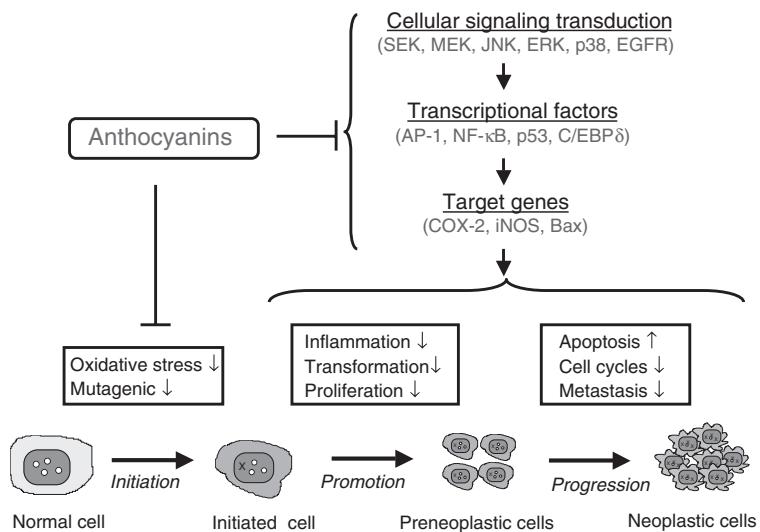
##### Inducible Nitric Oxide Synthase

Nitric oxide (NO) is a diatomic free radical produced from L-arginine by constitutive and inducible nitric oxide synthase (cNOS and iNOS) in numerous mammalian cells and tissues. NO may be generated in excess during the host response against viral and bacterial infections. Excessive and prolonged iNOS-mediated NO generation has been demonstrated to link to inflammation and tumorigenesis (Akaike et al. 2000; Maeda and Akaike 1998). Our group found that delphinidin and anthocyanin-rich extracts from bilberry could inhibit iNOS expression and NO production in LPS-activated mouse RAW264.7 cells (Hou 2006). The inhibitory effects of crude extracts of selected berries on NO production showed significant correlation with anthocyanin contents, suggesting that the consumption of anthocyanin-rich diet may reduce the oxidative stress of NO and increase the protective effects against cardiovascular and chronic inflammatory diseases (Wang and Mazza 2002).

##### Apoptosis-Related Genes

The balance between survival and death often tips toward the former in cancer cells. Thus, apoptosis is considered to play an important role in the elimination of seriously damaged cells or tumor cells by antitumor and chemopreventive agents (Galati et al. 2000; Thompson 1995). The cells that have undergone apoptosis are typically shown in chromatin condensation and DNA fragmentation. They are rapidly recognized by macrophages before cell lysis and can be removed without inducing inflammation (Steller 1995; Thompson 1995). Therefore, apoptosis-inducing agents are expected to be ideal drugs for cancer therapy.

Several reports indicate that anthocyanidins, anthocyanins, and certain anthocyanin-rich extracts could induce apoptosis in several carcinoma cells including CaCo-2 (Lazze et al. 2004), HeLa S3 (Lazze et al. 2004), HL-60 (Hou et al. 2003, 2005>; Katsube et al. 2003), Jurkat cells (Fimognari et al. 2004a), U937 (Hyun and Chung 2004), and HCT116 (Katsube et al. 2003). Our group has investigated the molecular mechanisms of apoptosis induced by delphinidin (Hou et al. 2003) and delphinidin-3-sambubioside (Hou et al. 2005a) in HL-60 cells. Both delphinidin and delphinidin-3-sambubioside



**Figure 16.2.** Possible molecular targets for cancer-preventive effects of anthocyanins on multistage carcinogenesis.

(100  $\mu$ M) stimulated generation of reactive oxygen species (ROS), JNK phosphorylation, *c-jun* gene expression, Bid truncation, mitochondrial membrane potential ( $\Delta \Psi_m$ ) loss, cytochrome *c* release, and activation of caspase-9, -8, and -3. Antioxidants including *N*-acetyl-L-cysteine (NAC) and catalase could effectively block these actions. Thus, anthocyanins might trigger an apoptotic death program in HL-60 cells through ROS/JNK-mediated mitochondria death pathway. In Jurkat cells, cyanidin-3-glucoside could induce apoptosis by enhancing p53 and Bax levels (Fimognari et al. 2004b).

These molecular data revealed that anthocyanins could modulate oncogenic signaling transduction pathways (e.g., MAPK, EGFR), transcriptional factor activations (e.g., AP-1, NF- $\kappa$ B, p53), and downstream gene expressions (e.g., COX-2, iNOS, Bax), which are involved in the actions for the inhibition of carcinogenesis, inflammation, metastasis, and for apoptosis induction in cancer cells (Figure 16.2).

## CONCLUSION

Population-based survey hinted at cancer-preventive effects of anthocyanin-containing diets in humans. Animal model experiments revealed that anthocyanins and anthocyanin-containing mixtures could protect against carcinogenesis of colon, skin, and mammary glands. Culture cell investigations exhibited potential inhibition of anthocyanins and anthocyanin-containing mixtures on the growth of

many malignant cells. Recent molecular data demonstrated that anthocyanins could modulate oncogenic signaling transduction pathways, transcriptional factor activations, and downstream gene expressions, which are involved in the actions for anticarcinogenesis, anti-inflammation, apoptotic induction, and antimetastasis. Therefore, anthocyanins might be potential phytochemicals for cancer prevention.

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# 17

## Food Components Activating Capsaicin Receptor TRPV1

*Tatsuo Watanabe, Yusaku Iwasaki, Akihito Morita, and Kenji Kobata*

### INTRODUCTION

Capsaicin is a pungent principle of hot pepper. Capsaicin exerts several biological activities such as causing burning sensation, stimulating primary afferent neurons conducting chemical pain or hotness, enhancing energy metabolism, showing protection against stomach mucosa, inducing apoptosis in some cancer cells, and so on. Many of them are exerted through capsaicin receptor activation.

Capsaicin receptor was cloned in 1997 from rat dorsal root ganglion (DRG) (Caterina et al. 1997). Capsaicin receptor was first called vanilloid receptor subtype 1 (VR1) according to the common structural parts of agonists, capsaicin and resiniferatoxin, both having vanillyl group (Figure 17.1). Now, capsaicin receptor is called transient receptor potential vanilloid subtype 1 (TRPV1; Figure 17.2) because it has homology to TRP ion channels (Clapham et al. 2001).

Because obesity is one of the serious factors of lifestyle-related diseases such as hypertension, stroke, diabetes, and hyperlipidemia, we focus on the thermogenic action or body-fat-lowering effect of capsaicin. Thermogenic action of capsaicin is thought to exhibit through activation of TRPV1 (Iwai et al. 2003). Chemical dysfunction of capsaicin-sensitive nerves (Watanabe et al. 1988) or pretreatment with a TRPV1 competitive antagonist capsazepine (Watanabe et al. 2001) results in disappearance or decrease in capsaicin-induced adrenaline secretion. Initial and the important points of capsaicin to energy metabolism are thought to be the stimulation of TRPV1. So, we are searching food components activating TRPV1 as candidates for energy consumption-stimulating compounds.

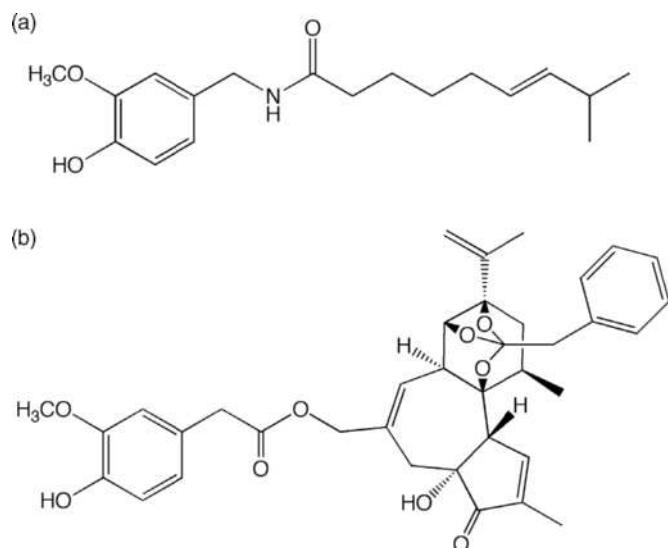
In this chapter, we introduce food components having TRPV1 activity.

From the discovery of TRPV1 gene in 1997, food components activating TRPV1 have been vigorously investigated. Calixto et al. (2005) of Brazil reviewed food components having TRPV1 activity up to the late 2004. They listed up capsaicinoids of hot pepper, piperine of black pepper, eugenol of clove, ginsenosides of Asian ginseng, evodiamine of *Evodia rutaecarpa*, and so on (Figure 17.3). Here, we describe other compounds and their list except for hot pepper compounds.

### HOW TO EVALUATE ACTIVITY TO TRPV1

In *in vitro* assay, native cells or exogenously TRPV1-expressed cells are used. DRG cells of rats or mice are one of the native cells having TRPV1 originally. In case of mice, the spinal column was excised after cervical dissection. DRGs were extirpated from the centrally divided spinal column. DRG cells in MEM (Eagle's minimal essential medium)-complete solution were treated with collagenase P and then dispersed by gentle pipettings. Resulting cell dispersion was sowed to poly-lysine-treated cover glass and cultured in CO<sub>2</sub> incubator. Primary cultured DRG cells, as mentioned above, were used for measurement of TRPV1 activity of native cells. TRPV1 activity was measured by calcium imaging technique with fluorescent microscope.

Human embryonic kidney (HEK) cells or *Xenopus* oocyte cells are used for exogenous expression of TRPV1. In our case, rat TRPV1 cDNA was prepared from mRNA of rat C6 glioma cells by polymerase chain reaction. The obtained cDNA was inserted into



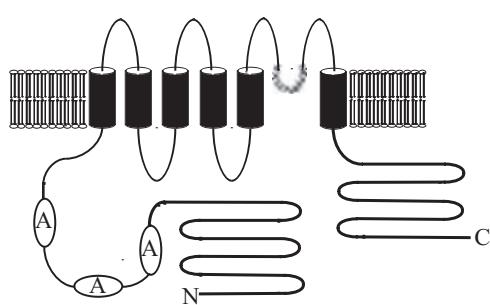
**Figure 17.1.** Structure of capsaicin (a) and resiniferatoxin (b).

pcDNA3 (Invitrogen) and transfected into HEK 293 cells by the use of SuperFect transfection reagent (Qiagen). G418-resistant and capsaicin-sensitive cells which express TRPV1 stably were picked up and used for measurement of TRPV1 activation (Morita et al. 2006).

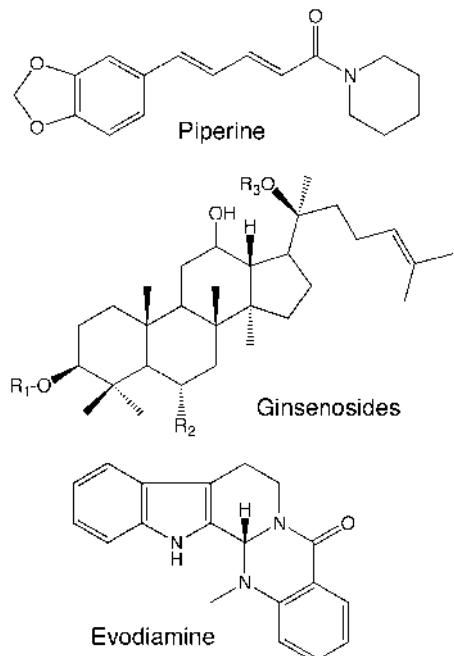
After taking intracellular  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ) indicator such as Fluo-2 AM or Fluo-4 AM into the cells expressing TRPV1, increment of  $[\text{Ca}^{2+}]_i$  after adding the test samples are monitored as an index of TRPV1 activity by the use of fluorescent microscope or fluorescent spectrophotometer (calcium imaging). Another technique for measuring TRPV1 activity is patch clamping. Patch clamping

records ion microcurrents when ion channels such as TRPV1 pass ion after channel activation.

As in vivo assay, subcutaneous injection of samples into rodent's hind paw and number or duration of pain-relating behavior after injection is used. By



**Figure 17.2.** Predicted membrane topology and domain structure of TRPV1. (Adapted from Caterina et al. 1997. A, ankyrin repeat domain; C, C terminal; N, N terminal.)



**Figure 17.3.** Structure of natural vanilloids.

this method, sample compounds are directly injected in the vicinity of sensory nerve endings. Tominaga et al (Iida et al. 2003) showed that capsiate or olvanil that has same affinity as capsaicin to TRPV1 but no pungency and higher lipophilicity than capsaicin induces pain after subcutaneous injection into mouse hind paw and suggested that the development of pungency needs not only TRPV1 activity but also moderate lipophilicity to pass endothelial cells.

## PUNGENCY-RELATED COMPOUNDS IN CAPSIUM FRUITS

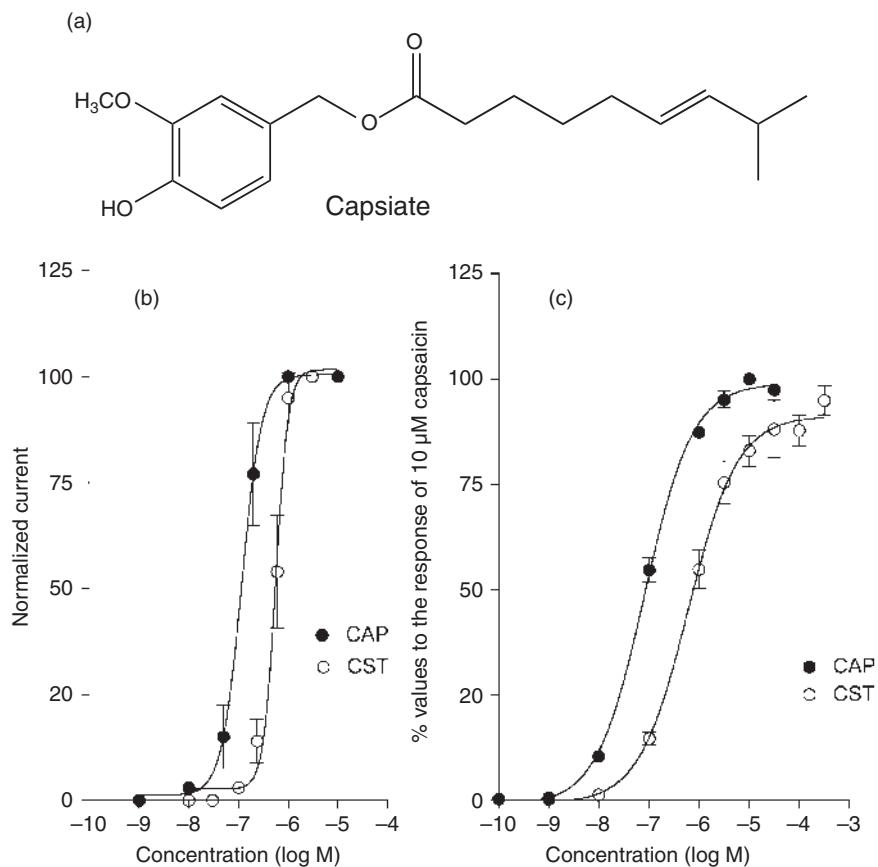
### CAPSIATE

Capsiate is a compound structurally close to capsaicin. Capsaicin is an amide of vanillylamine with 8-methyl-6E-nonenanoic acid (8-methylnon-6E-enoic acid). On the contrary, capsiate is an ester of vanil-

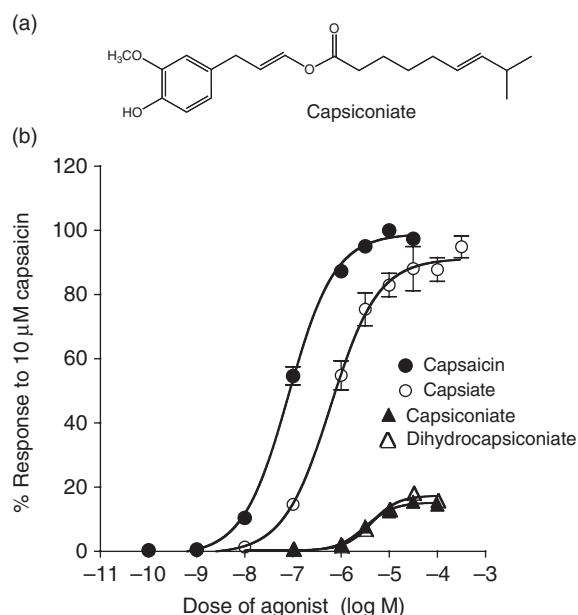
yl alcohol with the same fatty acid (Figure 17.4) (Kobata et al. 1998). It was found in “CH-19 Sweet” that is selected and fixed cultivar from pungent variety “CH-19” from Thailand (Yazawa et al. 1989).

Capsiate is distributed to various *Capsicum* varieties including *C. annuum*, *C. baccatum*, and *C. chinense* (Yazawa et al. 2004). Many very pungent cultivars synthesize and deposit capsaicin and small amount of capsiate. Both capsaicin and capsiate are biosynthesized from the same precursors, phenylalanine and valine (Sutoh et al. 2006). But the precise regulation of the synthesis ratio of capsaicin and capsiate are not known.

Capsiate stimulates rat TRPV1 with the same potency as capsaicin both in vitro (Iida et al. 2003; Morita et al. 2006) and in vivo (Iida et al. 2003). These results indicate that capsiate can enhance whole-body energy metabolism. Actually, ingestion



**Figure 17.4.** Structure of capsiate (a) and capsaicin- and capsiate-induced changes in membrane potential and  $[Ca^{2+}]_i$  of TRPV1-expressing HEK293 cells (b, c). (b) Data from patch-clamp, (c) data from calcium imaging. (Adapted from Morita et al. 2006.)



**Figure 17.5.** Structure of capsiconate (a) and dose–response curves for agonists in  $[Ca^{2+}]_i$  of TRPV1-expressing HEK293 cells (b). (Adapted from Kobata et al. 2006.)

of capsiate or capsiate-containing pepper CH-19 Sweet increase core temperature (of mice: Ohnuki et al. 2001b; of human: Ohnuki et al. 2001c) and enhance the oxygen consumption, an index for energy metabolism (of mice: Ohnuki et al. 2001a; of human: Ohnuki et al. 2001c). Ingestion of CH-19 Sweet, for 2 weeks resulted in decrease in body fats in human (Kawabata et al. 2006). These results clearly show that capsiate stimulating TRPV1 with the same potency as capsaicin inhibits body fat accumulation in human.

To date, other biological activities by capsiate such as antioxidant activity (Rosa et al. 2002), increase in production of dermal insulin-like growth factor after topical application (Harada and Okajima 2007), and inhibition of angiogenesis induced by VEGF (Pyun et al. 2008) have been reported.

### CAPSICONATE

Capsiconate is capsiate-like substance found from *C. baccatum* (Kobata et al. 2008). In contrast to capsiate, which is an ester of vanillyl alcohol with 8-methylnon-6E-enoic acid, capsiconate is a coniferyl ester of the same fatty acid as capsiate or capsaicin.

TRPV1 activity of capsiconate was evaluated by an analysis of  $[Ca^{2+}]_i$  in HEK 293 cells expressing TRPV1. The  $EC_{50}$  value was only 50 times larger than that of capsaicin, but the maximum response was 20% of that of capsaicin. Accordingly, capsiconate

is a partial agonist of TRPV1 with relatively high affinity (Figure 17.5).

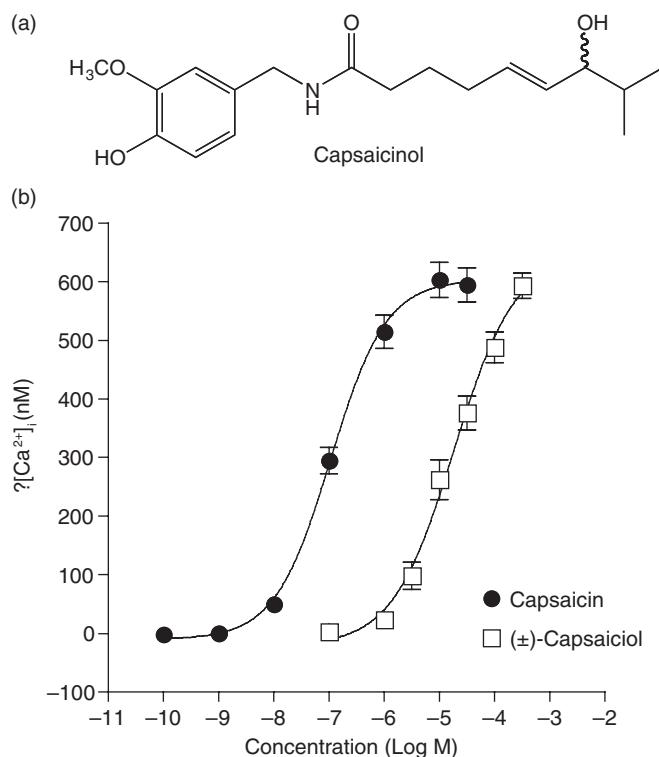
### CAPSAICINOL

Capsaicinol is a low pungent compound having strong antioxidant activity originally from *C. frutescens* (Masuda et al. 1990). It has an additional hydroxy group to acyl moiety and the position of double bond is different from capsaicin (Figure 17.6). The small difference in the structure results in lowering the pungency to one hundredth. We developed a simple synthetic method of capsaicinol and evaluated TRPV1 activity and adrenaline secretion from rats (Kobata et al. 2006). The affinity value to TRPV1 and the extent of adrenaline secretion after intravenous injection of capsaicinol were also one hundredth to capsaicin as human organoleptic results.

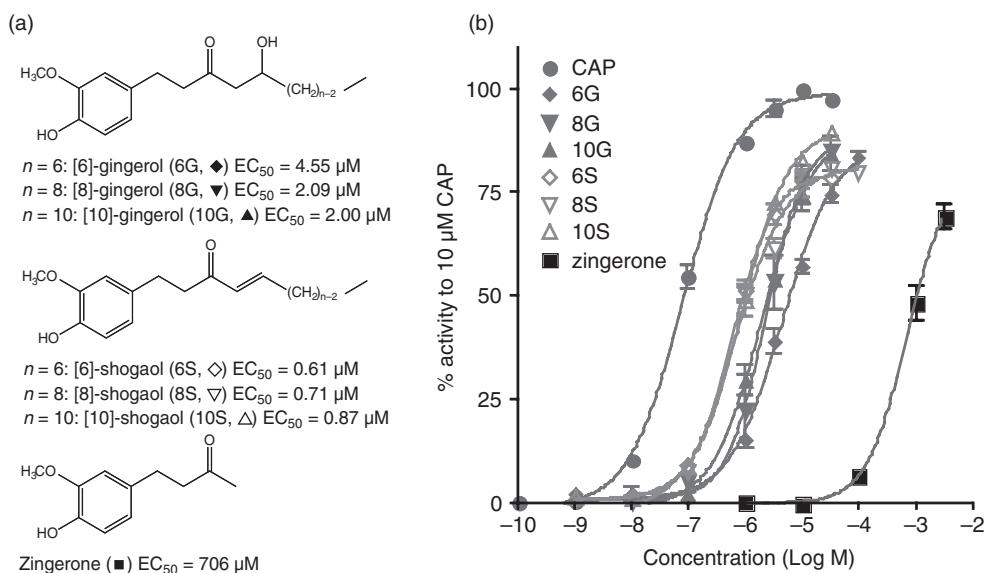
### GINGER COMPOUNDS

As pungent compounds, raw ginger contains mainly gingerols. In steamed and heated ginger, main pungent compounds are shogaols. Zingerone is the degradation product of these compounds.

In HEK cells expressing rat TRPV1, both gingerols and shogaols activated TRPV1 as the same potencies and no difference was found on potencies of such compounds having different length of acyl chains (Figure 17.7) (Iwasaki et al. 2006). In



**Figure 17.6.** Structure of capsaicinol (a) and capsaicin- and capsaicinol-induced changes in  $[\text{Ca}^{2+}]_i$  of TRPV1-expressing HEK293 cells (b).



**Figure 17.7.** Structure of pungency-related compounds of ginger (a) and dose-response curves for compounds in  $[\text{Ca}^{2+}]_i$  of TRPV1-expressing HEK293 cells (b). (Adapted from Iwasaki et al. 2006.)

these compounds [10]-shogaol showed no irritancy in eye-wiping test but induced pain-relating behavior in subcutaneous injection into hind paw in rats. These results suggest that [10]-shogaol is no pungent TRPV1 agonist.

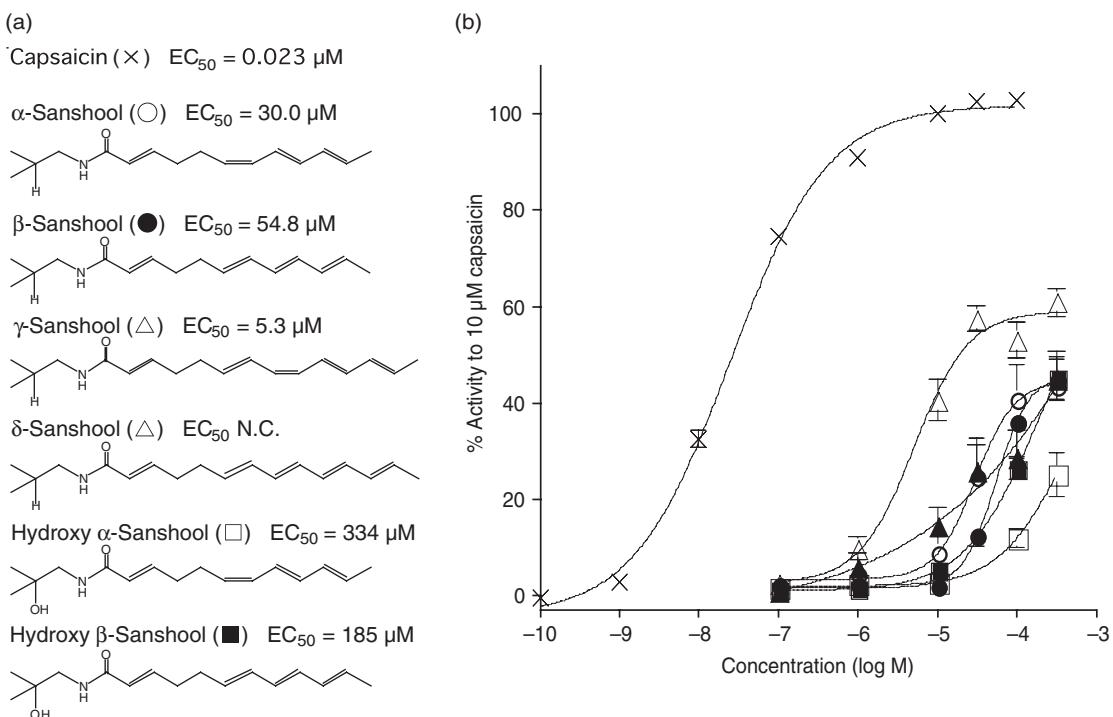
### SANSHO COMPOUNDS

Sansho (Japanese pepper, *Zanthoxylum piperitum*) is a spice of characteristic flavor and pungency. Pungency-related compounds of sansho are  $\alpha$ -,  $\beta$ -,  $\gamma$ -, and  $\delta$ -sanshools and hydroxy derivatives of  $\alpha$ - and  $\beta$ -sanshools. Scoville value (one of the human organoleptic assays for pungency) of capsaicin is 16,000,000. Those of sanshools and hydroxy derivatives were around  $10 \times 10^4$  and  $1 \times 10^4$  to  $2 \times 10^4$ , respectively, indicating that they are low pungent (Sugai et al. 2005). In HEK cells expressing TRPV1, EC<sub>50</sub> values of sansho compounds were two or three order higher (Figure 17.8). Hydroxy derivatives showed further one or two order higher value than sanshools. Maximal responses to TRPV1 of these compounds were about half of that of capsaicin.

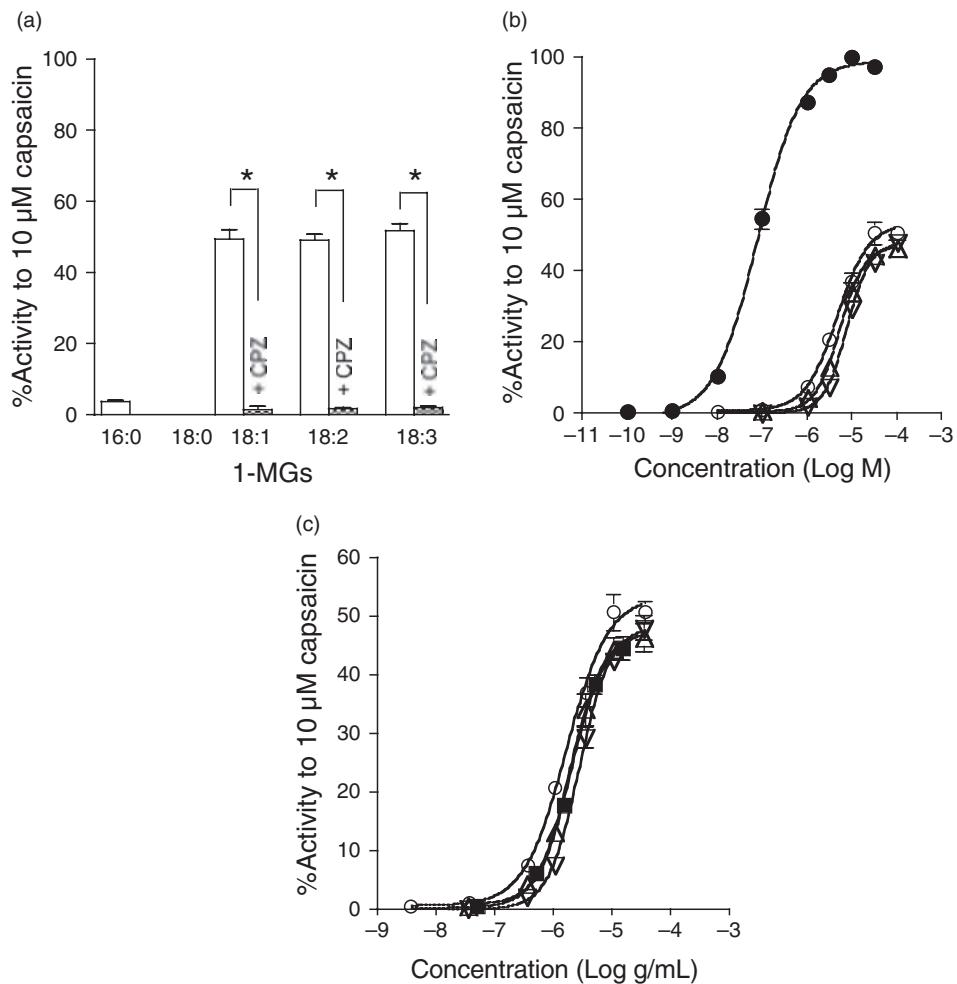
Scoville values and TRPV1 activities were roughly correlated. The pungency of sansho is not strong and somewhat paralytic. Therefore, sansho compounds may stimulate not only TRPV1 but also other TRP channels.

### NONPUNGENT COMPOUNDS FROM FOODS OF HOT OR WARM NATURE AND COOL OR COLD NATURE

Nineteen foods from the groups of hot or warm nature and cool or cold nature in traditional Chinese medicine were screened as candidates for TRPV1-activating compounds (Iwasaki et al. in press). Foods were successively extracted with hexane, ethyl acetate, and methanol. Among extracts, hexane extracts of all *Allium* species showed activities to TRPV1. Foods of *Allium* species belong to foods of warm or hot nature. Much of food extracts of cool or cold nature exhibited low TRPV1 activities. Interestingly, hexane extract of wheat powder, which is of cool or cold nature, had relatively high activity. We chose onion, myoga (*Zingiber mioga*), and wheat, as sauces



**Figure 17.8.** Structure of pungency-related compounds of sansho (a) and dose-response curves for compounds in  $[Ca^{2+}]_i$  of TRPV1-expressing HEK293 cells (b). (Adapted from Sugai et al. 2005.)

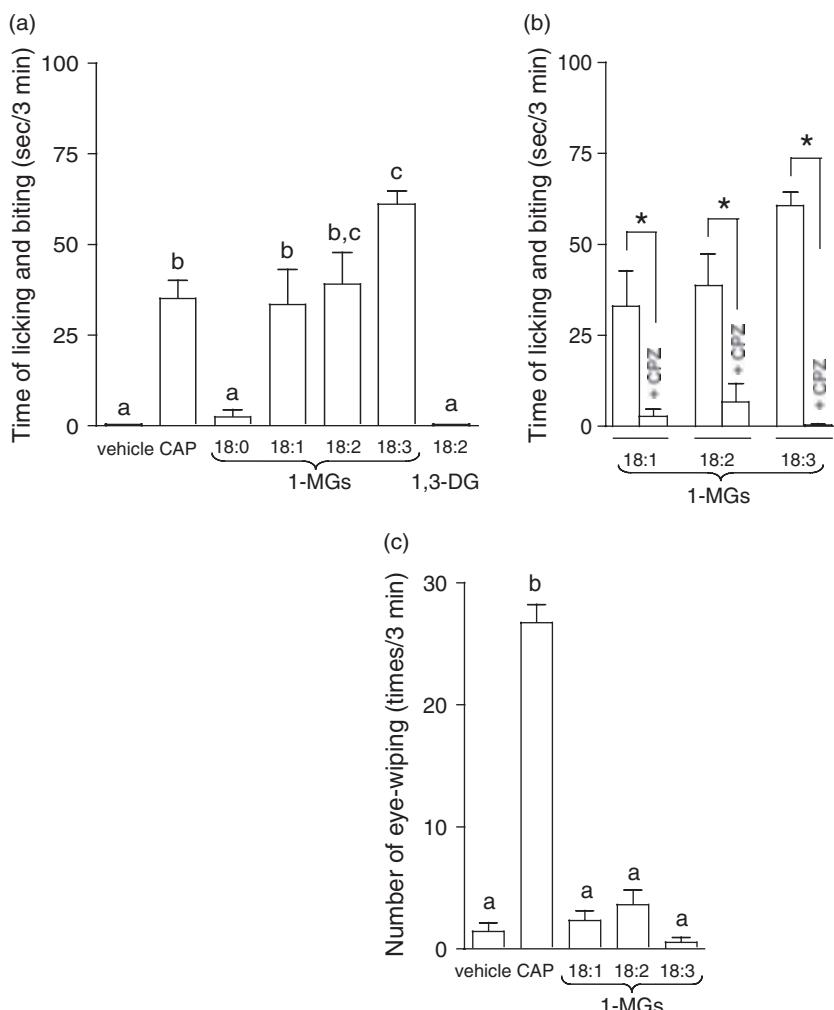


**Figure 17.9.** TRPV1 activities by monoacylglycerols (MGs) as measured by intracellular  $\text{Ca}^{2+}$  concentration in TRPV1-expressing HEK293 cells. (a) White bars indicate TRPV1 activities by  $100 \mu\text{M}$  MGs having 16:0, 18:0, 18:1, 18:2, and 18:3 fatty acids, respectively. Checked bars indicate TRPV1 activities by these MGs after pretreatment with  $100 \mu\text{M}$  capsazepine (CPZ). These values are mean  $\pm$  SEM ( $n = 3$ ). \* indicates a significant difference between each cell type ( $p < 0.01$ ). (b and c) Dose-response curves of capsaicin (CAP, closed circle), 1-MGs of 18:1, 18:2, and 18:3 (open circle, open triangle, and open inverted triangle, respectively), and Fr. 4-2-M of wheat flour extract (filled square). These values are mean  $\pm$  SEM ( $n = 3-10$ ). (Adapted from Iwasaki et al. 2008.)

of TRPV1-activating compounds from these foods showed high activities to TRPV1.

After several chromatographic separations, TRPV1 activity of wheat was determined to be 1-monoacylglycerols (1-MGs) having oleic, linoleic, and alpha-linolenic acids as acyl components (Figure 17.9). Activities in onion and myoga also contained the same 1-MGs. Additionally, myoga had 2-MGs containing C18 and C20 unsaturated fatty acids as activities.

In vivo experiments for TRPV1 activity of 1-MG were performed by the use of 1-oleoylglycerol as representative 1-MG from wheat, onion, and myoga. As expected, 1-oleoylglycerol did not cause scratching-wiping movement after instillation into rat's eye, showing nonpungency of 1-oleoylglycerol. Subcutaneous injection of 1-oleoylglycerol into rat hind paw induced pain reaction as the extent predicted by the TRPV1-expressing cell experiment (Figure 17.10). These results clearly show that



**Figure 17.10.** Aversive responses of mice induced by TRPV1-activating monoacylglycerols (MGs) after the subcutaneous hind paw injection or eye instillation. (a) The total time spent on licking and biting the injected hind paw was measured for 3 min after the injection of the vehicle, capsaicin (CAP, 0.5 mM), MGs (50 mM), and 1,3-dilinoleoylglycerol (1,3-DG) (18:2), 50 mM. (b) The total time spent on licking and biting was measured for 3 min after the injection of the TRPV1-activating MGs (50 mM) with or without capsazepine (CPZ, 10 mM) administration. (c) The number of eye-wiping movements was counted for 1 min after the administration of vehicle, CAP (0.5 mM), or TRPV1-activating MGs (50 mM). The values are mean  $\pm$  SEM (a,  $n = 5$ ; b,  $n = 4$ –6; c,  $n = 6$ –12). Different letters indicate significant differences,  $p < 0.05$ . \* indicates a significant difference between the groups with or without CPZ,  $p < 0.05$ . (Adapted from Iwasaki et al. 2008.)

1-oleoylglycerol is a nonpungent TRPV1 agonist *in vivo*.

## CONCLUSION

It has been hypothesized that TRPV1 agonists from foods may work like capsaicin to reduce excessive body fat in human. Some compounds such as capsi-

ate, [10]-shogaol, and 1-monoacylglycerol are non-pungent TRPV1 activators. Actually, capsiate inhibited accumulation of body fat in human. For [10]-shogaol and 1-MGs, feeding studies in animals and human are necessary to show the effectiveness of these compounds. But they are candidates of non-pungent energy metabolism enhancer for preventing obesity.

We feel there are some mechanisms on the energy metabolism enhancement through TRPV1 activation. Capsaicin induces significant adrenaline secretion from the adrenal gland, meanwhile capsiate causes only a small amount of adrenaline secretion (Iwai et al. 2003). But, both compounds inhibit fat deposition at the same extent. Integrative studies on the mechanisms of TRPV1 agonist from foods must be done from the point of view of action mechanism.

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# 18

## New Therapeutic Effects of Anthocyanins: Antiobesity Effect, Antidiabetes Effect, and Vision Improvement

Takanori Tsuda and Hitoshi Matsumoto

### INTRODUCTION

Obesity is defined as the accumulation of excess adipose tissue and may be due to various metabolic disorders. The “metabolic syndrome” is characterized by a group of metabolic risk factors in one person. Abdominal obesity is one of the central causal components in metabolic syndrome, and it is strongly associated with insulin resistance, including hardening of the arteries and an increased risk for cardiovascular disease (Kahn and Jeffrey 2000).

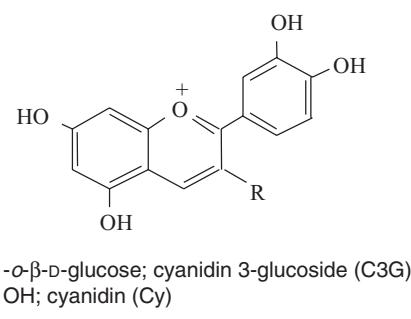
Adipocyte dysfunction plays an important role in the development of metabolic syndrome. Adipocytes synthesize and secrete biologically active molecules called adipocytokines (Matsuzawa 2005). For example, leptin (a product of the *ob* gene) is secreted by adipocytes. It reduces food intake and increases energy expenditure (Friedman and Halaas 1998). Adiponectin is one of the most important adipocytokines, and is specifically and highly expressed in adipocytes. The plasma adiponectin concentration and mRNA expression level are decreased in the obese and insulin-resistant state (Arita et al. 1999; Maeda et al. 1996). The administration of adiponectin improves insulin action accompanied by increase in fatty acid oxidation and decrease in triacylglycerol level in muscle (Fruebis et al. 2001; Yamauchi et al. 2001).

Anthocyanins are the largest group of water-soluble pigments in the plant kingdom. In the human diet, they are derived primarily from a wide variety of plant sources including crops, beans, fruits, vegetables, and red wine (Harborne and Grayer 1988). In general, anthocyanin pigments are stable under

acidic conditions, but are unstable and rapidly broken down under neutral conditions (Brouillard 1988). Therefore, anthocyanins have not been recognized as physiological functional food factors (Brouillard 1988). However, our *in vitro* and *in vivo* studies demonstrated that cyanidin-3-glucoside (C3G) (Figure 18.1), which is a typical anthocyanin, had antioxidative and anti-inflammatory activities (Tsuda et al. 1998, 1999, 2002a, b). These findings suggest that the beneficial effects of C3G extend beyond its antioxidant activity.

In the previous decade, the rapid spread of computers and visual display terminals (VDTs) in the home and workplace has led to an increase in ocular and visual problems, including eye discomfort, blurring of distant objects, and eye strain (asthenopia) (Murata et al. 1996; Tyrrell and Leibowitz 1990). Regarding nutritional mitigation of visual function problems, several dietary constituents, such as carotenoids (Snodderly 1995), long-chain polyunsaturated fatty acids (Neuringer et al. 2000), and anthocyanins (ACs) (Morazzoni and Bombardelli 1996), have been shown to enhance visual acuity.

We focused on black currant (*Ribes nigrum* L.) anthocyanins (BCAs) because black currant is rich in AC, has characteristic AC components, and is consumed in many countries. The composition of BCAs and flavonoids is summarized in Figure 18.2. The typical AC profile in black currant fruits is 47% delphinidin-3-rutinoside (D3R), 13% delphinidin-3-glucoside (D3G), 35% cyanidin-3-rutinoside (C3R), and 5% C3G (Matsumoto et al. 2001a). Additionally, BCAs have high bioavailability compared with other berry anthocyanins. D3R and C3R, which are the



**Figure 18.1.** Chemical structure of cyanidin 3- $\beta$ -D-glucoside.

major AC of BCA, are directly absorbed, distributed to the blood, and excreted in urine as intact forms. Furthermore, D3R and C3R have high bioavailability compared with their glucosides (Matsumoto et al. 2001b). Also in the distribution to ocular tissues, BCAs are absorbed and enter intact into the cornea, aqueous humor, iris, ciliary body, choroid, sclera, and retina. Additionally, the concentration of total ACs is higher in some ocular tissues than in plasma. These results suggest that ACs are concentrated in the ocular tissues (Matsumoto et al. 2006). Therefore, the oral intake of BCA is potentially effective for relieving visual disturbances (e.g., asthenopia).

In this part, we review the newly discovered effects of anthocyanins including their antihypertension effect, antidiabetes effect, and vision improvement.

## ANTIOBESITY EFFECT OF ANTHOCYANINS

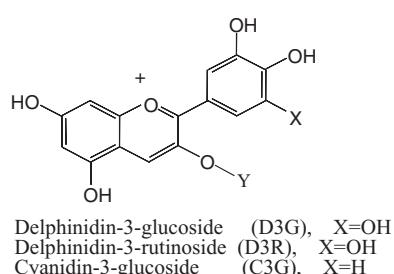
### EFFECT OF ANTHOCYANINS ON MICE

No prior studies have shown that anthocyanins themselves prevent obesity and ameliorate insulin resis-

tance. Our study was designed to examine the effect of anthocyanin-rich food color on the development of obesity and hyperglycemia induced by feeding a high-fat (HF) diet. “Purple corn color” (PCC) is made from purple corn (*Zea mays* L.). In Japan, about 50,000 kg per year of PCC (containing a large amount of C3G and other anthocyanins) is used for coloring foods, such as soft drinks, confections, and other foods. We used this C3G-enriched food color for our experiments.

Four-week old, male C57BL/6 mice were used. The mice were divided into four groups and fed one of the four diets (a control diet, PCC diet, HF diet, and HF and PCC diet) for 12 weeks. The HF diet contained 30% lard. PCC was added to each diet at a C3G concentration of 0.2%.

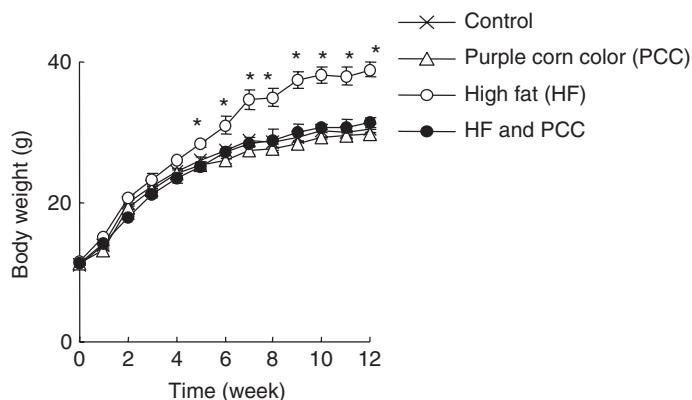
The body weight of the HF group was significantly higher than that of the control, PCC, and HF and PCC groups between the 5th and 12th week of feeding. On the other hand, the body weight of the control and HF and PCC groups did not differ throughout the experimental period (Tsuda et al. 2003) (Figure 18.3). Although the data are not shown, PCC itself did not affect food intake. Also, the energy intake and fecal lipid content did not differ among the groups. These data suggest that the suppression of body weight gain was not due to inhibition of dietary fat digestion and reduction of energy intake. The weights of all adipose tissues were significantly greater in the HF group than the control group. However, dietary PCC clearly suppressed HF-diet-induced increase in tissue weight. The data indicate that dietary PCC has a significant antihypertension effect (Tsuda et al. 2003) (Table 18.1). In the liver, lipid accumulation induced by the HF diet was also cancelled out by the administration of PCC. In the HF group, lipid accumulation was marked, indicating that the HF diet induced the typical fatty liver. However, PCC completely suppressed the accumulation of liver lipids (Tsuda et al. 2003) and significantly decreased fatty acid synthase expression, which can potentially cause body fat and liver lipid accumulation (Tsuda et al. 2003).



**Figure 18.2.** Structure of the four anthocyanins in black currants. Delphinidin-3-rutinoside (D3R; X = OH, Y = glucose-rhamnose), delphinidin-3-glucoside (D3G; X = OH, Y = glucose), cyanidin-3-rutinoside (C3R; X = H, Y = glucose-rhamnose), and cyanidin-3-glucoside (C3G; X = H, Y = glucose).

### ANTHOCYANINS REGULATE ADIPOCYTE FUNCTIONS INCLUDING ADIPOCYTOKINE EXPRESSION

Obesity is the key factor that predisposes to the development of metabolic syndrome. Also, amelioration of adipocyte dysfunction is crucial for preventing this syndrome. Nutrigenomics is the application of high-throughput genomic technology to nutrition



**Figure 18.3.** Body weight changes of mice fed the control, purple corn color (PCC), high-fat (HF), or HF and PCC diets during 12 weeks. Values are means  $\pm$  SE,  $n = 6$ . \*, the HF group differed ( $p < 0.05$ ) from all other groups. (This figure is reprinted from Tsuda et al. (2003), with permission from the American Society for Nutrition.)

research. DNA microarray technology has significantly advanced. Its use will help us discover how anthocyanins regulate the genes responsible for preventing obesity and ameliorating insulin resistance. Therefore, microarray profiling of gene expression in human adipocytes was carried out to determine the effect of anthocyanins.

Human preadipocytes obtained from subcutaneous adipose tissue were cultured and allowed to differentiate into adipocytes. Thirteen days after differentiation, the adipocytes were treated with anthocyanin for 24 hours, and then total RNA was obtained. The procedures were as described in the Affymetrix Technical Manual. Hierarchical clustering display of data for potentially significant genes showed nine clusters each containing from 19 to 234 genes (Tsuda et al. 2006) (Figure 18.4). These profiles indicated that 32% of the genes in human adipocytes did not have the same response to C3G and cyanidin (Cy)

treatment (Tsuda et al. 2006) (Figure 18.4). The array data identified the significantly upregulated or downregulated adipocytokines. Adiponectin (one of the most important adipocytokines) was upregulated, and plasminogen activator inhibitor (PAI)-1 and interleukin (IL)-6 were downregulated (Tsuda et al. 2006). Elevation of adipose PAI-1 expression is associated with both obesity and type 2 diabetes, suggesting that the regulation of the PAI-1 expression is also an important therapeutic target for metabolic syndrome (Eriksson et al. 1998; Uchida et al. 2004; Zirlik et al. 2004). IL-6 is an important regulator of the acute phase response. Plasma IL-6 is elevated in obese subjects and type 2 diabetes patients (Fried et al. 1998; Pickup et al. 2002; Pradhan et al. 2001). The administration of anthocyanins downregulated the expression of these adipocytokines. The gene expression level obtained using quantitative real-time polymerase chain reaction was consistent with

**Table 18.1.** Fat pad weight in mice fed control, PCC, HF, or HF and PCC diets after 12 week.

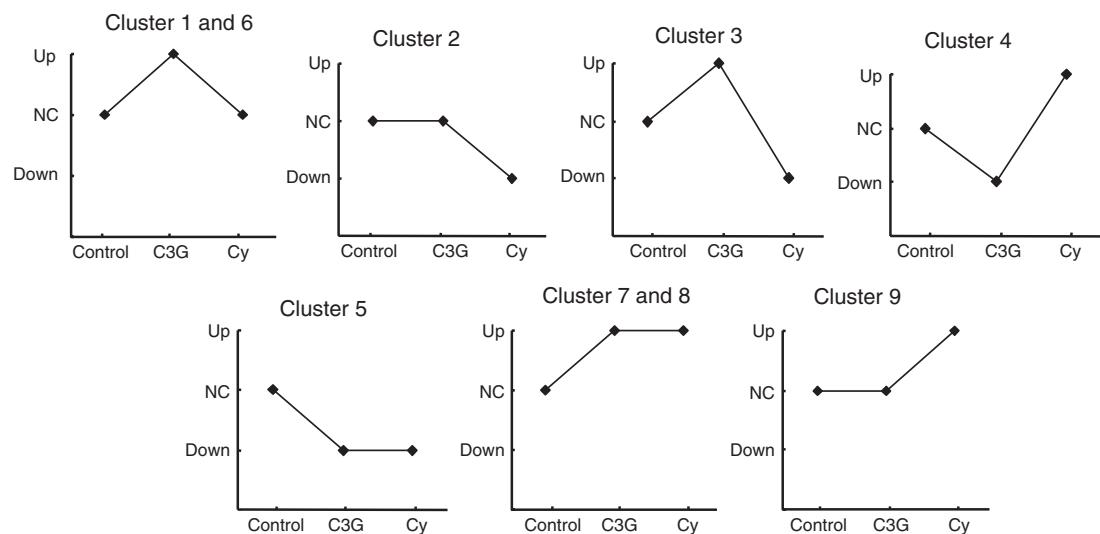
	Control	PCC	HF	HF and PCC
Subcutaneous WAT (g)	$0.53 \pm 0.07^*$	$0.45 \pm 0.08^*$	$1.24 \pm 0.13^{\dagger}$	$0.52 \pm 0.08^*$
Epididymal WAT (g)	$0.81 \pm 0.08^{*\ddagger}$	$0.62 \pm 0.07^{\ddagger}$	$2.16 \pm 0.15^{\dagger}$	$0.99 \pm 0.12^*$
Mesenteric WAT (g)	$0.34 \pm 0.02^*$	$0.29 \pm 0.16^*$	$0.74 \pm 0.11^{\dagger}$	$0.28 \pm 0.02^*$
Retroperitoneal WAT (g)	$0.25 \pm 0.03^{*\ddagger}$	$0.20 \pm 0.03^{\ddagger}$	$0.70 \pm 0.06^{\dagger}$	$0.35 \pm 0.04^*$
Interscapular BAT (g)	$0.17 \pm 0.02^*$	$0.16 \pm 0.02^*$	$0.24 \pm 0.03^{\dagger}$	$0.13 \pm 0.01^*$

PCC, purple corn color; HF, high fat, WAT, white adipose tissue; BAT, brown adipose tissue.

Data were obtained from 6 mice in each group and represent the means  $\pm$  SE.

The values with different superscript symbols are significantly different ( $p < 0.05$ ).

This table was reprinted from Tsuda et al. (2003), with permission from American Society for Nutrition.



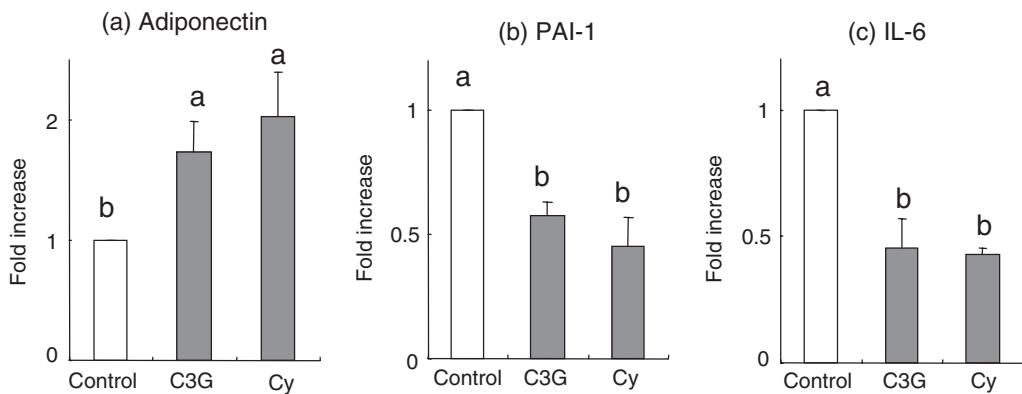
**Figure 18.4.** Profiles of each cluster (clusters 1–9) summarized in a hierarchical clustering display of data in adipocytes treated with cyanidin-3-glucoside (C3G) or cyanidin (Cy) for 845 potentially significant genes. These profiles indicate whether genes in a given cluster are significantly upregulated (up), downregulated (down), or not changed (NC) by the treatment with C3G or Cy. (This figure was reprinted from Tsuda et al. (2006), with permission from Elsevier.)

that obtained using microarray analysis (Tsuda et al. 2006) (Figure 18.5).

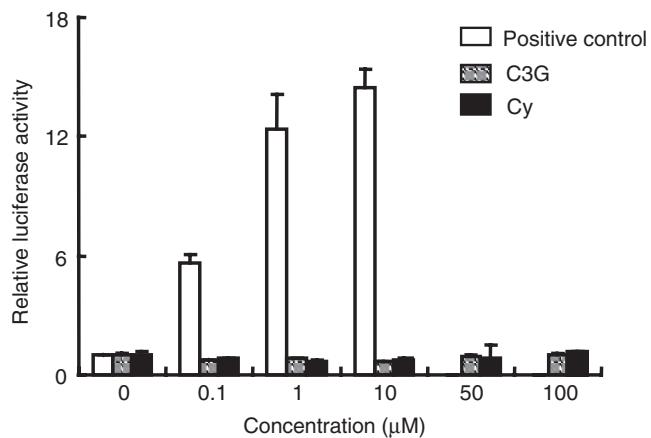
#### ANTHOCYANINS AND PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR- $\gamma$

The gene expression of adiponectin is regulated by peroxisome proliferator-activated receptor-gamma (PPAR $\gamma$ ) (Maeda et al. 2001). PPAR $\gamma$  is a ligand-

activated transcription factor and a member of the nuclear hormone receptor superfamily that functions as a heterodimer with a retinoid X receptor. Agonists, such as thiazolidinediones, induce activation of PPAR $\gamma$ , which causes adipocyte differentiation and insulin sensitivity. The liver receptor homologue-1 (LRH-1) enhances the PPAR $\gamma$ -mediated transactivation of the adiponectin gene in adipocytes (Iwaki et al. 2003). Anthocyanins may act as a PPAR $\gamma$



**Figure 18.5.** Gene expression level of adiponectin (a), PAI-1 (b), and IL-6 (c) in human adipocytes treated with cyanidin-3-glucoside (C3G) or cyanidin (Cy) determined by real-time polymerase chain reaction analyses. Values are means  $\pm$  SE,  $n = 3$ . Means without a common letter differ,  $p < 0.05$ . (This figure was reprinted from Tsuda et al. (2006), with permission from Elsevier.)



**Figure 18.6.** PPAR $\gamma$  transcriptional activity of cyanidin-3-glucoside (C3G), cyanidin (Cy), or positive control (T-174). The activity of a vehicle control (0  $\mu$ M) was set at 1.0 and the relative luciferase activities were presented as fold induction to that of the vehicle control. Values are means  $\pm$  SEM,  $n = 4$ . (This figure was reprinted from Tsuda (2008), with permission of American Chemical Society.)

ligand, resulting in increased adiponectin gene expression. To test this mechanism, the PPAR $\gamma$  ligand and activity of anthocyanins was assayed. These anthocyanins did not induce luciferase activity even if their concentration was increased to 100  $\mu$ M (Tsuda 2008; Tsuda et al. 2004) (Figure 18.6). The data indicated that anthocyanins induce adiponectin gene expression without stimulating PPAR $\gamma$  ligand activity, that is, induction is via a PPAR $\gamma$ -independent mechanism.

## ANTHOCYANINS AND TYPE 2 DIABETES

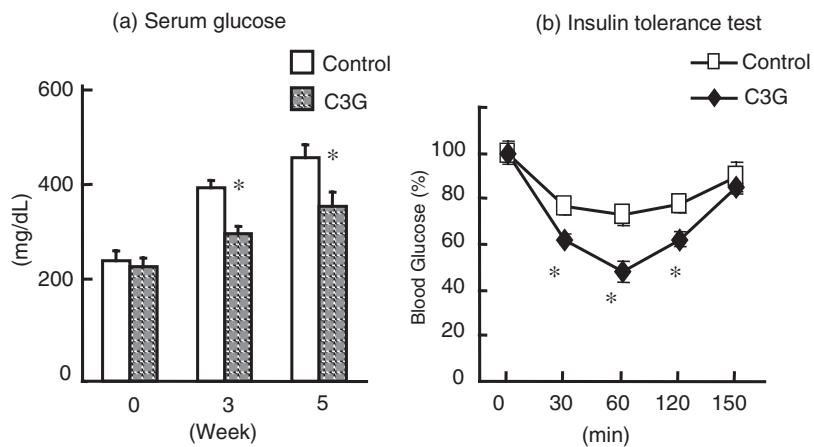
Our prior studies showed that anthocyanins enhanced the gene expression of adiponectin in adipocytes (Tsuda et al. 2004, 2006), increasing interest in the effect of C3G on adipocytokine expression and those adipocytokines responsible for amelioration of insulin resistance in type 2 diabetes.

Male KK- $A^y$  mice, an animal model of type 2 diabetes, were divided into two groups (one fed a control and the other an experimental diet, containing 0.2% purified C3G, for 5 weeks). The blood glucose concentration was significantly suppressed in the C3G group compared to that in the control group during weeks 3–5 (Sasaki et al. 2007) (Figure 18.7). The result of the insulin tolerance test clearly showed that dietary C3G ameliorates insulin resistance (Sasaki et al. 2007) (Figure 18.7).

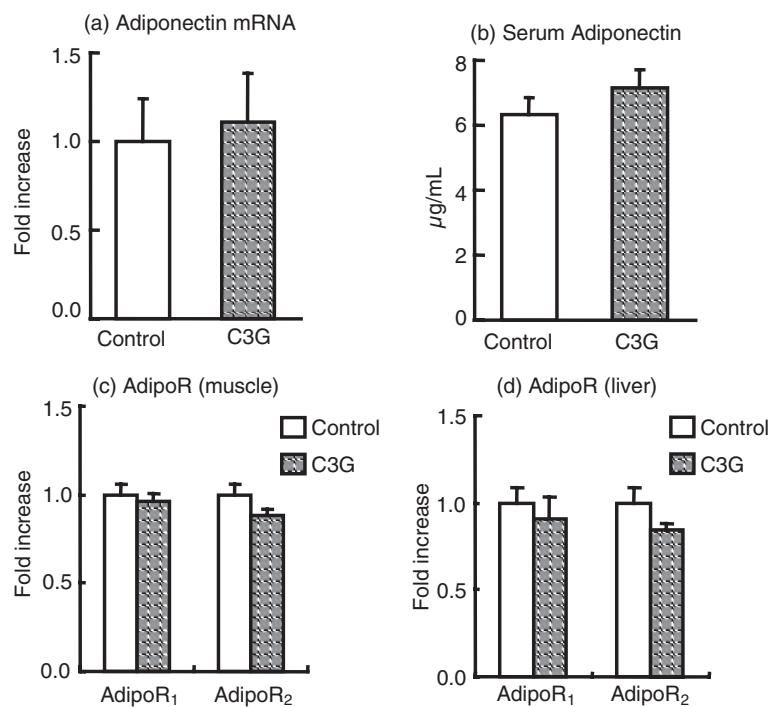
In our previous study, the administration of C3G upregulated the adiponectin gene expression in

adipocytes. We expected that the amelioration of hyperglycemia and insulin resistance would be due to upregulation of adiponectin. Contrary to our expectations, there was no significant between-group difference in adiponectin gene expression. The serum adiponectin concentration was also not affected by the administration of C3G. Furthermore, no significant between-group difference was observed in the expression of the adiponectin receptor genes, AdipoR<sub>1</sub> and R<sub>2</sub>, in both the skeletal muscle and liver (Sasaki et al. 2007) (Figure 18.8).

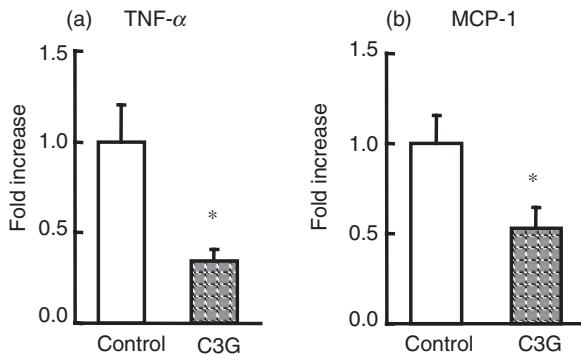
Obesity is associated with macrophage infiltration into adipose tissue and the activation of the inflammatory pathway in conjunction with the development of insulin resistance (Weisberg et al. 2003; Xu et al. 2003). Inflammatory molecules including monocyte chemoattractant protein-1 (MCP-1), TNF- $\alpha$ , IL-6, and PAI-1 are expressed and upregulated in the adipose tissue of obese individuals including those with type 2 diabetes (Alessi et al. 2000; Fried et al. 1998; Hotamisligil et al. 1993; Pickup et al. 2002; Pradhan et al. 2001; Sartipy and Loskutoff 2003). Recent studies have clearly demonstrated that an increase in MCP-1 expression in adipose tissue contributes to the development of insulin resistance and is a significant signal that triggers inflammation characterized by macrophage infiltration into the tissue (Kamei et al. 2006; Kanda et al. 2006; Sartipy and Loskutoff 2003). The gene expression level of TNF- $\alpha$  and MCP-1 in the white adipose tissue was significantly decreased in the C3G group compared to the control group (Sasaki et al. 2007) (Figure 18.9).



**Figure 18.7.** Serum glucose concentration (a) and insulin tolerance test (b) in KK- $A^{\gamma}$  mice fed the control or cyanidin-3-glucoside (C3G) diet for 4 weeks. Values are means  $\pm$  SE,  $n = 6$ . \*, significantly different at  $p < 0.05$  compared to the control in each period. (This figure was reprinted from Sasaki et al. (2007), with permission from Elsevier.)



**Figure 18.8.** Gene expression level of adiponectin in the white adipose tissue (a), serum adiponectin concentration (b), and the gene expression level of adiponectin receptors (Adipo R<sub>1</sub> and R<sub>2</sub>) (c and d) in KK- $A^{\gamma}$  mice fed the control or cyanidin-3-glucoside (C3G) diet for 5 weeks. Values are means  $\pm$  SE,  $n = 6$ . (This figure was reprinted from Sasaki et al. (2007), with permission from Elsevier.)



**Figure 18.9.** Gene expression level of tumor necrosis factor (TNF)- $\alpha$  (a) and monocyte chemoattractant protein-1 (MCP-1) (b) in the white adipose tissue in KK- $A^{\gamma}$  mice fed the control or cyanidin-3-glucoside (C3G) diet for 5 weeks. Values are means  $\pm$  SE,  $n = 6$ . \*, significantly different at  $p < 0.05$  compared to the control. (This figure was reprinted from Sasaki et al. (2007), with permission from Elsevier.)

We have a discrepancy between our prior study using adipocytes and this study using type 2 diabetes model mice concerning adiponectin expression, suggesting that the antidiabetic effect of C3G is through another mechanism. It has been observed that some polyphenols inhibit  $\alpha$ -glucosidase activity. However, this effect of C3G and cyanidin (an aglycone of C3G) occurred at an extremely low level, and the amelioration of insulin resistance by dietary C3G was not due to inhibition of  $\alpha$ -glucosidase activity (Iwai et al. 2006; Matsui et al. 2001).

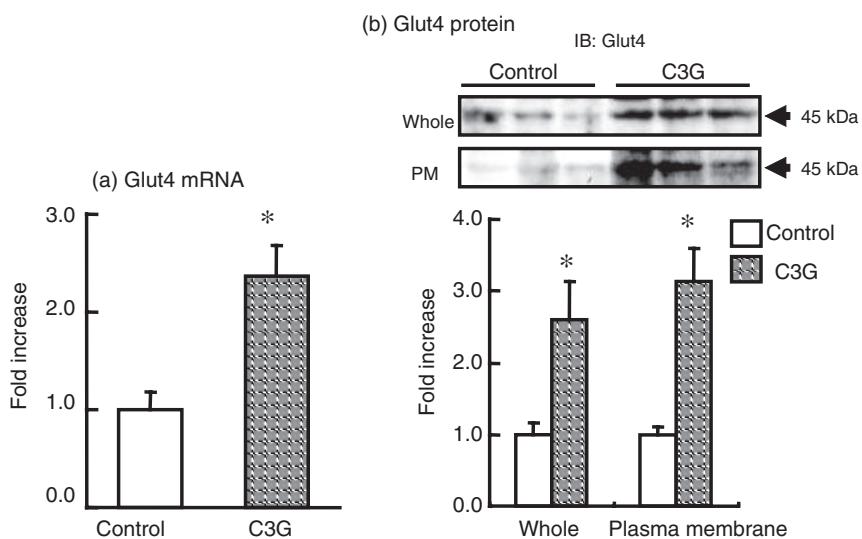
Yang et al. (2005) recently showed that retinol binding protein 4 (RBP4) is an adipocytokine and its expression and secretion in adipose tissue is closely associated with glucose uptake and insulin sensitivity. In the obese or diabetic state, the expression of glucose transporter 4 (Glut4) is reduced in adipocytes, and the reduction is accompanied by an increase in RBP4 expression and secretion into the blood. This increase causes impairment of insulin signaling in skeletal muscle and stimulates glucose production in liver. These changes lead to a high glucose concentration in the blood. Therefore, dysregulation of the adipocyte Glut4–RBP4 system is strongly associated with metabolic syndrome-related type 2 diabetes, and lowering RBP4 is a new important target molecule for the prevention and therapy of type 2 diabetes.

Recent studies also demonstrate association of RBP4 with insulin resistance and association of single nucleotide polymorphism in the RBP4 gene with type 2 diabetes in human subjects (Cho et al. 2006; Craig et al. 2006; Gavi et al. 2007; Graham et al. 2006; Munkhtulga et al. 2007). However, some reports found no correlation of RBP4 level with obesity (Janke et al. 2006; Takashima et al. 2006).

In obesity and type 2 diabetes, Glut4 expression is decreased in adipose tissue but not in skeletal muscle. The expression of Glut4 changes glucose influx, which in turn regulates RBP4 expression and secretion from adipocytes. In our study, Glut4 gene expression in white adipose tissue was significantly higher in the C3G group. Also, protein expression was significantly enhanced in the C3G group in both whole-cell lysate and plasma membrane (Sasaki et al. 2007) (Figure 18.10).

Interestingly, the RBP4 gene expression level in white adipose tissue was significantly suppressed in the C3G group, but in the liver, it did not differ between groups. Serum RBP4 concentration was significantly reduced in the C3G group. Glucose-6-phosphatase is one of the rate-limiting gluconeogenic enzymes, and the gene expression level of glucose-6-phosphatase was significantly suppressed in the C3G group by administration of C3G (Sasaki et al. 2007) (Figure 18.11).

The antidiabetic mechanism of dietary C3G in the type 2 diabetic model involves elevation of Glut4 expression, which contributes to the suppression of RBP4 in white adipose tissue. These decrease glucose output into the blood and increase insulin sensitivity. A decrease in MCP-1 expression mediated by C3G can contribute to inhibition of downregulation of Glut4 expression (Sartipy and Loskutoff 2003). Another possible mechanism for regulation of Glut4 expression or translocation to the plasma membrane is related to the PPAR $\gamma$  or AMP-activated protein kinase (AMPK) (Yamaguchi et al. 2005). Interestingly, although the data are not shown, AMPK activation in adipocytes has been induced by administration of anthocyanin (Tsuda et al. 2004). C3G may stimulate AMPK activation and enhance Glut4



**Figure 18.10.** Gene expression level (a) and protein level (b) of Glut4 in the white adipose tissue of KK- $A^{\gamma}$  mice fed the control or cyanidin-3-glucoside (C3G) diet for 5 weeks. Values are means  $\pm$  SE,  $n = 5$ –7. \*, significantly different at  $p < 0.05$  compared to the control. (This figure was reprinted from Sasaki et al. (2007), with permission from Elsevier.)

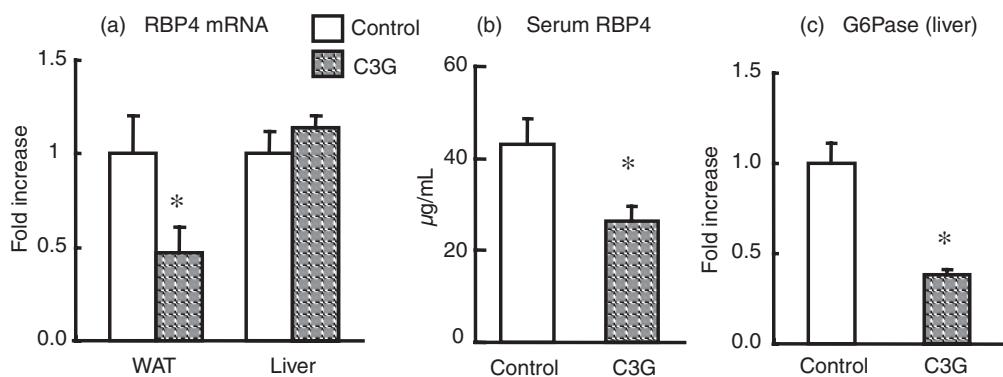
expression in adipose tissue (Sasaki et al. 2007) (Figure 18.12).

## VISION IMPROVEMENT

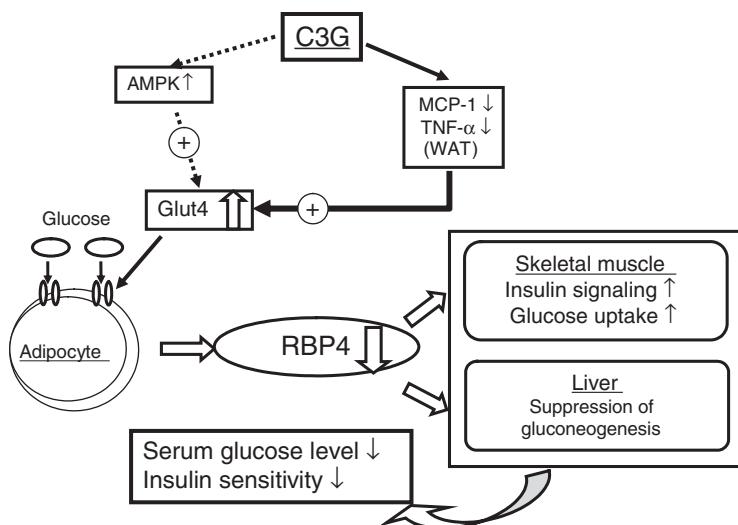
### TRANSIENT REFRACTIVE ALTERATION STUDY

The degradation of refraction and accommodation is an important diagnostic feature of asthenopia. The

effect of BCA on visual display terminal (VDT) task-induced refractive and accommodative alteration was examined in a randomized double-blind placebo-controlled, crossover study with young human subjects (Nakaishi et al. 2000). Test sample (200 mL of juice containing 50 mg of BCAs, including D3R (25.1 mg), D3G (7.4 mg), C3R (15.4 mg), and C3G (2.2 mg)) or a placebo with taste and color equivalent to that of the test sample was administered to 21



**Figure 18.11.** Gene expression level of RBP4 in the white adipose tissue or liver (a), serum RBP4 concentration (b), and gene expression level of glucose-6-phosphatase (G6Pase) (c) in the liver in KK- $A^{\gamma}$  mice fed the control or cyanidin-3-glucoside (C3G) diet for 5 weeks. Values are means  $\pm$  SE,  $n = 6$ . \*, significantly different at  $p < 0.05$  compared to the control. (This figure was reprinted from Sasaki et al. (2007), with permission from Elsevier.)



**Figure 18.12.** Proposed scheme for amelioration of hyperglycemia and insulin sensitivity by the cyanidin-3-glucoside (C3G). (This figure is reprinted from Sasaki et al. (2007), with permission from Elsevier.)

healthy subjects (aged 20–25 years; mean 20.9 years old). They were confirmed to be free of any ocular diseases, refractive errors (high myopia of more than 4 diopters (D), hyperopia more than 1 D, or astigmatism at the steepest meridian of more than 1.5 D), or presbyopia at the time of enrollment. All the subjects gave written informed consent according to the Declaration of Helsinki prior to the start of the study.

The refraction values for the dominant eye were measured by autorefractometry (Nidek AR-600A, Japan), flicker values (i.e., critical flicker fusion (CFF) frequency) using a Flicker 501 (Takei Kiki Kogyo, Japan), and visual analogue scale (VAS) questionnaire for the assessment of subjective asthenopia symptoms (Kirshner and Guyatt 1985). The 2-hour long near visual task consisted of performing a simple calculation on a VDT, which is a modification of the task used in the Kraepelin test method (Japan Psychotechnology Institute 1990). All measurements were performed before and after intake of the test sample (BCA concentrate or placebo). Comparison was made by means of a paired *t*-test. Refraction values (in diopters) for the dominant eye were measured before and after the visual task, and the changes are summarized in Table 18.2. The refraction values of those receiving BCA were unaffected by the visual task, whereas the values of those receiving the placebo exhibited a borderline significant decrease ( $p = 0.064$ ). Average changes following intake of BCA and placebo ( $-0.030 \pm 0.252$ D and  $0.119 \pm 0.278$ D, respectively) were significantly different ( $p = 0.006$ ).

The flicker values and assessment of subjective visual fatigue symptoms before and after the task in subjects given BCA or placebo are summarized in Table 18.3. Statistical analysis found no significant difference in flicker value between the BCA and placebo groups. Assessment of subjective asthenopia symptoms using a five-question questionnaire yielded higher average values after the task than before in both the BCA and placebo groups. Furthermore, the average value obtained for each of the five questions was smaller after BCA intake than after placebo intake. Statistical analysis showed a significant between-group difference in eye and lower back fatigue. These results suggest that the intake of dietary anthocyanins may help prevent myopic refractory shift due to visual tasks or promote visual recovery.

As shown in Table 18.3, all average values were smaller after the task than before the task; thus, the task was considered to be an adequate inducer of visual fatigue. In our study, BCA intake significantly reduced eye and lower back fatigue—a result that supports the finding that visual fatigue is mainly due to a temporary shift in refraction (Grandjean et al. 1980).

#### CILIARY MUSCLE RELAXATION INDUCED BY ANTHOCYANIN

One theory for the development of refractive myopia is that the ciliary muscle (CM) becomes spastic as a result of excessive contraction during

**Table 18.2.** Refraction values for the dominant eye, measured before and after the task in subjects given BCA or placebo, and the change in transient refractive alteration.

Subject eye number	BCA			Placebo		
	Before	After*	Change*	Before	After*	Change*
1	-0.91	-1.14	0.23	-0.99	-1.10	0.11
2	-0.08	-0.07	-0.01	0.04	-0.60	0.64
3	-1.13	-1.01	-0.12	-1.29	-0.96	-0.33
4	-0.77	-0.83	0.06	-0.85	-0.86	0.01
5	0.57	0.59	-0.02	0.33	0.27	0.06
6	-0.96	-0.95	-0.01	-0.74	-1.18	0.44
7	-0.22	-0.81	0.59	-0.14	-0.68	0.54
8	-0.01	0.53	-0.54	-0.28	0.07	-0.35
9	-0.47	-0.30	-0.17	-0.50	-0.45	-0.05
10	-0.20	-0.44	0.24	-0.28	-0.58	0.30
11	-0.80	-0.73	-0.07	-0.66	-0.85	0.19
12	0.47	0.43	-0.04	0.66	0.46	0.20
13	-0.81	-0.67	-0.14	-0.61	-0.77	0.16
14	-0.08	0.11	-0.19	-0.08	-0.25	0.17
15	-0.15	-0.28	0.13	-0.21	-0.25	0.04
16	0.40	0.20	0.20	0.34	0.11	0.23
17	0.05	0.21	-0.16	0.19	0.49	-0.30
18	-0.58	-0.51	-0.07	-0.32	-0.58	0.26
19	-1.75	-1.63	-0.12	-1.13	-1.44	0.31
20	-1.29	-1.37	0.08	-1.20	-1.42	0.22
21	-0.35	0.22	-0.57	-0.35	0.00	-0.35
Mean $\pm$ SD	-0.432 $\pm$ 0.602	-0.402 <sup>†</sup> $\pm$ 0.643	-0.030 <sup>‡</sup> $\pm$ 0.252	-0.384 $\pm$ 0.536	-0.503 <sup>§</sup> $\pm$ 0.579	0.119 <sup>¶</sup> $\pm$ 0.278

\*Statistical analysis was independently carried out for each dominant eye comparing the black currant anthocyanin (BCA) and placebo groups.

Values with different superscript symbols (<sup>†,‡,§,¶</sup>) are significantly different ( $p < 0.05$ ).

close-up work, leading to abrupt change in the refractive power of the lens. As a consequence, the myopic CM cannot relax sufficiently to allow the lens to focus on distant images (Tokoro et al. 1998). This led us to hypothesize that this ophthalmologic beneficial effect resulted from anthocyanin-induced relaxation of spasmoidically contracted CMs. The cause of asthenopia (visual fatigue) is considered to be this transient change in refraction. To clarify the mechanism of transient change in refraction, we examined the effect of D3R on endothelin-1 (ET-1)-induced contraction of bovine ciliary smooth muscle. We attempted to clarify the relevant signal-transduction pathways affected by D3R (Matsumoto et al. 2005). Specimens of ciliary smooth muscle from bovine eye (strips approximately 2-mm wide and 5-mm long from the lateral portion of the ciliary body) were isolated and muscle tension was measured according to a previously described method (Kamikawatoko et al. 1995). After reaching steady-state isometric tension (i.e., the

baseline), the CM was precontracted twice at 20-min intervals using physiological saline solution (PSS) containing 65 mM KCl. After washing and returning to the baseline tension, the muscle was treated with a pharmacological concentration of a muscle stimulant ( $10^{-8}$  M ET-1) and the resulting change in isometric tension (approximately 150 mg) was measured and recorded. When the tension had stabilized (20 min after the addition of ET-1), a single aliquot of D3R ( $3 \times 10^{-5}$  M), C3R, myricetin-3-rutinoside (M3R), or quercetin-3-rutinoside (Q3R) was added and the resulting relaxation within 40–60 min was measured (Figure 18.13). The values for D3R were significantly different ( $p < 0.01$ ) from the spontaneous decrease in tension that occurred without the addition of any compound. Both C3R ( $3 \times 10^{-5}$  M) and BCA (50 mg/ml) powder also had significant relaxation activity ( $p < 0.05$ ), while Q3R and M3R only induced relaxation 60 min after addition of the compound (Figure 18.14). In contrast, none of these

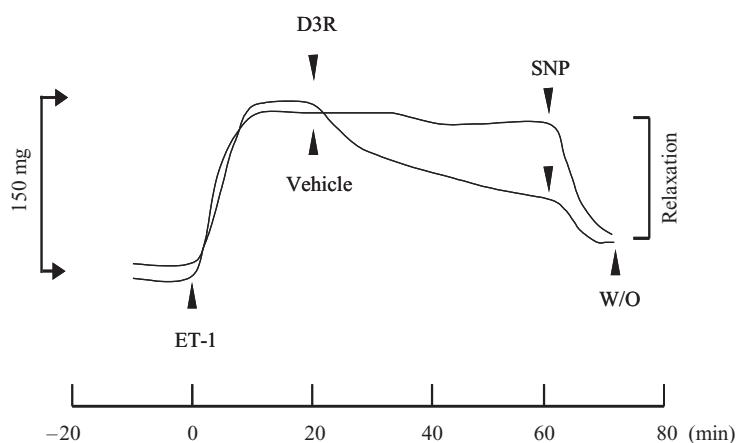
**Table 18.3.** Flicker values and assessment of subjective asthenopia symptoms, measured before and after the task in subjects given BCA or placebo.\*

Item and statement	BCA			Placebo		
	Before	After <sup>†</sup>	Change <sup>†</sup>	Before	After <sup>†</sup>	Change <sup>†</sup>
Flicker value, Hz	34.95 ± 3.16	34.39 ± 3.51	0.56 ± 1.15	34.72 ± 2.99	34.13 ± 2.90	0.59 ± 1.22
Asthenopia symptoms, (VAS mm)						
Head & neck	12.70 ± 13.45	40.08 ± 24.86	27.38 ± 18.39	8.34 ± 11.87	44.09 ± 26.09	35.75 ± 24.96
Arm	10.21 ± 17.82	36.15 ± 25.86	25.94 ± 29.61	4.32 ± 6.27	41.76 ± 29.33	37.44 ± 28.43
Eye	14.72 ± 15.55	47.31 ± 24.72	32.59 ± 18.94 <sup>§</sup>	14.59 ± 17.98	56.72 ± 25.24	42.14 ± 19.52 <sup>  </sup>
Shoulder	15.12 ± 15.65	49.66 ± 27.97	34.54 ± 25.75	10.95 ± 17.42	54.31 ± 29.31	43.36 ± 30.91
Low back	10.63 ± 15.92	29.79 ± 27.15 <sup>‡</sup>	19.16 ± 22.74 <sup>§</sup>	7.35 ± 9.25	42.83 ± 33.55 <sup>¶</sup>	35.48 ± 30.87 <sup>  </sup>

\*Expressed as mean ± SD.

<sup>†</sup>Statistical analysis comparing the values after the test, and the change in values was independently carried out in each horizontal row.

Values with different superscript symbols (<sup>‡</sup>, <sup>§</sup>, <sup>¶</sup>, <sup>||</sup>) are significantly different ( $p < 0.05$ ).

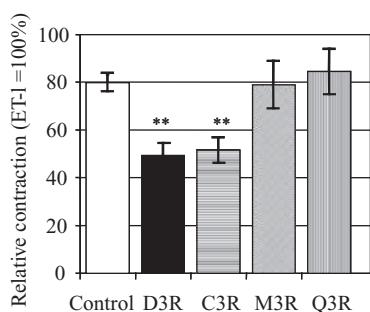


**Figure 18.13.** Ciliary muscle relaxation effect by delphinidin-3-rutinoside (D3R) against endothelin-1 (ET-1)-induced contraction.

polyphenolic compounds had an effect on contraction induced by  $10^{-5}$  M carbachol.

#### INHIBITORY EFFECT OF D3R AND PHARMACOLOGICAL ANALYSES

Using pretreatment inhibition, we attempted to clarify the signal-transduction pathways in ciliary smooth muscle affected by D3R. The amount of contraction was measured and expressed as a percentage of contraction induced in KPSS (Krebs' solution in which NaCl is replaced by 65 mM KCl).



**Figure 18.14.** Summary of data obtained from relaxation during a 60-min treatment with four flavonoids compared to control. \*\*, significantly different at  $p < 0.01$  compared to the control. The data represent the force generated in the presence of endothelin-1 (ET-1) ( $10^{-8}$  M) set to 100% with the values expressed as mean  $\pm$  SEM from six replicate specimens. D3R, delphinidin-3-rutinoside; C3R, cyanidin-3-rutinoside; M3R, myricetin-3-rutinoside; Q3R, quercetin-3-rutinoside.

The results were expressed as percentage decreases in maximal contraction induced by ET-1, with 100% relaxation being the point at which baseline tension was reached. Pretreatment with  $10^{-4}$  M D3R significantly reduced  $10^{-8}$  M ET-1-induced contraction from  $54.9 \pm 15.0\%$  (the control value) to  $42.2 \pm 3.2\%$  ( $p < 0.05$ ). In contrast, pretreatment with  $10^{-4}$  M D3R had no effect on carbachol-induced ( $10^{-9}$  to  $10^{-5}$  M) contraction.

The influence of several inhibitors and an antagonist of D3R was examined in order to investigate the relaxation mechanism. The addition of propranolol ( $\beta$ -adrenergic receptor blocker), iberiotoxin ( $10^{-7}$  M;  $K^+$  channel blocker), or indomethacin ( $10^{-4}$  M; cyclooxygenase inhibitor) to the bath 20 min prior to the addition of D3R resulted in no significant change in the inhibitory effect of D3R on ET-1 ( $10^{-8}$  M) induced contraction (Table 18.4), suggesting that the relaxation mechanism of D3R does not involve  $\beta$ -adrenergic or maxi- $K^+$  channels, nor the PGI2 pathway (Table 18.4).

Pretreatment with  $10^{-4}$  M NOARG (NG-nitro-L-arginine), an inhibitor of nitric oxide synthase, increased the contraction ratio and reversed the inhibitory effect of D3R (Table 18.4). The contraction ratio due to D3R was increased from  $42.2 \pm 3.2\%$  to  $63.7 \pm 7.1\%$  in the presence of NOARG ( $p < 0.01$ ) and was decreased from  $63.7 \pm 7.1\%$  to  $42.7 \pm 4.2\%$  in the presence of NOARG plus L-arginine ( $10^{-4}$  M) ( $p < 0.05$ ). The guanylyl cyclase (GC) inhibitor, 1H-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ) ( $10^{-4}$  M), added to the bath 20 min prior to the addition of D3R increased the contraction ratio from  $42.2 \pm 3.2\%$  to  $58.5 \pm 5.5\%$  ( $p < 0.05$ ), but ODQ alone had no effect on contraction ( $56.1 \pm$

**Table 18.4.** The comparison of contraction by ET-1 ( $10^{-8}$  M) after treatment of reagent.

Treatment	Concentration (M)	n	Contraction (%) (average $\pm$ SE)
Control	—	21	54.9 $\pm$ 3.3*
D3R	$10^{-4}$	21	42.2 $\pm$ 3.2
D3R + NOARG	$10^{-4} + 10^{-4}$	12	63.7 $\pm$ 7.1*
D3R + NOARG + L-Arg	$10^{-4} + 10^{-4} + 10^{-4}$	12	42.7 $\pm$ 4.2
Carboxy-PTIO	$3 \times 10^{-4}$	10	54.4 $\pm$ 2.1
D3R + Carboxy-PTIO	$10^{-4} + 3 \times 10^{-4}$	10	55.2 $\pm$ 3.2*
ODQ	$10^{-4}$	11	56.1 $\pm$ 5.4
D3R + ODQ	$10^{-4} + 10^{-4}$	11	58.5 $\pm$ 5.5*
BQ788	$10^{-7}$	12	81.0 $\pm$ 8.2
D3R + BQ788	$10^{-4} + 10^{-7}$	12	73.4 $\pm$ 9.6*
Propranolol	$10^{-4}$	9	61.3 $\pm$ 3.6
D3R + propranolol	$10^{-4} + 10^{-4}$	8	48.7 $\pm$ 3.4†
Iberiotoxin	$10^{-7}$	12	64.6 $\pm$ 3.0
D3R + iberiotoxin	$10^{-4} + 10^{-7}$	12	45.7 $\pm$ 4.8‡
Indomethacin	$10^{-4}$	12	70.3 $\pm$ 5.3
D3R + indomethacin	$10^{-4} + 10^{-4}$	12	52.4 $\pm$ 5.2§

NOARG, NG-nitro-L-arginine; ET-1, endothelin-1; D3R, delphinidin-3-rutinoside; ODQ, 1H-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one.

\*Significant difference at  $p < 0.05$  versus D3R treatment.

†Significant difference at  $p < 0.05$  versus propranolol treatment.

‡Significant difference at  $p < 0.05$  versus iberiotoxin treatment.

§Significant difference at  $p < 0.05$  versus indomethacin treatment.

5.4%). In another set of experiments, the addition of carboxy-PTIO ( $3 \times 10^{-4}$  M; an NO scavenger) to the bath 20 min prior to the addition of D3R increased the contraction ratio from  $42.2 \pm 3.2\%$  to  $55.2 \pm 3.2\%$  ( $p < 0.05$ ), but carboxy-PTIO alone could increase contraction ( $54.4 \pm 2.1\%$ ) (Table 18.4). These results indicate that the inhibitory effect induced by D3R was caused by enhanced synthesis of NO in the endothelium. Addition of BQ788 ( $10^{-7}$  M; a selective antagonist of the ET<sub>B</sub> receptor) to the incubation system augmented the contraction ratio from  $42.2 \pm 3.2\%$  to  $73.4 \pm 9.6\%$  ( $p < 0.01$ ), but BQ788 alone could increase contraction ( $81.0 \pm 8.2\%$ ) (Table 18.4). These results confirmed that D3R enhances synthesis of NO that in turn stimulates the ET<sub>B</sub> receptor in CMs.

#### MYOSIN REGULATORY LIGHT CHAIN PHOSPHORYLATION AND CYCLIC GMP PRODUCTION

Myosin light chain phosphorylation–dephosphorylation regulates smooth muscle contraction, and we were able to show changes in this ratio during D3R-induced relaxation. To confirm the inhibitory

effect of D3R pretreatment on contraction, we measured myosin regulatory light chain phosphorylation in bovine CM during stable contraction following the addition of ET-1 ( $10^{-8}$  M).

After the specific treatments, mounted muscles were quick-frozen using tongs prechilled in liquid nitrogen. The frozen muscles were then weighed and placed in a frozen slurry of 10% w/v trichloroacetic acid (TCA) in acetone containing 10 mM 1,4-dithiothreitol (DTT). Using a previously published method to measure phosphorylated and nonphosphorylated forms of smooth muscle myosin and calculate phosphorylation ratio (Lau et al. 1998), we found that this ratio decreased significantly from  $47.2 \pm 2.9\%$  to  $36.0 \pm 3.0\%$  ( $p < 0.01$ ). Pretreatment with NOARG increased the ratio from  $36.0 \pm 3.0\%$  to  $50.3 \pm 2.5\%$  ( $p < 0.01$ ), while pretreatment with an excess amount of L-arginine decreased the ratio from  $50.3 \pm 2.5\%$  to  $38.2 \pm 3.8\%$  ( $p < 0.05$ ) (Table 18.5).

Cyclic GMP was measured in quick-frozen CM. The dry extract was pretreated with IBMX ( $1 \times 10^{-5}$  mol/L) to inhibit degradation of cyclic GMP content, which was quantified by radioimmunoassay according to a previously published method (Lau et al. 1998). Basal cyclic GMP level (i.e., CM strips

**Table 18.5.** Comparison between contraction induced by ET-1 and phosphorylated myosin light chain.

Pretreatment	Concentration (M)	n	Contraction (%) (average $\pm$ SE)	Phosphorylated ratio (%) (average $\pm$ SE)
Control	—	21	54.9 $\pm$ 3.3*	47.2 $\pm$ 13.1*
D3R	10 <sup>-4</sup>	21	42.2 $\pm$ 3.2	36.0 $\pm$ 13.6
D3R + NOARG	10 <sup>-4</sup> + 10 <sup>-4</sup>	12	63.7 $\pm$ 7.1*	50.1 $\pm$ 4.2*
D3R + NOARG + L-Arg	10 <sup>-4</sup> + 10 <sup>-4</sup> + 10 <sup>-4</sup>	12	42.7 $\pm$ 4.2†	38.2 $\pm$ 3.7†

NOARG, NG-nitro-L-arginine; ET-1, endothelin-1; D3R, delphinidin-3-rutinoside.

\*Significant difference at  $p < 0.05$  versus D3R treatment.

†Significant difference at  $p < 0.05$  versus D3R + NOARG.

‡Significant difference at  $p < 0.01$  versus D3R + NOARG.

at rest without contraction) was determined to be  $13.4 \pm 0.6$  pmol/mg (Kamikawatoko et al. 1998). ET-1 decreased cyclic GMP level up to  $6.58 \pm 0.78$  pmol/mg (Table 18.6). The presence of  $10^{-5}$  M IBMX significantly decreased ET-1-induced contraction, while D3R decreased  $10^{-8}$  M ET-1-induced contraction and increased cyclic GMP production ( $9.39 \pm 0.96$  pmol/mg) (Table 18.6). These changes were again abolished by  $10^{-4}$  M NOARG ( $5.09 \pm 0.37$  pmol/mg).

#### RADIOLIGAND RECEPTOR BINDING ASSAY

To confirm the localization of ET receptor, receptor binding assay were carried out in CM and ciliary epithelium cells (CE), according to a method described previously (Matsumoto et al. 2005). The radiolabeled ligand used for saturation analysis was [<sup>125</sup>I]ET-1. Specific binding was defined as total binding minus nonspecific binding measured in the presence of 125 nmol/L unlabeled ET-1. Specific binding of [<sup>125</sup>I]ET-1 (50 pmol/L) was displaced by BQ123 ( $1 \times 10^{-10}$  to  $7.81 \times 10^{-6}$  mol/L) and BQ788 ( $1 \times 10^{-10}$  to  $7.81 \times 10^{-6}$  mol/L).

The [<sup>125</sup>I]ET-1 binding was saturated with high affinity. Scatchard plot analysis revealed that the

binding sites of [<sup>125</sup>I]ET-1 constituted a single population. Hill coefficients were not different from unity ( $n_H = 0.99 \pm 0.02$  in EC and  $n_H = 1.04 \pm 0.04$  in CM). The dissociation equilibrium constant ( $K_d$ ) and receptor density ( $B_{max}$ ) values were determined to be  $54.5 \pm 4.6$  nM ( $n = 4$ ) and  $168.4 \pm 25.4$  fmol/mg protein ( $n = 4$ ), respectively, in the CE, and  $141.7 \pm 18.0$  nM ( $n = 4$ ) and  $357.7 \pm 35.8$  fmol/mg protein ( $n = 4$ ), respectively, in the CM. The specific [<sup>125</sup>I]ET-1 binding in bovine CM or CE membranes was partially inhibited (approximately 40%) by BQ123 as a selective ET<sub>A</sub> receptor antagonist, whereas the binding was completely inhibited by BQ788 as a selective ET<sub>B</sub> receptor antagonist. The  $K_i$  values for BQ788 were calculated to be  $56.7 \pm 10.8$  pM in CE and  $93.4 \pm 23.3$  pM in CM. The ET<sub>B</sub> receptor was the predominant subtype in CE and CM. However, kinetics of the binding was different between CM and CE.

#### MECHANISM OF CILIARY MUSCLE RELAXATION BY ANTHOCYANIN

D3R had a preventive effect on ET-1 induced contraction in ciliary smooth muscle with a concomitant increase in cyclic GMP production and decrease

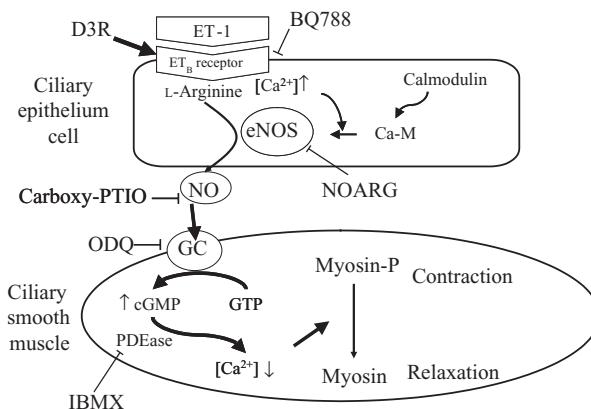
**Table 18.6.** A comparison between contraction induced by ET-1 and changes in the cyclic GMP levels.

Treatment	Concentration (M)	n	Contraction (%) (mean $\pm$ SE)	cGMP levels (pmol/mg tissue) (mean $\pm$ SE)
Control( $10^{-8}$ M ET-1)	—	12	40.7 $\pm$ 3.5*	6.58 $\pm$ 0.78*
+D3R	10 <sup>-4</sup>	12	28.2 $\pm$ 3.5	9.39 $\pm$ 0.96
+D3R + NOARG	10 <sup>-4</sup> + 10 <sup>-4</sup>	12	38.2 $\pm$ 3.5*	5.09 $\pm$ 0.37†

ET-1, endothelin-1; D3R, delphinidin-3-rutinoside.

\*Significant difference at  $p < 0.05$  versus D3R treatment.

†Significant difference at  $p < 0.01$  versus D3R treatment.



**Figure 18.15.** Schematic diagram of relaxation induced by delphinidin-3-rutinoside (D3R). GC, guanylyl cyclase; NOARG, NG-nitro-L-arginine; NO, nitric oxide; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one.

in phosphorylation ratio of myosin regulatory light chain. The inhibitory effect of D3R was significantly attenuated in the presence of NOARG (a nitric oxide synthase inhibitor), carboxy-PTIO (an NO scavenger), ODQ (an inhibitor of GC), or BQ788 (a selective ET<sub>B</sub> receptor antagonist).

Taken together, these results led us to speculate that D3R may stimulate ET<sub>B</sub> receptors to produce and release NO, which in turn results in relaxation of ciliary smooth muscles.

However, iberiotoxin (a Ca<sup>2+</sup>-activated K<sup>+</sup> channel inhibitor), propranolol (a  $\beta$ -adrenoceptor antagonist), and indomethacin (a cyclooxygenase inhibitor) all failed to modify D3R-induced relaxation. These results suggested that  $\beta$ -adrenoceptors, the PGI<sub>2</sub> pathway, and K<sup>+</sup> channels are not involved in the relaxation mechanism of D3R. In summary, D3R relaxed or induced reduction in the contraction of CM stimulated with ET-1 (Figure 18.15).

While Q3R and M3R have no effect on CM contraction, the  $\beta$ -ring structure of anthocyanins appears to be important to the relaxation effect. The results of our series of experiments suggest a potential beneficial effect of black currant or their anthocyanins in the prevention of myopia and glaucoma.

#### OTHER EFFECTS OF BCAs ON VISION

The effect of oral intake of BCAs on dark adaptation was examined in a double-blind, placebo-controlled, crossover study with healthy human subjects. Intake of BCA at three dose levels (12.5, 20, and 50 mg per subject,  $n = 12$ ) appeared to bring about dose-dependent lowering of the dark adaptation threshold (50-mg dose,  $p = 0.011$ ) (Nakaishi et al. 2000). To clarify the role of anthocyanins in improving dark

adaptation, the effects of four anthocyanins from black currant fruits on the regeneration of rhodopsin in frog reactive oxygen species membranes were examined. C3G and C3R but not the corresponding delphinidins stimulated regeneration (Matsumoto et al. 2003).

The effect of BCA intake on accommodation in response to visual fatigue caused by staying awake for 24 h was examined in a randomized double-blind placebo-controlled, crossover study with 19 healthy human subjects. Accommodation was measured using a computer-assisted infrared optometer. At the near point (refractive value +2 D) and far point of accommodation, BCA intake significantly ( $p = 0.040$  and  $p = 0.025$ ) improved refractive outcome compared with placebo control (Iida et al. 2008).

## CONCLUSION

Clearly, anthocyanins have an important role as functional nutrients in preventing obesity and diabetes, and improving vision. Long-term clinical studies are necessary to establish the beneficial effects of anthocyanins.

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# 19

## Licorice Flavonoids

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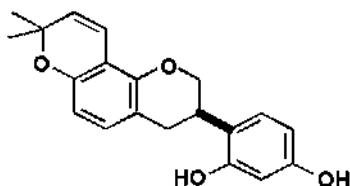
### INTRODUCTION

Licorice, the root of the leguminous *Glycyrrhiza* plant species, is one of the most useful and popular plants in both Asia and Europe, and the history of its consumption as a traditional medicine and food goes back for over 4,000 years to the era of ancient Mesopotamia and Egypt (Davis and Morris 1991; Fenwick et al. 1990; Shibata 2000). In the leguminous *Glycyrrhiza* plant genus, there are three major varieties: *Glycyrrhiza glabra* Linne, *Glycyrrhiza uralensis* Fischer, and *Glycyrrhiza inflata* Batalin (Kondo et al. 2007b; Nomura and Fukai 1998). Among these varieties, *G. glabra* is the most popular and is used as a food ingredient. In Japan and China, licorice root and its extracts have been generally used as herbal medicines or as a sweetening agent for foods. In Europe, these varieties have been routinely consumed as licorice confectionery, flavorings, herbal teas, and beverages. Furthermore, in the United States, licorice has generally recognized as safe (GRAS) status. In view of the fact that licorice has been consumed over such a long period, it could be regarded as a typical functional food, which is defined as a substance situated between foods and pharmaceuticals.

Licorice contains triterpenes and phenolic constituents (Nomura and Fukai 1998), such as glycyrrhizin, a well-known typical active constituent of licorice, and the species-specific constituents, glabridin, glycy coumarin, and licochalcone A in *G. glabra*, *G. uralensis*, and *G. inflata*, respectively (Kondo et al. 2007a). In *G. glabra*, the species specific compound is glabridin (Figure 19.1), a prenylated flavonoid, which is a major flavonoid of *G. glabra* and found exclusively in the roots (Hayashi et al. 1996).

Various studies have shown the biological effects of glabridin, licorice, or its extracts. These include antioxidative (Belinky et al. 1998; Wojcikowski et al. 2007), estrogen-like (Nomura et al. 2002), anti-inflammatory (Yokota et al. 1998) and anti-*Helicobacter pylori* (Fukai et al. 2002) activities. They have also been shown to have clinical efficacy in the treatment of conditions such as gastric ulcer, chronic hepatitis, diabetes, and allergic inflammation (Davis and Morris 1991; Shibata 2000). It has also been reported that licorice ethanolic extract has antioxidative properties and clinical effects against hypercholesterolemia (Fuhrman et al. 1997, 2002).

Recently, human lifestyles and behavior have been changing dramatically. These changes have resulted in a steadily increasing number of obese individuals worldwide, and have created a new kind of problem that needs to be solved not only at the personal level but at the social level as well. Obesity results from an energy imbalance due to excessive energy intake and insufficient energy expenditure. Excessive body fat often results in significant health problems, such as cardiovascular diseases, arteriosclerosis, osteoarthritis, and certain cancers. Metabolic syndrome is a condition that can eventually develop into arteriosclerotic disease, and is now defined as a condition characterized by clusters of multiple risk factors, such as dyslipidemia, hypertension, and hyperglycemia, in addition to visceral fat obesity (Zimmet 2005). Accumulation of visceral fat is considered to be a major cause of metabolic syndrome (Matsuzawa 2006). Reducing the accumulation of visceral fat and maintaining an optimal body weight are very important in the prevention and treatment of metabolic syndrome. In combination with lifestyle modification, such as appropriate diet or exercise, there is an expectation that the ingestion of evidence-based functional



**Figure 19.1.** The structure of glabridin.

foods may help to prevent or ameliorate lifestyle-related diseases such as metabolic syndrome caused by a high-fat diet.

A lot of studies have shown antioxidant effects and the pharmacological profiles of polyphenols or flavonoids (Sanbongi et al. 1997; Scalbert and Williamson 2000), and these substances, as components of typical functional foods, are considered to have actions that maintain or improve health conditions, particularly affecting body-modulating functions, such as adjustment of the metabolic and immune systems for latent diseases. In the research history of licorice, the efficacy of licorice has been reported in various research papers; however, there are no reports on DNA microarray analysis in relation to the effects of licorice. In previous studies, we have found that the hydrophobic flavonoids of licorice extract in the form of licorice flavonoid oil (LFO) were effective in the suppression of abdominal fat accumulation and weight gain in both animal models (Aoki et al. 2007a; Nakagawa et al. 2004) and human studies (Tominaga et al. 2006). In light of these results, we investigated the effects of LFO on the hepatic gene expression profile in high-fat diet-induced obese mice using DNA microarray technology (Aoki et al. 2007a).

## PREPARATION OF LICORICE FLAVONOID OIL

Licorice contains triterpenes or phenolic constituents, which are thought to confer health benefits. Glycyrrhizin, also called glycyrrhizic acid, is a water-soluble triterpenoid, and has useful medicinal properties. However, as glycyrrhizin is responsible for hypertension, sodium and water retention, or edema, where licorice containing glycyrrhizin is ingested over a prolonged period, care needs to be taken to avoid excessive daily intake of this substance (Stormer et al. 1993). On the other hand, licorice phenolic compounds, such as polyphenols or flavonoids, are also active components of licorice, and can be characterized by prenyl compounds.

The root of *G. glabra* contains glabridin, which is the major constituent and species-specific compound of *G. glabra*. Although LFO contains a variety of hydrophobic polyphenolic flavonoids from *G. glabra*, glabridin is the most abundant flavonoid. In this study, we focus not on glycyrrhizin but on licorice hydrophobic polyphenolic flavonoids from *G. glabra*. To extract and enrich a fraction of hydrophobic flavonoids, we prepared LFO, a new functional food ingredient consisting of licorice hydrophobic polyphenolic flavonoids with medium-chain triglycerides (MCT). Hydrophobic flavonoids from *G. glabra* are extracted and concentrated, and the resulting extract is referred to as LFO.

To obtain a fraction enriched in hydrophobic flavonoids, LFO was prepared by further extracting licorice ethanolic extract with MCT (Tominaga et al. 2006). First, ethanol was used to obtain an extract from the roots of licorice, *G. glabra*. The extract was dissolved in an MCT containing octanoic acid and decanoic acid (99:1), evaporated to remove ethanol, and then the glabridin concentration was adjusted to 3% (w/w) with MCT. In this solution, glycyrrhizin was present at a concentration of less than 0.005%; therefore, LFO is considered to be free from the side effects typically caused by glycyrrhizin. The solution is defined as "LFO concentrate solution; KANEKA GLAVONOID™." The LFO concentrate solution was diluted with additional MCT to adjust the concentration of glabridin to 1%. The obtained oil containing 1% of glabridin was used referred to as LFO. Accordingly, LFO comprises approximately 10% (w/w) of a licorice extract-derived solid fraction made up of hydrophobic polyphenols or flavonoids and 90% (w/w) of MCT.

## EFFECTS OF LFO ON DIET-INDUCED OBESITY MICE

To evaluate the ability of LFO to suppress abdominal fat accumulation, we applied LFO to diet-induced obese mice (Aoki et al. 2007a). A diet-induced obese model has been frequently used to investigate human obesity since it reproduces human obesity better than genetic obese models. C57BL/6J mice are obesity-prone and, when fed a high-fat diet, develop obesity, hyperglycemia, and hyperlipidemia (Collins et al. 2004; Lin et al. 2000). This model has thus frequently been used for investigation of obesity, diabetes, and dyslipidemia.

By feeding a high-fat diet for 8 weeks, obesity was induced in female C57BL/6J mice, and their average body weight was  $26.9 \pm 2.3$ g. The energy

**Table 19.1.** Body weight gain, adipose tissue weights, and serum parameters for C57BL/6J mice fed a high-fat diet containing LFO for 8 weeks\*.

	Control	0.5%	1%	LFO 2%
Body weight gain (g per 8 weeks)	6.2 ± 2.2	5.9 ± 1.7	2.4 ± 1.8 <sup>†</sup>	0.3 ± 1.9 <sup>†</sup>
Adipose tissue weight(g)				
Mesenteric WAT	0.611 ± 0.160	0.572 ± 0.189	0.419 ± 0.117 <sup>‡</sup>	0.284 ± 0.115 <sup>†</sup>
Perirenal WAT	0.958 ± 0.248	0.894 ± 0.297	0.589 ± 0.230 <sup>†</sup>	0.324 ± 0.187 <sup>†</sup>
Periuterine WAT	1.500 ± 0.415	1.444 ± 0.465	0.980 ± 0.360 <sup>‡</sup>	0.541 ± 0.238 <sup>†</sup>
Serum parameters				
Insulin (ng/ml)	1.69 ± 0.88	1.73 ± 1.23	0.80 ± 0.50 <sup>‡</sup>	0.70 ± 0.42 <sup>†</sup>
Leptin (ng/ml)	35.3 ± 12.6	35.4 ± 13.5	17.0 ± 7.6 <sup>†</sup>	6.54 ± 6.04 <sup>†</sup>
Adiponectin (μg/ml)	70.1 ± 9.6	72.8 ± 16.3	67.5 ± 12.2	57.7 ± 13.5

LFO, licorice flavonoid oil.

Reprinted with permission from Aoki et al. (2007a).

\*Each value is the mean ± SD.

<sup>†</sup>*p* < 0.01.<sup>‡</sup>*p* < 0.05.

ratio of the high-fat diet for fat, carbohydrate, and protein was 53, 27, and 20%, respectively, with total energy adjusted to 21 MJ/kg. The mice were then fed with a high-fat diet containing LFO at 0.5, 1.0, or 2.0% for an additional 8 weeks. In the control group, body weight increased to 33.1 ± 2.6 g, and body weight gain was 6.2 ± 2.2 g per 8 week, whereas LFO dose-dependently suppressed the body weight gain (Table 19.1). The body weight gains in the 1 and 2% LFO groups were significantly lower than that in the control group.

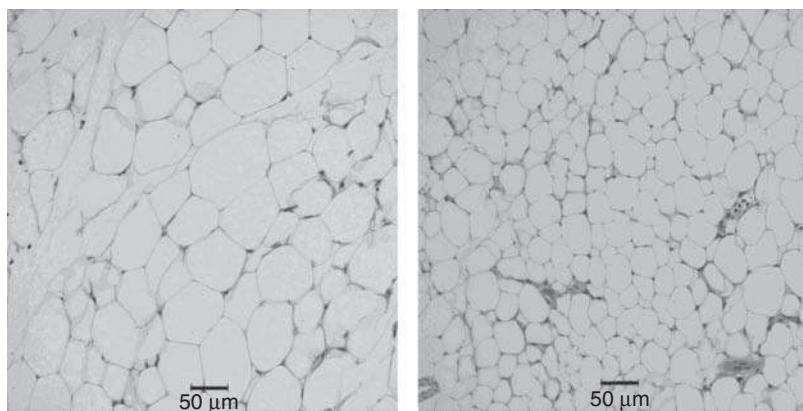
In addition, LFO did not affect food intake. The weights of mesenteric, perirenal, and periuterine white adipose tissues (WAT) were significantly lower in the 1 and 2% LFO groups than in the control group (Table 19.1). A comparison of liver, kidney, and spleen weights among the test groups showed no significant differences. LFO did not affect locomotor activity. Serum leptin and insulin levels were lower in the 1 and 2% LFO groups than in the control group, whereas there were no significant differences in serum adiponectin levels between the control and any of the LFO groups (Table 19.1).

To investigate the effects of LFO, we performed microscopic examinations. The effects of LFO consumption were also demonstrated by a histopathological examination of mesenteric WAT and of the liver. The adipocytes in the 2% LFO group were small in diameter compared with those in the control group, being approximately one-half or one-third the size of

those in the control group (Figure 19.2). Moreover, lipid droplets in the hepatocytes were abundant in the control group, but not in the 2% LFO group (Figure 19.3), suggesting that LFO prevented the development of fatty liver. No adverse histological findings such as inflammation due to LFO consumption were apparent in mesenteric WAT and the liver. It would appear that LFO may be able to promote lipolysis or suppress fat accumulation without any toxic effects.

LFO consumption dose-dependently and significantly suppressed the increase of abdominal adipose tissue mass comprising the mesenteric, perirenal, and periuterine WAT masses induced by the high-fat diet. Furthermore, body weight gain was also reduced in a dose-dependent manner (Table 19.1). No significant differences were observed in the weights of the liver, kidney, and spleen, suggesting that the effect of LFO was not due to any toxicity.

The serum insulin level dose-dependently decreased, the insulin level of the 2% LFO group being less than half of that of the control group (Table 19.1). Feeding a high-fat diet has been reported to increase the serum insulin level and elicit insulin resistance in C57BL/6J mice (Harte et al. 1999). LFO consumption clearly suppressed an increase in insulin levels, even when a high-fat diet was administered. Therefore, the decreases in visceral fat and insulin level brought about by LFO consumption would reflect an improvement in insulin resistance. These findings are consistent with the previous results that LFO



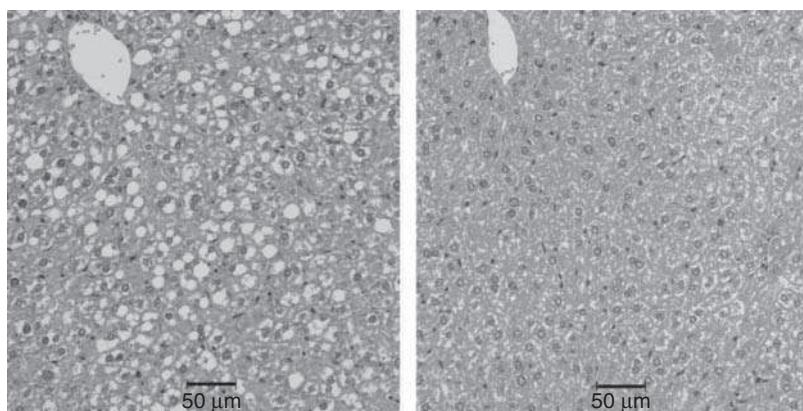
**Figure 19.2.** Histological staining of mesenteric white adipose tissue (WAT). Mesenteric WAT of the diet-induced obese C57BL/6J mice fed a high-fat diet containing licorice flavonoid oil (LFO) at 0% (control, left panel) and 2% (right panel) for 8 weeks was stained with hematoxylin–eosin (HE) and examined microscopically. The representative sections of mesenteric WAT in the control and 2% LFO groups are shown. (Reprinted with permission from Aoki et al. 2007a).

consumption suppressed abdominal fat accumulation and inhibited an increase in blood glucose level in a type 2 diabetes model (KK- $A^y$  mice) (Nakagawa et al. 2004).

Several studies have revealed that adipocytes secrete adipocytokines such as leptin or adiponectin as well as storing energy (Matsuzawa 2006). Leptin plays a number of roles in regulating food intake and energy expenditure, and serum leptin levels increase proportionately with increase in adipose tissue mass (Lin et al. 2000). Feeding of a high-fat diet to C57BL/6J mice has been reported to increase the serum leptin level by about sixfold and to cause leptin

resistance (Moraes et al. 2003). Compared with the control group, the average serum leptin level in the 2% LFO group decreased from 35.3 to 6.54 ng/ml in proportion to the decrease in adipose tissue mass (Table 19.1). This result seems to have been due to the leptin resistance induced in the control mice. Insulin has been reported to be involved in the regulation of serum leptin levels (Harte et al. 1999). Therefore, it is likely that a decrease in the serum leptin level would have resulted from decreases in the adipose tissue mass and the serum insulin level.

Adiponectin contributes to the regulation of energy homeostasis and thereby also regulates glucose



**Figure 19.3.** Histological staining of the liver. The liver of diet-induced obese C57BL/6J mice fed a high-fat diet containing licorice flavonoid oil (LFO) at 0% (control, left panel) and 2% (right panel) for 8 weeks was stained with hematoxylin–eosin (HE) and examined microscopically. The representative sections of the liver in the control and 2% LFO groups are shown. (Reprinted with permission from Aoki et al. 2007a).

and lipid metabolism. An increase in the circulating adiponectin level would lower hepatic glucose production (Berg et al. 2001), and diet-induced obesity would lead to a reduction in the expression of adiponectin and its corresponding serum level (Yamauchi et al. 2001). On the other hand, the thiazolidinediones (TZDs) as peroxisome proliferator-activated receptor-gamma (PPAR $\gamma$ ) ligands are reported to cause increased adiposity and body weight in animal models of insulin resistance (Yamauchi et al. 2001), and TZDs increased the serum adiponectin level (Maeda et al. 2001). Our previous study has demonstrated that LFO stimulated human adipocyte differentiation in vitro and had abdominal fat-lowering and hypoglycemic effects on KK-A $y$  mice that were possibly mediated via the activation of PPAR $\gamma$  (Nakagawa et al. 2004). However, there was no significant difference in serum adiponectin levels between the control and any of the LFO groups (Table 19.1), suggesting that LFO might have exerted an abdominal fat-lowering effect on C57BL/6J mice by some other mechanism.

The reduction in lipid droplet levels in the hepatocytes of the 2% LFO group indicates an improvement in fatty liver induced by the high-fat diet (Figure 19.3). It appears that LFO consumption activated fatty acid catabolism or inactivated fatty acid synthesis. Ashakumary et al. (1999) have reported that the antiobesity mechanism in the liver involves both the suppression of fatty-acid synthesis and the activation of fatty-acid metabolism. MCT is absorbed directly into the portal circulation and is rapidly oxidized in the liver unlike long-chain triglycerides (LCT); in addition, MCT consumption increases energy expenditure and decreases body weight gain. However, MCT has only a limited effect in preventing obesity where dietary LCT is replaced by MCT (St-Onge and Jones 2002). In fact, MCT as a control for LFO was not effective in any of our studies when the same amount of MCT was fed with a high-fat diet to all groups including the control group that did not receive LFO. The effect of adding MCT to licorice hydrophobic flavonoids appears to be negligible in our animal model and this finding is confirmed in human studies.

### STUDY OF MECHANISM OF ACTION OF LFO USING DNA MICROARRAY ANALYSIS OF THE LIVER

C57BL/6J mice fed a high-fat diet differ from those fed a normal diet in terms of the expression levels

of various genes (de Fourmestraux et al. 2004; Kim et al. 2004; Moraes et al. 2003). Genes encoding enzymes involved in lipid metabolism as markers of adipocyte differentiation, enzymes involved in detoxification, and structural components of the cytoskeleton are upregulated in adipose tissue of C57BL/6J mice fed a high-fat diet (Moraes et al. 2003). The genes involved in fatty acid catabolism, ketone body synthesis, and stress responses are upregulated, while those involved in lipogenesis and cholesterol synthesis are downregulated in the liver of C57BL/6J mice fed a high-fat diet (Kim et al. 2004). Since DNA microarray technology became available, it has provided insights into the possible mechanisms behind diet-induced obesity (Matsui et al. 2005; Tachibana et al. 2005; Turuoka et al. 2005).

To elucidate the mechanism whereby LFO has an effect on diet-induced obesity in C57BL/6J mice, the level of gene expression in the liver was compared between the control and 2% LFO groups by DNA microarray (Table 19.2). The liver is the major organ of lipid metabolism and insulin action for fat storage and release in response to the energy balance. We demonstrated the abdominal fat-lowering effect of LFO in diet-induced obese C57BL/6J mice and the effects of LFO on hepatic gene expression by DNA microarray technology. To determine the difference in hepatic gene expression between the control and the 2% LFO groups, the isolated total RNA was submitted to DNA microarray analysis (G4121A Mouse Oligo microarray; Agilent Technologies) covering more than 20,000 genes. Three mice in each group were selected by tissue weight. Equal amounts of isolated total RNA from these three mice in each group were then combined and used for the DNA microarray analysis. The data were analyzed using Feature Extraction software (Agilent Technologies).

LFO consumption upregulated the expression of genes involved in beta-oxidation and acyl-CoA degradation, which included enoyl-CoA, hydratase/3-hydroxyacyl CoA dehydrogenase (Ehhadh) (fold change, 2.09), acyl-CoA thioesterase 1 (Acot1) (fold change, 2.72), acyl-CoA oxidase (Aco) (fold change, 1.35), and medium-chain acetyl-CoA dehydrogenase (Mcad) (fold change, 1.37). In contrast, the expression of genes involved in acetyl-CoA synthesis, such as ATP citrate lyase (Acly) (fold change, 0.47) and acetyl-CoA synthetase 2 (Acas2) (fold change, 0.49), was downregulated. Similarly, the expression of genes involved in acyl-CoA synthesis, acetyl-CoA carboxilase (Acc) (fold change, 0.71), fatty acid synthase (Fas) (fold change, 0.58), and stearoyl-CoA desaturase 1 (Scd1) (fold change, 0.59) was also downregulated.

**Table 19.2.** Genes regulated in the liver of C57BL/6J mice fed a high-fat diet containing 2% LFO.

GenBank	Symbol	Gene name	Fold change
NM_023737	Ehhadh	Enoyl-CoA, hydratase/3-hydroxyacyl CoA dehydrogenase	2.09
NM_012006	Acot1	Acyl-CoA thioesterase 1	2.72
NM_015729	Aco	Acyl-CoA oxidase	1.35
NM_007382	Mcad	Medium-chain acetyl-CoA dehydrogenase	1.37
AF332052	Acly	ATP citrate lyase	0.47
NM_019811	Acas2	Acetyl-CoA synthetase 2	0.49
AF374169	Acc	Acetyl-CoA carboxilase	0.71
Mm.268690	Fas	Fatty acid synthase	0.58
Mm.267377	Scd1	Stearoyl-CoA desaturase 1	0.59
NM_013631	Pkrl	Pyruvate kinase in the liver and red blood cell	0.45
NM_130450	Elov16	Long-chain fatty acid elongase	0.55
NM_009117	Saa1	Serum amyloid A1	0.44
NM_011314	Saa2	Serum amyloid A2	0.25
NM_011315	Saa3	Serum amyloid A3	0.25

LFO, licorice flavonoid oil.

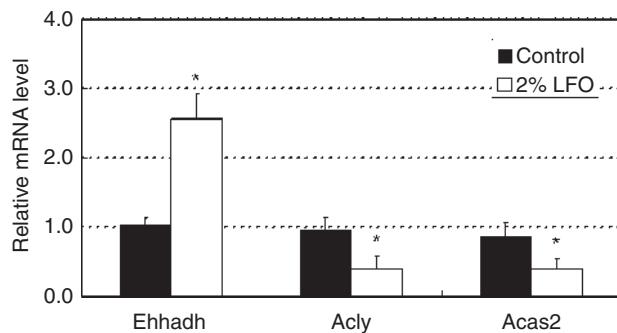
In addition, the expression of pyruvate kinase in the liver and red blood cells (Pkrl), a rate-limiting enzyme involved in glycolytic lipogenesis, was downregulated (fold change, 0.45) by LFO consumption. A long-chain fatty acid elongase (Elov16) was also downregulated (fold change, 0.55). Furthermore, the expression of various genes involved in acute inflammation, such as serum amyloid A1 (Saa1) (fold change, 0.44), Saa2 (fold change, 0.25), and Saa3 (fold change, 0.25), was also downregulated. It is interesting that downregulation of mRNA of proteins involved in acute inflammation appeared to be caused by licorice hydrophobic flavonoids not glycyrrhizin.

A quantitative real-time polymerase chain reaction (PCR) analysis was performed to confirm the expression changes of some genes selected by DNA microarray analysis. We determined the gene expression levels of Ehhadh, Acly, and Acas2 in each of the seven mice (except for the three mice subjected to DNA microarray analysis). The gene expression level of Ehhadh in the 2% LFO group was significantly increased by a factor of 2.49 (Figure 19.4). On the other hand, the gene expression levels of Acly and Acas2 were significantly decreased to 0.40 and 0.44, respectively (Figure 19.4).

LFO consumption upregulated the gene for Ehhadh, a bifunctional enzyme, which is the key enzyme at the late stage of peroxisomal beta-oxidation. On the other hand, LFO consumption downregulated Acly and Acas2 involved in the synthesis of acetyl-

CoA from citrate and acetate. The results obtained from the quantitative real-time PCR analysis correlated well with those from the DNA microarray analysis, suggesting that LFO consumption suppresses fatty-acid synthesis and activates peroxisomal beta-oxidation. SCD1 catalyzes the conversion of saturated fatty acids to monounsaturated fatty acids. A high-fat diet increase SCD1 gene expression in the liver, and inhibition of SCD1 expression by SCD1-specific antisense oligonucleotide reduces fatty acid synthesis in C57BL/6 mice fed a high-fat diet, indicating that SCD1 could be a target for obesity or metabolic syndrome (Jiang et al. 2005).

A change in the expression of these genes may thus be related to the decrease in the amount of fatty acids in the liver due to LFO consumption. The Elov16 is the gene encoding the elongase that catalyzes the conversion of palmitate to stearate. It has been reported that hepatic fatty acid composition is important for insulin sensitivity (Matsuzaka et al. 2007). Inhibition of this elongase could be a new approach for improving diabetes. The effect of LFO on the amelioration of hyperinsulinemia remains an area for future study. These results may support the finding from the histological examination that LFO consumption improved the status of fatty liver. We therefore conclude that the change in fatty acid metabolism in the liver due to a change in the expression of these genes reflects in whole or in part the antiobesity mechanism resulting from LFO consumption. A further study of gene expression in other tissues such as the muscle and



**Figure 19.4.** Verification of the expression changes of Ehhadh, Acly, and Acas2 by using quantitative real-time polymerase chain reaction (PCR). The changing expression of Ehhadh, Acly, and Acas2 was quantified by a quantitative real-time PCR analysis. The relative expression level of each sample was calibrated by the comparative threshold cycle (Ct) method, using Gapdh as an endogenous control. Each value is the mean  $\pm$  SD. Significant differences ( $p < 0.01$ ) from the control group are asterisked. LFO, licorice flavonoid oil. (Reprinted with permission from Aoki et al. 2007a).

adipose tissue would provide more important information about the effect of LFO.

## EFFICACY AND SAFETY OF LFO IN CLINICAL STUDIES

We showed the efficacy of LFO in the suppression of mesenteric fat accumulation and weight gain through the modification of lipid metabolism in high-fat diet-induced obese C57BL/6J mice. Before beginning the clinical studies of LFO in overweight subjects, we ascertained the safety of LFO in non-clinical studies, including a 90-day repeated dose toxicity study in rats, genotoxicity studies (Ames test, chromosome aberration test, bone marrow micronucleus test, and liver and peripheral blood micronucleus test), and a rat medium-term liver bioassay for carcinogens (Ito's method).

Furthermore, to ascertain the safety of LFO in humans, clinical studies were conducted using a step-by-step approach; a single-dose study, followed by 1-week and 4-week multiple-dose studies using a placebo-controlled single-blind approach (Aoki et al. 2007b). Prior to the 12-week efficacy study, an 8-week placebo-controlled efficacy dose-finding study was conducted in overweight subjects. No clinically significant adverse effects were observed in those studies.

In a 12-week efficacy study (Tominaga et al. 2006), a total of 103 overweight subjects (body mass index (BMI): 24–30) completed the study and were analyzed in a placebo-controlled, double-blind, long-term (12-week) ingestion study at 300 mg/day of

LFO, which was the minimum effective dose observed in the dose-finding study. Body weight increased in the placebo group, but was maintained at close to pre-ingestion levels in the LFO group, resulting in significant differences in the changes in body weight and BMI between the LFO group and the placebo group ( $p < 0.05$ ). Dual-energy X-ray absorptiometry (DXA) measurement of body fat indicated that the suppression in body weight gain was attributable to reduced body fat. No clinically significant adverse effects were observed during the 12-week ingestion period.

To confirm the safety of LFO for practical use, we also conducted a placebo-controlled, double-blind safety study in 40 overweight subjects involving the ingestion of a very high dose of LFO of 1,800 mg/day for 4 weeks. This dose was six times higher than the dose used in the efficacy study (300 mg/day). No clinically significant adverse effects were observed during the 4-week ingestion period. On the basis of these findings from human studies, it appears that LFO can be safely used as an ingredient in functional foods even when it takes over long periods, and that it has potential weight-reducing effects.

LFO contains a variety of hydrophobic polyphenolic flavonoids from *G. glabra*, and these flavonoids are absorbed into the body through the intestinal wall. To get a better indication of the potential beneficial effects in animals or humans brought about by LFO ingestion, pharmacokinetics studies of flavonoids are needed. The biological properties of dietary polyphenols or flavonoids depend on their bioavailability. Glabridin is the most abundant flavonoid among all licorice hydrophobic flavonoids and the most

characteristic compound in *G. glabra* and in LFO. As a result, glabridin is standardized at 1% (w/w) in LFO. Glabridin is thus a major compound among such flavonoids, and can be considered an appropriate marker compound for any study on pharmacokinetics of licorice hydrophobic flavonoids.

We developed and validated an analytical method for the determination of the concentration of glabridin in animal plasma and tissues and in human plasma using solid-phase extraction and HPLC equipped with an electrochemical detector or LC-MS/MS (Aoki et al. 2005; Ito et al. 2007). Dietary glabridin, prenyl flavonoid, was detected in plasma as an aglycon form in animal and human studies, suggesting the absorption of glabridin into the body (Aoki et al. 2007b; Ito et al. 2007). For example, based on the pharmacokinetics of glabridin in healthy humans, mean trough concentrations of plasma glabridin at the steady state in the 4-week multiple-dose study of LFO in male and female subjects was 0.30 to 1.75 ng/mL with single daily administration of 300, 600, and 1,200 mg of LFO. Plasma glabridin levels reached steady-state levels within 2 weeks. Prenyl flavonoids have been reported to escape conjugation (Shimizu et al. 2004), and we considered that glabridin was incorporated into the body in the active free form. The efficacy of LFO could be considered to be mainly due to the high bioavailability of prenylated licorice hydrophobic flavonoids as represented by glabridin.

## CONCLUSION

LFO is a unique food ingredient prepared by further extracting licorice ethanolic extract with MCT to concentrate the hydrophobic polyphenolic flavonoids. LFO consumption suppressed increases in abdominal adipose tissues and body weight, hyperinsulinemia, and hyperleptinemia induced by feeding a high-fat diet to C57BL/6J mice. Furthermore, after ascertaining the safety of LFO in animal and human studies, in overweight subjects in a randomized, double-blind, placebo-controlled trial, we found that LFO suppressed body weight gain by reducing body fat mass. In the study on the mechanism of action of LFO, the gene expression profiles were determined by a DNA microarray analysis, and the results suggest that the antiobesity effects of LFO are attributable to suppressed fatty acid synthesis and activated fatty acid catabolism in the liver. This appears to be one of the major mechanisms of action of LFO in controlling obesity. Considering that an accumulation of visceral fat induces metabolic syndrome, our findings suggest the possibility that LFO,

consumed in the form of a functional food, may be able to suppress the accumulation of visceral fat to ameliorate or prevent metabolic syndrome, a typical lifestyle-related disease, caused by an excessive intake of fat in the diet. LFO also received FDA approval as a new dietary ingredient (NDI) in the United States in 2006. Therefore, further studies that elucidate the mechanism of LFO containing licorice hydrophobic flavonoids would contribute to the efficient application of LFO in the treatment of metabolic syndrome.

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# 20

## Isoprenols

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### DIVERSITY OF ISOPRENOIDS IN NATURE

Nature relies on an intricate network of biosynthetic pathways to produce a lot of small organic molecules needed to support life. The isoprenoids constitute one of the largest families of natural products accounting for more than 30,000 individual compounds of both primary and secondary metabolism. Most of them are of plant origin, and hundreds of new structures are reported every year (Peñuelas and Mummé-Bosch 2005; Sacchettini and Poulter 1997).

Isopentyl diphosphate (IPP) and its isomer dimethylallyl diphosphate are the universal five-carbon precursors of isoprenoids. After the discovery of mevalonate (MVA) pathway in yeast and animals, it was assumed that IPP was synthesized from acetyl coenzyme A (CoA) via MVA and then isomerized to dimethylallyl diphosphate in all organisms (Chappell 2002). However, an alternative MVA-independent pathway for the biosynthesis of IPP was later identified in bacteria (Rohmer et al. 1993) and plants (Lichtenthaler et al. 1997). This pathway was named methylerythritol phosphate (MEP) pathway. Plants use both pathways although they are compartmentalized: MVA—to cytoplasm and possibly to mitochondria to provide sterols, sesquiterpenes, and ubiquinones; and MEP—to plastids giving plastidial isoprenoids, for example, isoprene, phytol, and carotenoids (Swiezewska and Danikiewicz 2005). Moreover, there is evidence that a certain degree of cross talk between the MVA and MEP pathways can occur, implying that these pathways are not completely autonomous (Laule et al. 2003).

In addition to universal physiological, metabolic, and structural functions, many specific isoprenoids function in various situations, including communica-

tion and defense. Members of the isoprenoid group also include industrially useful polymers (rubber, chicle) and agrochemicals (pyrethrins, azadirachtin).

It is known that several herbal plants improve medical conditions. Such plants contain many bioactive phytochemicals. Especially, isoprenoids are contained in many herbal plants, and several isoprenoids have been shown to be available for pharmaceuticals, for example, artemisinin and taxol as drugs to treat malaria and cancer, respectively. Various isoprenoids are contained in many plants for not only herbal but also dietary use (Mo and Elson 1999).

In this chapter, we describe several bioactive isoprenoids contained in herbal or dietary plants that have possibilities to ameliorate metabolic disorders via activation of ligand-dependent transcription factors: nuclear receptors.

### PPARs: THERAPEUTIC TARGET OF METABOLIC SYNDROME

#### NUCLEAR RECEPTORS

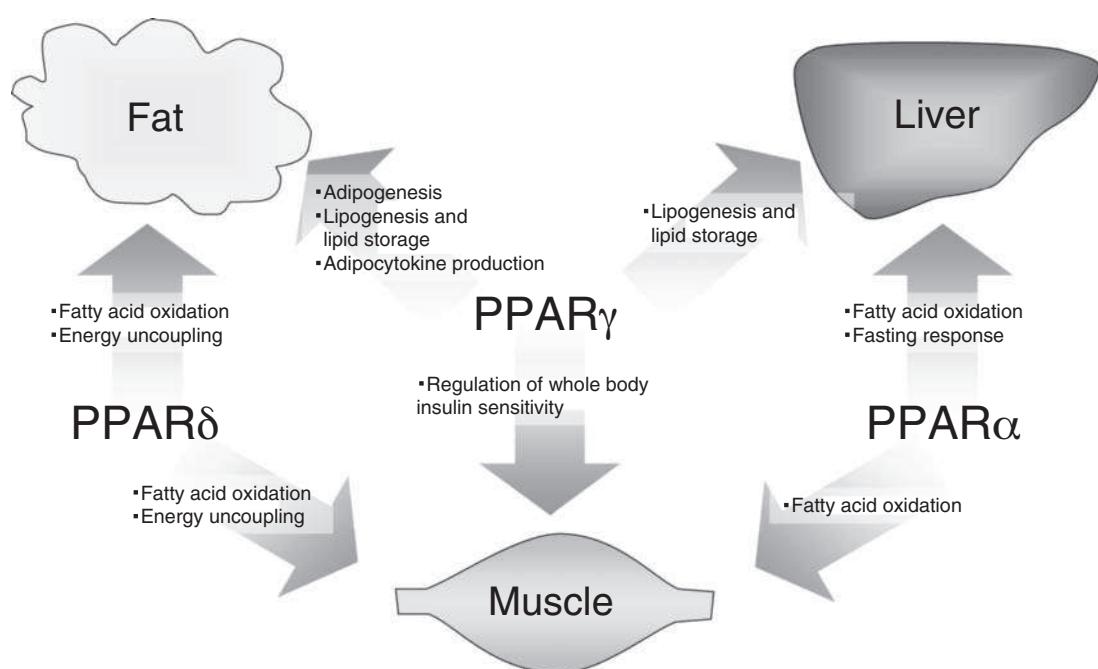
Members of the nuclear receptor superfamily of ligand-dependent transcription factors play a multitude of essential roles in development, homeostasis, reproduction, and immune function (Evans 1988; Giguère 1999; Glass 2006; Mangelsdorf and Evans 1995; Mangelsdorf et al. 1995). Nuclear receptors include the classical steroid hormone receptors (estrogen, progesterone, androgen, glucocorticoid, and mineralocorticoid receptors); “orphan receptors,” which exhibit conserved features of the nuclear receptor family, but have not been linked to endogenous ligands; and the so-called “adopted orphan receptors,” which were initially identified as orphan receptors but were subsequently linked to endogenous

ligands. The adopted orphan receptors include the thyroid hormone receptors, retinoic acid receptors, peroxisome proliferator-activated receptors (PPARs), and liver X receptors. Nearly all members of this family contain a highly conserved DNA-binding domain that mediates sequence-specific recognition of target genes and a C-terminal domain that determines the specific ligand-binding properties of each receptor and mediates ligand-regulated transcriptional activation and/or repression functions (Evans 1988).

Nuclear receptors are frequent biological targets of active compounds contained in herbal and dietary plants. This is perhaps not surprising, since nuclear receptors evolved to be regulated by lipophilic molecules derived from diet and the environment (Lazar 2004; Chawla et al. 2001). At least 10 of these receptors have been shown to be directly activated by compounds purified from herbal remedies (Lazar 2004). Some compounds have a complex pharmacology; for example, grapeseed-derived resveratrol is a ligand of estrogen receptor and PPARs (Inoue et al. 2003; Klinge et al. 2003), but has also been suggested to activate SIRT1, an NAD<sup>+</sup>-dependent protein deacetylase enzyme implicated in the biology of aging (Howitz et al. 2003). Other phytochemicals target multiple nuclear receptors.

## PPARs

PPARs are members of nuclear receptor superfamily that are activated by fatty acid and its derivatives. PPARs are dietary lipid sensors that regulate lipid and carbohydrate metabolism (Figure 20.1; Evans et al. 2004). In 1990, the  $\alpha$  type was subjected for the first time to cloning from a mouse liver cDNA library. Subsequent cDNA cloning experiment identified several PPAR subtype genes from various animal species and organs. These PPAR subtype genes form a family. In mammals, three subtype genes,  $\alpha$ ,  $\delta$  (called NUC I in human, fatty acid-activated receptor in mouse, and PPAR $\beta$  in frog), and  $\gamma$ , were found (Kawada et al. 2008). PPARs directly regulate a lot of genes involved in energy homeostasis. PPARs form heterodimers with retinoid-X receptors (RXRs) and bind to consensus DNA sites composed of direct repeats of hexameric DNA sequences (AGGNCA) separated by 1 bp (direct repeat 1). In the absence of ligand, PPAR–RXR heterodimers recruit corepressors and associated histone deacetylases and chromatin-modifying enzymes, silencing transcription by the so-called active repression (Jepsen and Rosenfeld 2002; Ordentlich et al. 2001; Privalsky 2004). Ligand binding induces a conformational change in PPAR–RXR complexes, releasing repressors in exchange



**Figure 20.1.** The three PPAR isoforms regulate lipid and glucose homeostasis through their coordinated activities in liver, muscle, and adipose tissue.

of coactivators. Ligand-activated complexes recruit the basal transcriptional machinery, resulting in enhanced gene expression.

PPAR $\alpha$  is highly expressed in the liver, cardiac muscle and digestive tract, and regulated expression of target genes involved in lipid catabolism. Activators of PPAR $\alpha$ , such as fibrates, lower the levels of circulating lipid and are commonly used to treat hypertriglyceridemia and other dyslipidemic states (Lefebvre et al. 2006). PPAR $\delta$  is expressed in many tissues including skeletal muscle and brown adipose tissue. Recently, it was suggested that PPAR $\delta$  activation attenuates obesity and type 2 diabetes (Tanaka et al. 2003; Wang et al. 2003). PPAR $\gamma$  is abundant in adipose tissues, functioning as the key transcription factor for adipogenesis. Synthetic ligands for PPAR $\gamma$ , such as thiazolidinediones, are increasingly used to treat type 2 diabetes (Semple et al. 2006).

PPARs are involved in not only energy homeostasis but also inflammation. PPAR $\alpha$  regulate inflammatory processes, mainly by inhibiting inflammatory gene expression. In recent years, several molecular mechanisms responsible for the immunosuppressive effects of PPAR $\alpha$  have been uncovered (Vanden Berghe et al. 2003). These include interference with several proinflammatory transcription factors including signal transducer and activator of transcription, and NF- $\kappa$ B by PPAR $\alpha$  (Delerive et al. 1999). The number of studies that have addressed the role of PPAR $\delta$  during inflammation is limited. So far, an anti-inflammatory effect has been observed in macrophages, suggesting possible role for PPAR $\delta$  in the process of atherogenic inflammation. It appears that PPAR $\delta$  acts as an inflammatory switch in which inactivated PPAR $\delta$  is proinflammatory and activated PPAR $\delta$  promotes an anti-inflammatory gene expression profile. Recently, it was suggested that switch of PPAR $\delta$  is linked to the B cell lymphoma-6 protein that functions as inflammatory suppressor protein (Lee et al. 2003). Similar to PPAR $\alpha$ , PPAR $\gamma$  is involved in governing the inflammatory response, especially in macrophages. Currently, two different molecular mechanisms have been proposed by which anti-inflammatory actions of PPAR $\gamma$  are effectuated: (1) via interference with proinflammatory transcription factors including signal transducer and activator of transcription, NF- $\kappa$ B, and AP-1 (Ricote et al. 1998), and (2) by preventing removal of corepressor complexes from gene promoter regions, resulting in suppression of inflammatory gene transcription (Pascual et al. 2005). This mechanism involves ligand-dependent SUMOylation of PPAR $\gamma$  followed by binding of PPAR $\gamma$  to nuclear receptor corepressor-histone deacetylase-3

complexes localized on inflammatory gene promoters.

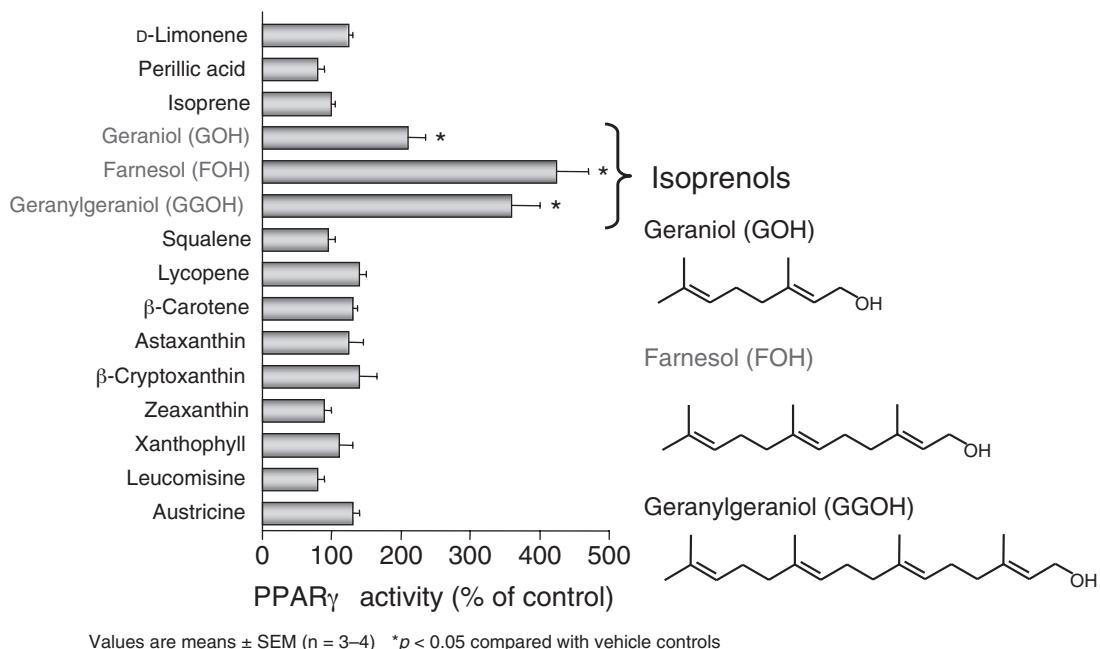
Recently, it has been indicated that obesity is associated with a low-grade chronic inflammation state (Fernández-Real and Ricart 2003). The inflammatory condition in obesity is increasingly being recognized as an important contributor to the development of the metabolic syndrome and of its associated complications. Adipocytes can secret cytokines involved in inflammation, such as adiponectin, monocyte chemoattractant protein-1 (MCP-1), and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) (Yu and Ginsberg 2005). MCP-1, a member of CC chemokine superfamily, plays a pivotal role in monocyte/macrophage trafficking and activation (Yu et al. 2006). Macrophages also produce various proinflammatory factors including MCP-1 and TNF- $\alpha$ . Macrophage-derived TNF- $\alpha$  establishes a vicious cycle that augments the inflammatory changes and insulin resistance in obese adipose tissues (Suganami et al. 2005). Therefore, to prevent obesity-related inflammation, it is important to decrease the production of obese adipose tissue-derived proinflammatory factors such as MCP-1 and TNF- $\alpha$ .

It is known that several herbal and dietary plants improve medical conditions including diabetes mellitus, hyperlipidemia, and cardiovascular disease with the abnormality of lipid metabolism (Elson et al. 1989; Miller 1998). To find novel natural ligands for PPARs, we have evaluated the ligand activity of PPARs for various isoprenoids in an advanced, highly sensitive system with the coexpression of a coactivator for nuclear receptors, the cAMP-response element-binding protein-binding protein, developed by modifying the luciferase reporter assay system (Takahashi et al. 2002). Hereinafter, several compounds are described, identified as novel PPAR ligands, in our PPAR ligand screening.

## NOVEL FUNCTIONS OF DIETARY ISOPRENOIDS AS PPAR LIGANDS

### ISOPRENOLS

We carried out the screening for novel PPAR ligands in natural compounds contained in medicinal plants. We used several isoprenoids including carotenoids and polyisoprenoid alcohols (isoprenols) for this screening, because these compounds are contained in many medicinal and dietary plants (He et al. 1997). These isoprenoids have multifunctions such as the suppression of tumor proliferation (Burke et al. 2002; He et al. 1997; Yu et al. 1995), apoptosis-inducing activity (Mo and Elson 1999), and cation channel



**Figure 20.2.** Effects of various isoprenoids on the activation of the PPAR $\gamma$  and the chemical structures of isoprenols.

regulation (Rouillet et al 1997). Some isoprenoids that are intermediates in cholesterol synthesis regulate the activity of 3-hydroxy-3-methylglutaryl-CoA reductase, a key enzyme in cholesterol synthesis by controlling the degradation of the enzyme (Bradfute and Simoni 1994; Correll et al. 1994). Such functions of dietary isoprenoids are significant for the trials to manage disease conditions such as cancers or cardiovascular diseases by food factors.

As shown in Figure 20.2, some isoprenols such as geraniol, farnesol, and geranylgeraniol (their chemical structures as shown in Figure 20.2) activated PPAR $\gamma$  at a concentration of 100  $\mu$ M (Takahashi et al. 2002). At this concentration, the activations by geraniol, farnesol, and geranylgeraniol were about 2.2-, 4.1-, 3.7-fold, respectively, as compared to vehicle control. The effects of isoprenols showed in a dose-dependent manner. On the other hand, squalene had no effect on PPAR $\gamma$  transactivation (Figure 20.2).

We tested whether isoprenols had the activity of PPAR $\alpha$  stimulation in PPAR $\alpha$  ligand assay system. Although geraniol had no effect, farnesol and geranylgeraniol dose-dependently activated PPAR $\alpha$  (10- and 8.6-fold increase, respectively, at a concentration of 100  $\mu$ M as compared to vehicle controls) (Takahashi et al. 2002). These activities were as potent as

that of 10  $\mu$ M fenofibrates, one of the fibrates (anti-hyperlipidemia drugs) used as a positive control for PPAR $\alpha$  (data not shown). In this regard, these isoprenols have the effects of dual activation in both PPAR $\gamma$  and PPAR $\alpha$ .

PPAR $\gamma$  activation in adipose tissues results in the improvement of insulin resistance (Lehmann et al. 1995) and PPAR $\alpha$  activation in liver induces lowering of circulating lipid levels (Staels et al. 1998). These effects are due to the regulation of target genes of PPARs at the mRNA level. The addition of these isoprenols upregulated several PPAR target genes in 3T3-L1 adipocytes and HepG2 hepatocytes (Takahashi et al. 2002). Therefore, it is possible that isoprenols could regulate insulin resistance and/or circulating lipid levels. Finding the dual effect of isoprenols to activate both PPAR $\gamma$  and PPAR $\alpha$  is very important for finding out the mechanisms of the effects of medicinal plants and valuable for managing diabetic and hyperlipidemic conditions in herbal medicine.

Actually, our preliminary study indicates that farnesol, one of the isoprenols, ameliorated hyperglycemia and glucosuria in KKAY obese diabetic mice. In this study, not PPAR $\gamma$  target genes in white adipose tissue but PPAR $\alpha$  target genes in liver were upregulated in mice fed 0.5% farnesol-containing

diets. Moreover, a 0.5% farnesol-containing diet ameliorates hepatic lipid accumulation in PPAR $\alpha^{+/+}$  mice but not in PPAR $\alpha^{-/-}$  mice. These data indicated that farnesol improved obesity-associated metabolic disorders mainly dependent on PPAR $\alpha$  activation.

These results suggested not only a significant molecular basis as to how herbal plants containing phytochemicals such as isoprenols induce the improvement of diabetes or hyperlipidemia, but also the possibilities that phytochemicals might have therapeutic applications in lipid abnormalities, such as obesity, diabetes mellitus, and hyperlipidemia.

### PHYTOLS

Phytol, a branched fatty alcohol, which is a carbon side chain of chlorophyll molecule, functions as a biomolecule that is involved in the production of energy from light. Phytol is one of plastidial isoprenoids and synthesized via MEP pathway in plastids (Swiezewska and Danikiewicz 2005). Since almost all photosynthetic organisms use chlorophyll, phytol is also abundantly present in nature, including various vegetables. It is suggested that in animals chlorophyll is digested partially and the phytol moiety is released. Then released phytol is converted to phytanic acid.

Phytanic acid is a branched-chain, isoprenoid-derived fatty acid constituent of diet. In surveys of phytanic acid content of a variety of food products, high levels were indeed found in foods such as milk, butter, cheese, meat from cows and sheep, and some kinds of fish and fish oils, while no phytanic acid is present in vegetables (Brown et al. 1993). Phytanic acid can also result from the conversion of dietary phytol in the body (van den Brink and Wanders 2006).

Recently, phytanic acid has been reported to activate PPAR $\gamma$  and the RXR (Kitareewan et al. 1996; Schlüter et al. 2002a) so that differentiation is stimulated in both white (Schlüter et al. 2002c) and brown adipocytes (Schlüter et al. 2002b; Schlüter et al. 2002). In addition, phytanic acid stimulates PPAR $\alpha$  to regulate lipid metabolism in some types of cells (Ellinghaus et al. 1999). Therefore, the intake of phytol as a precursor of phytanic acid is valuable for the management of lipid metabolism through the activation of PPARs. Actually, phytol-enriched diet increased the plasma and liver levels of phytanic acid and induced the expression of PPAR $\alpha$  target genes involved in peroxisomal and mitochondrial  $\beta$ -oxidation and fatty acid metabolism.

However, since the conversion of phytol into phytanic acid is not so fast, phytol-enriched diet also induced accumulation of phytol in the liver (Baxter 1968). Moreover, as described previously, several isoprenols, which resemble phytol in their structures, activate PPARs in adipocytes and hepatocytes (Takahashi et al. 2002). In this sense, it must be valuable to analyze the effects of phytol itself as an activator of PPARs.

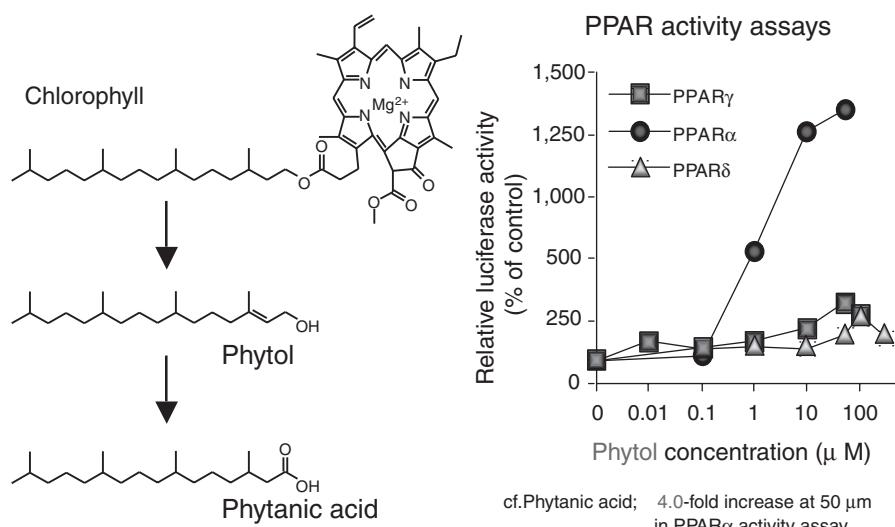
So, we evaluated the effects of phytol on PPARs activity in our advanced highly sensitive luciferase assays. Of the PPAR isoforms, PPAR $\alpha$  was activated the most markedly by the addition of phytol (Figure 20.3). The PPAR $\alpha$  activation level with 50- $\mu$ M phytol was approximately 13-fold higher than that of the vehicle control. The effects of phytol on PPAR $\alpha$  activation were larger than those of phytanic acid under our experimental conditions (about 4.0-fold increase at 50  $\mu$ M). Phytol induced the expression of PPAR-target genes in a manner dependent on the level of PPAR $\alpha$  expression in HepG2 hepatocytes (Goto et al. 2005).

To confirm the direct effects of phytol on PPAR $\alpha$  activation, we performed in vitro binding assay. A coactivator, steroid receptor coactivator-1 (SRC-1), interacts with PPAR proteins when ligands of PPARs bind to the PPAR ligand-binding domain (Krey et al. 1997). As well as PPAR $\alpha$  synthetic agonist, fenofibrate, in the presence of phytol, SRC-1 protein was recruited onto PPAR $\alpha$ . The degrees of binding of SRC-1 to PPAR $\alpha$  in the presence of 100  $\mu$ M fenofibrate, and 50 and 100  $\mu$ M phytol increased 27-, 8.9-, and 26-fold, respectively, relative to that of the vehicle control (Goto et al. 2005). These findings indicate that phytol itself can directly bind to PPAR $\alpha$  as its ligand.

Because the activation of PPAR $\alpha$  is one of the most important factors in lipid metabolism in peripheral tissues including the liver and muscles, the ability of phytanic acid and phytol to activate PPAR $\alpha$  is very important in the management of lipid metabolism by food factors. Upregulation of PPAR $\alpha$  target genes in mice fed phytol-enrich diet is likely due to not only phytanic acid but also phytol. It is thought that such effects of phytol are valuable for control of lipid abnormalities in common diseases including obesity, diabetes, and hyperlipidemia through PPAR $\alpha$  activation in the liver.

### ABIETIC ACID DERIVATIVES

The amount of variety of hydrocarbons and their derivatives used in industrial and commercial activities has been increasing over the years. Abietic



**Figure 20.3.** Phytol-activated PPARs as determined by luciferase ligand assays using the GAL4/PPAR chimera system.

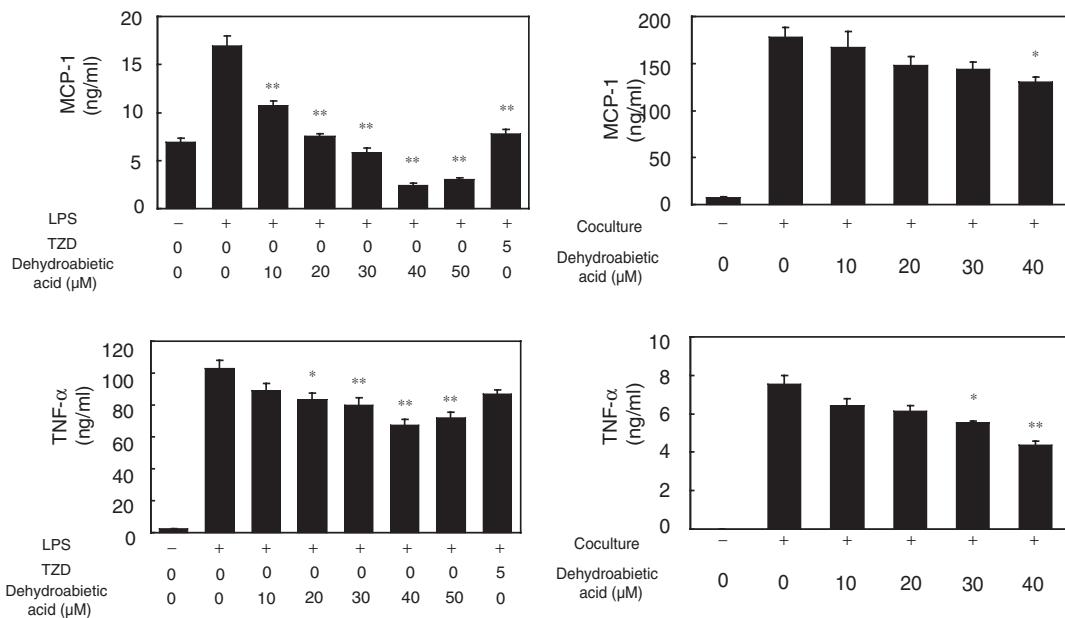
acid is a tricyclic, diterpene carboxylic acid, and is the main component of the rosin fraction of oleoresin synthesized by conifer species, such as grand fir (*Abies grandis*) and lodgepole pine (*Pinus contorta*) (Aranda and Villalaín 1997). Abietic acid is commonly used as fluxing agents in solder, as paper-sizing agent to make paper more water resistant, and in printing inks, adhesives, and plasticizers (Mitani et al. 2007). Moreover, it was reported that abietic acid is a bioactive compound, and has an anti-inflammatory effect. In lipopolysaccharide (LPS)-stimulated macrophages, abietic acid suppresses production of prostaglandin E2 in vitro and in vivo (Fernández et al. 2001).

To investigate whether the activation of PPARs is related to the anti-inflammatory effects of abietic acid and its derivatives, we evaluated the effects of abietic acid and its derivatives on PPAR activity. Abietic acid and dehydroabietic acid, one of major components of colophony as well as abietic acid, potently activate both PPAR $\alpha$  and PPAR $\gamma$  but not PPAR $\delta$  (Kang et al. 2008; Takahashi et al. 2002). Like thiazolidinedione, a synthetic PPAR $\gamma$  ligand, abietic acid suppressed expressions of TNF- $\alpha$  and cyclooxygenase 2, which is induced in inflammatory reactions, in LPS-stimulated macrophages (Takahashi et al. 2003). Dehydroabietic acid stimulated PPAR $\alpha$  and PPAR $\gamma$  more potently than abietic acid (Kang et al. 2008). Dehydroabietic acid significantly suppressed the production of proinflammatory mediators such as MCP-1, TNF- $\alpha$ , and NO in

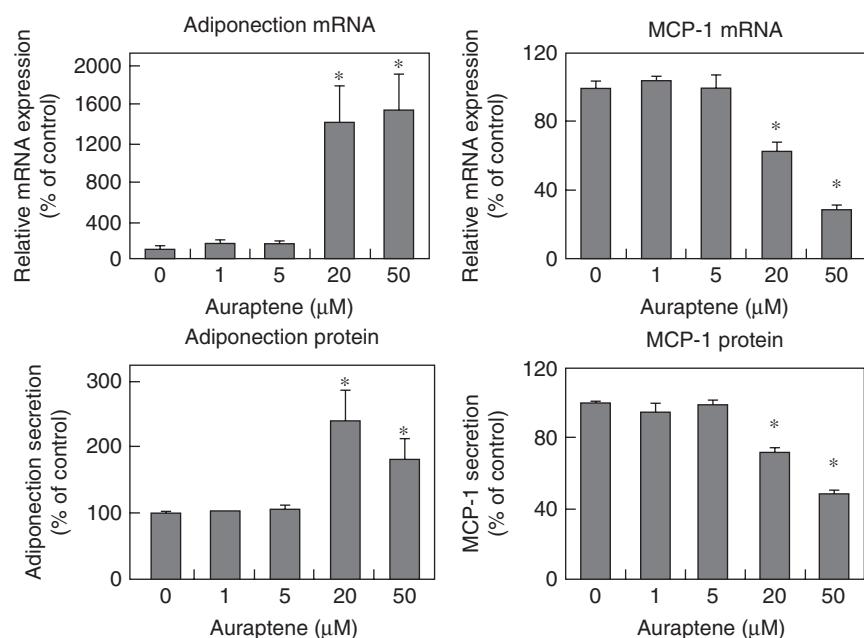
LPS-stimulated macrophages and in the coculture of macrophages and adipocytes (Figure 20.4; Kang et al. 2008). In our preliminary data, in obese diabetic KKAY mice, dehydroabietic acid repressed obesity-associated elevation of circular MCP-1 and TNF- $\alpha$  levels and mRNA expression of these genes in WAT. Moreover, in this experiment, dehydroabietic acid improved carbohydrate and lipid metabolism. These data indicated that anti-inflammatory effect of abietic acid and dehydroabietic acid is at least partly due to activation of PPARs. Additionally, it is suggested that these compounds can be used not only for anti-inflammation but also for regulating carbohydrate and lipid metabolism and atherosclerosis.

#### AURAPTENE

Citrus fruit compounds have many beneficial bioactivities (e.g., anti-carcinogenesis, antihypertension, and antocardiac effects) (Ito et al. 2005; Mononen et al. 2005). Through our screening for PPAR ligands in citrus fruit compounds, we identified auraptene, one of isoprenoid coumarins, as a novel PPAR $\alpha$  and PPAR $\gamma$  ligand (Kuroyanagi et al. 2008). Auraptene occurs in a variety of citrus fruits. It was reported that auraptene has anti-inflammatory and anticarcinogenic activities. In cultured adipocytes, auraptene upregulated an antiatherosclerotic, antidiabetic, and anti-inflammatory cytokine adiponectin, while downregulated proinflammatory cytokine MCP-1 (Figure 20.5). These



**Figure 20.4.** Dehydroabietic acid suppressed the production of MCP-1 and TNF- $\alpha$  in LPS-stimulated macrophages and in the coculture of macrophages and adipocytes. Values are means  $\pm$  SEM ( $n = 3-4$ ). \*,  $p < 0.05$ , \*\*,  $p < 0.01$  compared with culture treated with LPS alone or nontreated coculture.



**Figure 20.5.** Auraptene upregulated adiponectin while downregulated MCP-1 in cultured adipocytes. Values are means  $\pm$  SEM ( $n = 3-4$ ). \*,  $p < 0.05$  compared with vehicle controls.

effects disappeared in the presence of GW9662, a PPAR $\gamma$  antagonist. In addition, several PPAR $\alpha$  target genes, such as those encoding human liver carnitine palmitoyltransferase-1, acyl-CoA synthetase, and acyl-CoA oxidase, were also induced at the mRNA level in PPAR $\alpha$ -expressing HepG2 hepatocytes by auraptene treatment. It is likely that auraptene regulates the expressions of both PPAR $\alpha$  and PPAR $\gamma$  target genes as a dual agonist.

## CONCLUSIONS

In this chapter we mentioned diverse isoprenoids, function of PPARs, and several isoprenoids activating PPARs. The prevalence of obesity worldwide has progressively increased over the past decades. This unabated rise has spawned proportionate increases in obesity-associated metabolic disorders. Currently, synthetic PPAR agonists are widely used for the treatment of metabolic disorders. Dietary isoprenoids that activate PPARs as described in this chapter may be valuable for controlling carbohydrate and lipid disorders by eating daily. Moreover, because the isoprenoids constitute one of the largest families of natural products, more potent and useful PPAR activators may exist.

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# 21

## Anti-inflammatory and Anticarcinogenesis Potentials of Citrus Coumarins and Polymethylated Flavonoids

*Akira Murakami and Hajime Ohigashi*

### INTRODUCTION

#### BIOACTIVE PRINCIPLES OF CITRUS FRUITS

Citrus fruits are well known to contain an array of secondary metabolites in terms of their chemical structure and biological activities, which biosynthesize monoterpenes (D-limonene, etc.), triterpenes (limonoids), flavonoids (nobiletin, hesperidin, etc.), coumarins (auraptene, bergamottin, etc.), and carotenoids ( $\beta$ -carotene,  $\beta$ -cryptoxanthin, etc.). There is also ample evidence that these citrus components have physiological functions such as antioxidant, anti-inflammatory, and anticarcinogenesis activities. For example, D-limonene was revealed to be an inhibitor of the oncogenic Ras farnesyl transferase (Gould et al. 1994), while limonoids induced apoptosis in human neuroblastoma cells (Poulou et al. 2005). Also, hesperidin and naringin, both flavanone-*O*-glycosides, were shown to have functions that counter hypoglycemic (Jung et al. 2004) and anti-*Helicobacter pylori* (Bae et al. 1999) activities, as well as attenuate experimental colitis (Crespo et al. 1999) and prevent tumor promotion, but not initiation, in mouse skin (Berkarda et al. 1998).

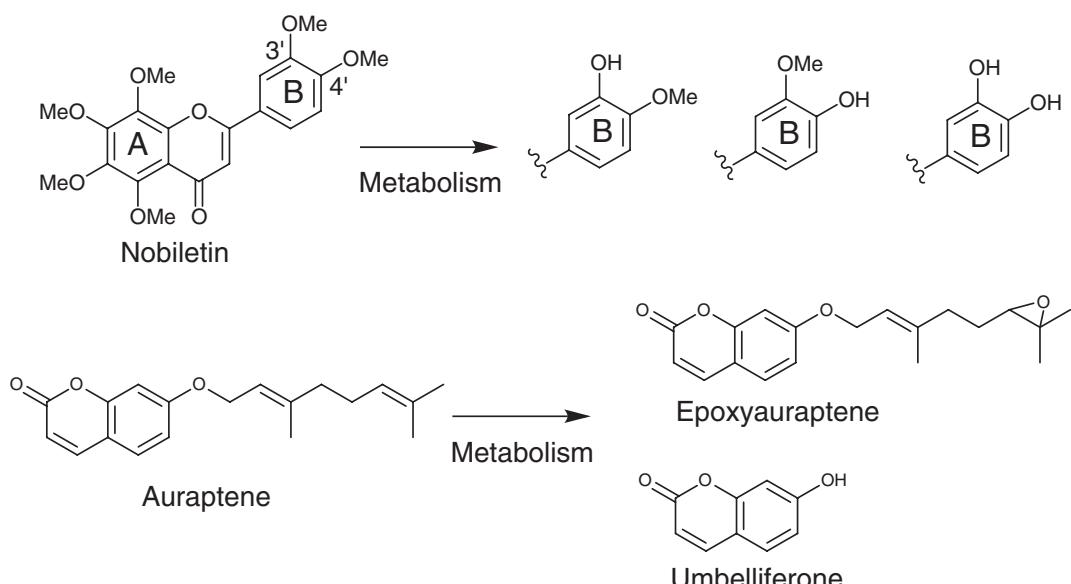
More strikingly,  $\beta$ -cryptoxanthin has attracted the attention of many researchers ranging from basic scientists to epidemiologists. Nishino et al. were the first to suggest that  $\beta$ -cryptoxanthin has a more pronounced cancer preventive potential than  $\beta$ -carotene (Tsushima et al. 1995), which was initially believed to play a major role in the chemopreventive activity of green-yellow vegetables and fruits, though the outcome of a clinical trial was puzzling (Nowak 1994). Some epidemiological surveys have also implied

that  $\beta$ -cryptoxanthin is beneficial for cancer prevention (Stram et al. 2007; Yuan et al. 2003; Zhang et al. 2007), while rodent experiments that explored the chemopreventive potential of  $\beta$ -cryptoxanthin also provided encouraging findings that this unique carotenoid markedly suppressed chemical carcinogenesis in the urinary bladder (Miyazawa et al. 2007), lung (Kohno et al. 2001a), and colon (Narisawa et al. 1999; Tanaka et al. 2000b).

In addition to the above-mentioned citrus components, both alkyloxyated coumarin (e.g., auraptene) and polymethylated flavonoids (PMFs, e.g., nobiletin) (Figure 21.1) are highlighted in this chapter because of their physiological activities (anti-inflammatory and anticarcinogenesis, modulation of drug metabolism, etc.), underlying molecular mechanisms, effects on metabolism (in vitro and in vivo bioconversion), and potential side effects.

#### CHEMICAL CHARACTERISTICS

Coumarins, phytochemicals that are widely distributed throughout the plant kingdom, have a two-piece ring consisting of a C6-C3 structure with an ester moiety. Oxidation of the coumarin ring, for example, by introduction of epoxy and hydroxyl groups, results in a chemical variety by extending the side chains of the alkyl and alkenyl groups. One of the prominent side-chain groups has a tandem connection to an isopentenyl group, which is the chemical unit for terpenoids. The *Citrus* genus has been well described as an abundant source of coumarins with isopentenyl moieties. As described later, coumarins with the isopentenyl moiety have distinct properties



**Figure 21.1.** Chemical structures of nobiletin, auraptene, and their metabolites.

as compared with simple coumarins in terms of biological activities, which is a characteristic presumably derived from their higher molecular hydrophobicity.

Flavonoids are a large group of plant secondary metabolites. Chemically, it is notable that they consist of C3-C6-C3 units and are classified into several subgroups according to oxidation status, number of aromatic rings, carbon skeleton, and presence or absence of positive charge, as well as other characteristics. One common chemical characteristic among flavonoids is the presence of phenolic and hydroxyl groups. Further, PMFs have unique chemical properties, that is, hydroxyl groups that are mostly methylated, as well as an A-ring oxidation status distinct from that of common flavonoids, since the 6- or 8-position is oxidized in addition to the 5- and 7-positions, where common flavonoids have hydroxyl groups. Similar to coumarins with a side chain, these hydrophobic characteristics provide PMFs with notable biological activities and metabolism properties, which will be described later.

## ANTIMUTAGENIC ACTIVITIES

Coumarins and their isopentenyl derivatives are not considered to be effective antimutagens. Nevertheless, one coumarin was reported to show chemoprotection against aflatoxin B1 in a 6-thioguanine resistant mutation assay with liver S9 from rats and 19-day-old chick embryos for aflatoxin B1 bioactivation

(Goeger et al. 1998). In addition, it should be noted that linear furanocoumarins showed a greater inhibitory effect on dimethylbenz[a]anthracene DNA adduct formation in mouse mammary glands as compared with simple coumarins (Prince et al. 2006). In contrast, another study found that marmesin, isolated from Nigerian medicinal plants, exhibited mutagenicity in a conventional Ames test (Uwaifo 1984), whereas another identified coumarin as a co-mutagen with aflatoxin B1 upon activation by human liver S9 (Goeger et al. 1999). These results indicate that the mutagenic and antimutagenic activities of natural coumarins are dependent on their chemical structures and the experimental systems used for evaluation.

On the other hand, the antimutagenic properties of PMFs have long been demonstrated by various groups using in vitro models (Calomme et al. 1996; Miyazawa et al. 1999). Interestingly, Miyazawa et al. (1999) performed activity-guided separation of *Citrus aurantium* and isolated some active constituents, including PMFs (tetra-O-methylscutellarein, sinensetin, and nobiletin). Also, nobiletin in *Aspergillus niger* was metabolized to 4'-hydroxy-5,6,7,8,3'-pentamethoxyflavone and found to be antimutagenic (Okuno and Miyazawa 2004), while Iwase et al. (2001) showed the efficacy of PMFs using in vivo experiments, in which nobiletin and 3,3',4',5,6,7,8-heptamethoxyflavone exhibited anti-tumor-initiating activities toward mouse skin tumors induced by a nitric oxide (NO)

donor  $(+/-)-(E)$ -methyl-2-[ $(E)$ -hydroxyimino]-5-nitro-6-methoxy-3-hexenamide, as the initiator, and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) as the promotor.

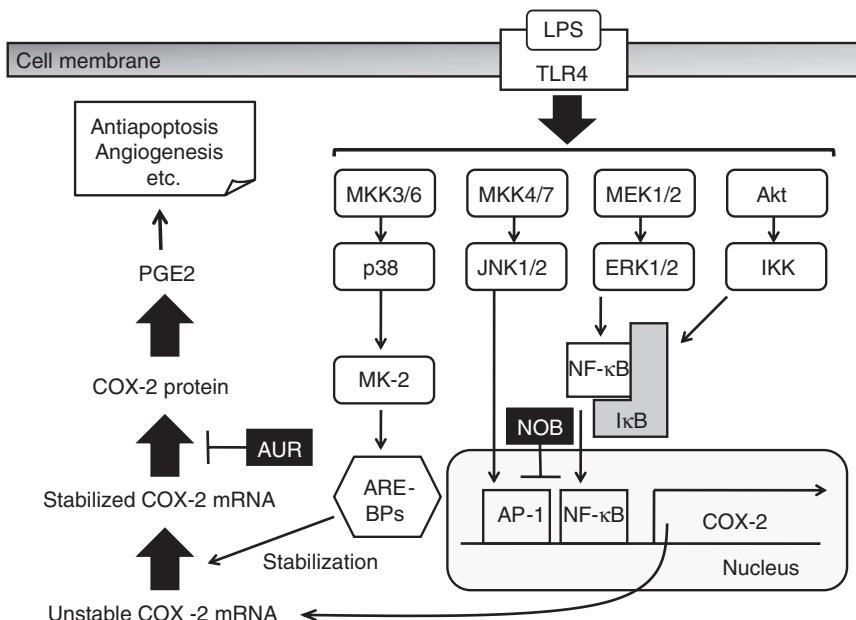
## ANTIOXIDATIVE AND ANTI-INFLAMMATORY ACTIVITIES

Inflammation is a pathophysiological phenomenon that is involved in numerous diseases and each human organ has the potential for a disease with an inflammatory condition that is essential to its etiology. Of importance, a considerable proportion of chronic inflammatory diseases display an overlap with the onset and development of cancer, such as ulcerative colitis reflux esophagitis, Barrett's esophagus, hepatitis, and gastritis. It is also well documented that infection with microorganisms is closely related to inflammation-derived carcinogenesis (Coussens and Werb 2002). In that process, neutrophils and monocytes mature and are recruited, and then infiltrate the inflamed tissue for inducing and producing proinflammatory molecules, such as reactive oxygen and nitrogen species, prostaglandins (PGs), inflammatory cytokines, and chemokines, as well as others. NO is released at high levels by inducible NO synthase (iNOS) with the formation of stoichiometric amounts of L-citrulline from L-arginine. iNOS-mediated excessive and prolonged NO generation has attracted attention on account of its relevance to epithelial carcinogenesis (Nathan and Xie 1994; Ohshima and Bartsch 1994). On the other hand, there is a large body of data showing that cyclooxygenase (COX)-2 expression is involved in the development of certain cancers (Taketo, 1998a, b). In contrast to COX-1, COX-2 activity is inducible and its elevation enhances the biosynthesis of PGs, including PGE<sub>2</sub>, which is one of the physiologically active and stable PGs produced in the pathways downstream of COX enzymes. PGE<sub>2</sub> is known to stimulate Bcl-2 activity and thereby prevent apoptosis (Fosslien 2000). Superoxide anion (O<sub>2</sub><sup>-</sup>) is a free radical generated through NADPH oxidase, dominantly present in leukocytes, as well as from xanthine oxidase in epithelial cells, and may be subsequently converted into more reactive intermediates such as the hydroxyl radical responsible for DNA mutations.

We previously found that both auraptene and nobiletin are able to efficiently attenuate phorbol ester- and endotoxin-activated leukocytic activation (Murakami and Ohigashi 2006; Murakami et al. 2005). For instance, those phytochemicals suppressed phorbol ester-induced O<sub>2</sub><sup>-</sup> generation in differentiated

HL-60 cells, which are the model for neutrophils (Murakami et al. 1997, 2000c). It is important to note that they have no potential for free radical-scavenging activity, while they have properties for suppressing oxidative stress by preventing their generation from stimulated leukocytes. Of interest, they suppress both iNOS and COX-2 protein expression in lipopolysaccharide (LPS)-challenged macrophages via different molecular mechanisms (Figure 21.2). In previous studies, auraptene did not disrupt iNOS/COX-2 mRNA expression, but prevented protein synthesis (Murakami et al. 1997, 2000c), and such translational regulation was also seen for the protein production of matrix metalloproteinase (MMP)-7 in human colon cancer cells, as described hereafter. On the other hand, nobiletin appears to target the transcriptional factors activator protein (AP)-1 and nuclear factor (NF)- $\kappa$ B, without modulating upstream protein kinases, including extracellular signal-regulated protein kinase (ERK)1/2 and c-Jun N-terminal kinase1/2 (Murakami et al. 2005). This finding is consistent with our recent findings that nobiletin attenuated constitutive (Kawabata et al. 2005) and phorbol ester-induced (Eguchi et al. 2006) AP-1 transcriptional activity in HT-29 human colon cancer and THP-1 human monocytic cells, respectively, which were supported by the results of an independent group (Sato et al. 2002). Along a similar line, nobiletin was shown to inhibit the DNA-binding activity of NF- $\kappa$ B in LPS-activated RAW 264.7 murine macrophages (Choi et al. 2007). It is also noteworthy that both mono- and di-demethylated nobiletin, urinary metabolites of nobiletin, suppressed LPS-induced COX-2 expression in macrophages more potently than nobiletin (Li et al. 2007). On the other hand, nobiletin inhibited IL-1-induced production of PGE<sub>2</sub> and proMMP-9 in rabbit synovial cells, suggesting that it is useful for the treatment of matrix degradation of the articular cartilage, and pannus formation in osteoarthritis and rheumatoid arthritis (Ishiiwa et al. 2000).

Wei et al. (1993) previously reported that double applications of phorbol ester to mouse skin led to excessive reactive oxygen species production and induction of proinflammatory molecules. Ji and Mar nett (1992) termed such successive applications as "priming" (the first stage, illustrated by leukocyte recruitment, maturation, and infiltration of inflammatory leukocytes such as polymorphonuclear leukocytes and macrophages into inflamed lesions) and "activation" (the second stage, illustrated by ROS production from accumulated leukocytes). We previously attempted to determine whether auraptene and nobiletin inhibit the priming and/or activation stages in this double application model, and found that



**Figure 21.2.** Proposed molecular mechanisms by which nobletin and auraptene attenuate the expression of LPS-induced COX-2 protein production in RAW264.7 mouse macrophages. LPS stimulation leads to simultaneous activation of multiple protein kinases, including IKK, ERK1/2, JNK1/2, and p38, thereby inducing transcription activities of AP-1 and NF- $\kappa$ B, critical transcription factors for COX-2 expression. With an adenine and uracil-rich element in the 3'-untranslated region, COX-2 mRNA is unstable and degraded. However, activation of p38 and resultant phosphorylation of MK2 results in a complicated binding of numerous ARE-BPs to ARE, which are the determinant of mRNA half-life, thereby stabilizing it. Nobletin may interfere with co-activators that enhance the transactivation of NF- $\kappa$ B and AP-1, but does not disrupt the activities of upstream protein kinases. Auraptene may disturb the translation step of COX-2 mRNA, as it suppresses COX-2 protein but not the production of mRNA, and this translational mechanism may be involved in the suppression of proMMP-7 protein production (see Figure 21.3). AP-1, activator protein-1; ARE-BP, AU-rich element-binding protein; AUR, auraptene; COX, cyclooxygenase; ERK, extracellular signal-regulated kinase; I $\kappa$ B, inhibitor of NF- $\kappa$ B; JNK, c-Jun NH<sub>2</sub>-terminal kinase; LPS, lipopolysaccharide; NOB, nobletin; MK-2, mitogen-activated protein kinase-activated protein kinase-2; MKK/MEK, mitogen-activated protein kinase kinase; NF- $\kappa$ B, nuclear factor- $\kappa$ B; IKK, I $\kappa$ B kinase; PG, prostaglandin; TLR, toll-like receptor.

auraptene selectively inhibited the activation stage while nobletin inhibited both stages (Murakami et al. 2000b, c). The anti-inflammatory effect of nobletin was also examined in a phorbol ester-induced mouse ear edema formation test and its inhibitory potency was shown to be higher than that of a synthetic COX inhibitor, indomethacin (Murakami et al. 2000a). Lin et al. (2003) also demonstrated that nobletin downregulates IL-1-induced gene expression, as well as the production of proMMP-1 and proMMP-3 in human synovial fibroblasts. Recently, Takeda et al. (2007) presented an interesting finding that feeding of auraptene for 7 weeks suppressed gastric inflammation induced by *Helicobacter pylori* infection in *Mongolian gerbils*. On the other hand, topical application of nobletin prevented ultraviolet B-induced increases in transepidermal water loss

and hyperplasia of the epidermis in hairless mouse skin (Tanaka et al. 2004). In addition, intraperitoneal injection of nobletin antagonized eosinophilic airway inflammation in asthmatic rats (Wu et al. 2006). Meanwhile, we recently reported that dietary administration of auraptene at 100 ppm ameliorated dextran sulfate sodium-induced colitis in mice, accompanied by reduced production of MMPs (Kawabata et al. 2006a).

## ANTITUMOR PROMOTING AND CHEMOPREVENTIVE ACTIVITIES

There is a growing body of evidence that PMFs are superior for cancer prevention as compared with

general flavonoids (Walle 2007). The 15-lipoxygenase inhibitory activities of several flavonoids were investigated by Malterud and Rydland (2000), and the strongest inhibition was shown by 3,5,6,7,3',4'-hexamethoxyflavone, while sinensetin, nobiletin, tangeretin, tetramethylscutellarein, and 3,3',4',5,6,7,8-heptamethoxyflavone were less active. It is known that cell-cell communication mediated via gap junctions is suppressed by tumor-promoting treatments (Rivedal et al. 1994). Chaumontet et al. (1994) showed that two flavones, apigenin and tangeretin, inhibited blockade of TPA-induced gap junctional intercellular communication (GJIC) in REL rat liver epithelial cells, while other flavonoids tested, including naringenin, myricetin, catechin, and chrysin, did not enhance GJIC or counteract the TPA-induced inhibition (Chaumontet et al. 1997), suggesting higher antitumor promotional potentials of PMFs than those of general flavonoids. Of note, Iwase et al. (2000) selected 13 flavones from the peels of citrus fruits and examined their inhibitory effects on TPA-induced Epstein-Barr virus early antigen induction, and found that 3,5,6,7,8,3',4'-heptamethoxyflavone was a significant inhibitor.

We previously reported that topical applications of nobiletin (Murakami et al. 2000b) and auraptene (Murakami et al. 1997) were able to suppress the tumor promotion stage in mouse skin in two-stage carcinogenesis experiments. Moreover, oral administration of those compounds (100 and 500 ppm in basal diet) inhibited azoxymethane-induced formation of aberrant crypt foci (Kohno et al. 2001b; Tanaka et al. 1997), a histological tumor marker, as well as adenomas/adenocarcinomas (Suzuki et al. 2004; Tanaka et al. 1998a) in rats. Along a similar line, auraptene and collinin, another prenyloxy-coumarin, inhibited colitis-related colon carcinogenesis in mice (Kohno et al. 2006). In addition, Hayashi et al. (2007) found that dietary auraptene (250 ppm) reduced the numbers of azoxymethane-induced aberrant crypt foci and  $\beta$ -catenin-accumulated crypts in C57 BL/KsJ-db/db (db/db) mice with obese and diabetic phenotypes, as well as in wild-type mice. On the other hand, auraptene (100 and 500 ppm) suppressed 4-nitroquinoline 1-oxide-induced oral carcinogenesis in rats (Tanaka et al. 1998b), while dietary supplementation of auraptene inhibited *N,N*-diethylnitrosamine-induced rat hepatocarcinogenesis (Sakata et al. 2004). In addition to the results of chemical carcinogenesis experiments, Tang et al. (2007) used transgenic rats that developed adenocarcinomas of the prostate bearing the SV40T antigen transgene and reported that a diet containing

500 ppm nobiletin or auraptene decreased those carcinomas.

### ANTICANCER CELL PROLIFERATION AND APOPTOSIS/DIFFERENTIATION-INDUCING ACTIVITIES

PMFs are notable for their abilities to inhibit cancer cell proliferation, as well as induce apoptosis and differentiation. One of the earliest in vitro findings showing the anticancer activity of PMFs were reported by Kandawaswami et al. in 1991, in which two PMFs, nobiletin and tangeretin, markedly inhibited cancer cell growth, whereas quercetin and taxifolin did not. They speculated that the differences in anticancer cell proliferation activity were due to the greater membrane update of PMFs as compared with that of quercetin and taxifolin. Kawaii et al. (1999a) performed some extensive investigation of the anticancer cell proliferating activities of natural flavonoids, in which 27 citrus flavonoids were examined for their antiproliferative activities against several tumor and normal human cell lines, and reported the following order of inhibitory potency: luteolin > natsudaidain > quercetin > tangeretin > eriodictyol > nobiletin > 3,3',4',5,6,7,8-heptamethoxyflavone. Regarding the structure-activity relationship of PMFs, the C-3 hydroxyl and C-8 methoxyl groups were found to be essential for high activity in that same report. Along a similar line, Rodriguez et al. (2002) found that while quercetin, hesperetin, 7,3'-dimethylhesperetin, and eriodictyol did not produce any effect on B16F10 or SK-MEL-1 melanoma cell lines, tangeretin was the most effective among the flavonoids investigated in inhibiting cell growth, and also that the presence of at least three adjacent methoxyl groups conferred a more potent antiproliferative effect.

In addition, an apoptotic cell death-inducing activity of tangeretin was reported by Hirano et al. (1995) using HL-60 cells. Further, Kawaii et al. (1999b) found PMFs, including nobiletin and tangeretin, to induce differentiation of mouse myeloid leukemia cells, and, on the basis of the results of structure-activity relationship studies, they concluded that the hydroxyl group at the 3-position and methoxyl group at the 8-position enhanced the differentiation-inducing activity. Mak et al. (1996) also isolated nobiletin and tangeretin from *Citrus reticulata*, and then utilized them as antileukemia compounds by exploiting their proliferation suppression- and differentiation-inducing activities toward a murine

myeloid leukemic cell clone, WEHI 3B. Meanwhile, Jun et al. (2007) recently revealed the apoptosis-inducing effect of auraptene on Jurkat T cells by the endoplasmic reticulum stress-mediated activation of caspase-8, and the subsequent induction of mitochondria-dependent or -independent activation of the caspase cascade.

### ANTITUMOR INVASION AND METASTATIC ACTIVITIES

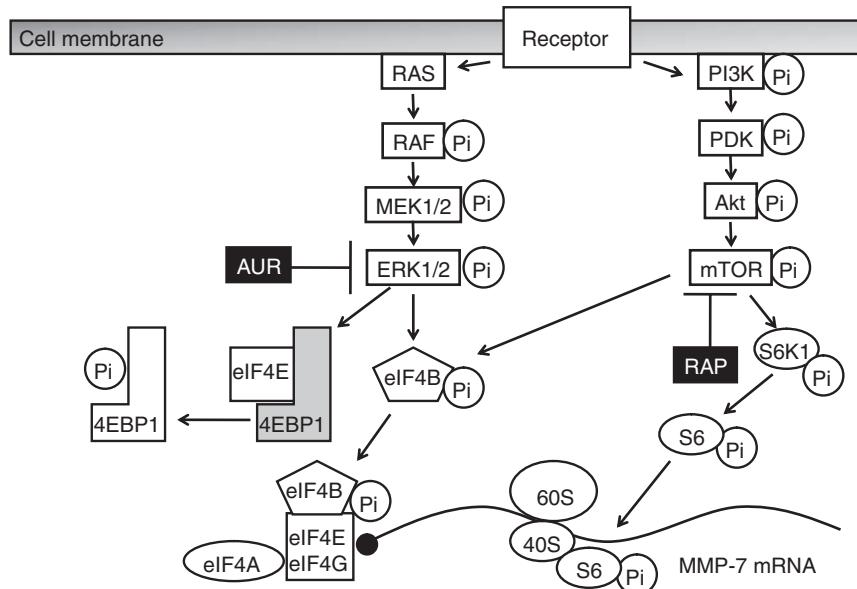
The most thoroughly characterized biological activity of PMFs may be their antitumor invading property. The MMP super family plays some pivotal roles in not only tumor cell invasion, but also angiogenesis and oncogenesis (Egeblad and Werb 2002). Further, Bracke et al. (1989, 1991, 1999) reported the potential activity of PMFs for suppressing tumor invasion and demonstrated an in vitro model, in which some flavonoids inhibited the invasion of mouse MO4 cells into embryonic chick heart fragments, with potencies in the following order—tangeretin > nobiletin > hesperidin = naringin (Bracke et al. 1989)—indicating a higher activity of PMFs than common flavonoids. Unlike (+)-catechin, tangeretin bound poorly to the extracellular matrix and did not alter the fucosylated surface glycopeptides of MO4 cells. There is also an in vivo study of an SCID mouse model showing that nobiletin inhibited the formation of peritoneal dissemination nodules from a cancer cell line, TMK-1, and the total weight of the dissemination nodules was significantly lower in the treated group than in the vehicle control group, as was the total number disseminated (Minagawa et al. 2001). In addition, the enzymatic activity of MMP-9 expressed in culture medium obtained from a coculture of TMK-1 and mouse fibroblastic cells was inhibited by nobiletin in a concentration-dependent manner. Rooprai et al. (2001) investigated the effects of 4 anti-invasive agents, swainsonine (a locoweed alkaloid), captopril (an antihypertensive drug), tangeretin, and nobiletin, on various parameters of brain tumor invasion, such as MMP secretion, migration, invasion, and adhesion, and found that nobiletin was the most effective agent. Also, the E-cadherin/catenin complex is a powerful invasion suppressor in epithelial cells that is expressed in the human MCF-7 breast cancer cell line family, though it is functionally defective in the invasive MCF-7/6 variant. Vermeulen et al. (1996) showed that tangeretin is able to upregulate the function of this complex in MCF-7/6 cells, implicating an action mechanism of PMFs for suppressing tumor

invasion. Recently, Miyata et al. (2008) presented an interesting finding that TPA-augmented MEK activity was diminished by nobiletin treatment in HT-1080 cells, which may be related to its antitumor metastatic activity *in vitro*.

In contrast to nobiletin and its related PMFs, reports on the antitumor progressive activity of auraptene are limited. However, Tanaka et al. (2000a) presented an *in vivo* finding that dietary supplementation of auraptene led to marked suppression of lung metastasis by melanoma cells in mice. Also, auraptene remarkably inhibited the production of a tumor invasive and metastatic protein, proMMP-7, in HT-29 human colon cancer cells, without affecting its mRNA expression level (Kawabata et al. 2006b). Our mechanistic studies using siRNA revealed that the phosphorylation levels of 4E binding protein (4EBP)1 at Thr37/46 and Thr70, and eukaryotic translation initiation factor (eIF)4B at Ser422 were substantially decreased (Figure 21.3), though it markedly dephosphorylated constitutively activated ERK1/2 for reducing the phosphorylation of eIF4B at Ser422. Further, auraptene attenuated the protein, but not mRNA, expression of proliferation- and apoptosis-associated genes, such as c-myc, cyclin-dependent kinase 4, and Bcl-xL. Together, these findings indicate that auraptene targets the translation stop for several tumor progression-related molecules including proMMP-7 by disrupting the ERK1/2-mediated phosphorylation of 4EBP1 and eIF4B (Kawabata et al. 2006b), and this mechanism may also be involved in the suppression of COX-2 protein production, as noted.

### INFLUENCE ON DRUG METABOLISM

It is known that exogenous substances are metabolized by drug-metabolizing enzymes, consisting of those in the phase 1 (e.g., cytochrome P450s that add a hydrophilic functional group) and phase 2 (e.g., glutathione-*S*-transferase (GST), which provides glutathione to that metabolized by phase 1 enzymes) groups. For instance, most polycyclic aromatic hydrocarbons, called procarcinogens, are biologically inactive in their native structure and activated by a phase 1 enzyme, leading to the formation of ultimate carcinogens that can bind to cellular DNA. Alternatively, activated carcinogens are subjected to the reaction by phase 2 enzymes to be inactivated and then excreted from the body. On the other



**Figure 21.3.** Proposed mechanism by which auraptene and rapamycin suppress the production of MMP-7 protein in HT-29 human colon adenocarcinoma cells. One of the critical steps for protein translation is the activation of eIF4B, which is induced by both RAS/RAF/MEK/ERK and PI3K/PDK/Akt/mTOR pathways. In HT-29 cells, those translational machineries are constitutively active. Both auraptene and rapamycin affected the level of MMP-7 mRNA and suppressed its protein production. Of interest, auraptene did not disrupt the mTOR pathway, though it dephosphorylated ERK1/2 and thereby decreased the phosphorylation of eIF4B. Rapamycin is a well-known mTOR inhibitor and blocked S6 phosphorylation. AUR, auraptene; eIF, eukaryotic initiation factor; EBP, eIF4E-binding protein; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase kinase; MMP, matrix metalloproteinase; mTOR, mammalian target of rapamycin; PDK, phosphoinositide-dependent kinase; PI3K, phosphatidylinositol 3-kinase; RAP, rapamycin; S6K, S6 kinase.

hand, P-glycoprotein, known as a plasma membrane glycoprotein, confers multidrug resistance to cells by virtue of its ability to exclude cytotoxic drugs in an ATP-dependent manner.

The effects of tangeretin on cytochrome P450 activity were first demonstrated in 1995 by Obermeier et al., in which 7-ethoxyresorufin-*O*-deethylase (classified as CYP 1A) and nifedipine oxidase (CYP 3A4) in human liver microsomes were inhibited by tangeretin in a noncompetitive manner. Conversely, Lake et al. (1999) showed the effects of tangeretin, naringin, and naringenin on xenobiotic-induced genotoxicity using precision-cut rat and human liver slices. Tangeretin was also shown to be a potent inhibitor of cooked food mutagen (2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine)-induced unscheduled DNA synthesis in human liver slices, whereas naringenin was ineffective and naringin only inhibited genotoxicity at a high concentration. In rat liver slices, tangeretin inhibited 2-acetylaminofluorene-

induced unscheduled DNA synthesis, whereas both naringenin and naringin were ineffective (Lake 1999).

Contradictory data have also been presented by Canivenc-Lavier et al. (1996), who showed that flavone and tangeretin increased cytochrome P450 1A1, 1A2, and 2B1 and 2 B2 forms in rat liver specimens, whereas flavanone only enhanced the cytochrome P450 2B isozymes. A Northern blot analysis in the same study showed that flavone and tangeretin increased the level of the cytochrome P450 1A2 mRNA. Flavone and, to a lesser extent, tangeretin increased the activities of ethoxyresorufin *O*-deethylase, methoxyresorufin *O*-demethylase, and pentoxyresorufin *O*-dealkylase, whereas flavanone mainly enhanced only pentoxyresorufin *O*-dealkylase activity (Canivenc-Lavier et al. 1996). Together, the modifying effects of PMFs on phase 1 enzyme activity may depend on the experimental conditions, for example the tissues, organs,

and species used. To our knowledge, there is no known report on the effect of PMFs on phase 2 enzyme activity.

As mentioned above, an important mechanism of multidrug resistance involves the multidrug transporter P-glycoprotein, which confers upon cancer cells the ability to resist lethal doses of certain cytotoxic drugs by pumping the drugs out of the cells and thus reducing their cytotoxicity (Ueda et al. 1999). It has been reported that nobiletin, tangeretin, and 3,3',4',5,6,7,8-heptamethoxyflavone (PMFs) enhanced vinblastine uptake by specifically inhibiting drug efflux via P-glycoprotein, but did not affect cytochrome P450 isozyme CYP3A4 activity, suggesting that PMFs have potentials as agents for reversing multidrug resistance or recovering the bioavailability of certain drugs (Ikegawa et al. 2000; Takanaga et al. 2000). While it has been established that grapefruit juice has some potential to accelerate the intestinal absorption of certain drugs (Bailey et al. 1998), Takanaga et al. (2000) found that orange juice extract, which includes PMFs, had a higher activity to inhibit P-glycoprotein than grapefruit juice.

On the other hand, coumarins in grapefruit and pummelo juice are also known to be substantial modulators of drug metabolizing enzymes (Fukuda et al. 1997). For example, 6',7'-dihydroxybergamottin is a potent inhibitor of CYP3A (Edwards et al. 1996), 2B6 and 3A5 (Lin et al. 2005), and 2C9 (Girennavar et al. 2007), while naringin and naringenin do not play a major role (Edwards and Bernier 1996). In addition, CYP2D6 was inhibited by five different furocoumarins in the following order of inhibitory potency: paradisin A > dihydroxybergamottin > bergamottin > bergaptol > geranylcoumarin (Girennavar et al. 2007). In particular, bergamottin, a component of grapefruit juice, was found to be a mechanism-based inactivator of the cytochromes P450 2B6 and 3A5 by covalently binding to them (Lin et al. 2005).

## BIOAVAILABILITY AND METABOLISM

We previously investigated the in vitro absorption and metabolism of nobiletin and compared the results with luteolin (Murakami et al. 2002). Nobiletin preferentially accumulated in a differentiated Caco-2 cell monolayer, which was used as a model for small intestinal epithelial cells, whereas luteolin did not (Murakami et al. 2001). Treatment of nobiletin with a rat liver S-9 mixture led to the formation of 3'-demethylnobiletin, whereas that of luteolin did not (Figure 21.1). In support of our in vitro data, Nielsen

et al. (2000) recently reported the in vivo biotransformation and excretion of tangeretin by analyzing urine and feces samples from rats after repeated administrations of the flavone at 100 mg/kg body weight/day. The metabolites identified were either demethylated or hydroxylated derivatives, and metabolic changes were found to primarily occur in the 4'-position of the B-ring (Nielsen et al. 2000). Further, tangeretin is metabolized in rat and human liver microsomes by an *O*-demethylation reaction involving cytochrome P450 (Canivenc-Lavier et al. 1993). Recently, Koga et al. (2007) reported results from extensive studies on nobiletin metabolism and found that constitutive P450s, such as CYP2C11, CYP2D1, CYP3A1, CYP3A2, and CYP2C12, are responsible for demethylation at the 6-, 7-, 3'- and 4'-positions of nobiletin, whereas 3-methylcholanthrene-inducible P450s, CYP1A1 and CYP1A2, preferentially catalyzed demethylation at the 3'- and 4'-positions. In addition, the research group of C.T. Ho identified the major nobiletin metabolite in mouse urine as 4'-demethylnobiletin (28.9  $\mu$ g/mL), whereas 3'-demethylnobiletin was a minor metabolite (Li et al. 2006), while the former metabolite has also been detected in rat urine (Yasuda et al. 2003).

Auraptene also has notable characteristics of metabolism and absorption. We previously reported that 7-ethoxycoumarin permeated the basolateral (portal vein) side of differentiated Caco-2 cells in a time-dependent manner, while auraptene slightly permeated the cells, though intracellular accumulation was remarkable (Murakami et al. 2000a). In addition, epoxyauraptene and umbelliferone were detected when auraptene was treated with the rat liver S-9 mixture (Figure 21.1). In addition, 7-ethoxycoumarin was also converted to umbelliferone, though its  $t_{1/2}$  value of 2 h was much shorter than that of auraptene (more than 24 h) (Murakami et al. 2000d). This suggests that auraptene, bearing a geranyloxy side chain, is a relatively metabolism-resistant substrate for cytochrome P450 enzymes and, thus, is stable in the liver as compared with 7-ethoxycoumarin. We also explored the metabolism characteristics of auraptene and 7-ethoxycoumarin in male SD rats (Kuki et al. 2008), and found that a single gastric intubation of auraptene, but not 7-ethoxycoumarin, led to significant localization in the liver from 1 to 4 h, with comparable contents in the gastrointestinal tract. Following 7-ethoxycoumarin administration, treatments of serum and urinary samples with glucuronidase/sulfatase led to a greater formation of umbelliferone as compared with auraptene. Collectively, these results indicate that auraptene has a longer life span than 7-ethoxycoumarin.

## POTENTIAL SIDE EFFECTS AND TOXICITY

Using GST placental form-positive foci, Chaumontet et al. (1996) compared the effects of various flavonoids (quercetin, tangeretin, flavone, and flavanone, each at 1,000 ppm in the diet) with the effects of phenobarbital on the occurrence of liver preneoplastic lesions, together with the effects of flavonoids on GJIC in liver samples and in two cell lines. No significant differences in the number and area of GST placental form-positive foci were found between any flavonoid group and the control group. Further, phenobarbital decreased GJIC by 60%, whereas tangeretin, in contrast to the other three flavonoids, decreased dye transfer *in vivo*, suggesting a tumor-promoting property. Inversely, antitumor promotional properties of tangeretin and flavone were seen in REL cells *in vitro* (Chaumontet et al. 1996), indicating some discrepancies between the *in vivo* and *in vitro* data for tangeretin.

The *in vitro* effects of several flavonoids, including tangeretin, on nonenzymatic lipid peroxidation in rat brain mitochondria were studied by Ratty and Das (1988). The flavonoids apigenin, flavone, flavanone, hesperidin, naringin, and tangeretin each promoted ascorbic acid-induced lipid peroxidation, while the other 18 nonmethoxylated flavonoids were found to be suppressive (Ratty and Das 1988). Tamoxifen is a promising agent for the prevention and cure of breast cancer (Bentrem and Craig Jordan 2002). However, Bracke et al. (1999) presented surprising data showing that the antitumor effect of tamoxifen in female nude mice inoculated subcutaneously with human MCF-7/6 mammary adenocarcinoma cells was completely masked by tangeretin via unknown mechanism(s).

Phototoxicity has been noted for furocoumarin derivatives in some citrus fruit juices such as lemon (Naganuma et al. 1995). For example, it has long been known that 8-methoxysoralen causes photosensitization for inducing sunburn cells, a reliable marker for skin photodamage, while bergamot oil has similar effects (Yasui and Hirone 1994). The major metabolism pathway of coumarins, most frequently used as a fragrance enhancer or stabilizer, in rodents has been clarified to be 3,4-epoxidation, which results in the formation of toxic metabolites, though the risk toward humans is yet to be investigated (Lake 1999). Carlton et al. (1996) administered a coumarin in the diet to Sprague-Dawley rats at doses of 0, 333, 1,000, 2,000, 3,000, and 5,000 ppm and to CD-1 mice at doses of 0, 300, 1,000, or 3,000 ppm for more than 100 weeks, and noted no dose-related abnormalities

in regard to clinical signs, clinical pathology, hematology, or gross or microscopic pathology.

## CONCLUSION

There is a great body of evidence from epidemiological surveys showing the beneficial effects of fresh fruits on human health promotion and disease prevention. On the other hand, other approaches directed specifically toward citrus fruits are still limited and thus highly encouraged (see Sugiura et al. 2004). On the basis of accumulated results, there is no doubt that citrus fruits and their constituents are attractive and useful materials from the viewpoints of both basic and applied sciences, as well as for the development of physiologically functional foods. One of the distinct characteristics of citrus fruits, as compared with other foods, is the variety of active constituents in terms of chemical characteristics and bioactivities. Thus, combination studies using different types of citrus components for enhancing the efficacy of each are warranted, such as combining nobiletin (targeting COX-2 transcription) and auraptene (targeting COX-2 translation) to determine their additive or even synergistic effects.

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## 22

# Probiotics: Food for Thought

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### INTRODUCTION

In simple terms, humans would be dead without the presence of microbes. Outnumbering human cells at least 10 to 1, these microbes play a role in establishing the immune system and affecting a wide range of functions. With the uncovering of the human genome, in due course it will become evident which genes are particularly influenced by microbes at different stages of life. The mere presence of certain genes is invariably not the determining factor in health and disease. Rather, environmental factors play a major role in gene expression. Thus, it will be challenging to prove the cause and effect of a particular microbe being associated with a specific human function or health outcome as distinct from a disease for which Koch's postulates showed how a pathogen can cause illness.

Studies in animals can help provide an understanding of nutrigenomic effects, as illustrated by the work which showed that *Bacteroides thetaiotaomicron* plays a role in intestinal angiogenesis in newborns (Stappenbeck et al. 2002). The organism regulated the elaboration of microvasculature by signaling through a bacteria-sensing epithelial cell. This provided an important example of a health benefit derived from a bacterium, although the finding does not rule out other organisms also being able to induce this effect.

The ability to manipulate the microbiota and host responses using administration of microbes led to the term probiotics—"live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO 2001). Most probiotics are food and dietary supplements, and therefore they can have nutrigenomic effects. In addition, by-products of these organisms are often protein-

aceous, and so the use of probiotics is also relevant to the topic of proteomics. In this chapter, examples of how indigenous and probiotic microbes and their by-products contribute to health is discussed using references to select studies. A range of reviews on probiotics per se are already available for interested readers (Boirivant and Strober 2007; Deshpande et al. 2007; Lenoir-Wijnkoop et al. 2007; Pineiro and Stanton 2007; Reid et al. 2006; Szajewska 2007), and where possible overlap with these will be kept to a minimum in this chapter.

Nutrigenomics involves the interaction between dietary factors and host genes, either directly or indirectly, and how genes and their products metabolize these constituents into nutrients, antinutrients, and bioactive compounds (Kaput 2007). Nutrigenomic science seeks to understand the variability of the individual's response to food and the underlying mechanisms whereby foods exert their health-promoting or disease-causing activities.

The bioactive chemicals in food affect health by altering the molecular expression and/or structure of an individual's genetic makeup. Recently a 5-year European Union (EU)-Integrated Project GEnetics of Healthy Aging (GEHA) was constituted by 25 partners (24 from Europe plus the Beijing Genomics Institute from China) to identify genes involved in healthy aging and longevity, which allow individuals to survive to advanced old age in good cognitive and physical function and in the absence of major age-related diseases (Franceschi et al. 2007). The project is designed to identify gender-specific genes involved in healthy aging and longevity in both women and men, stratified for ethnic and geographic origin. Although not part of the project, it would be interesting to collect samples such as feces to investigate whether changes in microbiota

coincide with specific changes in gene expression in the host.

The Chinese study will be particularly interesting for several reasons. Some communities have not altered their main diet for centuries, while others have changed to a more Western diet including fast food. It is not uncharacteristic for some Chinese people to live beyond 100 years, and yet there are also infants who die from malnutrition and diarrheal disease; thus, again the study should uncover important lifespan differences.

Human ancestors, mostly native Africans, were noted for eating fermented food products, such as plants and plant roots high in fructo-oligosaccharides that stimulate lactic acid bacterial growth in the gut. Modern day Africans are not noted for longevity, mainly because of political strife, wars, malnutrition, and diseases such as HIV/AIDS, tuberculosis, and malaria. Thus, this makes studying their gut microbiota with a view to nutrigenomics and longevity more difficult. Africans who have since moved to North America or North Americans descended from Africans have recently been shown to be at higher risk of colorectal cancer and mucosal proliferation than Native Africans, through an association with higher dietary intakes of animal products and higher colonic populations of potentially toxic hydrogen and secondary bile-salt-producing bacteria (O'Keefe et al. 2007). Pinning down the precise dietary and microbial components and human genes that influence the risk of diseases such as colon cancer is an extremely difficult task, and a problem that is not likely to be resolved soon. For now, attempts to use probiotics to reduce the risk of intestinal and other diseases is at a relatively primitive level, especially not knowing the composition of the gut microbiota of a given individual at any one time, nor fully comprehending the mechanisms of action of probiotics. Nevertheless, there are signs that this therapy has great promise without the side effects so prominent with pharmaceutical agents.

## PROBIOTICS

Although several definitions have been ascribed to probiotics, the one used by the Food and Agricultural Organization of the United Nations (FAO) and the World Health Organization (WHO) is now preferred (FAO/WHO 2002). The majority of probiotics are strains of lactobacilli or bifidobacteria, and they are administered in food products such as yogurt, milk drinks, and cheese, as well as capsules and tablets.

In terms of associating probiotic effects with health, it is first worth stating what is meant by the term health. In 1946, representatives of 61 nations signed and ratified the preamble to the constitution of the WHO, which defined health as “a state of complete physical, mental, and social well-being and not merely the absence of disease or infirmity” (WHO 1946). Although this definition is still used today, it has generated heated debate with regard to the parameters that can be used to measure “complete,” “physical,” “social,” well-being (Declaration of Alma-Ata 1978).

The unveiling of the human genome exposed the differences inherent in biological entities including humans. It is important to note that there are variations even among healthy human populations. A single nucleotide polymorphism (SNP) allele that is common in one geographical or ethnic group may be much rarer in another. SNPs may fall within coding sequences of genes or noncoding regions of genes, or in the intergenic regions between genes. SNPs within a coding sequence will not necessarily change the amino acid sequence of the protein that is produced, due to degeneracy of the genetic code. Variations in the DNA sequences of humans can affect how humans develop diseases and respond to pathogens, chemicals, and drugs, including biotherapy involving probiotics. In short, nutrigenomics and “health” are a long way from being intertwined, and until such time it will be impossible to comprehend the influence of probiotics on health through genomic or nutritional modulation. Furthermore, with access to the normal gut functioning during the processing of food being all but impossible in real time, the use of animals is currently the only way to try and understand these complex events. Given the differences between animals and humans, such experiments have limitations.

## THE GUT AND VAGINAL MICROBIOTA

The human gut is home to an estimated  $10^{14}$  microorganisms including bacteria, yeast, and viruses. This constitutes over 1,500 species, with 30–40 comprising up to 99% of the total population (Guarner and Malagelada 2003). Fecal sampling has been primarily used to identify the bacterial constituents, but this has many flaws, not the least of which is the inability to grow most of the clones. The feces, which comprises mostly bacteria, does not provide a reliable readout of which microbes are active in the upper reaches of the intestine, in the mucin, or on epithelial cells. Recent molecular methods have increased

detection of nonculturable organisms. These techniques mostly comprise isolating DNA and examining 16S ribosomal segments that provide footprints for different species. By using polymerase chain reaction (PCR) and techniques such as denaturing gradient gel electrophoresis (DGGE) (Simpson et al. 1999), real-time PCR with DGGE (Bibiloni et al. 2006), rapid amplified ribosomal DNA restriction analysis (ARDRA) (Ventura et al. 2000), and a combination of broad-range PCR amplification of 16S rDNA with clone analysis, bacterium-specific PCR assay of 16S rDNA, and fluorescence in situ hybridization (FISH) (Fredricks et al. 2005), it has been possible to better describe the variety of species in human sites. Understandably, the gut microbiota is complex and fluctuates over time, while the vaginal flora comprises relatively few species and mostly lactobacilli in healthy women (Fredricks et al. 2005; Heinemann and Reid 2006).

These techniques along with culture provide insight into the stepwise colonization of the host after birth. Interestingly, a study of several hundred babies showed no support for the hypothesis that sensitization to foods or atopic eczema in European infants in early life was associated with lack of any particular culturable intestinal commensal bacteria (Adlerberth et al. 2007). Care must be taken in interpreting this conclusion. First, most species are not culturable, and thus the report cannot conclude that foods do not influence the microbiota. Second, even if species-specific probes are used in combination with quantitative PCR, it is still not possible to accurately detect all microbes present, and thus the finding of relatively small numbers (of bacteria, phages, yeast, or other organisms) may be missed, and at a given niche any one of them could be important in health or pathogenesis.

A very careful DGGE study of the microbiota inherited by two babies was carried out in 2002 and showed clear effects with weaning (Favier et al. 2002). The exact species identified is not so important, as a sample size of two is not sufficiently large to make conclusions. However, the fact that changes were easily observed shows that the effect on diet is measurable. This was further confirmed in a study of 10 healthy newborn infants during their first days of life, and at 6 months of age, where bacterial cellular fatty acid (CFA) were found to fluctuate from hour to hour during the first days of life and resembled those for both the mothers and the nurses, while gut colonization also fluctuated markedly from hour to hour (Favier et al. 2003). If the babies are fed breast milk, formula, or fructo-oligosaccharide supplemented formula, the microbiota also changes,

with the latter associated with higher numbers of bifidobacteria and bacteroides and fewer *Escherichia coli* and enterococci (Kapiki et al. 2007). Taking these studies one step further, it would have been interesting to have obtained intestinal cell readouts as the microbiota was being established. This would represent nutrigenomics at its best. As humans age, the complexity of the diet and microbes makes it much more difficult to make conclusions on genomic modulation caused by the organisms and/or the food.

The process of determining a nutrigenomic or bacterial-human genomic print out in the gut of newborns is further complicated by the process of birthing and feeding. During vaginal birth, the mother's fecal and vaginal organisms pass into the baby, while those born by caesarian acquire their microbes through handling and kissing. Recent studies have shown that breast milk also plays a role in delivery of microbes. Martin's group (Martín et al. 2003), then others (Gueimonde et al. 2007; Perez et al. 2007), reported the presence of lactobacilli in breast milk, ostensibly taken to the mammary duct via intestinal translocation or sampling by dendritic cells. If this is confirmed by others, it will be intriguing to find out if, and how, the dendritic cells choose certain bacterial types among the plethora of species present. It seems inconceivable that such selection occurs, but if it does not, then how is it that lactobacilli are the only species to survive in the mammary gland? Many questions and, right now, few answers.

There are well-known protective factors in breast milk, but if lactobacilli are part of the defenses passed onto the newborn in milk (Parracho et al. 2007), it would be helpful to differentiate their role. Other species clearly play a role in host immunity. It has been reported that *Bacteroides fragilis* produces a zwitterionic polysaccharide (ZPS) that activates CD4+ T cells and directs the development of a normal immune system (Mazmanian et al. 2005). However, in two studies of sequential colonization by bacteria in newborns, Favier et al. (2002, 2003) did not detect *B. fragilis*, thus raising the question did another *Bacteroides* species undertake this role?

The urogenital tract has long been assumed to have sterile areas (bladder, prostate, uterus) and regions where the microbiota is relatively simple in types of organisms present (vagina, distal urethra, cervix). However, recent studies have altered our understanding of this region. In women attempting to conceive, studies have shown that those with bacterial vaginosis (BV) and a decreased vaginal count of lactobacilli have decreased conception rates and increased early pregnancy loss (Eckert et al. 2003).

There is no universal agreement on this, with one example in the United Kingdom showing that BV was more prevalent in patients with tubal (31.5%,  $n = 149$ ) versus with nontubal (19.7%,  $n = 152$ ) infertility, but this did not have an adverse effect on the fertilization rate (Liversedge et al. 1999). The mechanism whereby lactobacilli might reduce the failure of in vitro fertilization is theoretically twofold. By reducing inflammation caused by BV, it may make conception more possible. Or, the presence of lactobacilli may inhibit infection that would otherwise lower conception rates.

Once pregnant, other possible roles for lactobacilli exist. If they ascend the uterus from the cervix, which is not an unlikely occurrence given the ability to survive in mucin and in an anaerobic or low oxygen environment, they could produce molecules that cross the placental wall and influence fetal development. Or, they could produce molecules that influence fetal health via the maternal–fetal bloodstream. Such concepts may appear far fetched, but the study showing that maternal intake of lactobacilli led to reduced incidence of atopic dermatitis (Kalliomäki et al. 2003) suggested that some signaling process was happening that affected the fetus. This finding has not been repeated to date. However, to offset some criticism of their findings, the Finnish group undertook a study on infants with IgE-associated atopy. Treatment with *Lactobacillus rhamnosus* GG induced higher C-reactive protein levels than in the placebo group as well as higher IL-6 levels and soluble E-selectin, which was indicative of a skew toward a Th1 response, as distinct to a Th2 allergic response (Viljanen et al. 2005). This supported their initial conclusions that lactobacilli influenced atopy. It is also feasible that the probiotic therapy in the mother led to increased lactobacilli counts in the vagina, and these were transferred to the newborn and led to less severe atopy. To date, this has not been investigated.

The vaginal and urethral microbiota form early in life and change notably with hormonal levels at puberty and menopause. The early composition is high in *E. coli*, *Klebsiella*, *Proteus*, enterococci, staphylococci (Bollgren and Winberg 1976a; Senses et al. 2007), coinciding with an increased risk of urinary tract infection (Bollgren and Winberg 1976b). There are very few studies on the urethral microbiota in healthy males, and of those, one study of 50 subjects showed that enterococci and *E. coli* were prevalent (Günşar et al. 2004), while another showed the additional presence of *Staphylococcus epidermidis*, *Corynebacterium*, lactobacilli, and *Haemophilus* sp. (Bowie et al. 1977).

## HOW THE HOST RESPONDS TO MICROBES

Dendritic cells are believed to be critical to innate and adaptive immunity, as specialized antigen-presenting cells. Niess et al. (2005) showed that lamina propria of dendritic cells forms transepithelial dendrites that enable the cells to directly sample antigens, such as commensal or probiotic bacteria. In the gut, anecdotal evidence has revealed the commensal-specific TLR/MyD88 pathways, which permits the symbiotic relationship between the microflora and the host, while pathogen-specific virulence factors are required to trigger proinflammatory responses via the usage of additional TLR coreceptor and/or adaptor molecules for disease-causing organisms (Sirard et al. 2006).

The interaction between microbes and the human host can, of course, lead to infection and inflammation. Kleessen and colleagues (Kleessen et al. 2002) demonstrated that more bacteria are present on the mucosal surface of inflammatory bowel disease (IBD) patients than on those of non-IBD controls. Bacterial invasion of colonic specimens has been demonstrated from ulcerative colitis patients, but not in tissues from controls. The specimens were colonized by a variety of organisms such as *Proteobacteria*, the *Enterobacteriaceae*, the *Bacteroides/Prevotella* cluster, the *Clostridium histolyticum/C. lituseburense* group, the *C. coccoides/Eubacterium rectale* group, high G + C Gram-positive bacteria, and sulfate-reducing bacteria.

Molecular genetic studies of IBD samples have led to the application of genome-wide linkage analysis involving multiple affected families. This, in turn, has led to the identification of a number of susceptibility loci, such as the IBD1 locus on chromosome 16 that heralded the discovery of the NOD2/CARD15 gene as the first susceptibility gene in Crohn's disease (Ogura et al. 2001).

NOD2 has been shown to recognize intracellular peptidoglycan fragments (e.g., muramyl dipeptide) through its leucine-rich region, leading to proinflammatory responses through activation of NF- $\kappa$ B. NOD2 serves as an intracellular pattern recognition receptor to enhance host defenses by inducing the production of antimicrobial peptides (AMPs) such as human  $\beta$ -defensin-2 (Voss et al. 2006). Genetic studies have revealed three common disease susceptibility variants (DSV) in the NOD2 gene associated with IBD in Caucasians, but not in Asians. The response of these genes to intake of probiotics or dietary compounds has not been investigated, to our knowledge. Such a nutrigenomics study would be very interesting

especially to determine if bacteria or food were involved in either induction of the IBD condition or its remediation.

Native Indians have been the focus of a series of studies because of apparent increased risk of IBD, diabetes, and other diseases that may be influenced by diet. A recent case-controlled study screened NOD2 variants and examined susceptibility for IBD in North Indians. Confirmed cases of ulcerative colitis and Crohn's disease and healthy controls matched for age ( $+/- 10$  year) and ethnicity were studied. In addition to genotyping the three DSV (SNP8, SNP12, and SNP13), all 12 exons were resequenced to determine other potential SNPs. The study showed that all three DSV were either monomorphic or rare in the population. Sequencing revealed two SNPs involving SNP5 (268 Pro/Ser) and rs2067085 (178 Ser/Ser). The frequency of SNP5 was higher among UC (17% versus 12.0% in controls,  $p = 0.016$ ) and CD cases (20% versus 12%,  $p = 0.28$ ). SNP5 carriers had elevated risks for UC (OR = 1.72, 95% CI = 1.17–2.52,  $p = 0.005$ ). Besides, the absence of known IBD, DSV and potential associations between SNP5 and UC in North Indians suggest the presence of allelic heterogeneity for UC susceptibility (Juyal et al. 2007). This landmark finding has led to a redirection of basic research in IBD with interest focused principally on regulation of the innate immune response and mucosal barrier function. This is another reason why probiotic interventions may have a place in therapy. However, to date, the use of probiotics to treat IBD has had limited success.

One study showing that *L. rhamnosus* GG failed to retain remission in moderate-to-active Crohn's disease was too small (only 11 subjects) to make definitive conclusion (Schultz et al. 2004). In a trial of 32 patients, 6 weeks of VSL#3 treatment (a probiotic product that contains strains of *L. casei*, *L. plantarum*, *L. acidophilus*, *L. delbrueckii* ssp. *bulgaricus*, *Bifidobacterium longum*, *B. breve*, *B. infantis*, and *Streptococcus salivarius* ssp. *thermophilus*) did show some maintenance of remission (Bibiloni et al. 2005). The positive results are more credible when there is a possible mechanism identified. An animal study showed that VSL#3 could increase basal luminal mucin content by 60%, with MUC2 gene expression particularly upregulated (Caballero-Franco et al. 2007).

The anti-inflammatory effects of VSL#3 have been explored. The mixture produces soluble factors that inhibit the chymotrypsin-like activity of the proteaseome in gut epithelial cells and inhibit the proinflammatory NF- $\kappa$ B pathway and induce the expression of cytoprotective heat shock proteins (Petrof et al.

2004). However, modulation of immunity is not a simple process, and a rat study showed that VSL#3 upregulated hBD-2 via induction of proinflammatory pathways including NF- $\kappa$ B, AP-1, and MAPKs (Schlee et al. 2008). In other words, the opposite conclusion to the Petrof et al. (2004) paper. Clearly, more studies are needed to understand if gene expression changes in immune regulators differ between people or animals, and if a net anti-inflammatory process is the mechanism that leads to clinical benefits.

Gene expression changes are being investigated and helping to understand the role of molecules such as IL-23 and autophagy-related 16-like 1 (ATG16L1) strongly implicated in IBD (Van Limbergen et al. 2007). IL-23 is a key cytokine in the differentiation of T-helper cells, especially to Th17 T cells, a subset of which mediate chronic and autoimmune inflammatory response (Hue et al. 2006). To reemphasize that different populations respond differently to treatment or prevention of IBD, in some populations, rs1004819 is the major IL23R variant associated with CD, while the p.Arg381Gln IL23R variant is a protective marker for CD and ulcerative colitis (Glas et al. 2007).

To assess the effect of nutrients and probiotics on IBD, biomarkers need to be found. A recent study compared the performance of fecal markers lactoferrin, calprotectin, polymorphonuclear neutrophil elastase, and serum C-reactive protein in IBD patients, and reported that they could differentiate active from inactive disease as well as IBS (Langhorst et al. 2008). Lactotransferrin, a member of the iron-binding transferrin proteins known to have antimicrobial properties, plays an important role in the mucosal immune response (Spik and Montreuil 1983), with an ability to inhibit the growth of *E. coli* (Nakshatri and Badve 2007).

With respect to irritable bowel syndrome (IBS), a recent review suggested there is a rationale for using probiotics to correct a dysfunctional relationship between the indigenous microbiota and the host (Quigley and Flourié 2007). The authors were involved in the development of *B. infantis* 35,624, an organism that showed relief of some symptoms of IBS (O'Mahony et al. 2005; Whorwell et al. 2006). This is a complex condition that presents itself in different ways to different people. Pain, abdominal discomfort, excess gas, and urgency in defecation are some of the symptoms. Formally, IBS is diagnosed by a Rome III criterion (Lacy and Lee 2005), which requires at least 12 weeks of abdominal pain or discomfort in the past year, with two of the following three symptoms: relieved with defecation; and/or change in the frequency of stool; and/or change in

the form of stool. Symptoms that cumulatively support the diagnosis of IBS include the following: abdominal frequency of stools ( $>3$  per day or  $<3$  per week); abnormal form; abnormal passage; presence of mucus; bloating with distension. The mechanisms of action of *B. infantis* 35,624 have been somewhat investigated in animals, with a proposed upregulation of anti-inflammatory IL-10 (Sheil et al. 2006). This seems a dubious conclusion, given that inflammation is not the main factor that presents in IBS, unless it plays a role in abdominal pain. Of more sense, in terms of correlation with clinical outcome, is that *B. infantis* 35,624 does not function through an anti-inflammatory process, but using some other regulatory pathway, it reduces abdominal pain and bloating. Other effects, such as reducing bowel dysfunction affecting incomplete evacuation, straining, and the passage of gas needs to be investigated for mechanisms of action.

The finding in a rat study that *L. acidophilus* NCFM induced an antinociceptive effect similar to subcutaneous administration of 1 mg morphine per kg (body weight) provided a mechanism that could reduce pain in IBS patients after probiotic use (Rousseaux et al. 2007). Human studies are needed to test this, but the findings are very interesting. The induction of analgesia was significantly inhibited by peritoneal administration of the cannabinoid receptor 2 (CB2)-selective antagonist AM-630 but not by the opioid receptor antagonist naloxone methiodide, providing indirect evidence for a physiological role of CB2 in the control of intestinal pain.

A new multispecies probiotic supplementation (*L. rhamnosus* GG, *L. rhamnosus* Lc705, *Propionibacterium freudenreichii* ssp. *shermanii* JS, and *Bifidobacterium animalis* ssp. *lactis* Bb12) has been shown to be effective and safe in humans, and is able to alleviate symptoms of IBS (Kajander et al. 2007). More strains and product formulations are being tested, and if combined with the use of microarrays, RNA interference, and nanotechnologies, it will hopefully become apparent that these therapies and the molecules they produce directly or indirectly affect individual phenotypes (Trujillo et al. 2006).

## ORAL CAVITY AND UROGENITAL TRACT

Microorganisms provide benefits to regions of the body in addition to the gastrointestinal tract. The most widely studied are the mouth and urogenital tract. The oral environment is surrounded by saliva, recently described as a “dynamic proteome” because

salivary protein secretion is under neurologic control, with protein output being dependent on the stimulus (Helmerhorst and Oppenheim 2007). Also, extensive salivary protein modifications occur in the oral environment, where a plethora of host- and bacteria-derived enzymes act on proteins emanating from the glandular ducts. Many modified proteins occur and influence the microbiota. Uncovering the proteome of whole saliva will help decipher the key factors in oral health.

A Bioinformatics Resource for Oral Pathogens (BROP) has been created to help analyze and determine the growing number of complete genomic sequences of oral organisms (mostly pathogens to date). The system has been designed to provide side-by-side visual comparison of independently annotated datasets for the same genome (Chen et al. 2005). Such a system could prove useful for studying probiotic strains from the same species.

Some applications of probiotics to the oral cavity have been tested. A two-strain *L. reuteri* lozenge taken once daily for 10 days significantly reduced salivary *S. mutans* (Caglar et al. 2008), while a study using *S. salivarius* K12 showed reduction in halitosis (Burton et al. 2006). One mechanism of action appears to involve displacement of pathogens, but they might also influence AMPs, including defensins, which are part of innate immunity (Abiko and Saitoh 2007). Human  $\beta$ -defensins 2 and 3 (hBD-2 and hBD-3) are expressed in response to bacterial stimuli or inflammation, and these differ for oral commensal *S. gordonii* and oral pathogens *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans* (Chung and Dale 2004). Thus, it is possible that probiotic lozenges increase host defensin activity and help reduce pathogen load.

The urogenital microbiota is a relatively simple one in comparison to the mouth and gut, with a few strains, most commonly lactobacilli, present during a healthy state, and a more diverse grouping of 4–12 species associated with illness (Heinemann and Reid 2006). In women, the normal and pathogenic microbiota mostly originates in the person’s own intestine, and then migrates along the skin to the vagina.

Two recent gene expression studies have been done on vaginal samples. In the first, a method was developed to assess epithelial cells gene expression without the need to collect a biopsy (Kirjavainen et al. 2008). This showed an upregulation of antimicrobial factors including defensins following administration of probiotic *L. rhamnosus* GR-1, but results are preliminary due to the small sample size. In the second study of 20 postmenopausal women, the absence of lactobacilli and presence of BV was associated

with a sevenfold downregulation of host antimicrobial colony stimulating factor:  $-9.83$ -fold for IL-1 $\alpha$ ,  $-8.33$  for IL-1 $\beta$ , and  $-3.63$  for IL-6 (Dahn et al. 2008). In this study, quantitative PCR was performed on selected molecules to confirm that Affymetrix genomic array data from chips displayed 54,675 different probesets corresponding to 54,613 transcripts.

These are the first genomic readouts of vaginal cells associated with the presence or absence of lactobacilli. The expense of arrays makes it prohibitory for large-scale studies, and thus to date, only clinical end points have been reported following the use of probiotics in the vagina.

*L. rhamnosus* GR-1 and *L. reuteri* (formerly fermentum) RC-14 are the most documented for repopulating the vagina and reducing the risk of infection (Burton et al. 2003; Cadieux et al. 2002; Morelli et al. 2004; Reid et al. 2001, 2003). The two *Lactobacillus* strains were detected in 80% of 10 healthy premenopausal women 1 week after daily vaginal instillation of  $10^9$  viable cells (Reid et al. 2001).

In a randomized, double-blind, placebo-controlled trial, Reid et al. (2003) showed that oral administration of *L. rhamnosus* GR-1 and *L. reuteri* RC-14 significantly improved the vaginal microenvironment. In the study, 32 women (19–46 years old) received oral freeze-dried capsules of *Lactobacillus* GR-1/RC-14 ( $>10^9$  cfu per strain) once daily for 60 days and the other 32 women received placebo. Cultures of vaginal fluid showed a significant increase in lactobacilli ( $p = 0.01$ ), a decrease in yeast ( $p = 0.01$ ), and a reduction of coliforms ( $p = 0.001$ ) at day 28 in the *lactobacillus* group compared with those in the placebo group. More women in the *lactobacillus* group reported improvement in vaginal health (vaginal itching and odor). Morelli et al. (2004) demonstrated independently the ability of these two lactobacilli to pass through the intestine and reach the vagina when given orally.

Another strain, *L. crispatus* CTV-05, has been shown to populate the vagina after local administration. After 30 days, the vagina of 62% women had the strain present compared to 2% in the placebo group (Antonio and Hillier 2003). The use of strains GR-1, RC-14, and CTV05 provide hope for reducing the burden of illness caused by urogenital pathogens in women (Falagas et al. 2006).

The failure of pharmaceutical agents to adequately prevent, or in some cases effectively treat, infections, without side effect has led to increased interest in alternatives, such as probiotics (Boskey 2005). BV is the most frequent of urogenital infections in women, but it is a condition that is often difficult to diagnose, and one in which treatment is not always given

or effective. For many years, BV was regarded as a condition in which pH was elevated ( $>4.5$ ), fishy discharge was present, and lactobacilli were replaced by Gram-negative anaerobic bacteria. However, the realization that odor and discharge do not always occur (Klebanoff et al. 2004) and aerobic (Donder et al. 2002) and Gram-positive anaerobic (Burton et al. 2004) bacteria are associated with BV has made it clear that diagnosis of BV is far from being simple.

The use of metronidazole or clindamycin is the primary treatment of BV. The efficacy is reasonable, but far from ideal, with the condition recurring commonly. One problem is that neither agent was designed to be effective against biofilms, multilayered, dense clumps of bacteria indicative of BV (Swidsinski et al. 2005). Also, growing resistance to clindamycin (Austin et al. 2005) and inability of both these agents to cure aerobic BV constituents make other approaches worthy of testing.

On the basis of the ability of lactobacilli to disrupt BV biofilms (Burton et al. 2003; Saunders et al. 2007), it was hypothesized that by combining probiotics with antibiotics the cure rate of BV could be improved. To test this, a randomized, double-blind, placebo-controlled trial of 125 premenopausal women diagnosed with BV was performed. Subjects were given a single oral dose of metronidazole (500 mg) twice daily from days 1–7, plus oral *L. rhamnosus* GR-1 and *L. reuteri* RC-14 or placebo twice daily from days 1–30. Primary outcome was cure of BV as determined by Normal Nugent score, negative sialidase test and no symptoms or signs of BV at day 30. Out of 106 subjects that returned for 30-day followup, 88% were cured in the antibiotic/probiotic group compared to 40% in the antibiotic/placebo group ( $p < 0.001$ ) (Anukam et al. 2006a). Of the remaining subjects, 30% in the placebo group and none in the probiotic group had BV, while 30% in the placebo and 12% in the probiotic group fell into the intermediate category based on the Nugent score, sialidase result, and clinical findings. High counts of *Lactobacillus* sp. ( $>10^5$  cfu/ml) were recovered from the vagina of 96% probiotic-treated subjects compared to 53% controls at day 30 (Anukam et al. 2006a).

In a study comparing clinical utility of probiotics on their own versus vaginal metronidazole gel for BV, the lactobacilli treatment resulted in 90% cure of BV compared to 33% in the metronidazole group (Anukam et al. 2006b). The use of two dried capsules containing *L. rhamnosus* GR-1 and *L. reuteri* RC-14 each night for 5 days proved better than 0.75% metronidazole gel, applied vaginally twice a day (in the morning and evening).

## HOW THE MICROBES RESPOND TO THE HOST

The genetic makeup of bacteria provides a means to respond to different environments. Thus, bifidobacteria have genes that can utilize fructo-oligosaccharides (Schell et al. 2002) and their numbers in the colon increase with the ingestion of prebiotics (Puccio et al. 2007; van den Broek et al. 2007). Short-chain fructo-oligosaccharides and other prebiotics are used to selectively stimulate the growth and activity of lactobacilli and bifidobacteria in the colon.

The genomic basis of short-chain fructo-oligosaccharides' metabolism in *L. plantarum* WCFS1, expressing different genes when grown on scFOS compared to glucose has been studied. A significant upregulation (8- to 60-fold) was observed with a set of only five genes located in a single locus and predicted to encode a sucrose phosphoenolpyruvate transport system, a beta-fructofuranosidase, a fructokinase, an alpha-glucosidase, and a sucrose operon repressor (Saulnier et al. 2007). These findings suggest that it may be feasible to select probiotic strains because they express specific functions when delivered with dietary products. Another example is the use of *Lactococcus lactis*, which utilizes either a lactococcal aspartate aminotransferase gene or a *B. longum* alpha-galactosidase gene as selectable markers (Sridhar et al. 2006).

Also, strains of lactobacilli have been shown to modulate immunity in different ways (Díaz-Ropero et al. 2007), suggesting not only differences between the organisms but also the ability to sense and react to their environment. Thus, a probiotic could theoretically help alleviate constipation and diarrhea (Guyonnet et al. 2007; Koebnick et al. 2003).

The ability of bacteria to sense their environment is termed "quorum sensing." This is essentially a cell–cell communication function that uses autoinducer peptides (AIs) to sense the environment and utilize genes that best allow adaptation to the conditions. For example, several inducible class II AMPs function as AIs. The AIs induce their own structural genes, and genes required for production of, and immunity to, the class II AMPs (Quadri 2002). AI-2 is regarded as a universal bacterial signaling molecule synthesized by the LuxS enzyme, which forms an integral part of the activated methyl cycle. Probiotic *L. rhamnosus* GG, GR-1, and RC-14 all produce AI-2-like molecules. It appears that the lactobacilli send out and receive signals that induce metabolic changes and, for example, might lead to

a more dense presence, like a biofilm (Lebeer et al. 2007).

A number of bacteriocins are involved in quorum sensing in lactobacilli. The presence of certain bacteria can act as an environmental signal that switches on bacteriocin production in *L. plantarum* NC8 via a quorum-sensing mechanism mediated by an induction factor PLNC8IF (Maldonado et al. 2004). Such switching on of bacteriocins could be critical in vivo. An animal study has shown that a bacteriocin from *L. salivarius* UCC118 can protect mice against *Listeria* infection (Corr et al. 2007).

An intestinal anaerobic firmicute organism, *Roseburia inulinivorans*, produces butyric acid during growth on glucose, starch, or inulin. It contains a set of genes coding for fucose utilization, propanediol utilization, and the formation of propionate and propanol that are upregulated during growth on fucose. In this organism, a typical Gram-positive, agr-type, quorum-sensing system was upregulated during growth on fucose (Scott et al. 2006), again demonstrating the importance of signaling in probiotic and commensal bacteria.

AI-2 production can affect virulence genes in pathogens such as enterohemolytic *E. coli* (EHEC) O157, which is responsible for lethal gastroenteritis that leads to multiorgan failure. *L. acidophilus* La-5 has been shown to secrete a molecule that either acts as a quorum-sensing signal inhibitor or directly interacts with bacterial transcriptional regulators, controlling the transcription of EHEC O157 genes involved in colonization (Medellin-Peña et al. 2007). In another series of experiments, the activity of Shiga-like toxin 2, critical to *E. coli* O157:H7's lethal infectivity, was significantly inhibited by *L. acidophilus* A4 and 30SC cell lysates (Kim et al. 2006).

Such findings from in vitro experiments do not necessarily mean that the use of probiotics at early stage *E. coli* O157:H7 infection will reduce the incidence of death or serious renal damage. In an effort to examine the correlation between in vivo and in vitro findings, Denou et al. (2007) investigated the transcription of the *L. johnsonii* NCC533 genome during in vitro and in vivo growth, using the microarray technology. During broth growth, 537, 626, and 277 of the 1,756 tested genes were expressed during exponential phase, "adaptation" (early stationary phase), and stationary phase, respectively. In these three phases, 101, 150, and 33 genes, respectively, were specifically transcribed. In vivo transcription of *L. johnsonii* NCC533's genome in mice indicated the largest number of transcribed genes were in the stomach, followed by cecum and the jejunum, while only 26 were transcribed in the colon. In vitro and

in vivo transcription programs overlapped only partially. One hundred ninety-one of the transcripts from the lactobacilli in the stomach were not detected during in vitro growth; 202 and 213 genes, respectively, were transcribed under all in vitro and in vivo conditions; but the core transcriptome for all growth conditions comprised only 103 genes. The study revealed major differences between in vitro and in vivo expression of genes in this *Lactobacillus* gut commensal organism, and also marked changes in the expression of genes along the digestive tract. This finding is significant and may provide a clue to why differences exist in response to certain probiotic activities in the gut.

Animal studies continue and provide some insight into how probiotic organisms are influenced by the host, and affect the host. Using microarray analysis, Shima et al. (2008) demonstrated that *L. casei* Shirota and *B. breve* Yakult noticeably affected gene expression in the ileal and colonic epithelial cells of mice, more so than segmented filamentous bacteria. In addition, *L. casei* Shirota enhanced gene expression involving defense/immune functions and lipid metabolism more strongly than *B. breve* Yakult. It would be interesting to understand what triggers the Shirota strain to do this, and what benefit it derives from the host's response.

Using Affymetrix microarrays, Nerstedt et al. (2007) comparatively characterized the differences in gene transcription in the distal ileum of normal microbiota and germ-free mice evoked by oral administration of two *Lactobacillus* strains used in fermented dairy products—*L. paracasei* ssp. *paracasei* F19 or *L. acidophilus* NCFB 1748. Although no wild-type animal is germ free, the study suggested that changes in intestinal gene expression caused by *Lactobacillus* were complex and indicative of a dynamic host–bacterial relationship.

## CONCLUDING REMARKS

The use of probiotics has gained momentum over the past 10 years. The gastrointestinal microbiome project now underway will uncover valuable information on the genetic readout of these organisms. If analyzed along with genomic information from different hosts, it will help us better understand the importance of microbes for our health. The key to such analysis will be tracking the acquisition of microbes in newborns, as well as changes associated with specific diseases. Real-time readouts, when technologically possible, will help to explain how different nutrients influence the microbiota and host. Over the next

10 years, this dynamic field offers much hope in creating new, natural probiotic, prebiotic, and proteomic solutions to restoration and maintenance of human and animal health. Deciphering local and distant site genomic and proteomic readouts will be challenging, to say the least, especially for the latter that can differ from cell to cell, can constantly change, and be overshadowed by different physiological and environmental factors.

The identification of biomarkers that reflect the outcome of probiotic and prebiotic use will greatly benefit the field. Reliable markers that distinguish disease from health or different stages of a disease or recovery will be the core element of progress. Access to tissues and real-time analysis are challenging, and will require engineering technologies not yet ready for human use. The use of recombinant probiotics will increase, and depending upon regulatory acceptance, they will bring the food–medicine disciplines closer together, and expand the extent to which beneficial microbes impact well-being. Hopefully, new products will reach the high standards set by the FAO/WHO (2002). Meanwhile, the creation and universal access to “omic” databases will be a major step in allowing the global scientific community to take a chance to make sure progress is optimal.

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## Section IV

# Advanced Analytical Techniques for Nutrigenomics and Proteomics

# 23

## Microarrays: A Powerful Tool for Studying the Functions of Food and Its Nutrients

*Hiroshi Mano, Jun Shimizu, and Masahiro Wada*

### INTRODUCTION

#### MICROARRAYS AS A TOOL FOR STUDYING GENE EXPRESSION

A microarray is a high-throughput genomic tool (Elliott et al. 2007; Mutch et al. 2005). It can be used for profiling and monitoring the expression levels of tens and thousands of genes (entire genomes). It can also be used to determine the influence of food nutrients and/or bioactive compounds (food factors) on metabolic pathways and to understand how food nutrients and factors maintain homeostatic control of gene expression levels. The traditionally accepted central dogma of molecular biology and general schematic of “ome” analysis are shown in Figure 23.1. Microarray technology is a “nutrigenomics” tool and can be used to investigate the levels of transcripts in particular (Davis and Hord 2005; Goodacre 2007).

A microarray is an arrayed series of microscopic spots of DNA oligonucleotides of specific sequences (Müller and Kersten 2003). A microarray is used to measure the mRNA abundance of a sequence in a sample relative to that in the control (Figure 23.2). mRNA is extracted from each animal or cell sample treated with food factors or nutrients. The mRNA is reverse transcribed to yield cRNA probes. The labeled mRNA of the samples functions as probes that hybridize only with the correct target sequence under high-stringency conditions. In microarray analysis, the probes covalently bind to a specific sequence on the surface of the slide. The abundance of the bound-labeled probes is measured using microarray scanners such as the GeneChip Scanner (Affymetrix), GenePix (Axon Instruments), and ScanArray (Perkin Elmer). The following microar-

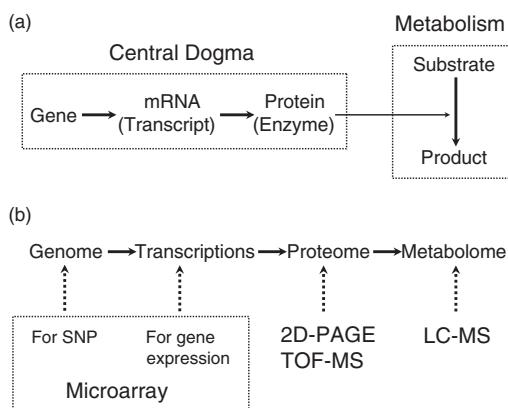
rays are suitable platforms for gene expression analyses: GeneChip (Affymetrix), Agilent Oligo Microarray Kit (Agilent Technologies), Illumina Microarray Core (Oregon Health & Science University), and CodeLink (GE). The expression data of tens and thousands of genes are visualized and statistically analyzed using specific software programs such as Expression Consol (Affymetrix), GeneSpring (Agilent Technologies), and Spotfire (Spotfire Inc.). The obtained expression data are interpreted using software programs that analyze biological information, such as KeyMolnet (originally developed by Institute of Medical Molecular Design Inc.) or Pathway Studio (Ariadne Genomics). Thereafter, these data are used for the analysis of metabolic pathways and in bioinformatics.

#### OTHER MICROARRAYS

Microarrays are now being used for applications other than the analysis of gene expression. Complex features such as chromosomal additions and deletions, introns and exons, gene regulatory sequences, single nucleotide polymorphisms (SNPs), and translation modifiers such as microRNAs are now being analyzed with microarrays.

“SNP arrays” identify an SNP in alleles, and these SNPs can, in turn, be compared within a population or between populations (Beaudet and Belmont 2008; Heinrichs and Look 2007). In other words, it identifies the genetic variations among individuals and across populations. An SNP array can also be used to profile somatic mutations and discover or measure genetic predisposition to diseases.

“ChIP-on-chip” (chromatin immunoprecipitation on chip), which is also known as location analysis,



**Figure 23.1.** Traditionally accepted central dogma of molecular biology (a). A schematic of the “ome” analysis and the method used are provided in the upper field (b).

provides an insight into the key mechanisms of methylation; histone modification; and DNA replication, modification, and repair (He et al. 2008; Trelle and Jensen 2007). It has been used to understand diseases such as those related to lifestyle, cancer, and cell proliferation and differentiation. ChIP-on-chip uses chromatin immunoprecipitation along with a

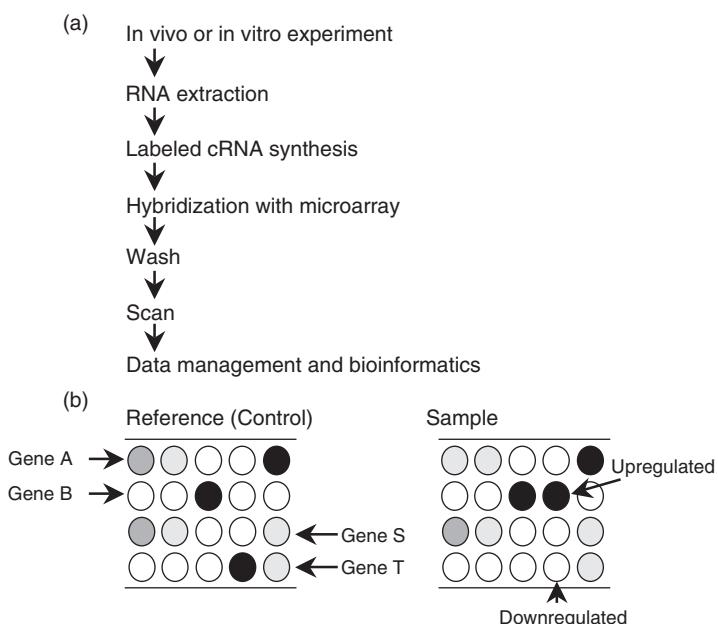
glass-slide microarray (chip) to analyze the mechanism underlying the interaction between regulatory proteins and the genome of living cells. Regulatory proteins control chromosome replication and gene activity by binding to genomic DNA. The protein–chromatin (DNA fragment) complexes are collected by immunoprecipitation, and the DNA fragments are labeled and hybridized using microarray technology.

## METHODS

In this chapter, we focus on the use of microarrays for analyzing gene expression to determine the influence of food factors and/or nutrients on metabolic pathways and to understand the homeostatic control exerted by these food factors and/or nutrients on gene expression (Reiter et al. 2005; Salsberg and Ludwig 2007; van Breda et al. 2005a, 2005b; Weindruch et al. 2001; Xie et al. 2007). A general flowchart of the procedure followed for microarray analysis is shown in Figure 23.2.

## EXPERIMENTAL DESIGN

In general, whole animals (in vivo) and cultured cells (in vitro) are used as samples for analyzing the functions of food factors and nutrients (Mano et al. 1994; Nakatani et al. 2007). However, animal experiments



**Figure 23.2.** Schematic of the typical procedures of microarray analysis (a). Model of the resulting images of microarray (b).

should be ethically performed, and animal use in the experiments should be in keeping with the guidelines of the “Institutional Animal Care and Use Committee.” Animal experiments are suitable for analyzing all metabolic pathways involving food factors and nutrients.

Whole animals (in vivo) are used as analytical samples: Rodents such as mice and rats, including gene-modified (transgenic and knockout) mice, are frequently used in microarray analysis. This is because ready-made microarray chips for these animals can be obtained from a number of platforms, and considerable information regarding the influence of food factors and nutrients on metabolic pathways and their homeostatic control of gene expression is also available. Other species such as human, chicken, *Caenorhabditis elegans*, and *Drosophila* may also be used in microarrays.

We used adult male mice (4-week-old C57BL/6J or model mice) or adult male rats (4-week-old [Sprague-Dawley] SD or model rats) in our in vivo experiments. The animals were housed individually in an environment-controlled room under constant temperature ( $25 \pm 3^{\circ}\text{C}$ ) and a 12-h light/dark cycle. The animals were divided into two groups: the first group was treated with food factors or nutrients and the other group was the control group. Each group comprised five to seven animals. In the long-term dose experiment, food factors or nutrients were continually administered for several weeks (1–4 weeks) to clarify the late and/or secondary biological effects of the administered food factors or nutrients (Figure 23.3). In the short-term dose experiment, the animals were administered a food factor or nutrient for

2–24 h, and the other food factors or nutrients were administered subsequently to clarify the early biological effect of the administered food factor or nutrient. Dosage was decided according to human consumption (Chávez et al. 2003). The mice were killed by cervical dislocation either with or without overnight food deprivation. They were carefully dissected to obtain tissues for isolating total RNA.

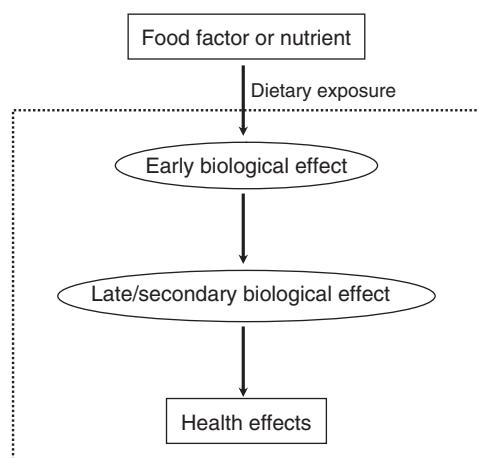
Cultured cells (in vitro) are used as analytical samples. Common cell lines and primary cultures are frequently used in microarrays. The microarray procedures should be decided depending on the cell type. The concentrations of food factors or nutrients to be administered should be determined according to their serum concentration in human treated with these food factors/nutrients. Moreover, a time-course study is important. Short-term (about several hours) treatment with the compounds leads to early gene regulation, while long-term (about several days) treatment with the compounds leads to late and/or secondary gene regulation. This mechanism, and not direct gene regulation, may be related to cell differentiation and maturation. Short-term experiments are more suitable for assessing direct gene regulation by food factors and/or nutrients.

## SAMPLE PREPARATION

Total RNA was isolated using Trizol reagent (Invitrogen) or an RNeasy kit (QIAGEN) according to the manufacturer’s instructions (Mano et al. 2000). The RNA was dissolved in diethyl pyrocarbonate-treated distilled water. The concentration of the total RNA was estimated from the absorbance at 260 nm, and the quality of the sample RNA was determined by agarose electrophoresis and reverse transcriptase polymerase chain reaction (RT-PCR). If RNA degradation was detected by agarose electrophoresis and/or no amplification of genes such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or actin was detected by RT-PCR, the RNA samples were not used for microarray analysis. High-quality RNA isolated from the cells and tissues of the experimental animals were used for analysis.

## MICROARRAY HYBRIDIZATION AND SCANNING

Microarrays enable the analysis of each individual RNA obtained from an animal from a group or that of pooled RNA obtained from all animals in a group. For the analysis of pooled RNA, equal amounts of the total RNA from several animals in each group were pooled. All experiments and analyses were



**Figure 23.3.** The effects of dietary intake of a food factor or nutrient on health.

performed according to the protocol of the microarray platform (Mano et al. 2006).

Our experiments were conducted according to the protocol provided in the GeneChip Expression Analysis Technical Manual (Affymetrix). The isolated mRNAs were reverse transcribed with the T7-(dT)<sub>24</sub> primer and copied to yield double-stranded cDNAs (SuperScript Choice System, Invitrogen). Further, biotin-labeled cRNAs were synthesized (High Yield RNA Transcript Labeling Kit; Affymetrix) and fragmented by heating at 94°C for 35 min. The fragmented cRNAs were hybridized using GeneChip (Affymetrix). After hybridization, washing and staining were performed using the Affymetrix Fluidics Station 400. Thereafter, these arrays were scanned using an Affymetrix array scanner, and the fluorescence intensity was measured with Microarray Suite 5.0 (Affymetrix).

“One-color” or “two-color” microarrays from Agilent Technologies may also be used. A one-color microarray is almost the same as the GeneChip microarray from Affymetrix. A two-color microarray uses a single microarray slide for the comparison of two groups. In a two-color microarray, the cRNA of one group is labeled with Cy5 (green fluorescence) and that of the other group is labeled with Cy3 (red fluorescence). Common slide scanners such as GenePix (Axon Instruments) and ScanArray (Perkin Elmer), which can detect fluorescence, can scan slides prepared using several platforms. The relative intensities of each type of fluorescence may then be used for ratio-based analysis to identify the upregulated and downregulated genes.

#### DATA MANAGEMENT AND BIOINFORMATICS

The scanned hybridized microarray image data need to be converted to numerical data for biological analysis. The considerable, noisy, and complex numerical data are visualized and statistically analyzed using suitable software programs (Werner 2008; Zhang et al. 2008).

The data management cycle comprises quality control (filtering), biological hypothesis (normalization, determination of parameters, and interpretation), statistical analysis (*t*-test, analysis of variance [ANOVA], and clustering), and biological interpretation (construction of pathway maps or similar lists).

In the quality control step, the unreliable data are eliminated using different filters such as “filter on expression level,” “filter on error,” and “filter on flags.” In the biological hypothesis step, reliable numerical data are normalized for comparative analysis by using the guided steps recommended by each platform.

The step “fold change” in comparative analysis is the simplest method for comparing the gene expression level between a sample and reference. For statistical analysis, *t*-test and ANOVA were used. Clustering analysis can group similar genes. The resulting gene expression data may be used for biological analyses. In the biological interpretation step, pathway maps and similar lists are usually constructed. Pathway maps such as metabolic maps and binding and signal transduction maps are constructed by using the genes from similar lists that have been filtered using several parameters.

The data obtained by bioinformatics analysis may also be mined for biological information by using platforms such as KeyMolnet, GeneSpring, Spotfire, and Rosetta Biosoftware. Moreover, bioinformatics database and bioinformatics tools can be used from DAVID Bioinformatics Resources at National Institute of Allergy and Infectious Diseases (NIAID) Web site. Further, the gene expression profiles determined by other scientists can be accessed from databases such as the “Gene Expression Omnibus (GEO)” that may be accessed through the National Center for Biotechnology Information (NCBI) Web site and “ArrayExpress” that can be accessed through the European Bioinformatics Institute (EMBL-EBI) Web site.

## APPLICATIONS

### EFFECTS OF THE DIETARY INTAKE OF “NIGANA” (*Crepidiastrum lanceolatum*, AN EDIBLE PLANT FROM OKINAWA)

Microarray technology was used to evaluate the effect of the dietary intake of nigana (*Crepidiastrum lanceolatum*), which is an edible plant from Okinawa, on the gene expression pattern in the liver.

C57BL/6J mice (8 weeks old) were fed a modified fat diet (oriental yeast) for 3 days (Mano et al. 2006). After overnight fasting, they were orally administered crude plant powders (0.3 g/kg). After 4 h, the mice were sacrificed, and mRNA was extracted using Trizol reagent (Invitrogen). Microarray analysis was carried out using the Murine Genome U74Av2 array (Affymetrix).

The expression levels of several genes changed in the liver of mice that had consumed nigana or spinach (Table 23.1). Although the dietary intake of spinach and water (control) did not affect the mRNA levels of erythropoietin (EPO), nigana consumption increased the gene expression of EPO in the mouse liver (Table 23.2). To obtain high-quality vegetables, we modified the conditions for growing nigana. Three

**Table 23.1.** Genes whose expressions were up- or downregulated by at least 2-fold in the liver of mice fed with "nigana" or spinach relative to their expressions in the liver of control mice (fed only water):

Genes (12621)	c (Water)	Nigana	Spinach
Expressed	5046	4778	5318
Upregulated	—	163	113
Downregulated	—	296	43
Stress (31)			
Upregulated	—	2	0
Downregulated	—	2	0
Energy (11)			
Upregulated	—	1	0
Downregulated	—	0	0
Transcription (821)			
Upregulated	—	19	4
Downregulated	—	19	22
Inflammatory (52)			
Upregulated	—	1	0
Downregulated	—	2	5
Cell Cycle (240)			
Upregulated	—	10	4
Downregulated	—	3	7
Ribosomal (151)			
Upregulated	—	0	0
Downregulated	—	2	5

**Table 23.2.** Comparison between the gene expression levels in the liver of mice fed with nigana or spinach and that in the liver of the control mice (fed only water):

Gene	Nigana	Spinach
Growth factor		
HGF	1.4	1
IGF	0.83	0.9
EGF	nd	nd
TGF $\beta$ 1	nd	nd
TGF $\beta$ 2	nd	nd
TGF $\beta$ 3	1.8	0.87
TGF $\alpha$	1.1	0.7
Hematopoiesis		
CSF1	1.6	1.3
G-CSF	1	0.83
GM-CSF	nd	nd
EPO	5.3	1.3
TPO	0.97	0.45

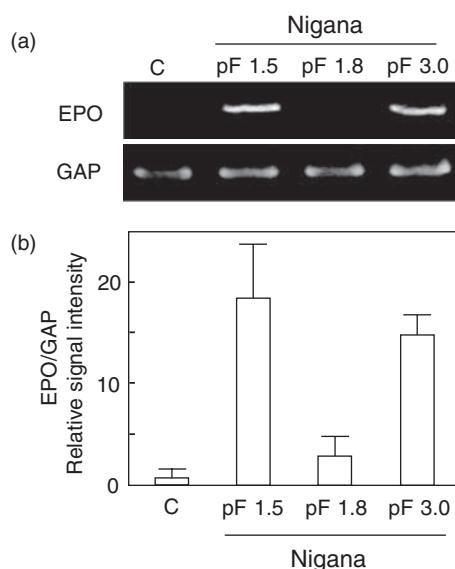
Abbreviations: HGF, hepatocyte growth factor; IGF, insulin-like growth factor; EGF, epidermal growth factor; TGF, transforming growth factor; CSF, colony stimulating factor; G-CSF, granulocyte-colony stimulating factor; GM-CSF, granulocyte-macrophage colony stimulating factor; EPO, erythropoietin; TPO, thrombopoietin; nd; not detected.

irrigation-rate conditions (potential free energy [pF] 1.5, pF 1.8, and pF 3.0) were used for nigana hydroculture. The irrigation condition affected the amounts of the nutrition factors in nigana as well as the gene expression level of EPO in the liver of mice that ingested the plant (Figure 23.4). The pF 1.5 condition did not afford the highest levels of nutrients. However, the low levels of potassium and nitric acid found in plants grown under this condition are a positive finding since these chemicals are known to adversely affect vegetable quality (data not shown). Further, EPO mRNA induction was highest under the pF1.5 condition. Therefore, this condition is considered beneficial (Figure 23.4).

This study indicates that high-quality food may be produced by using microarray technology to study the effect of ingested food on experimental animals.

#### EFFECTS OF THE DIETARY INTAKE OF GLYCOSPHINGOLIPIDS FROM RICE (*ORYZA SATIVA*)

Microarray technology was used to evaluate the effect of the dietary intake of glycosphingolipids (GSLs) from crude rice (RG) on the gene expression pattern in the liver. GSLs are a subtype of glycolipids



**Figure 23.4.** The mRNA levels of erythropoietin (EPO) in the liver of mice fed cultured nigana. (a) RT-PCR analysis of EPO mRNA levels in the liver. (b) Relative signal intensity calculated after measurement with “Image J.” Mean and standard deviation (SD) of the samples that were examined in triplicate are shown.

that contain the amino alcohol sphingosine. The cell membrane and bran of grain contain high concentrations of GSLs. A well-known function of GSL is as a cellular signal. For example, GSLs function as signals that regulate the differentiation, proliferation, programmed death, and apoptosis of cells.

C57BL/6J mice (6 weeks old) were fed an AIN-93G diet containing 0.5% RG and 0.5% konjac glycosphingolipid (KG) or 0.5% cyclodextrin (control) for 2 weeks (Shimizu et al. 2007). mRNA was extracted using Trizol reagent (Invitrogen). Microarray analysis was performed using a CodeLink UniSet Mouse 20 K Bioarray (GE).

The expression levels of several genes changed in the liver of mice that consumed RG or KG (Table 23.3). Dietary intake of RG did not affect the expression of drug metabolism-related genes, showing that interactions between RG and some drugs is unlikely (Table 23.4). The expression of glycolysis-related genes was slightly increased, while that of tricarboxylic acid cycle-related genes was slightly decreased (Table 23.3 and Figure 23.5). With regard to lipid metabolism-related genes, the expression of carnitine acetyltransferase (Crat), which reversibly catalyzes the production of acetyl carnitine from acetyl-CoA, was markedly increased. As for the

**Table 23.3.** Genes whose expressions were up- or down-regulated by least 2-fold in the liver of mice fed experimental diets relative to their expressions in the liver of the control mice.

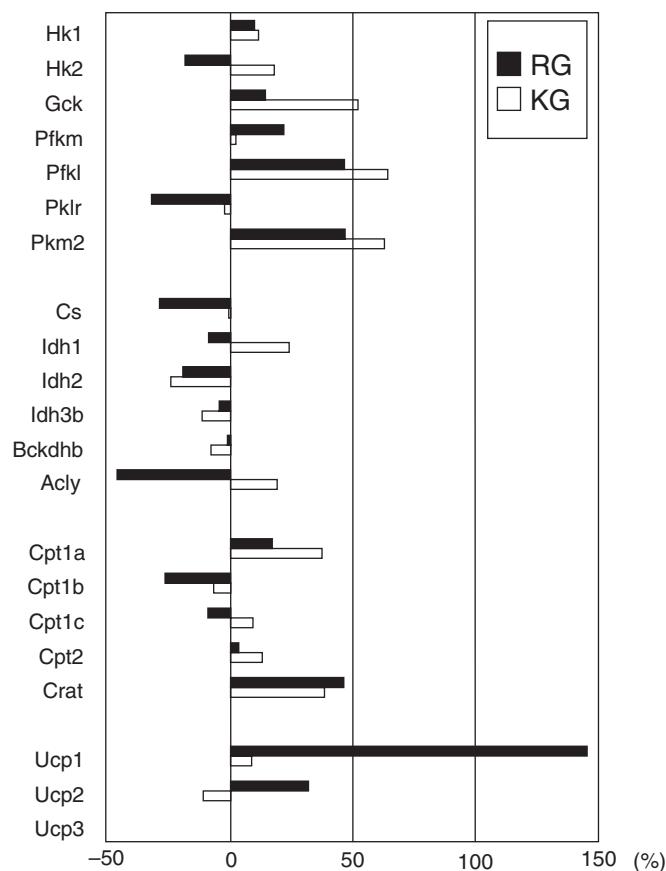
Genes (12,419) expressed	C (4,886)		RG (4,856)		KG (4,868)	
	Up	Down	Up	Down	Up	Down
Total	—	—	233	265	195	287
Apoptosis (153)	—	—	6	4	4	6
DNA repair (39)	—	—	1	2	1	4
DNA replication (34)	—	—	1	4	1	1
Transcription (618)	—	—	27	33	22	35
Cell cycle (149)	—	—	2	11	2	7
Cell adhesion (167)	—	—	8	10	4	16
Regulation cell cycle (59)	—	—	1	2	0	3
Regulation of transcription (584)	—	—	27	34	20	33
Carbohydrate metabolism (42)	—	—	0	1	2	0
Glucose metabolism (8)	—	—	0	1	1	1
Amino acid metabolism (42)	—	—	0	0	0	0
Fatty acid metabolism (20)	—	—	0	1	0	0
Immune response (41)	—	—	1	3	2	5
Lipid metabolism (47)	—	—	3	4	3	1
Protein biosynthesis (106)	—	—	4	1	5	5
Steroid biosynthesis (10)	—	—	1	0	1	0
Electron transport (111)	—	—	2	4	4	0

Abbreviations: C, control; RG, rice glycosphingolipid; KG, konjac glycosphingolipid.

**Table 23.4.** Drug-related genes whose expressions were up- or down-regulated by least 2-fold in the liver of mice fed experimental diets relative to their expressions in the liver of the control mice:

Drug-related genes (133)	C		RG		KG	
	Up	Down	Up	Down	Up	Down
Phase I metabolizing enzymes	—	—				
P450 gene family (25)	—	—	0	0	1	0
Phase II metabolizing enzymes	—	—				
Acetyltransferases (7)	—	—	0	0	1	0
Epoxide hydrolases (3)	—	—	0	0	0	0
Glutathione S-transferases (16)	—	—	0	0	0	0
Methyltransferases (7)	—	—	0	0	0	0
Sulfotransferases (21)	—	—	0	0	0	3
UDP-glycosyltransferases (4)	—	—	0	1	0	1
Drug-related transporters	—	—				
P-glycoprotein family (33)	—	—	1	2	2	1
Other (14)	—	—	1	0	0	1

Abbreviations: C, control; RG, rice glycosphingolipid; KG, konjac glycosphingolipid.

**Figure 23.5.** Relative gene expression in the liver of mice fed experimental diets. Increase or decrease (expressed as a percentage) in the expression level of genes in the experimental group relative to their expression level in the control group.

genes involved in energy metabolism, the expression of P450 (Cyp) and cytochrome 7a1 was increased.

These findings imply that RG plays the role of a functional food material by promoting fatty acid and cholesterol metabolism by altering Crat and Cyp7a1 gene expressions.

## CONCLUSION

Studying the functions of foods and nutrients is rather difficult. Typically, food is a complex and variable mixture of nutrients and other components. Most food factors are weak dietary signals and must be considered in the context of chronic exposure (Lee and Go 2005).

Microarray analysis clearly indicates the effects exerted by food factors and nutrients on metabolic pathways via transcriptome modifications. Moreover, the results of microarray analysis suggest that food factors and nutrients influence the metabolome because alterations in the transcriptome cause changes in the metabolome (Figure 23.1). So microarray analysis is one of the most convenient tools for inferring the proteome and metabolome (Endo et al. 2002; Kato and Kimura 2003).

Microarray technology is a typical tool used in nutrigenomics. This technology will enhance the understanding of the manner in which food and nutrition influence metabolic pathways and how these factors maintain homeostasis under normal conditions or diet-related or non-diet-related disease conditions.

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# 24

## Challenges and Current Solutions in Proteomic Sample Preparations

*Feng Tao*

### OVERVIEW

Biochemical studies in nutrition and human metabolisms have contributed an enormous amount of knowledge of nutrients obtained from food and drugs to human health. The understanding of food–health relationship is growing with the use of advanced science and technology. The societal benefit of such knowledge is evident by the increase in human lifespan and better quality of living. However, poverty is still one of the major problems in the world. It was reported that 63% of children living in developing countries die due to lack of sufficient nutrients in their food. There is hope that knowledge gained in the study of food–health relationship will not only lead to better health by providing guidance for taking balanced and sufficient food, but also help in learning more about diseases that are caused by improper food consumptions. One of the most promising technological developments in food–health studies is the proteomic approach that complements with the genomic information gained by the successful human genome project which was completed in 2003.

Proteomics is a discipline of relatively short history, but it holds great promise in elucidating biochemical information via quantitative determinations of the whole collection or representative proteins. In 1995, Marc Wilkins, a PhD student in Keith Williams' laboratory at Macquarie University, Sydney, Australia, proposed a downstream word of *genomics*, the word *proteomics*, to illustrate protein complements in an entire organism. One of the common objectives in proteomic studies is the discovery of biomarkers. Although biological systems are ex-

tremely complex and the technological challenges are still many, hundreds and thousands of biomarker candidates are being discovered with advancements made in proteomic technologies. One of the major huddles in proteomics is the identification of *true biomarkers* via analytical and clinical validation studies. Research studies in nutrigenomics and proteomics may not only be viewed for the investigation of food–health correlations, but is also useful for drawing “baselines” of genomic and proteomic components in a healthy human being. Deviation from these baselines may then be investigated in the pathological state.

The primary steps in biomarker discoveries and establishments of their clinical utilities include (1) selecting samples and quantitative determination of the proteome or representative proteins as many as possible, (2) utilizing protein sequence determination methods and bioinformatic tools to identify interesting protein targets or biomarker candidates—this step is sometimes called prevalidation of biomarkers, (3) further analyzing the potential biomarker candidates using validated analytical methods, and searching for their biological and clinical relevance, and (4) developing diagnostic or therapeutic products by establishing proper biomarker assay; some of the clinical diagnostic tests will need to be submitted for Food and Drug Administration's approvals, prior to marketing as clinical assays. This chapter highlights some critical aspects of biomarker determination using proteomic methods and some examples of new developments in the proteomic sample preparation techniques, particularly the pressure cycling technology (PCT).

## SELECTIONS OF SAMPLES FOR PROTEOMIC STUDIES AND BIOMARKER DISCOVERIES

It is critical to make wise decisions on what sample types and sampling strategies should be used in proteomic biomarker discoveries.

Biomarkers are target proteins that are present either in their host cells or secreted in circulation. There are good examples that biomarkers were utilized in the clinical diagnostics. For example, prostate surface antigen is being used in early detection of prostate malignant neoplasm since FDA approval in the early 1990s (Conrads et al. 2004). However, biomarkers discovered through proteomic techniques have not been seen, nor there is one close to be confirmed being a clinical significant marker for diagnostics.

One of the major questions in biomarker discoveries is the identification of the correlation between a physiological or pathological state and apparent concentration of a biomarker candidate. Sometimes the concentration of such a biomarker is in the detectable concentration range from blood samples selected for analysis. However, it is most likely that the maximum concentrations of biomarkers are found in their host cells, except biomolecules that are closely related to the cardiovascular functions, and immunological reactions. This may be an exaggerated statement and may turn out to be incorrect in many cases. However, this being said, it may be easier analytically that large number of biomarkers of high biochemical significance may be identified directly from the source, that is, the host cells and tissues.

Although, in some cases, biomarkers are secreted and may be detectable in circulation, it would be intuitive to say that one may still have better chances to discover biomarkers by protein profiling using their source or the host cells. Once a biomarker is found being statistically correlated to a physiological/pathological condition, assays can be established by targeting circulating/secretion types of samples, for example, blood, serum, and urine. There are many laboratories that focus on the most accessible sample types. There have been successes, but they have been limited. Thus, protein profiling using circulating types of samples may not necessarily be the best approach in biomarker discoveries. This is because the complexity of proteomes and the large cost associated with the discovery studies may well outweigh the cost in obtaining samples that appear hard to get and are expensive. The point is that like many biological studies, proteomic projects need to begin with serious considerations in selecting the sample source(s). Once this is determined, there will be sev-

eral available sample preparation techniques and analytical tools to choose from and data will be generated based on the samples. The lesser the mistakes made in sample selection, the lower will be the risks and costs, thus avoiding wasting resources necessary for these studies.

Are proteomic studies in nutrition–health–disease relationship any different from those for clinical diagnostics of diseases, personal medicine, and drug discoveries? It is hard to say. This is because biomarkers to be used in monitoring the nutritional impact are similar, and some are the same as the ones that are used for both physiological and pathological studies. However, there may be more accessible and less expensive samples or specimens available, simply because the subjects of these studies may be healthy individuals, and the major experimental variants are nutritious food. This may be advantageous compared to studies of diseases, where samples are sometimes harder to obtain, largely depending on the type of disease, rare or common. The other side of the coin is what will be the most obvious benefits from these studies, for example, the consumers, food manufactures, and/or public health in general. Another question is where the funding for these studies will come from—public or private, or both.

Biomarker studies can be more than the identification of the protein sequences using techniques such as immunoreaction or mass spectrometry. There are posttranslational modifications (PTMs), assemblies of multimeric complexes, and rapid, sometimes transient, conformation or association of proteins and protein complexes that result in significant physiological/pathological events. These features need to be determined in order to dissect contributions of proteins in biological systems.

PTMs are the chemical modifications of proteins after their translations. Forms of PTMs involving addition include ([wikipedia.org](http://en.wikipedia.org), searched using “post-translational modification” in May 2008):

- acylation
- acetylation
- alkylation
- demethylation
- amidation
- biotinylation
- formylation
- $\gamma$ -carboxylation
- glutamylation
- glycosylation
- glycation
- heme moiety covalently attached
- hydroxylation

- iodination
- isoprenylation
- lipoylation
- prenylation
- GPI anchor formation
- myristylation
- farnesylation
- geranylgeranylation
- nucleotides or derivatives covalently attached
- ADP-ribosylation
- flavin attachment
- oxidation
- palmitoylation
- pegylation
- phosphatidylinositol covalently attached
- phosphopantetheinylation
- phosphorylation
- polysialylation
- pyroglutamate formation
- racemization of proline by prolyl isomerase
- tRNA-mediated addition of amino acids such as arginylation
- selenoylation
- sulfation

Since protein–protein and protein–substrate interactions are responsible for essential biological events and processes in cells, one of the tasks of proteomic studies is to learn about these complexes and the interactions of the components in these complexes. Based on the association energy in the formation of the complexes, these molecular complexes may be categorized as static (high affinity) to transient (low affinity) complexes. The dynamic and kinetic energy required for the dissociation and association of protein complexes are dependent on the physiological/pathological conditions. Thus, some of the proteomic studies are aimed to characterize the components and their biophysical properties.

Some posttranslational forms of the biomarkers may be present dynamically at a particular state governed by the host cell and the metabolism processes. Again, it may be necessary to discover these PTM proteins from their root source, that is, the host cells. From the sample accessibility point of view, it is indeed easier to obtain samples in a form of body fluid, for example, blood, serum, urine, sweat, and sputum, instead of tissues. However, proteomic studies using body fluid would eventually need to be interpreted and verified for their correlations with cellular reactions in host cells and tissues. Some biomarkers analyses from body fluids are not always possible, since some biomarkers are only present transiently or hydrolyzed by proteases quickly and are extremely

difficult to determine in their secreted forms. So, getting back to the previous point, there are good reasons that proteomic research in many cases should be using host cells and tissues as the primary sample source other than peripheral sample types, such as body fluids. The next section will discuss experimental methods that are designed to process these samples, as well as some strategies that may be helpful in the studies of protein complexes.

## SAMPLE COLLECTION AND PREANALYTICAL TREATMENT

The technological challenges in proteomic sample preparations are many. One of the major challenges is how to preserve and demonstrate the proteomes using technologies that begin with precious samples and end with meaningful interpretation. There is a saying that everyone must have heard over and over: “garbage in, garbage out.” Here, it means that a bad selected sample gives unavoidable bad data downstream. Thus, one must pay great attention to the issue of sample, selection, collection, and preanalytical methods. It carries absolutely no less weight than the efforts in target analysis and data interpretation. One should know what the levels of signal and noise are in the preanalytical sample treatment, including controlled timing for sample collection. In the practice of proteomic studies, analysis at cellular level is often preferred. This is particularly true when the tissue samples contain heterogeneous cell populations.

Sample collections are steps that involve preanalytical sample treatment from time zero, including dissection, fixation, record keeping, and repository. Readers are encouraged to review a document of “National Cancer Institute Best Practices of Biospecimen Resources” at <http://biospecimens.cancer.gov/>. The National Cancer Institute (NCI) of the National Institutes of Health has established the Office of Biorepositories and Biospecimen Research (OBBR); in author’s opinion, this is one of the organizations in the world that have taken into account many issues in sample handling prior to analysis. From this Web site, one may find many useful information including latest references and presentations made by the field experts who discuss issues in current preanalytical practices.

### TISSUE SAMPLES

Tissue samples may be collected in the form of blocks or sections, fine-needle aspirates, and biopsies. Only fine-needle aspirates may be repeatedly

collected from a subject for follow-up studies, such as laser scanning cytometry (LSC) and immunohistochemistry (IHC) (Alaiya et al. 2005; Emmert-Buck et al. 1996). Using these samples, cells of interest can often be enumerated and quantitative analyses of some proteins can be possible. However, there are challenges in the sensitivity and specificity of this method and its applications due to the limitations in the number of cells available for analysis. It is sometimes possible to collect biopsy samples more than once at different time points, such as skin, fat, muscle, and tumor, but it is infrequent and difficult. Analytical techniques often applied to biopsy tissues include ELISA, LSC, enzymatic assays, and messenger RNA (mRNA) transcript analysis. In nutrigenomic and proteomic studies of food factor–gene–health correlations, collection of human tissues may also be a hard-to-accomplish task. However, studies using animal models may be practical. In any event, it is necessary to protect samples, including the morphology of tissues in some cases, and ultimately keeping molecules intact and biological function preserved prior to analysis.

Generally, proteomic studies are more difficult than those targeting nucleic acids and small molecules. Protein turnover, modification, or hydrolysis may take longer time than mRNA molecules, but still, proteolysis can cause significant loss of intact proteins due to the released endogenous proteases from proteasome. In common practice, freshly collected tissue should be immediately preserved cryogenically, such as keeping sample in liquid nitrogen at the collection site as soon as possible, and then stored at  $-80^{\circ}\text{C}$  or in liquid nitrogen prior to analysis. If liquid nitrogen is not accessible at the sample collection site, the sample should be kept on dry ice mixed with ethanol. After dissection, samples can be used on ice temporarily, but this can be a significant source for variation of sample itself, due to proteolysis, dissociation of protein complexes, oxidation, and other degrading reactions. If the dissected sample is large, for example, over 1 g, it is recommended that the sample be fragmented into a smaller size, e.g., 10–100 mg. Each sample is stored in separate containers. The container should have labels that record the date of sample collection, size, sample type, and a special code for information tracking. The code and sample information should be recorded in great details and kept in a safe place for future retrieval of the sample information.

Specimens from human or animal models are precious samples. These samples are often fixed using chemicals, such as formalin, and embedded using paraffin. The formalin-fixed paraffin-embedded

tissue samples can be stored for years under ambient conditions. They can be sliced, stained, and investigated using microscopic methods. Tissue fixation makes proteomic studies more difficult, because the fixation reaction introduces covalent linkages among proteins and other molecules within the cells. Artifacts are common due to these reactions. Another challenge in fixation is that diffusion or penetration of chemicals, such as formalin, is a time-consuming process. It was estimated that it takes approximately an hour to allow formalin to penetrate in 1-mm-thick tissue. If the thickness of the tissue is large, it would not only take a lot of time for the fixation to occur, but also the diffusion would not be even across the tissue sections. There are also factors contributing to the quality of the sample and later variation in data collected downstream. How to recover proteins or peptides from formalin-fixed, paraffin-embedded tissues for proteomic analysis is still an important subject for thorough investigation.

During the recent years, an interesting laser capture microdissection (LCM) technology has been utilized for collecting abnormal or normal cells from thin layer tissue slides that are prepared in pathological laboratories (Emmert-Buck et al. 1996). This technique has been found useful and supported by mRNA and protein recovery methods, so that transcriptomic and proteomic information may be obtained. One of the advantages is that one may perform studies on correlating mRNA or protein analytes with a subgroup type of cells. The challenges include the possible introduction of artifacts during sample fixation, staining and subsequent microscopic investigations. When freshly collected tissue is used, difficulties in reconstituting the proteomic map may come from the limited number of collectable cells within the time frame when proteome is considered stable and good quality data may be derived. Despite these challenges, it is hopeful that biomarkers will be identified from samples collected using the LCM technology, simply because this method allows proteomes to be collected from the host cells—a root source of the sample of significance. Tissue array technology has been recently introduced in proteomics. This technology has been used and reported along with the LCM sample collection method. Cells are collected and deposited on a protein chip or tissue array chip. These chips may hold unique features as many as 96, 384, and so on. Then target recognition by antibodies, of over 100 kinds, can be applied to these chips. The parallel sample analysis may eliminate some of the experimental errors present in low-throughput individual sample analysis.

### **BLOOD, SERUM, PLASMA, AND OTHER BODY FLUID**

Blood samples may be analyzed in the form of whole blood, plasma, serum, peripheral blood mononuclear cells, and circulating tumor cells. These samples are often used as a surrogate tissue. Although they are often not the host cells of biomarkers, compared to other types of tissues, blood is often easier to obtain. Some biomarkers may still be detectable in these samples using conventional technologies, such as ELISA, enzymatic assays, flow cytometry, and transcript analysis. Serum proteins may be present in various concentrations over a range of greater than 10 billion-fold. Since proteins cannot be easily amplified like DNA, it is often difficult to detect trace amount of materials with satisfactory precision so that critical information can be missed. Some of the highly abundant serum proteins, such as albumin, may act as carriers of low-abundant proteins. Therefore, some important biomarkers may be difficult to detect and so be missed. Practical separation techniques are required to isolate these target proteins from their carriers prior to analysis. Second, separation of proteins and peptides from complex media such as serum is technically demanding and time-consuming. Third, identification of PTMs and splice variants requires specialized analytical techniques and sophisticated database searching. In conclusion, biomarker analysis requires the minimization and eliminations of significant variation in sample preparation and the downstream experimental protocols used by various laboratories and experimentalists. There are possibly significant differences between the blood samples, depending on when, where, and how the sample is collected and stored, and what kind of anticoagulant-containing Vacutainer® (Becton, Dickinson and Company, Franklin Lakes, NJ) blood collection tube is used (Luque-Garcia and Neubert 2006).

### **CULTURED CELLS**

There are numerous advantages and disadvantages using cultured cells for proteomic studies. One of the obvious advantages is that samples are more accessible than tissues. It is also easier to control variants and experimental conditions. Some cells may be cultured being homogeneous so that cells at various developmental stages can be obtained and monitored. There are examples where cells are collected from blood and cultured *in vitro*. These cultures would make some proteomic and genomic studies feasible, when it is impossible to detect biomarkers using cells

from their host tissues or other *in vivo* resources. Another major advantage in proteomics by studying cultured cells is that many matured cell biology research tools and resources are available. For example, specific biochemical information at special and temporal resolution may be obtained using confocal microscopy, *in vivo* PCR, and other cell biology techniques. Proteomic information of internal cellular structures, for example, organelles and membrane protein complexes, may be obtained by first extracting these subcellular structures, and then followed by enrichment and purification, for example, by ultracentrifugation in glucose gradient solution. Then the proteome of the subcellular structures may be analyzed to elucidate the majority of components, including some proteins in low copy numbers.

The disadvantage of cultured cells for proteomic studies is the limited availability of cultivable types of cells. The artificial culturing environment may be significantly different from the living cells found in the tissues. How much biased is the information buried in the obtained data can be questionable. Nonetheless, one can use cultured cells to evaluate a new analytical technique and identify variability, reproducibility, and sensitivity of the methods. Once the method is validated, data may be obtained using both cultured cells as well as tissues. Data from these two resources can then be compared.

As shown above, sample selection, collection, pre-analytical processing, documenting, repository, and retrieval require proper handling, so that entity and representation of the target proteome can be preserved. People have said that as soon as the sample is collected, the data of the study is pretty much set. It means that no matter how the downstream processes are chosen and run, data will not be much different once the sample is collected and stored. It tells us that one of the most important steps in proteomic study is sample handling. Standard operation procedures (SOPs) are necessary to minimize variations due to sample handling. Such SOPs would need to be envisioned and established during the initial phase of a study. These SOPs will need to recognize the variability of individual steps, sample themselves, and provide sufficient training to operators who are going to perform the study.

### **PROTEOME EXTRACTION AND PURIFICATION METHODS**

Once samples are selected for the determination of proteomes, that is, total proteins in a sample, the first sample process is extraction and purification.

The purpose of these processes is to separate proteins from other biomolecules in a sample so that proteins may be detected by a downstream analytical method, such as ELISA or other immunochemical-based reactions, or mass spectrometry. The common extraction methods are either chemical or physical based. For example, high concentrations of denaturants, such as guanidinium or urea solution, are typically used to dissolve proteins in cultured cells. For tissue blocks, mechanical disruption is often required. Soft tissues, such as brain, liver, and spleen, are usually homogenized using homogenizers, such as Dounce, Potter-Elvehjem, rotor-stator, or Polytron types. In some cases, mortar-pestle is required to break large blocks of tissue into smaller ones so that homogenization can be completed using the homogenizers mentioned above. These traditional tools are mostly manual and exhibit inconsistent efficiency in extraction. Thus, more robust and better-controlled extraction methods are needed for proteomic sample processing. A novel process using Pressure Cycling Technology Sample Preparation System (PCT SPS) has recently been introduced to the proteomic field as well as other biological sciences where extraction is needed (Schumacher et al. 2002; Tao et al. 2006). PCT SPS holds great promise in the field of sample preparation of proteomics and others due to its unique features. Although the PCT SPS is still a method under development, current knowledge supports the hypothesis that PCT may be established as a standardized extraction process that is applicable for a wide variety of samples.

#### TISSUE PROTEOME EXTRACTIONS USING PCT SPS

The PCT SPS was developed at Pressure BioSciences, Inc. (South Easton, MA) and is being used by a small group of users in pharmaceutical companies and research institutions. The primary uses of PCT SPS have been in the processes of biological samples for the release of DNA, RNA, and proteins (Schumacher et al. 2002; Tao et al. 2006). This system consists of a Barocycler™ instrument and specially designed, sealed, single-use devices—the PULSE™ Tube. The system provides a rapid, safe, and reproducible approach for processing many types of biological samples in either liquid or solid form (Tao et al. 2006). Results from several laboratories show excellent release of DNA, RNA, and protein, as compared to traditional methods such as mortar and pestle grinding, homogenization, bead beating, and enzymatic digestion. The quality and quantity of PCT-extracted molecules are comparable

or better than those obtained using classical methods (Smejkal et al. 2006, 2007; Tao et al. 2006). The released biomolecules, including labile RNA and enzymes, are suitable for further analyses, such as PCR, RT-PCR, microarray, immunoreactions, SDS-PAGE, Western blot, and 2D gels. It is one of the most versatile techniques in processing broad range of sample types from microbes to human tissues. Examples of data relating to proteomic studies are presented below. In addition to its capability, the use of instrument-controlled processes in sealed, single-use tubes facilitates a high level of operational safety and reproducibility, while concomitantly offering a process that is free from cross-contamination.

For example, total proteins from rat liver was extracted using either the popular mortar and pestle/Polytron method or by PCT. For comparison, traditional mortar and pestle/Polytron homogenization was used. Frozen pieces of rat liver were pulverized using mortar and pestle in the presence of liquid nitrogen. After grinding, samples were transferred to a 15-mL centrifuge tube. Three milliliters of COMS extraction buffer (7.0 M urea, 2.0 M thiourea, 1% C7BzO, 40 mM Tris, pH 8.0) were added, and samples were further homogenized with a Polytron. For extraction by PCT, whole pieces of rat tissue were placed directly into PULSE Tubes with COMS buffer and extracted by pressure cycling under the following conditions: 35,000 psi for 20 s, ambient pressure for 20 s, repeated for 10 cycles.

Table 24.1 summarizes the results of the above experiments performed in triplicate. The data clearly show that PCT-based extraction not only is less labor intensive, since it does not require pregrinding of the frozen tissue in a mortar and pestle, but is also more efficient and reproducible. Further, highly reproducible protein patterns from rat liver tissue extracted by PCT were shown by a set of two-dimensional gel electrophoresis (2DGE) experiment. In this experiment, all samples were reduced and alkylated, and then subjected to centrifugation to remove any insoluble material, once homogenized. Soluble protein was acetone precipitated, solubilized in a resuspension solution (7.0 M urea, 2.0 M thiourea, 2% CHAPS), and separated by 2DGE.

In another experiment, the feasibility of recovering biomarker candidate proteins from murine tumor tissue was demonstrated by PCT. Tissue samples were extracted either by the established method of pulverizing frozen tissue on liquid nitrogen (LNP method) or by PCT as follows: For PCT extraction, intact tissue pieces were placed directly into PULSE Tubes with RIPA buffer (20 mM Tris, 150 mM NaCl, 9% sucrose, 5% glycerol, 0.1% SDS, 1% Triton

**Table 24.1.** Comparison of PCT and pulverizer homogenization (LNP) for the extraction of total proteins from rat liver.

Method	Relative yield <sup>a</sup> (mg protein/mg tissue)	Mean yield	Standard deviation	Coefficient of variation
Barocycler (not pulverized)	0.172	0.162	0.016	10.2%
	0.143			
	0.171			
Polytron (pulverized)	0.147	0.181	0.035	19.4%
	0.178			

<sup>a</sup>Protein concentrations were determined by Bradford assay.

X-100, 2 mM EDTA, pH 7.5). PCT was performed for 30 cycles, each cycle consisting of 10 s at 35 kpsi followed by 5 s at atmospheric pressure. After PCT, each PULSE Tube was coupled to the insert of an Ultrafree<sup>®</sup>-CL centrifugal device with 5-μm filter (Millipore Corporation, Danvers, MA) and centrifuged at 1,000  $\times$  g to completely evacuate the contents of the PULSE Tubes. The total time for sample processing by PCT was 30 min. For protein extraction by pulverization (LNP), tissues were pulverized under liquid nitrogen, transferred to polypropylene tubes with 0.5 mL of RIPA buffer, and incubated on a rotator for 48 h at 4°C. Following this incubation, the samples were clarified by centrifugation in Ultrafree-CL centrifugal devices. The total time for sample processing by LNP was 48 h.

To compare protein recovery by these two methods, protein concentrations were estimated by BCA protein assay. The results shown in Table 24.2 indicate that PCT is a far more efficient method for protein extraction from tumor tissue, resulting in more consistent (coefficient of variation: 7.8% for PCT compared to 37.7% for LNP) and more efficient protein extraction (threefold increase in relative protein yield) in a fraction of the time required by the LNP protocol. The improved efficiency and reproducibility of PCT-based protein extraction over traditional methods such as LNP is predicted to facilitate biomarker discovery. To further investigate the potential of PCT in this area, total protein extracts were prepared from mouse tumor tissue (SJ38-4)

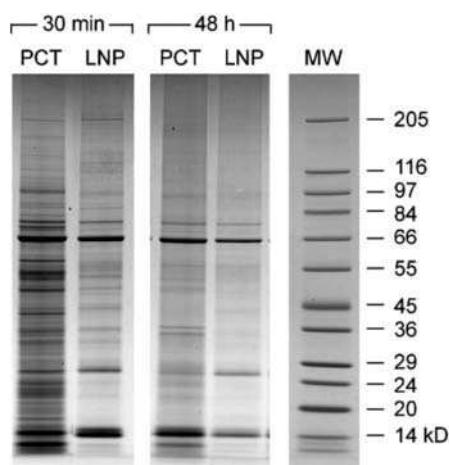
and compared with normal control tissues from the same animal. Protein profiles extracted by PCT were compared with profiles generated by LNP extraction as described above. As shown in Figure 24.1, several proteins were identifiable in the PCT-extracted samples that were not detectable in the samples extracted by LNP.

#### EXTRACTION OF MOLECULAR COMPLEXES

A particular challenge in proteomic studies is the preparation of extracts in which cellular complexes are preserved and ready for isolation. Although subcellular proteomics is still an emerging field, the advantages of research on subcellular proteomes rather than whole cells or tissues are obvious. This is of particular importance in proteomic studies, since the high complexity of proteomes may be greatly simplified by focusing on specific subcellular complexes. Currently, subcellular contents are either studied using microscopy or traditional biochemical techniques. Although conventional protocols and generic recipes are available for the release of many cellular complexes or organelles, there continues to be a great need for well-controlled, automated sample preparation systems that simplify the extraction process and satisfy diverse but important requirements. When subcellular contents need to be isolated from tissues, the extractions are typically done using a Dounce homogenizer, sometimes in combination with enzymatic digestion. While other common cell disruption

**Table 24.2.** Extraction of proteins from small subcutaneous murine tumors by PCT and LNP.

Extraction method	Time required	Relative yield (mg protein/mg tissue)	Coefficient of variation
PCT	30 min	4.41 $\pm$ 0.32	7.8%
LNP	48 h	1.47 $\pm$ 0.55	37.7%



**Figure 24.1.** Extraction of proteins from adipose tissue using PCT or LNP and buffer extraction for 30 min or 48 h, respectively. Prolonged incubation of the triturations in RIPA buffer resulted in the loss of proteins presumably due to the aggregation and precipitation of hydrophobic proteins.

techniques, for example, mortar–pestle, bead beater, sonicator, or French press, are considered more efficient in disintegration of cellular material, they are usually much less popular for extractions of intact subcellular components from cells or tissues due to the excessive nonspecific damage to the desired organelles or apparent lack of reproducibility. One attempt in mapping the mitochondrial proteome has revealed a need for complexity reduction and enrichment strategies in order to reliably detect and identify lower abundance components of the mitochondrial proteome (Lopez et al. 2000).

In developing a system that meets the needs, a comprehensive understanding of the various biological systems, characteristics of biomolecules, and the physical and chemical properties of the sample preparation process is required. Although a universal extraction method for all sample types may not be possible due to the vast diversity of organisms and target analytes, it is highly desirable to develop a system where physical and chemical parameters can be well controlled, so that the system can be applied to as wide a range of distinct samples and target types as possible. The PCT SPS has been investigated and optimized in the applications of extracting molecular complexes.

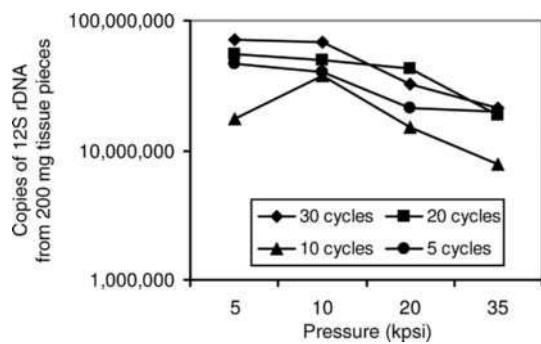
The PCT-based extraction is powerful, since it has been used in extracting a variety of molecules from wide selections of sample types. It has also been

shown to be a relatively gentle method. For example, DNA extracted by PCT tends to be larger in size compared to DNA extracted using more traditional techniques, indicating that less DNA shearing occurs during PCT-based extraction. As shown by 2DGE, high-molecular-weight proteins may also be more efficiently recovered after PCT than after extraction using other methods (Grove et al. 2006; Witzmann et al. 2006). Another important area of successful and unique application of PCT is in the extraction of hydrophobic molecules, such as proteins from lipid-rich tissue, where organic solvents are used instead of conventional detergents with greater recovery of membrane proteins (Lazarev et al. 2007; Romanovsky et al. 2007). Furthermore, PCT-based extraction tends to be more reproducible than conventional extraction techniques, most likely because this process is automated and programmable.

PCT was shown in gentle disruption of plasma membranes, allowing intact organelles to be released into solution. For some organelles such as mitochondria, the structural integrity and biological functions may be well maintained after PCT-induced cell disruption. It was determined that membrane-enclosed cells and subcellular components may be selectively disrupted using alternating cycles of hydrostatic pressure. The PCT SPS provides controlled levels of physical stress to selectively rupture plasma membranes of several types of mammalian cells, leaving most organelles intact. Thus, PCT holds great promise to be developed into a powerful tool for the extraction of subcellular contents.

PCT extraction of mitochondria was carried out by applying pressure cycles to pieces of fresh or frozen rat liver in HEPES buffer at 4°C. The processed samples were first centrifuged at  $800 \times g$  for 10 min to remove large particulates, and the mitochondria-containing supernatant was collected. Mitochondria were collected by centrifugation of the supernatant at  $8,000 \times g$  for 10 min at 4°C. The pellets were collected and washed with HEPES buffer. DNA was extracted from the pellets using the QIAGEN DNeasy kit. DNA was eluted in 200  $\mu$ L DEPC-treated double distilled water. From each sample, 1.0  $\mu$ L DNA was used in real-time PCR (Q-PCR) assay using primers and probes designed for mtDNA gene targets. DNA copy numbers were compared from samples processed under different conditions.

Using the Q-PCR assay and additional tests discussed below, PCT-based mitochondrial isolation was shown: (1) It causes little damage to mitochondrial morphology, and (2) results in mitochondrial yields comparable to, or better than, conventional protocols such as Dounce homogenization. To



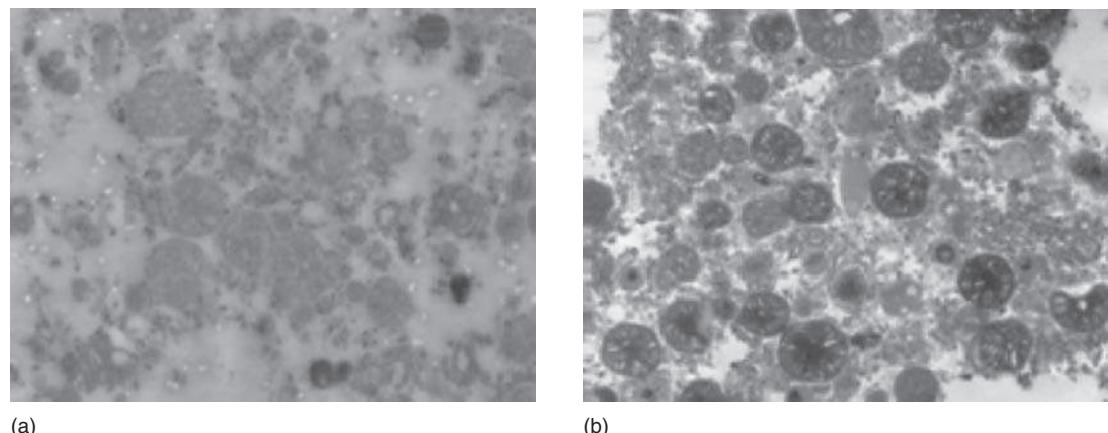
**Figure 24.2.** Mitochondrial recovery was estimated using Q-PCR to amplify the 12S rRNA gene from the mitochondrial genome. Samples were processed at different levels of pressure and various numbers of pressure cycles. Each pressure cycle comprises 20 s incubation at high pressure, 10 s at ambient pressure, and processed at 4°C.

quantify the number of mitochondria released by PCT, real-time Q-PCR assays were established targeting unique mtDNA genes. Figure 24.2 shows that by increasing the number of cycles, mitochondria yield from frozen–thawed rat liver can be increased, particularly when using 5 or 10 kpsi. With an equal number of pressure cycles, mitochondria yield drops off at higher pressures, which may indicate loss of intact mitochondria due to pressure-induced damage.

This observation was supported by a transmission electron microscopy image analysis with samples

prepared from freshly collected mouse liver (never frozen; Figure 24.3). High-pressure treatment at 35 kpsi resulted in an increased amount of dark matter at the centers of many mitochondria (image not shown). Thus, a PCT process using gentler conditions, as low as 5 kpsi and five cycles of pressure, seems to be sufficient for the recovery of good quality mitochondria from liver tissue. It is important to note that Dounce homogenization produces high-quality mitochondrial preparations when performed by highly skilled operators. However, it is very easy to either “over-Dounce” or “damage” the mitochondria by creating substantial vacuum when the homogenizer pestle is being pulled too fast—a possible explanation for the appearance of the sample in Figure 24.3. This further illustrates a necessity to improve the reproducibility of the mitochondrial preparation methods.

An extraction of mitochondrial proteome was carried out using PCT-extracted samples from fresh or frozen rat liver. 400 mg of samples were placed into an FT500 PULSE Tube, with 1.0 mL of HEPES buffer (20 mM HEPES, 5 mM MgCl<sub>2</sub>, 250 mM sucrose, 1 mM EDTA, pH 7.3, and 1 pellet of protease inhibitor cocktail tablet (Roche) dissolved immediately before experiment). These samples were treated with 30 cycles of pressure between ambient pressure (10 s) and 5 kpsi (20 s) at 4°C. The lysate was centrifuged at 1,000 × g at 4°C for 10 min, and supernatants were collected and loaded onto a BDA instrument (Alfa Wassermann Proteomic Technologies, AWPT, West Caldwell, NJ). Ninety-six buoyant



**Figure 24.3.** Transmission electron microscopy images of mitochondria extracted from fresh mouse liver tissues by (a) Douncing and (b) PCT. Dounce: four strokes on ice. PCT: five cycles of 5 kpsi (20 s) and ambient pressure (10 s), 4°C. Circular mitochondria are observed in both samples. Mitochondria at higher density and purity were seen in most PCT-extracted samples.

density accumulation (BDA) fractions were collected at the end of the BDA run. These fractions are analyzed using the BCA protein analysis kit (Pierce Biotechnology, Rockford, IL), followed by Western blotting for mitochondrial markers using the MS604 antibody panel (MitoSciences, Eugene, OR). The antibodies in this panel probe for outer and inner membrane-bound mitochondria proteins. The positive response shown by the Western blot suggests that mitochondria and its protein components were successfully extracted using the PCT method. After extracting either the total proteins or organelles from the tissues, further purification and enrichment of subgroup or specific proteins may be followed.

## ENRICHMENT AND DETECTION OF PROTEINS USEFUL IN PROTEOMIC STUDIES

### PROTEIN CAPTURE AND ENRICHMENT REAGENTS

The accessibility of proteins in body fluids and tissue extracts facilitates extensive protein-focused research. Only reliable methods for protein identification and measurement can be useful in generating reproducibility and comparability of research results. There is an urgent need in the field of proteomics for high-quality, standardized reagents that can improve the specificity and reproducibility of proteomic technologies. Capturing protein molecules are often needed for enriching the target from a complex biological sample so that one can make identification and quantification using a detection technology, for example, mass spectrometry. A capture reagent is a molecule, such as a monoclonal antibody or a nucleic acid aptamer, that binds tightly to a specific protein or peptide. The capture reagents enable one to detect and/or isolate proteins or peptides from a mixture of molecules present in biological samples. Generally speaking, the proteomic field still lacks qualified standards, reagents, and validated technologies that can permit the development, management, interpretation, and comparison among large quantities of proteomic data. This in turn slows the progress in transforming proteomic discoveries into clinical applications. Several popular methods are currently investigated for high-quality protein identification and measurement.

One of the broadly used capture reagents in proteomic research is antibodies, a naturally occurring serum protein whose biological role is high-antigen specificity. Both monoclonal or polyclonal antibodies have been used as detection and capture reagents in proteomics. Aptamer reagents show promise as an

adjunct to antibodies (Mosing and Browser 2007). These nucleic acid-based molecules possess protein-binding specificity, similar to antibodies that make them useful as protein capture and detection reagents. Since they are nucleic acid based, the technology for their synthesis and chemical modification is more mature than antibody production, and various mutation and selection protocols can be used to specify their binding properties.

Recently, new solid-state protein-binding microarray assays based on the conventional immunoassay principles have developed using a variety of immobilized “capture reagents” to detect and quantify proteins and protein fragments (peptides) extracted from tissues and dissolved from biological fluids. The capture reagents are usually immobilized on a solid support material, such as glass, synthetic membranes, mini or nano beads, or mass spectrometer plates. Because microarray elements can be miniaturized, tens of thousands of capture features may be arranged in a grid. Each element is selected being specific for a given protein or peptide. Using this microarray format, multiple assays can be conducted simultaneously. The high density of capture reagents permits a high-throughput system for measuring many proteins and their roles in the networks of biochemical and signaling interactions within a sample. In addition to capturing agents, reporter molecules are needed in detecting the presence of a target in a particular biological sample. Sometimes the reporter works by modifying target molecules. Reference materials are also required for calibrating instruments or comparing different proteomic platform technologies.

### MEMBRANE PROTEINS AND LIPOPHILIC PROTEINS

Membrane proteins are responsible and essential for cellular and subcellular metabolisms. As discussed in one of the recent NIH Protein Structure Initiative (PSI) meetings, the most challenging task that relates to the assessment of structures of proteins at a proteomic scale is the structural determination of membrane proteins. The biochemical importance of membrane proteins cannot be overstated. For example, ~30% gene-coded proteins are associated with membrane or members of membrane-bound proteins. Approximately 60% of the currently available drugs act on membrane proteins. The difficulties in the studies of membrane proteins include the following: (1) it is not easy to obtain enough materials for analysis; (2) it is difficult to mimic membrane environment in experimental conditions to allow membrane proteins stay structurally stable in an enriched form;

(3) it is difficult to crystallize membrane proteins as compared to other globular proteins; (4) membrane proteins often exert their function by partnering with other cellular components, such as organelles or protein complexes; (5) PTM can be critical in the functions of membrane protein; and (6) overexpression of membrane proteins is not easy, including folding of membrane protein correctly. There are studies that aim for improvements in solubilization and crystallization of membrane proteins. A funnel approach has been taken to select “easy” targets for the first structural determinations of membrane proteins.

To tackle this challenge, a ProteoSolve<sub>LRS</sub> kit is now available from Pressure BioSciences, Inc. By combining PCT SPS with the novel chemistry provided by the ProteoSolve<sub>LRS</sub> kit, it is possible to fractionate the lipid and protein components of a sample, resulting in higher protein recovery and greater reproducibility as compared to traditional, detergent-based extraction methods. In addition, proteins that are underrepresented in extracts obtained by conventional methods have been successfully extracted by the PCT/ProteoSolve<sub>LRS</sub> method. (More information regarding this product may be obtained at [www.pressurebiosciences.com](http://www.pressurebiosciences.com).) These novel proteins offer the potential to be important diagnostic or prognostic markers.

#### PROTEIN IDENTIFICATION BY MASS SPECTROMETRY

Mass spectrometry is a powerful tool used to identify, and in some cases quantify, the proteins or peptides in a sample. Mass spectrometers are designed to measure two properties: the mass-to-charge ratio ( $m/z$ ) of ions (particles with an electric charge) and the number of ions present at each  $m/z$  value. On a mass spectrum, each peak represents an ionized molecule, which can be a peptide originated from a protein in the sample, with the height of the peak proportional to the abundance of the peptide. Proteins may be identified by recording their “peptide mass fingerprint”—the pattern of peaks in the mass spectrum after fragmentation by specific enzymes—or by amino acid sequencing after breaking down the protein fragments further into a series of peptides differing by one amino acid. Prior to mass spectrometry analysis, technologies are required to reduce the complexity of protein isolates by enriching proteins of interest and to enhance the range and sensitivity of the instrumentation.

Multiple-reaction monitoring (MRM) assays are established for the quantification of candidate-based

protein markers in plasma (Broad Institute). MRM is currently the gold standard for identification and quantification of drug molecules and metabolites in clinically relevant plasma samples due to its extremely high sensitivity and specificity. For clinical proteomics, a method involving strong cation-exchange chromatographic fractionation of peptides and immunoaffinity enrichment on specific antipeptide antibodies (stable isotope standards and capture by anti-peptide antibodies, SISCAPA) is used. When SISCAPA is combined with MRM quantitation by mass spectrometry, reliable and reproducible quantitation of signature peptides may result from the protein digests.

Prior to mass spectrometry analysis, sample fractionation was investigated using magnetic beads for the capture of peptides (Whiteaker et al. 2007). The rationale of this method is that beads provide a larger surface area-to-volume ratio than do flat plate protein chip designs. At Purdue University, scientists use high-throughput immunoaffinity combined with mass spectrometry instrumentation and bio-fabrication methods for integrating and comparing proteomics data from different platforms. Another technology in development is a microarray platform employing interferometric analysis that offers the potential for label-free, high-throughput, and sensitive analysis of small amounts of biological fluids. This application has the potential, leading to the identification of useful antibody reagents and antibody arrays suitable for discovery and clinical proteomics. At the University of California, San Francisco/Lawrence Berkeley National Laboratory/Buck Institute, methods are evaluated to monitor the degree of degradation of banked biological samples.

#### NANOTECHNOLOGY

Nanotechnology devices have the potential to greatly expand the capabilities of proteomics. By definition, nanotechnology utilizes devices and components from 1 to 100 nm in at least one dimension. The size range is roughly between an antibody molecule and a viral particle. These devices may be useful in addressing current limitations in selectively reaching a target protein *in vivo* by penetrating through physical and biological barriers. They may also be utilized in detecting low-abundance targets and providing a “toolbox” to translate the discovery of protein biomarkers to novel therapeutics and diagnostic tests. Nanowires and nanocantilever arrays can be used in biosensors that measure minute quantities of biomarkers in biological fluids.

### PROTEIN STRUCTURE, DYNAMICS, AND FOLDING

When a draft sequence of the human genome was completed, knowing the genes was only the beginning. Indeed, it is the proteins made from these genes that actually carry out all the functions that keep us alive. Knowing sequence does not mean knowing the functions of a large fraction of human proteins and knowing how each protein affects human health. Some of the questions are closely related to proteomics in general, such as how protein folds, how the dynamic properties of proteins are in the relationship with their functions, the formation of protein complexes (including some that are transient), and the synthesis, inhibition/activation, degradation, and recycling of proteins in cells.

The National Institute of General Medical Science (NIGMS) of the National Institutes of Health (NIH) has invested substantial funding in support of a PSI since 2000. The long-range goal of the PSI is to make the three-dimensional atomic-level structures of most proteins easily obtainable from knowledge of their corresponding DNA sequences. The expected benefits from the PSI include structural descriptions to help researchers discover the functions of proteins, design experiments, and solve other key biomedical problems; faster identification of promising new structure-based medicines; better therapeutics for treating both genetic and infectious diseases; and development of technology and methodology for protein production and crystallography. When a piece of peptide or protein is found by proteomic techniques, it will be searched in protein sequence and protein structure databases. Based on the knowledge revealed in these databases, its biological function may be speculated. If it seems plausible, then more focused studies may be initiated to confirm the observation. These studies may include cytological investigations, such as correlation of the biomarker candidate and gene-expression, knockouts, life cycles, division, and death. Partners of this protein will be searched and molecular interactions of these molecules will be studied.

### DETECTION OF TRANSIENT PROTEIN COMPLEXES

In order to characterize the “transient” protein–protein interactions, two sets of complexes were collected and analyzed. The first set consists of 16 experimentally validated “weak” transient homodimers, which are known to exist as monomers and dimers at physiological concentration, with dissociation constants in the micromolar range. A set of 23

functionally validated transient (i.e., intracellular signaling) heterodimers comprise the second set. This set includes complexes that are more stable, with nanomolar binding affinities, and require a molecular trigger to form and break the interaction. In comparison to more stable homodimeric complexes, the weak homodimers demonstrate smaller contact areas between protomers and the interfaces are more planar and polar on average. The physicochemical and geometrical properties of these weak homodimers more closely resemble those of nonobligate heterooligomeric complexes, whose components can exist either as monomers or as complexes *in vivo*. In contrast to the weak transient dimers, “strong” transient dimers often undergo large conformational changes upon association/dissociation and are characterized with larger, less planar, and sometimes more hydrophobic interfaces. From sequence alignments, it was found that the interface residues of the weak transient homodimers are generally more conserved than surface residues, consistent with being constrained to maintain the protein–protein interaction during evolution. Protein families that include members with different oligomeric states or structures are identified, and found to exhibit lower sequence conservation at the interface (Nooren and Thornton 2003).

### BIOINFORMATICS

Bioinformatics provides tools needed for proteomic data modeling, database design and management, data interoperability and comparison, correlation of gene and protein expression analysis, structural predictions, vocabularies, annotations, ontologies, and systems biology modeling. In proteomic research, the development of new tools in these areas are necessary to drive the collaborative, multidisciplinary effort required to push proteomic research and discovery from the laboratory to clinical practice. One example of bioinformatics establishment is the NCI of National Institutes of Technology, in which bioinformatic programs include addressing the need for an IT infrastructure that enables collection, analysis and sharing of huge amounts of data for interinstitutional studies through its cancer Biomedical Informatics Grid™, or caBIG™, a voluntary network connecting individuals and institutions to enable the sharing of data and tools, creating a World Wide Web of cancer research.

### COMBINATIONAL STUDIES OF PROTEOMICS, GENOMICS, AND METABOLOMICS

Proteomic studies are often carried out in a combination of other biochemical methodologies, such

as genomics, transcriptomics, and metabolomics. The combined multidiscipline studies is sometimes known as *system biology* research. Thus, a proteomic project can well be a relatively broader study in which a wider scope than proteomics itself can be anticipated. The benefit of a wider scope of study includes complementary information that would help better understand the biochemical composition under special biological conditions. In addition, the cost of sample collections can be quite high and sometimes prohibitively expensive. Thus, it would be more cost-effective if more information is obtainable from the same sample. In these cases, sample preparation methods are desirable if they allow simultaneous extraction and/or purification of nucleic acids, proteins, and small molecules. This is also true in situations where samples are cells collected by LCM, and are formalin-fixed and paraffin-embedded tissues. As mentioned above, like in most biological studies, it is extremely important that each step in the data collection, beginning from the selection and collection of samples to reagents, instruments, and data processing, is well designed and quality controlled using internal or external reference standards, and proceeds using standardized operational procedures.

#### METHOD VALIDATION

There are many factors that may complicate the data interpretations, which include the individual genetic makeup, age, race, personal routine behavior, and so on. The behavioral factors, for example, daily routing habits, physical activities, food/nutrient intakes, and environmentally caused mutations and modifications, can also be significant. It will not be easy to reach a general conclusion if a relatively large collection of samples is selected, characterized, and compared. Technically speaking, at the time of sample collection, data are more or less determined, no matter what proteomic platforms or methods are used. So, sample selection and collection can be far more important than the downstream analysis. In general, any of the biomarker discoveries requires validations, which include a technological validation and a clinical or biological validation.

The technological validations are rigorous studies that demonstrate the reproducibility, precision, and sensitivity of the processing methods. The most reproducible, highly precise, and sensitive methods are desirable. Good methods or processes are those having small coefficient of variation and standard deviation values, for example,  $<5\%$  coefficient of variation and  $>0.95$  standard deviation. A detec-

tion method for quantifying proteins would preferably cover relatively large dynamic range (3–4 logs). This may be a challenging task due to the large span of protein concentrations found in samples. For example, serum proteins can vary in concentration over a range of greater than 10 billion-fold. Serum contains highly abundant as well as low-abundant proteins. There are research studies and commercial products that deplete highly abundant proteins, primarily by affinity column purification. However, there are studies that show significant loss of low-abundant species during the affinity purification or poor reproducibility of recovered low-abundant proteins.

Clinical validation of a biomarker is another major challenge, particularly in the applications of disease diagnostics. Till date, there is no such example that a biomarker is discovered and validated by clinical studies. The nutrigenomics and proteomics hold promise in the establishment of human baselines in normal genomic and proteomic compositions, which may be referenced and compared with abnormal variants, such as diseases or pathological conditions.

What the proteomic community has realized in recent years is that a biomarker cannot be found in the absence of rigorous validation studies in both technology and clinics. To best utilize resources, it is best to carry out such validation and standardization studies in an organized and collaborative manner. In some situations, more than one set of validated and standardized methods or platforms may need to achieve complimentary and critical information.

The good news is that technologies become more sensitive, quantitative, and reproducible than traditional techniques. The proteomic field is advancing at ever-faster rates. Although the costs of genomic and proteomic research remain pretty high now, technologies leading to reduced proteomic complexity are on the horizon. One would expect that the sample and process costs will be reduced so that a large enough number of assays may be carried out, and statistically significant interpretation can be made. Along with rapid advancements made in system biology and bioinformatics, it has been in a general agreement within the proteomic community that genomic and proteomic approaches will reveal novel biological information. Such knowledge will be gained at subcellular, cellular, and tissue levels. Based on the genomic and proteomic information, public health can be improved by tweaking human consumptions of therapeutics, natural food, and artificially enriched nutritional products.

## OUTLOOK OF SOLVING SAMPLE PREPARATION ISSUES IN PROTEOMICS

Proteomics is of at least three basic requirements: (1) appropriate technologies useful in data generation and management, (2) tools and know-how in biological information integration and interpretation, and (3) collaborative efforts among a team of scientists with different sets of skills. The quality of proteomic study relies on not only the precision and sensitivity of the analytical tools being used, but also sample quality, particularly the important components of the proteome in question. The ideal samples for proteomic researches are the ones from which reasonable amount of material can be relatively easy to obtain, data may be reproducibly produced, and the data are biochemically relevant to the biological questions being asked. One should keep in mind that the practical sample processing methods often are a trade-off among the requirements for the optimal method, the biological specimen, and the concentration of the targets. Although many of the considerations are interrelated, the crucial items often need to be selected to help define the others. This is particularly true for proteomic research—there is massive complexity of the sample and extraordinary complex answers to a simple biological question. Establishment and practical use of standardized protocols during data generation is the only way that can lead to the success in the proteomic study.

The success of proteomic study also needs substantial research funding. There are several major federal- and private-run proteomic programs and initiatives that will fuel the proteomic research studies in the near future. Some of them have been mentioned in the text above. However, there are many more relevant programs that fund proteomic research. For example, the NIH Roadmap is one of the initiatives. Under the Roadmap, it will support developments of protein capture/proteome tools. This was aimed to characterize protein function in health and disease and to monitor the markers of a disease in order to deploy early prevention efforts and to identify potential therapeutic targets. Another focused area of the Roadmap is the detection of transient molecular complexes. Understanding interactions within transient complexes is essential for robust modeling that can accurately describe how diseases develop and progress. It is plausible that these transient complex studies will need not only cell biology techniques, but also proteomic methods to elucidate the subcellular components that lead to the transient transformational changes, for example, impact of

small molecules, events of posttranslational protein modification, and induced gene expressions. Finally, proteomic sample preparation has many challenges. Technological improvements on solving these challenges will lead to good answers to big questions, for example, understanding the food–health relationship, disease, and new findings of therapeutic solutions.

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# 25

## Computational Methods in Cancer Gene Networking

*Edwin Wang*

### BIOLOGY IS A SCIENCE ABOUT RELATIONSHIPS

In the last few years, many high-throughput techniques have been developed and applied to biological studies. Techniques such as “next-generation” genome sequencing, chIP-on-chip, and microarray can be used to measure gene expression and gene regulatory elements in a genome-wide scale. Moreover, as these technologies are more affordable and accessible, they have become the driving force in modern biology. As a result, huge amount of biological data have been produced, with the expectation of increasing number of such data sets to be generated in the future. High-throughput data are more comprehensive and unbiased, but “real signals” or biological insights, molecular mechanisms, and biological principles are buried in the flood of data. In current biological studies, the bottleneck is no longer a lack of data, but the lack of ingenuity and computational means to extract biological insights and principles by integrating knowledge and high-throughput data.

To develop computational tools effectively, one must first understand what biology is. Biology deals with many kinds of relationships among genes, proteins, RNAs, cells, tissues, organs, and environmental factors. For example, biological relationships include those encompassing gene regulatory relationships, protein interaction, activation, genetic interaction, and inhibitory networks. In one word, biology is a science about relationships. Traditionally, biologists describe this as relationships between a limited number of genes or proteins using a descriptive language. With the huge amount of data produced by high-throughput techniques, biologists have to deal with thousands of biological relations in

a single experiment. In this situation, the traditional, descriptive ways for biological relations are not sufficient to deal with the huge number of relations under study. The only way to deal with a large amount of relations is through mathematical representations and computations. Taking into account that the readers of this book are biologists, I would like to first introduce basic computational concepts and then illustrate the procedures and computational techniques for high-throughput data analysis, using examples from cancer research.

### NETWORK BIOLOGY: CONCEPTS AND BIOLOGICAL MEANINGS

Graph theory, a branch of mathematics, is designed to describe all kinds of relationships and complexity in mathematical language. Networks represent the principles of graph theory. Thus, it is reasonable to use networks to represent biological relationships. By doing so, we are able to transform the biological/descriptive language into a mathematical language, which is computable and capable of handling a large number of relationships. In summary, network biology involves the use of networks to represent complexity, computes biological relationships, and seeks to uncover biological principles and insights. We can ask inspiring and fundamental questions and develop elegant computational methods using the principles of networks and statistics, which lead to new biological insights from high-throughput data. In turn, insightful results from these analyses can be used to ask new questions and design wet-lab experiments.

### **TYPES AND CATEGORIES OF CELLULAR NETWORKS**

Several types of cellular networks have been found in cells, such as protein interaction networks, metabolic networks, gene regulatory networks and signaling networks, genetic interaction network, and gene co-expression network (Wang et al. 2007), which can be further classified into two categories based on whether the networks capture biological relations in genome-wide or a specific cellular condition. Protein interaction networks encode the information of proteins and their physical interactions. Protein interaction information in the network ranges from basic cellular machinery, such as protein complexes for DNA synthesis, metabolic enzyme complexes, and transcription factor complexes, to protein complexes involved in cellular signaling. A genome-wide protein interaction network encodes all the protein interaction information across all biological processes in a cell. A gene regulatory network describes regulatory relationships between transcription factors and genes. Similar to protein interaction networks, a gene regulatory network encodes the gene regulatory information for all biological processes and activities in a cell. Genetic interaction networks describe genetic interactions between genes that lead to certain phenotypes. A genetic interaction network encodes the genetic interaction information for all kinds of cellular phenotypes. Therefore, we have classified protein interaction networks, genetic interaction networks, and gene regulatory networks into the first category: general cellular network. The second category of networks identified is cellular-specific network, which encompasses metabolic networks, gene coexpression networks, and signaling networks, describing relations in specific cellular conditions or specific cellular activities such as signaling and metabolism. A metabolic network collects all the metabolic reactions and metabolic flows, while a signaling network encodes signal information flows and biochemical reactions for signal transductions. Traditionally, both types of information are presented using pathways, for example, metabolic pathways and signaling pathways. In a metabolic network, metabolic pathways are intertwined so that metabolic flows are transferable across different pathways. Certain metabolites can be shared and used by many different pathways, while certain end-product metabolites can be produced via bypassing one or several pathways. Signaling networks illustrate inter- and intracellular communications and information processing between signaling proteins. In fact, pathway concept gets fuzzy and many pathways lose their identities

in networks (Patil and Nielsen 2005; Spirin et al. 2006). Gene coexpression networks capture the co-expressed genes during certain cellular conditions. Coexpressed genes often represent a collection of genes that are involved in similar biological functions and activities. In addition, other types of networks can be constructed based on different biological contexts, that is, disease gene networks, drug target networks, amino acid networks, and gene–environment factor network. Nevertheless, these networks are represented at either a genome scale or under certain cellular conditions or specific cellular activities such as signaling.

### **BIOLOGICAL FUNCTIONS AND MECHANISMS ARE ENCODED IN NETWORK PROPERTIES**

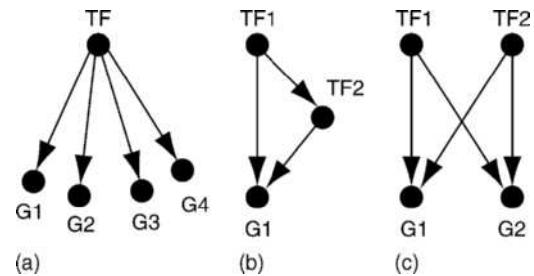
A particular phenotype is the result of collaborations of a group of genes. This notion provides a structured network knowledge-based approach to analyze genome-wide data in the context of known functional interrelationships among genes, proteins, and phenotypes. The biological relations and complexity are encoded in cellular networks (Wang et al. 2007). Therefore, a network view or a systems-level view of cellular events emerges as an important concept. Cellular networks can be presented as either directed or undirected graphs. Usually in these networks, nodes represent proteins or genes and the links represent the physical interactions between proteins, gene regulatory relationships, genetic interactions, gene coexpressions, activation/inactivation, or signaling reaction relationships. Notably, signaling networks contain the most complicated relationships between proteins, for example, nodes might represent different functional proteins such as kinases, growth factors, ligands, receptors, adaptors, scaffolds, and transcription factors, all of which have different biochemical functions and are involved in many different types of biochemical reactions that characterize a specific signal transduction machinery (Wang et al. 2007).

The topology or architecture of cellular networks is “scale-free,” meaning that the network contains a small fraction of nodes that act as highly connected hubs, whereas most nodes have only a few links. As an example, an air transportation map is an analogy for the scale-free network. In the U.S. air transportation map, only a few big airports (hubs) in big cities such as Boston, New York, Chicago, and Los Angles have many air routes (links) to other airports, while many small airports just have a few air routes to the nearby big airports. This common structural feature encodes a special property of these networks:

they are robust but are also very vulnerable to failure and attack (Barabasi and Albert 1999; Barabasi and Oltvai 2004). Random removal of a substantial fraction of the low-linked nodes will result in little damage to the network's connectivity and function; however, targeted removal of the hub nodes will easily disconnect and destroy the network completely. As illustrated by the air transportation map, disabling big airports (hubs) will wreak havoc in many ways, while damaging a few small airports will have little or no effect on the overall air transportation.

Hub genes in gene regulatory networks are generally global transcription factors that govern a large number of genes in response to internal and external signals. Furthermore, mRNAs of the hub transcription factors have significantly faster decay rates than nonhubs in *Escherichia coli* and yeast gene regulatory networks (Batada et al. 2006; Wang and Purisima 2005), suggesting that hub transcription factors facilitate a rapid response of the network to external stimuli (Wang and Purisima 2005). In protein interaction networks, hub proteins take part in many biological processes and play a more important role in an organism's survival. Removal of hub proteins from an organism would have a much broader effect on the organism than that of nonhubs (Babu et al. 2004; Jeong et al. 2001; Wuchty et al. 2003, 2006). Hub proteins are more evolutionarily conserved than nonhubs (Saeed and Deane 2006). One explanation of these phenomena is that hub proteins are subject to selection pressure and constraints, due to their involvements in many biological processes and their multiple interacting protein partners. In signaling networks, hub proteins are the proteins most commonly used by multiple signaling pathways. They become information exchanging and processing centers of the network (Wang et al. 2007). Hubs are one of the global properties of networks. Other global network properties include the following: network diameter, shortest path, network density, average links, clustering coefficient, network centrality (degree centrality, closeness centrality, radiality, betweenness, and PageRank), minimum spanning trees, network flows, and network bottlenecks. These measures have been described in detail in recent reports (Mason and Verwoerd 2007; Yu et al. 2007).

A complex network can be broken down into distinct regulatory patterns or basic units. Thus, local network properties can be defined. Network motif is such an example. Network motifs are the smallest functional modules in networks. These motifs are the statistically significant recurring structural patterns or small subgraphs or subnetworks that are found more often in a real network than would be expected



**Figure 25.1.** Network motifs in gene regulatory networks. (a) Single-input module (SIM): a transcription factor (TF) regulates a group of genes (G1, G2, G3, and G4). (b) Feed-forward Loop (FFL): a transcription factor (TF1) regulates the second transcription factor (TF2), both TF1 and TF2 regulate a target gene (G1). (c) Bi-fan: both transcription factors TF1 and TF2 regulate both target genes (G1 and G2).

by chance (Shen-Orr et al. 2002). These types of motifs have also been known as gene regulatory loops in biology. In gene regulatory networks, three major motifs are found in gene regulatory networks (Figure 25.1): single input module (SIM), bi-fan, and feed-forward loop (FFL).

Positive feedback loops lean to emergent network properties such as ultrasensitivity, bistability, and switch-like behavior, while negative feedback loops perform adaptation, desensitization, and preservation of homeostasis (Ferrell 2002). These motifs are enriched with the transcription factors whose mRNAs have fast decay rates, suggesting that motif structures encode a regulatory behavior: they are able to rapidly respond to internal and external stimuli and decrease cell internal noise (Wang and Purisima 2005). Network motifs also bear distinct regulatory functions and particular kinetic properties that determine the temporal program of gene expression (Mangan et al. 2003). Thus, the frequencies and types of network motifs that the cells use indicate the regulatory strategies that are selected under different cellular conditions (Balazsi et al. 2005; Wang and Purisima 2005). For example, FFLs are buffers that respond only to persistent input signals (Mangan and Alon 2003), which makes them well suited for responding to endogenous conditions, while the motifs whose key regulator's transcripts have a fast mRNA decay rate are preferentially used for responding to exogenous or environmental conditions (Wang and Purisima 2005). In signaling networks, network motifs such as switches (Bhalla et al. 2002), gates (Blitzer et al. 1998), and positive or negative feedback loops provide specific regulatory capacities in decoding signal strength, processing

information, and controlling noise (Dublanche et al. 2006). Network motifs often form large aggregated structures called network themes that perform specific functions by forming collaborations between a large number of motifs (Zhang et al. 2005). A higher level of aggregation of network themes can be regarded as network modules.

### A PRACTICAL GUIDE FOR NETWORK ANALYSIS IN CANCER BIOLOGY

Cancer is an extremely complex, heterogeneous disease and represents a typical example of biology gone awry. More and more efforts in “omics” are being made in the cancer research community. As a result, tremendous amount of money has been poured into cancer research in the last few years. Relatively speaking, more high-throughput data have been generated in cancer biology than in any other field of biology. However, the complexity of cancer forms a major obstacle for a comprehensive understanding of underlying molecular mechanisms of tumorigenesis. To crack the cancer code, computational methods have been developed and applied to cellular networks of cancer. Using a few examples of cancer-based studies, we will demonstrate the procedures and computational analysis involved in cancer cell networks.

#### DATA SOURCES AND QUALITY FOR CANCER CELL NETWORKS

Public databases collect and assemble literature-mined data sets describing human protein interactions; metabolic and signaling pathways; cancer-driving mutated genes such as COSMIC (<http://www.sanger.ac.uk/genetics/CGP/cosmic/>); and tumor gene expression profiles such as Oncomine (<http://www.oncomine.org/>); human protein interaction database, HPRD (<http://www.hprd.org/>); IntAct (<http://www.ebi.ac.uk/intact/site/index.jsf>); MINT (<http://mint.bio.uniroma2.it/mint/Welcome.do>); and DIP (<http://dip.doe-mbi.ucla.edu/>); and signaling pathway database such as BioCarta (<http://www.biocarta.com/>) and Reactome (<http://reactome.org/>). Data sets can be found in research articles that discuss high-throughput studies such as microarray profiling of tumors and genome-wide sequencing of tumor samples. Useful data sets are also found in research articles that manually curate data from literature for constructing cellular networks (Oda et al. 2005; Oda and Kitano 2006); that is, we have

manually curated a large human signaling network containing more than 1,600 proteins and 5,000 signaling relations (Cui et al. 2007). When using these data sets, the quality of data should be carefully examined. For example, it is well known that false positives are in the protein interaction data derived from high-throughput studies. Relevant computational methods have been developed to clean these false positives as much as possible (Mahdavi and Lin 2007). However, it is still a challenging task. On the other hand, data are often incomplete. To overcome these problems, sensitivity analysis can be applied: we can mimic false positives and false negatives by randomly adding or removing extra 10 and 20% of network nodes into the networks, and then reexamine the analysis (Cui et al. 2006).

#### NETWORK CONSTRUCTION AND VISUALIZATION

Networks can be constructed using the information contained in relationships and other information. Genes and proteins are often used as nodes; relations between them are edges (undirected links) or arcs (directed links). In gene regulatory network, links are directed and represent regulatory relationships. In protein interaction networks, links are undirected and represent physical interactions between proteins. In signaling networks, there are both undirected and directed links; furthermore, signs (i.e., activating and inhibiting) can be assigned to the directed links (Wang et al. 2007). In certain situation, links can be assigned numbers such as protein interaction affinity. The resulting network is called weighted network. Free software programs such as Pajek (<http://vlado.fmf.uni-lj.si/pub/networks/pajek/>) and Cytoscape (<http://www.cytoscape.org/>) are available to visualize networks.

#### DATA INTEGRATION ONTO NETWORKS

The integration of experimental techniques with the information technology provides a powerful approach to address and dissect the complexity of cancer and other biological problems at various levels in a systems-based manner.

It is often necessary to integrate different sources of data onto the network to perform analysis. The selection of data sources is based on the questions asked. For example, we integrated cancer driver-mutated genes determined by large-scale genome sequencing of tumors onto a human signaling network to understand cancer signaling mechanisms (Cui et al. 2007). Chuang et al. (2007) integrated tumor gene microarray expression profiles onto a human

protein interaction network to find network clusters as breast cancer biomarkers.

### NETWORK PROPERTY ANALYSIS

Global and local network properties can be analyzed. Almost all the network properties are analyzed using statistical methods. Global network properties can be investigated and biological insights drawn. For example, by integrating cancer driver-mutated genes and cancer-associated methylated genes onto a human signaling network, we discovered that cancer causal mutations most likely occur in signaling proteins that are acting as signaling hubs (i.e., actively sending or receiving signals) rather than in nodes that are simply involved in passive physical interactions with other proteins (Cui et al. 2007). These results can be interpreted in light of the fact that since signaling hubs are focal nodes that are shared by, and/or are central in, many signaling pathways, alterations of these nodes, or signaling hubs, are predicted to affect more signaling events, resulting in cancer or other diseases. We further showed that activation and inhibitory network flows enhance and alleviate oncogenic signaling flows, respectively. Network node connectivity is correlated with cancer biomarkers; that is, a protein that has more links with cancer mutated genes has a higher chance to be cancer-associated genes or a biomarker (Cui et al. 2007). We also found that the downstream cancer mutating genes of the network, especially the genes of the output layer of the network, have a higher mutation frequency, which complements with another finding that cancer-associated genes are enriched in the nuclear proteins (Awan et al. 2007). In contrast, the distributions of the cancer-associated methylated genes have no such preference, suggesting that genes subject to genomic silencing do not tend to directly affect the output layer of the network. These results strongly suggest that the genes in the output layer of the network, which play direct and important roles in determining phenotypic outputs, are frequent targets for activating mutations. The importance of this output layer is reinforced by another observation that the expression of the output layer genes of the signaling network is heavily regulated by microRNAs (Cui et al. 2006).

A study of integration of literature-mined cancer genes onto a human protein interaction network showed that cancer proteins have more interaction partners than other proteins in the network, suggesting that cancer proteins may be involved in significantly more biological processes and play a central role in the protein network (Jonsson and Bates 2006).

As another example, Platzer et al. (2007) systematically investigated 22 individual network measures for the cancer gene networks constructed by combining a human protein interaction network with tumor-associated, differentially expressed genes. Network measures range from closeness centrality, network diameter, index of aggregation, assortative mixing coefficient, connectivity, and sum of the Wiener number to modified vertex distance number, representing several key network properties: size, distribution, relevance, density, modularity, and cycles. The analysis showed that genes showing significant differential expressions in cancer appear to be interlinked on the human protein interaction network. These cancer gene networks show a low density, indicating that they bear high robustness.

Biological insights are also encoded in local network properties. We have found that cancer-associated mutated and methylated genes are enriched in positive and negative regulatory loops, respectively. These results showed not only an overall picture of the network architecture where the oncogenic stimuli are embedded but also the signaling regulatory mechanisms involving cancer mutated and methylated genes (Cui et al. 2007). Furthermore, cancer genes are enriched in the convergent target nodes of most network motifs, and form network motif clusters or cancer gene hotspots on the network (Awan et al. 2007). We have extracted a giant subgraph, which we named oncogenic map, containing connected cancer mutated and methylated genes (Cui et al. 2007). In the map, we further defined three network regions, which are enriched in cancer-dependent signaling events, based on the overall frequency of cancer-associated gene mutations. These results show an overall cancer signaling network architecture and highlight the most frequently used cancer signaling cascades. The map was further decomposed into 12 network community modules with different signaling functions: cell surface receptor-linked signaling, intracellular signaling cascades, and apoptotic signaling. Similarly, cancer genes on protein networks also form network communities (Jonsson and Bates 2006), representing distinct biological processes.

Most of the methods for network property analysis have been implemented: Pajek, Cytoscape, and Network Workbench (<http://nwb.slis.indiana.edu/>) are used to explore both global and local properties. Mfinder (<http://www.weizmann.ac.il/mcb/UriAlon/groupNetworkMotifSW.html>), FANMOD (<http://www.minet.uni-jena.de/~wernicke/motifs/index.html>), and MAVisto (<http://mavisto.ipk-gatersleben.de/>) are designed for finding network

motifs. CFinder (<http://www.cfinder.org/>) can be used to define network communities. More network measures and computational tools are expected to be developed to interpret biological meanings in the future.

#### NETWORK DYNAMICS AND MODELING

Dynamics of cancer gene networks can be constructed by mapping expressed cancer gene in different stages of tumor progression such as tumors in the early developmental stages and metastasis onto all kinds of cellular networks such as protein interaction networks. Chuang and coworkers mapped tumor-expressed genes onto a human protein interaction network to identify subnetworks as cancer biomarkers. The resulting cancer subnetwork markers are more reproducible than individual marker genes selected without network information, and they achieve higher accuracy in the classification of metastatic versus nonmetastatic tumors (Chuang et al. 2007).

Although cancer is considered a very heterogeneous disease, querying mutating genes in tumor samples using the network communities defined in a human cancer signaling map reveals that one common network module occurs in most of the tumor samples. Furthermore, the common module seems to collaborate with one or more tumor-specific modules for tumorigenesis in different tumor types (Cui et al. 2007). In particular, breast and lung cancers have more complex oncogenic signaling block collaborative patterns than other cancer types examined, highlighting their heterogeneous nature.

Tumor gene coexpression networks can be constructed using different types or subtypes of tumors. Weighted gene coexpression networks have been constructed using gene microarray profiles of glioblastoma samples (Horvath et al. 2006). Analysis of such networks provides a blueprint for leveraging genomic data to identify key control networks and molecular targets for cancer.

Tumor gene microarray profiles can also be used to reversely construct gene networks. We constructed a gene regulatory network using the time course microarray profiles from a mouse epithelial breast cell line (BRI-JM01) (Wang et al. 2007), which undergoes an epithelial-to-mesenchymal transition (EMT) when they are treated with TGF- $\beta$  (Lenferink et al. 2004). Notably, clusterin, one of the genes that is up-regulated in the middle and late time points, shows many regulatory links to other genes in the network. During the EMT process, clusterin is secreted by

the BRI-JM01 cells. Interestingly, by applying anti-clusterin antibodies to the TGF- $\beta$ -treated BRI-JM01 cells, we were able to block the TGF- $\beta$ -induced EMT (Wang et al. 2007). Reverse-engineered gene network approach has been applied to the expression profiles of prostate cancer to identify genetic mediators and mediating pathways (Ergun et al. 2007).

Oncogene predictions can be made by constructing networks and modeling of multiple sources of data. Oncogenes of the malignant B-cell phenotypes have been predicted by generating a network from multiple sources: a genome-wide compendium of human B-cell molecular interactions, in combination with a large set of microarray expression profiles (Mani et al. 2008). The resulting network contains protein–protein, protein–DNA, and posttranslational interactions. This approach allows capturing several different mechanisms of action associated with oncogenic lesions, specific tumor phenotypes, and biochemical perturbations.

In summary, the analysis of the cancer phenomenon using a network approach is still in its infancy. However, network approaches provide new ways to explore the complexity of biological systems and lead to discovering new insights into high-throughput data.

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# 26

## Peptidomics

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### INTRODUCTION

Completion of human genome project coupled with the advancement in “omic” technologies has enabled researchers to analyze the complex interplay of metabolism, gene expression, and function, and more broadly, genetic diversity within and between human populations. Nutrition science has broadened to a new discipline called nutrigenomics, which allows in-depth understanding of metabolism, health, and pathophysiology of disease that ultimately could be used to prevent or treat diseases (Lévesque et al. 2008). It is becoming increasingly obvious that an individual’s phenotype represents a complex interaction between the genetic background and environmental factors over the course of an individual’s lifetime. Therefore, food consumption and nutrient exposure are key environmental factors that play an important role in pathogenesis and progression of the diet-related diseases, namely, diabetes, obesity, atherosclerosis, insulin resistance, cardiovascular disease, and other metabolic syndrome (Phillips et al. 2008). Nutrigenomics utilizes new high-throughput technologies to provide detailed information about the composition and functions of the genome, mechanisms for regulation of gene expression, and the influence of nutrients on gene and protein expression (Afman and Mullet 2006). Proteomics is central to nutrigenomics and has the potential to explain many of the physiological changes associated with nutritional stimuli. In proteomics, all proteins expressed in a cell or tissue are analyzed to identify the presence or absence of some key proteins, which gives information about the early stages of disease or different conditions. However, a comprehensive analysis of peptides and small proteins of a biological system corresponding to the re-

spective genomic information was missing in proteomics. Hence, the concept of peptidomics was introduced.

The term *peptidomics* was first introduced as a subset of proteomics for the description of peptides as gene products in February 2000 at the ABRF conference “From Singular to Global Analysis of Biological Systems.” It was coined as a short version of “peptide proteomics” and is defined as the technology for comprehensive qualitative and quantitative description of peptides in a biological sample (Schulz-Knappe et al. 2001).

Peptides are short polymers of amino acids linked by peptide bonds. There is no clear-cut definition to differentiate between peptides and proteins; however, the term peptide was referred for oligo- and polypeptides ranging from dipeptides to molecules of about 20 kDa (Schrader and Selle 2006).

Peptides play a central role in many physiological processes. Although technically peptidomics includes analysis of all peptides present in the system including products of protein degradation, typically studies are focused on biologically active peptides. A large number of peptides function as intercellular messengers in the endocrine system (peptide hormone) and in the brain they function as neurotransmitters (Fricker 2007). Peptides that are involved in the intercellular communication between neurons are termed as neuropeptides. Neuropeptides influence various physiological processes, including feeding and body weight regulation, energy balance, thermogenesis, circadian rhythms, sleep/wake cycles, and reproduction (Fricker 2007; Strand 2003). However, neuropeptides are also implicated in the various neurological and psychiatric disorders such as Parkinson’s disease, depression, and eating and sleeping disorders (Hökfelt et al. 2000).

Other than neuropeptides, a number of endocrine peptides function as hormones. The physiological process of peptide hormones includes regulation of glucose levels, water retention, and food intake (Fricker et al. 2006). In addition, peptidomics applications include discovery of biologically active molecules, drugs, and novel drug targets (Schulte et al. 2005). Further, peptides appear in body fluids, and tissues function as potential diagnostic biomarker, which serves as a tool for early detection and monitoring of diseases (Adermann et al. 2004).

Interestingly, peptides have so far been neglected in most proteomic studies. The completion of genomic project has opened new opportunities for rapid identification and functional analysis of peptides (Baggerman et al. 2004). At this stage, introduction of "postgenomic" techniques such as cDNA arrays and proteomics has led to the discovery of genes and/or proteins that are up- or downregulated in certain physiological processes.

In proteomics, all the proteins and their posttranslational modifications (PTMs) are identified in tissues. In this approach, proteins are routinely separated by two-dimensional (2D) electrophoresis, and subsequently the expressed proteins are identified by mass spectrometric techniques. The limitation of this technique is that the proteins of molecular mass lower than 10 kDa are generally not retained (Baggerman et al. 2004). Nevertheless, the low-molecular-weight peptides and proteins belong to families of hormones, cytokines, growth factors, and neuropeptides. Standard proteomic approaches are not suitable for a systematic peptide analysis, since they do not cover the low-molecular-mass window. Therefore, peptidomics has been introduced.

The main aim of peptidomics is the simultaneous visualization and identification of whole peptidome of a cell or tissue. As in proteomics, the core technology in peptide identification is mass spectrometry (MS). MS allows a sensitive and precise detection of the total peptide content in complex mixture, and therefore, MS is the method of choice for peptide detection (Baggerman et al. 2004). Both matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) and electrospray MS have proven to be successful.

## AFFINITY PEPTIDOMICS

In affinity peptidomics approach, the composition of protein mixture can be determined by directly assaying the peptides from crude tryptic or otherwise digested protein using immobilized antibodies, rather than from full length native protein prepara-

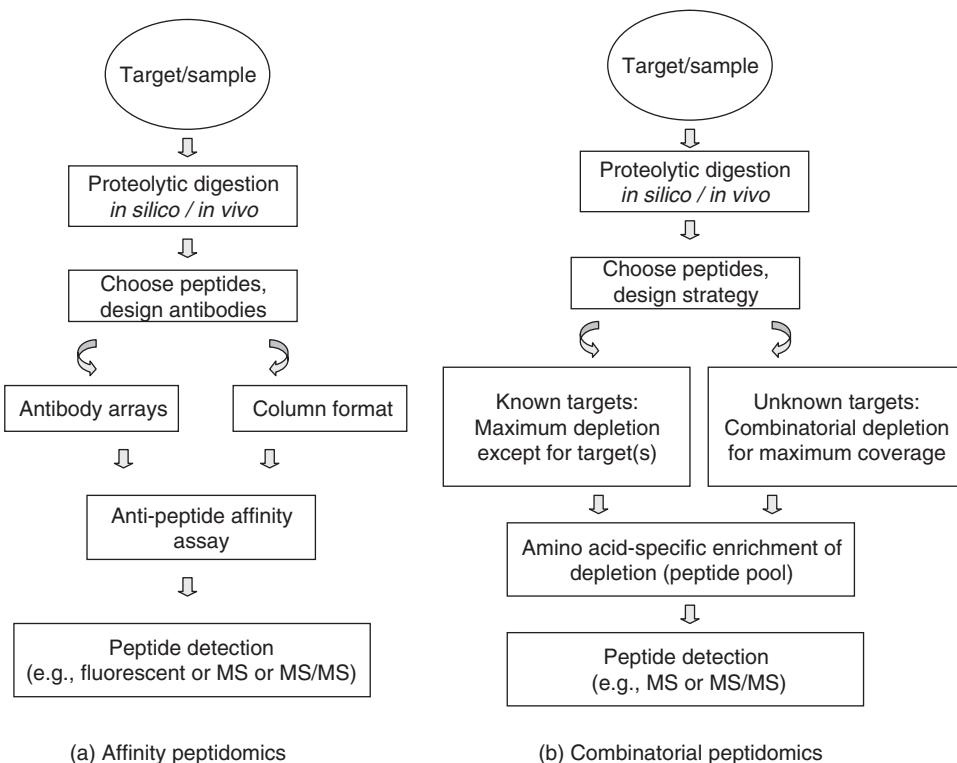
tions. Digestion of intact tissues or cellular fractions gives rise to many smaller components, resulting in the release of a range of peptides. The released peptides are most probably hydrophilic, and it enhances the performance of the affinity assay. Scrivener et al. (2003) reported that the best response was obtained using affinity agents developed against the most hydrophilic peptides. The less hydrophilic peptides showed weaker or no binding at all. Most human proteins (98.7%), including hydrophobic ones, yield sufficiently hydrophilic peptides (hydrophilicity 0.45–4.5) on tryptic digestion. Therefore, in affinity peptidomics approach, the antibody generation was facilitated through both better (more hydrophilic and immunogenic) peptide sequence and the availability of antigens (synthetic peptides). This helps in prediction of peptide sequence *in silico* on the basis of sequence alone (Soloviev and Finch 2005).

In affinity peptidomics, the chips are immobilized on a solid support with an array of captured reagents (antibodies) that specifically recognizes a region of a target peptide fragment. These target peptide fragments may be generated by enzymatic or chemical cleavage of the protein of interest (Scrivener et al. 2003). Identification of captured species may be carried out either by fluorescent scanning or by using MS (MALDI-TOF-MS).

In fluorescent method, peptides are labeled prior to the incubation, and it allows a spatially resolved image of fluorescence on scanning the chip. The advantage of this method is that faster scanning and information retrieval is possible. The disadvantages include the following: Fluorescence is unable to verify that the right peptides are captured, and it does not help in distinguishing different PTMs of the polypeptide fragment.

The MS (MALDI-TOF-MS) allows identification of *m/z* of the peptide by desorbing captured species directly from the array. Therefore, the resulting mass spectra help in clear identification of captured species. Further, MS could also help in the identification of PTMs. However, MS analysis also has considerable technical disadvantages. Importantly, as mentioned by Scrivener et al. (2003), the hydrogel pads mounted onto silicon are not suitable for MALDI-TOF-MS, and increased sensitivity may be obtained from antibodies immobilized on directly modified, polished metallic surfaces. Also, obtaining quantitative data from MALDI is not direct, and several factors influence the peak height.

Peptidomics approach has several advantages over affinity capture of intact proteins, which enables the high-throughput screening of proteins in a microarray format. Homogeneity of digested proteins



**Figure 26.1.** Scheme illustration of (a) affinity peptidomics and (b) combinatorial peptidomics. In affinity peptidomics, proteolytic peptides are captured by antibodies. In combinatorial peptidomics, peptides are selected by depleting (or enriching) crude proteolytic digests by chemical cross-linking of the peptides to solid support (e.g., beads). (Reproduced with permission from Soloview and Finch 2005.)

(typically in the form of tryptic peptides) results in a more uniform pool of target species. As peptides are much more stable and robust than proteins, they are less susceptible for degradation. In addition, antibody reagents can be more easily generated, such as by chemical synthesis of *in silico* predicted peptides against which antibodies are raised, for example, by phage display technique. Therefore, such affinity reagents can be obtained in a high-throughput manner, and their specificity can be more easily regulated (Scrivener et al. 2003). The affinity peptidomics approach also suits well with both monoclonal antibody and polyclonal antibody (Soloview and Finch 2005), and does not require labeling of proteins or peptides. In addition, it allows multiple antibody-peptide usage to assay the sample protein target, which in turn increases the reliability of the assay (Soloview et al. 2003).

The major disadvantages of this affinity-assay-based technique, including peptidomics, are availability and cost of the capture reagents (antibodies). Each protein requires the production of specific affin-

ity reagent (e.g., antibody), and its production may cost time and more money (Soloview and Finch 2005; Figure 26.1).

## COMBINATORIAL PEPTIDOMICS

The combinatorial peptidomics approach allows the determination of the composition of a protein mixture by assaying peptides directly from crude tryptic digests without using antibodies or any other affinity selection. This approach relies on the chemical reactivities of the side chains of amino acids and amino acid content (their information content) of the peptides and is not based on their physical properties. Also, combinatorial peptidomics does not depend on either chromatography or electrophoretic separation techniques. In this approach, firstly, protein samples are proteolytically digested using one or a combination of proteases or, alternatively, by chemical cleavage. Secondly, there is quantitative depletion or enrichment of peptide pools by chemical cross-linking of a subset of peptide (support through amino acid

side chains) to a solid support. This results in reduced complexity of a peptide pool and amino acid compositional complexity to a required degree to make it compatible with MS detection (Soloviev et al. 2003).

Unmodified peptides as well as proteins generally contain up to eight reactive groups, of which six are chemically reactive amino acid side chains, namely, guanidinyl groups of arginines, thioether groups of methionines, sulfhydryl groups of cysteines, imidazolyl groups of histidines, phenolic groups of tyrosines, and indolyl groups of tryptophans. Any of these chemically reactive amino acid side chains can potentially be used to deplete or enrich a sample of peptide, which contain them, in a specific and fully predictable manner with respect to amino acid content. The other two reactive groups—amino groups ( $\text{H}_2\text{N}-$ ) and carboxyl groups ( $\text{HOOC}-$ )—are present on every peptide as N- and C-terminal groups or epsilon amino groups of lysines or as parts of aspartic acid and glutamic acid side chains. These may be used for amino acid sequence and content-independent manipulations through chemical, radioactive, fluorescent, or isotopic labeling (Soloviev et al. 2003; Soloviev and Finch 2005). In depletion method, amino acid filters up to six different combinations that can be used repeatedly or until the amino acid complexity of the peptide pool and the amino acid complexity of the remaining peptides is reduced to the optimum level. Therefore, the peptides that do not contain an amino acid, which is not recognized by any of the amino acid filter(s), remain in the mixture. The depleted pool will have peptides of decreased amino acid complexity, and hence, it allows direct analysis by TOF-MS and TOF/TOF-MS (Soloviev and Finch 2005).

It is possible to bring maximum depletion in complex peptide mixtures by using all filters together, for example, whole cell proteomics. However, in the case of a simple microorganism proteomics where less-complex protein mixtures are found, individual amino acid filter or subset of filters may be used. The depletion rate varies depending on either the number of amino acid filters used or the length of peptide fragments. Longer peptides are more likely to be cross-linked due to higher occurrence probabilities of amino acids in long peptide fragments.

Combinatorial peptidomics approach uses enrichment selection technique in which peptides containing required amino acids are retained. In this method, the peptide mixtures are passed through individual or different combination of selected amino acid filters and these filters form bond with the peptides containing the respective amino acid. Then, filter assembly is washed to remove free peptides and chemical cross-

link is cleaved in order to free the bound peptides. Like depletion technique, in this approach too, the procedure can be repeated using single or multiple combinations of amino acid filters. However, both the methods are aimed to achieve the reduced complex mixture of the sample (Soloviev et al. 2003).

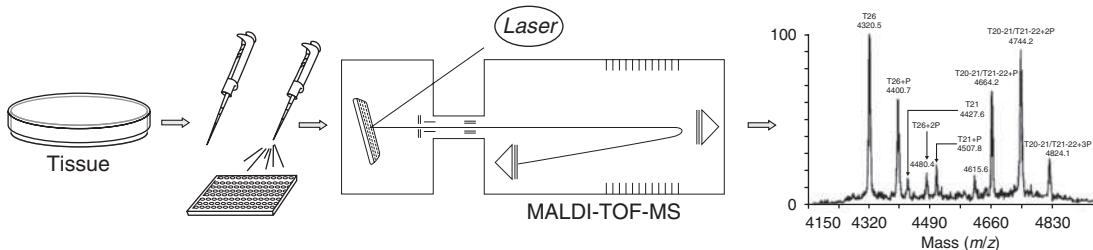
There are noted advantages and disadvantages with both the approaches. The depletion approach is straightforward, faster, and more suitable for peptides of 10 amino acids and longer, whereas shorter peptides (10 or less amino acid) are more suitable for the slower enrichment approach. The depletion approach helps in decreasing the compositional complexity of the remaining peptide pool over enrichment approach to support further MS analysis. Further, the depletion approach has advantage over enrichment approach toward incompletely digested peptides. Incompletely digested peptides are easily eliminated from the depleted samples due to their increased peptide length, which has more probability for being cross-linked (Soloviev et al. 2003).

## PEPTIDOMICS TECHNOLOGIES

Characterization of the genomics sequence of an organism coupled with technological advancements leads to a revolution in biology and medicine. Toward understanding life process at the molecular level, the research in proteomics is the next logical step after structural genomics. In a broad sense, proteomics encompasses the knowledge of the structure, function, and expression of proteins in the biological or biochemical context of all organisms. The key element of the classical proteomic research combines the multidimensional separation of proteins from a complex mixture through 2D gel electrophoresis (Barnouin 2004), and the protein identification is performed by MS (Smith 2002). However, in peptide analysis and peptidomics, this method does not provide adequate separation and is restricted to protein ranging from 10 to 20 kDa. Therefore, alternative methods such as HPLC-MS (high-performance liquid chromatography – mass spectrometry), MALDI-TOF-MS, and CE-MS (capillary electrophoresis–mass spectrometry), including Edman sequencing, have been developed (Soloviev and Finch 2005). In analogy with the proteomics, the core technology in peptide identification is MS.

## MASS SPECTROMETRY

MS is currently one of the important analytical techniques that allows sensitive and precise detection of the total peptide content even in complex mixtures



**Figure 26.2.** Schematic illustration of direct peptide analysis by MALDI-TOF-MS. A cell or tissue is directly placed on MALDI target plate or first on extraction solution. Sample is analyzed by MALDI-TOF-MS, and the result shows singly charged ions corresponding to the peptides present in the sample.

(Yates 1998). The success of MS in this field is partly due to its ability in obtaining the structural information from molecule, in addition to the relative mass. Therefore, MS is the method of choice for peptide identification. The main technologies used in peptidomics research today are MALDI-TOF-MS for direct peptide profiling, MALDI-TOF-MS in combination with liquid chromatography (LC) (off-line), and ESI-TOF-MS combined with nanoLC (online) (Clynen et al. 2003).

### MALDI-TOF-MS

MALDI-TOF-MS was introduced in the late 1980s and serves as a technique for analyzing complex protein and peptide mixtures (Tanaka et al. 1988). This can be used for direct analysis of the peptidome of a cell, cell organelle, or organ (Clynen et al. 2003). In this method, a small amount of sample is premixed in solution with a light-absorbing material known as the matrix ( $\alpha$ -cyano-4-hydroxycinnamic acid mostly used for peptides) and applied to a target plate. After drying, it is inserted into the ionization source of the mass spectrometer, where a pulse of UV light is directed toward the sample. Ionization of the sample results in singly charged molecules (Ashcroft 2003). MALDI is very popular because it is highly sensitive and there is formation of singly charged ions, which facilitates the analysis of a complex mixture (Boonen et al. 2008). Due to its pulsed nature, MALDI is more often coupled to TOF mass analyzer. MALDI-TOF-MS has many advantages compared to other MS technologies for the direct analysis of complex mixture. This is less susceptible to impurities and salt, and also results in formation of singly charged molecules. However, other ionization methods (like ESI) result in multiple charge states of larger peptides, which interfere in mass spectra data interpretation (Clynen et al. 2003).

MALDI-TOF instruments equipped with an ion selector fragmentation by postsource decay (PSD) helps in obtaining structural information. The major drawback of PSD is the time-consuming spectrum acquisition and the low resolution of the ion selectors. This may be problematic when working with complex mixtures like tissue extracts (Baggerman et al. 2004). The recent advances in instrument development such as MALDI-Q-TOF technique, which combines the advantages of MALDI ionization and Q-TOF tandem MS analysis, provide an excellent tool for *in situ* peptide analysis (Verhaert et al. 2001; Figure 26.2).

### NanoLC ESI-TOF MS

LC is regarded widely as the method of choice for separating peptides as well as other biomolecules. When combining these miniaturized separation techniques with MS, very powerful tools could be invented. ESI can be coupled with HPLC (online) since it transfers ions from solution directly into the gas phase. The advantage is that the peptides are separated and elute gradually while each peptide is sharply focused and concentrated in a chromatographic peak. Then peptides are analyzed mass spectrometrically as they elute from the column (Clynen et al. 2003). An important aspect of ESI is the production of multiply charged ions, which helps in analyzing proteins with masses up to 100 kDa in a small  $m/z$  window. Another useful feature, especially for peptides, is that fragmentation of multiply charged ions yields more detectable fragment ions (Boonen et al. 2008).

### PEPTIDE SEPARATION

Previously, it was reported that peptides can be analyzed by MS by placing samples directly on the

MALDI target plate (Clynen et al. 2001; Reyzer and Caprioli 2005; Verhaert et al. 2001) or by a brief extraction in the spraying solvent in the case of ESI (Clynen et al. 2001; Köllisch et al. 2000). In most instances, bioactive peptides concentration is not unique in tissues or cells. Organs like brain and fluids like blood and cerebrospinal fluid contain high concentration of peptides. In these conditions, mass spectrometric analysis of complex sample requires prior separation. However, some body fluids and organs contain very low concentration of peptides (e.g., hormones), which require preconcentration of samples before being subjected to MS analysis (Oosterkamp et al. 1998).

A successful peptidomics experiment requires a marked reduction in sample complexity before being subjected to MS analysis. Otherwise, detection techniques like MALDI and ESI suffer from competitive ionization. In addition, ionization efficiency also depends on the concentration of the analyte, because concentrated analytes are detected more sensitively than original sample (Wilm and Mann 1996). Further, separation of complex mixture decreases the negative influence of contamination, which improves the sensitivity and accuracy. Overall, the prior chromatographic separation of samples can enhance MS analysis and detection (Beavis and Chait 1990).

In proteomics, the primary tool is 2D gel electrophoresis in combination with MS. However, this method is restricted to proteins ranging between 10 and 200 kDa, which makes this technique obsolete for peptidomics study. In contrast, peptides can be separated with high resolution by column chromatography techniques. The most preferred method for peptide separation is reversed phase-high performance liquid chromatography, especially a combination of ion-exchange chromatography with reversed phase (Issaq et al. 2005). The advantage of chromatographic separation is that the analytes remain in the liquid phase. So, the loss of proteins seen during extraction from gel is not observed. This is because recovery of protein usually requires in-gel digestion. However, peptide recovery can be possible in an unchanged molecular form, since the majority of peptides do not contain complex secondary and tertiary structures, which are capable of spontaneous refolding (Schulz-Knappe et al. 2001).

Identification of peptides based on the separating power of chromatographic techniques also carries specific problems. Biological samples usually have higher concentration of larger proteins as compared to peptides. For example, in blood plasma, albumin content is higher than that of whole protein content, about 40–50 g/L, which corresponds to almost

1 mmol/L in molecular concentration. However, typical concentrations of peptides in blood vary between micromolar and picomolar concentrations for peptide hormones (Heine et al. 2002). Chromatographic separation is thus compromised in terms of resolution and loading capacity. Therefore, separation of peptides from proteins >20 kDa is a necessary preparation step to reduce the protein load for chromatography and MS (Schulz-Knappe et al. 2001). Further, it was reported that capillary electrophoresis with MS is becoming popular in peptide separation and identification. In combination with MS, it allows separation of peptides ranging from small molecule to large protein in the same analysis. The advantages of capillary electrophoresis are its high efficiency, fast analysis times, and low sample and reagent consumption (Herrero et al. 2008).

## DETECTION OF PEPTIDES

Prior to the launching of MS approaches, peptides were identified by Edman degradation. These studies were having problem of protein degradation, and productive results were obtained only when applied to peptide-containing vesicles (Sigafoos et al. 1993). Another approach was based on C-terminal amide group (Tatemoto and Mutt 1978), which is shared by many bioactive peptide hormones and some neuropeptides, but not in protein degradation fragments. Therefore, it was considered as the signature of bioactive peptides. Currently, the majority of the sequence information is generated through mass spectrometric methods followed by database comparison owing to the high-throughput nature of the technique compared with Edman sequencing or amino acid analysis (Aebersold and Mann 2003). Generally, trypsin is used to digest the proteins into several peptides and detected by comparison of such cleavage pattern with databases. But for peptides, the number of possible specific trypsin cleavage sites is typically too small for identification via this approach. So, the original peptide has to be purified to a high extent as a prerequisite. With the emergence of peptide MS/MS sequencing technology, MS-based peptide identification has largely replaced Edman degradation.

For detection of peptides from complex biological mixture, MS is used as a method of choice by Schulz-Knappe et al. (2001). They have used two different ionization procedures that allow the transfer of peptide into the mass analyzer. For smaller peptides, ESI-MS is the method of choice for analysis of tryptic fragments by combining LC and MS. This method is complicated when larger peptides are present in the samples. In this case, complex

mixtures are resolved better with the higher sensitivity by using MALDI-MS technology since it results in singly charged molecule but less often doubly charged molecules (Uttenweiler-Joseph et al. 1998). Further, Schulz-Knappe et al. (2001) introduced the concept of peptide trapping, in which peptide analysis was done using MS, the subsequent production of MS fingerprints, and sequence determination of all detected peptides. Using peptide trapping, they have done large-scale analysis of peptides from human blood and created peptide bank from more than 100,000 L of blood ultrafiltrate (Schulz-Knappe et al. 1996).

The amino acid sequence of peptides and small proteins are identified with a “top-down” approach, which involves high-resolution mass spectrometric measurement and fragmentation of intact ionized molecules in the gas phase (Kelleher 2004). This methodology is generally for small peptides (up to 9 kDa) from tissues, body fluids, and other biological samples. Therefore, top-down approach was successfully applied for the identification of endogenous peptides in cerebrospinal fluid (Heine et al. 2002; Mohring et al. 2005) and murine brain tissue (Mohring et al. 2005).

### PEPTIDE QUANTIFICATION

Quantitative peptidomics techniques are generally similar to the standard peptidomics approach, except that the samples are first labeled with one of the two related chemicals that differ isotopically. In quantitative peptidomics studies, an important issue is an accurate comparison of the quantitative level of the peptides of interest between two samples. When performing HPLC separations of peptides (LC-MS), the easiest way to compare the relative abundances of peptides is through the use of a UV detector placed between the LC system and the mass spectrometer. However, less reproducibility of peak area/height associated with MS signals is an important drawback to compare peptide levels. Nevertheless, for compounds having similar mass and functional groups, the relative ion intensities may correspond to their relative contents (Baggerman et al. 2004).

The direct quantification by MS is by using internal standards. An internal standard is often a copy of the molecule containing one or more heavy, stable isotopes. The addition of known concentrations of the internal standard to the biological sample can be used to obtain accurate quantification (Kirkpatrick et al. 2005). However, in peptidomics studies, a large number of peptides are detected in a single analysis. Direct quantification of every peptide present in

the tissue would be labor-intensive and too costly. Instead, it is easier to examine the relative level of a peptide in two different samples.

The isotopic labels can be incorporated into the peptides after their extraction. Each one of the two samples of peptides to be compared are labeled with one of the two isotopic forms of a given reagent (e.g., one sample may be labeled with the natural H-isotopic form of a given reagent such as the H6-acetic anhydride—H6-Ac2O, and the other one with its deuterated form D6-acetic anhydride, D6-Ac2O). Both samples are mixed and subjected to micro-/nano-HPLC-MS analysis. The relative intensity of the MS signals of the heavy and light forms of the labeled peptides reveals their relative amounts in both biological samples. These isotopic-labeling techniques do not provide an absolute quantitation of the peptide, and instead only provide a measure of the relative changes in the peptide levels between two samples (Saz and Marina 2008). In another approach, if working in a cell culture system or with an organism, the diet contains enriched isotope that can be fed and then label can be incorporated into the peptide during its biosynthesis. This approach has been used for analysis of the rate of peptide biosynthesis in neuroendocrine cell lines (Che et al. 2004). In this method also, relative abundance of the peptide in the two samples can be calculated from the ratio of the peak intensity or peak area of the two isotopic forms.

Many varieties of compounds have been used for proteomic analysis (Julka and Regnier 2004), among which the reagent named isotope-coded affinity tags (ICAT) that reacts with the sulphydryl group of cysteines has commonly been employed in proteomic studies. Peptides that are extracted from two groups of animals are labeled with either the heavy or light form of ICAT reagent. After enzymatic digestion, the labeled peptides from the two combined samples are purified by affinity chromatography and then peptides are identified by LC-MS/MS analysis. In both conditions, the relative abundance of peptides can be compared by comparing their relative signal intensities (Gygi et al. 1999). However, very few peptides contain cysteine residues, and therefore, ICAT technology can be used for only cysteine-containing peptides or proteins. To avoid such general labeling methods, other reagents that react with amines are introduced.

The best described isotopic labels for peptidomics studies are the active esters of trimethylammoniumbutyrate (TMAB) containing either nine deuteriums (heavy) or nine hydrogens (light) (Che and Fricker 2005; Fricker et al. 2006). The

*N*-hydroxysuccinimide ester of the TMAB reagent reacts with amines that are present on the N-terminus of the peptide and/or on the side chain of lysine residues. With this quantitative peptidomics method, it is possible to detect, quantify, and identify approximately 100 peptides in extracts of a single mouse hypothalamus (Che et al. 2005; Pan et al. 2006). Moreover, a large number of peptides are also detected in other brain regions such as prefrontal cortex, striatum, hippocampus, and amygdala (Lim et al. 2006). However, disadvantage of this method is that TMAB reagent showed instability of labeled peptides on some MS analysis depending on the experimental conditions (Che and Fricker 2005). Using quantitative peptidomics approach, a number of studies have been performed, some of which are relevant to the area of food intake/body weight regulation and/or which involve mice lacking peptide-processing enzymes. On the basis of the quantitative peptidomics analysis, Fricker (2007) showed that increased body weight of the Cpefat/fat mice is due to the reduced levels of peptides that decrease body weight, such as  $\alpha$ -melanocyte-stimulating hormone.

#### POSTTRANSLATIONAL MODIFICATIONS

The covalent processing events that change the properties of a protein or peptide, by cleavage or addition of one or more amino acids, are called PTMs (Mann and Jensen 2003). In general, most proteins and peptides require a modification to become functionally active or to improve its stability. The commonly involved processing enzymes are endopeptidases. In addition to the peptidase cleavage, other enzymes perform additional PTMs such as glycosylation, C-terminal amidation, acetylation, phosphorylation, and sulfation. Important protective PTM in peptides is amidation, which is carried out by peptidyl  $\alpha$ -amidating monooxygenase, a multifunctional enzyme that performs a two-step process of converting C-terminal glycine residues into an amide group (Prigge et al. 2000). Most neuropeptides carry a pyroglutamic acid at their N-terminus, which is formed by the cyclization of an N-terminal glutamine (Schoofs et al. 1997). Some peptides like melanotropins and beta-endorphins are acetylated at their N-terminus to increase their stability and to modulate their activity (O'Donohye et al. 1982). Peptides are not always processed the same way in different cell types or even within the same cell type under different conditions. This is because different types of peptides often bind to their receptor targets with different affinities. The posttranslational processing can serve to regulate the resulting bioactive prop-

erties of the peptide. An important example is the processing of proopiomelanocortin into adrenocorticotrophic hormone in the anterior pituitary and into  $\alpha$ -melanocyte-stimulating hormone in the intermediate pituitary (Eipper et al. 1986).

Usually PTM can be identified by comparison of experimental data to a known amino acid sequence. But it requires prior detection of peptide sequence before PTM analysis. In the case of labile modifications such as sulfation and glycosylation, modification will be lost before peptide fragmentation itself. In this condition, the peptide can be sequenced and identified, but the mass increment between the obtained sequence and the parent mass will be the same as that of PTM. Hence, in this condition the exact location of the PTM cannot be identified (Torfs et al. 2002). If the modification is stable, such as acetylation, methylation, oxidation of methionine, N-terminal amidation, or formation of a pyroglutamic acid, the fragmentation spectrum will be similar to that of the unmodified peptide. There is an exception for the modified amino acids, which will have a mass increment or decrement corresponding to the PTM. In this condition, the fragmentation pattern will allow the exact localization of the modification (Baggerman et al. 2004).

#### PEPTIDE BIOMARKERS IN DISEASES

Peptides play a central role in many physiological processes and are tightly regulated by proteolytic control (Sewald and Jakubke 2002). Often one precursor is cleaved into many biologically active peptides and occasionally this process varies in different types of cells or tissues. Due to their multiple involvements in the biological process, peptides serve as ideal biomarkers. The term *biomarker* has been defined as a characteristic that can be measured and evaluated as an indicator of normal biologic processes, pathologic processes, or pharmacologic responses to therapeutic intervention (Schulz-Knappe et al. 2001). Identification of biomarkers that are on the causal pathway, have a high probability of reflecting health or the progression to clinical disease, and have the ability to account for all or most of the variation in a physiological state of the specified clinical outcome have largely remained elusive (Davis and Milner 2007).

Currently, there is a continuous increase in the demand for new biomarkers due to lack of specific and sensitive diagnosis. Moreover, diseases usually occur in different types and stages, which need

**Table 26.1.** Examples of peptides used as diagnostic biomarkers.

Peptide	Molecular mass (kDa)	Disease/clinical condition	References
Gastrin	2.1	Ulcer, diarrhea	Schrader and Selle (2006)
Beta-amyloid peptide	4.8	Alzheimer's disease	
C-peptide	3.1	Diabetes	
Osteocalcin	5.8	Osteoporosis	
NT-proBNP	8.6	Cardiovascular diseases	
Procalcitonin	13	Sepsis	Whicher et al. (2001)
Procarboxypeptidase B activation peptide	10	Pancreatitis	Tammen et al. (2007)
Proinsulin	10	Diabetes	
Trypsinogen-activating peptide	1	Pancreatitis	

specific diagnostic markers and therapeutic intervention (Schrader and Selle 2006). Peptides appear in all body fluids, namely, blood, plasma, urine, or cerebrospinal fluid. Since the peptides have the ability to migrate between compartments of an organism, many pathogenic processes can be reflected via change in composition of the peptides in these body fluids. Therefore, peptides can play a significant role in diagnosis and therapy. In addition, peptides help in early detection and characterization of many diseases such as Parkinson's disease, Alzheimer's disease, and cancer (Table 26.1) that are crucial for control and prevention (Dorsey et al. 2006; Sunderland et al. 2006; Wulffkuhle et al. 2003). Further, peptide biomarkers bear the potential of adding value for the diagnosis and stratification of cancer. Cancer cells exhibit specific changes in protein expression and alterations in proteolytic activities; in turn, peptides are capable of reflecting these pathological changes (Tammen et al. 2007). Increased or altered expression and secretion of proteases, such as matrix metalloproteinases and cathepsins that exert proteolytic activity in the tumor tissue, are associated with tumor cell growth, invasion, angiogenesis, and metastasis (Overall and Dean 2006; Rubin 2005). As a result of these proteases activity, peptides reflect these changes in the body fluids. Calcitonin is an example of a peptide tumor marker (molecular weight: 3.5 kDa). It is expressed primarily by the C cells (parafollicular or ultimobranchial cells) of the thyroid. Serum calcitonin is a sensitive and accurate marker of medullary thyroid carcinoma. Currently, serum calcitonin measurement in patients with thyroid disorders is evaluated for early diagnosis (Vierhapper et al. 2005).

## CONCLUSION

Until recently, the field of nutritional science has suffered from lack of advanced techniques to study and understand the mechanisms of diet–disease associations. Genomics and the postgenomic technologies allow deeper understanding of the roles of food components at the cellular and molecular levels. Proteomics plays a significant role in functional genomics analysis, but currently represents a bottleneck to progress in this area because of the inability to capture small proteins and bioactive peptides. Studies of peptidomics cover peptides with low-molecular-weight and small proteins (0.5–15 kDa), since peptides, among them families of hormones, cytokines, and growth factors, play a central role in many physiological processes. Moreover, their roles as biomarkers bear the potential of adding value to the disease diagnosis and their therapeutic interventions. Besides this, application of peptidomics knowledge to the nutrient effect may yield potential information about the diet-induced peptide changes and may act as good biomarkers. However, the field of peptidomics is relatively new and has potential to progress in future with the advent of high-throughput MS-based technologies coupled with bioinformatics and genomic databases.

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