Interactions between carbohydrate and lipid metabolism in metabolic disorders

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Interactions between carbohydrate and lipid metabolism in metabolic disorders

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Aan mijn ouders

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Een vers geopend lichaam leert
hoeveel meer goed is dan verkeerd.
Organen glanzen als in lust
en leven door elkaar gekust.
Daar vindt wie kijkt in wie verteert
een ontrustbarende rust.

1

General introduction

Adapted from: European Journal of Pediatrics (2002) 161: S65-69

General aspects of regulation of metabolic fluxes

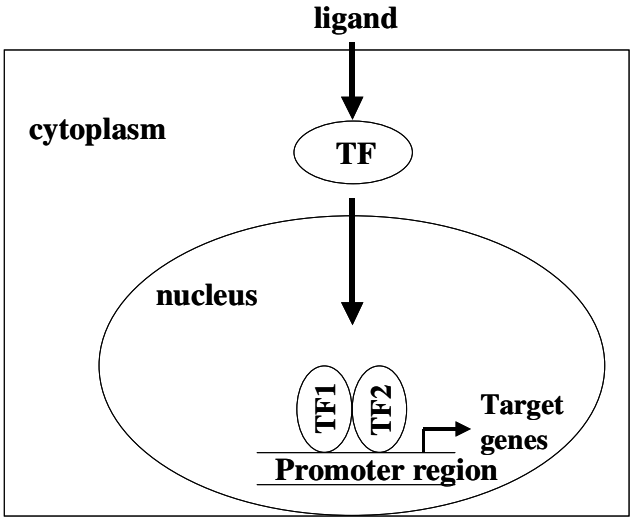
Eukaryotic cells derive energy from the oxidation of ”fuel molecules” to yield ATP. Oxidizable substrates include carbohydrates, lipids and proteins. Cells are also capable of synthesizing these three types of substrates. The processes of oxidation and synthesis are ingeniously regulated. This thesis focuses on the interactions between carbohydrate and lipid metabolism, particularly related to the pathophysiology of glycogen storage disease and type II diabetes.

Metabolic fluxes, which can be defined as the rate of flow of given molecules/substrates through defined biochemical processes that occur within a living organism, need to be regulated to maintain homeostasis at a cellular level. One can look at regulation of metabolism in many ways, but it is illustrative to group the several mechanisms that can be involved into classes. These classes can be separated according to the time needed for the regulatory change to occur. Some regulatory events can take place in a matter of seconds or less. Mechanisms operational at this time scale involve reversible bin ding of metabolites to enzymes. This binding is usually non-covalent and therefore relatively weak, but has the advantage to induce rapid changes in metabolic fluxes. Since it is not favorable from an energetic point of view to have large stores of enzymes available, which also decreases the possibility to slow down the rate of a metabolic flux, the maximum degree of stimulation of a metabolic flux via this fast route is limited.

Regulation can also take place on a time scale of a few seconds to minutes. The major mechanism for this kind of regulation is by cyclic activation and deactivation of enzymes. This activation and deactivation takes place by covalent modification of the enzymes involved. An important example of these modification processes is phosphorylation and dephosphorylation of enzymes, involving enzymes known as kinases.

In the order of hours to days, eucaryotes can changes the rate of metabolic fluxes by changing the amount of enzyme. This can be achieved either by modulation of the rate of enzyme degradation or the degree of gene expression of a particular enzyme, which in itself can be either production or degradation of mRNA. Since the first description of regulation of gene expression in the bacteria Eschereichia Coli, more than 40 years ago, scientific interest in this type of metabolic regulation has greatly expanded. Regulation of gene transcription usually involves the actions of specific transcription factors. Transcription factors are soluble proteins that are able to bind to DNA. Their binding to promoter sites of genes influences the transcription of these genes, leading to up- or down-regulation of gene expression. Some transcription factors need to be activated by ligands before they are targeted to the nucleus. Since a number of these ligand-activated transcription factors will be discussed throughout this thesis, a schematic model of this type of transcriptional regulation is given in Figure 1.

Figure 1. Example of regulation of gene transcription by transcription factors.
TF, transcription factor.



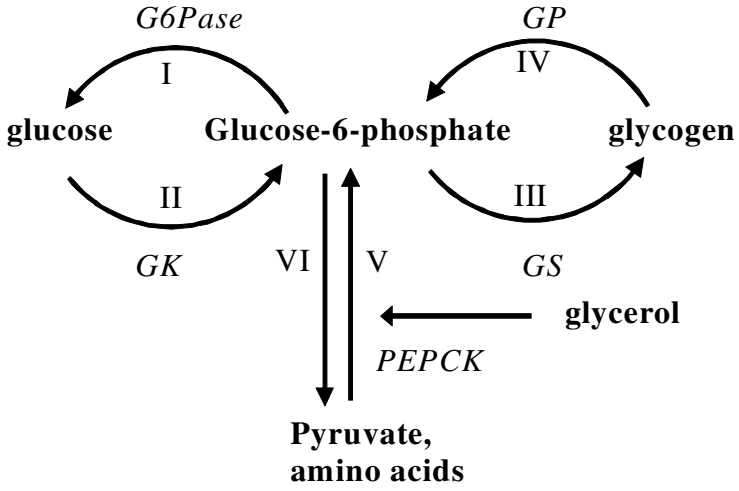
Glucose metabolism

Carbohydrates are a main source of energy and can be stored in the form of starch in plants and glycogen in animals. Carbohydrates are also part of the structural framework of both DNA and RNA and form structural elements in cell walls of bacteria and plants. An important group of carbohydrates comprises the so-called monosaccharides of which glucose is an example. Glucose is the prime fuel for the generation of energy. Monosaccharides are aldehydes or ketones with two or more hydroxyl groups, that can be described by the formula $(CH_2O)_n$. Glucose metabolism is tightly regulated in humans and animals to guarantee a sufficient glucose supply to glucose-dependent organs. The brain is the organ that is most dependent on an adequate supply of glucose, since it can only use ketone bodies as an alternative energy source and this only to a limited extent.

Carbohydrates are transported to and from various tissues through the blood compartment. Glucose can enter the blood via two routes, *i.e.*, dietary glucose derived from the intestine and glucose production by the liver and the kidney. During fasting, the organism will solely depend on the production of glucose, mainly by the liver. Glucose can be produced directly through gluconeogenesis from various substrates, such as certain amino acids, lactate and glycerol. The liver is also able to produce glucose indirectly through phosphorylation of glycogen, the storage form of glucose. This process is called glycogenolysis. Glycogen stores are, however, limited, *i.e.*, ± 100 g after an overnight fast in adult humans.[1] After a 24 h fast, about 55-65 % of the hepatic glucose production is through glycogenolysis.[2,3] Of course, this percentage is much lower after 24 h of fasting in

smaller animals with a higher metabolic rate. Glucose can also be taken up first by the blood, phosphorylated by glucokinase to form glucose-6-phosphate (G6P) and then be secreted again after dephosphorylation by glucose-6-phosphatase (G6Pase). This process is called glucose cycling and its importance in human physiology remains to be elucidated. A schematic model of the processes mentioned above is depicted in Figure 2.

Figure 2. Pathways of hepatic glucose metabolism. *GK*, glucokinase; *G6Pase*, glucose-6-phosphatase; *GP*, glycogen phosphorylase; *GS*, glycogen synthase; *PEPCK*, phosphoenolpyruvate carboxykinase; *I*, G6P hydrolysis; *II*, glucose phosphorylation, *III*, glycogen synthesis; *IV*, glycogen phosphorylation, *i.e.*, glycogenolysis; *V*, *de novo* G6P synthesis; *VI*, glycolysis; *I* + *V*, total gluconeogenesis.



The process of hepatic glucose production is tightly regulated by a variety of mechanisms. The two routes, *i.e.*, gluconeogenesis and glycogenolysis, seem to be interrelated in such a way that a decrease in gluconeogenesis is generally accompanied by an increase in glycogenolysis and *vice versa*.[4] This process of autoregulation is not under control of hormones. Hormones do play important roles in regulation of hepatic glucose production, however. Pancreatic β-cells respond very quickly to small variations in plasma glucose concentrations by secreting insulin. Insulin is mainly responsible for decreasing hepatic glucose production, by inhibiting glycogenolysis and gluconeogenesis, and increasing peripheral glucose uptake.[5] Specifically, insulin inhibits the transcription of the genes encoding phosphoenolpyruvate carboxykinase (PEPCK), G6Pase, and fructose-1,6-biphosphatase and increases transcription of the genes encoding glucokinase (GK) and pyruvate kinase (PK).[6] Glucagon is secreted by α-cells of the pancreas in response to low levels of glucose and induces hepatic glycogenolysis as well as gluconeogenesis.[7]

Epinephrine, secreted by the adrenal medulla, also stimulates glycogenolysis and gluconeogenesis in the liver.[8,9] Cortisol, a steroid hormone, influences carbohydrate metabolism by increasing glycogen synthesis[10], but conflicting data exists on its role in gluconeogenesis and hepatic glucose production.[10-13] Furthermore, fatty acids also seem to play important regulatory functions in hepatic carbohydrate metabolism and this issue will be addressed in paragraph 4 ('Physiological interaction between hepatic carbohydrate and lipid metabolism').

In recent years the transcription of genes encoding a number of enzymes involved in regulation of carbohydrate metabolism have been found to be regulated by specific transcription factors, either directly or through interaction with insulin. Since these transcription factors have regulatory functions in both carbohydrate and lipid metabolism, their mode of action and individual functions will be discussed in paragraph 4.

Physiology of lipid metabolism

Triglyceride metabolism

Apart from carbohydrates, lipids are the second major fuel for mammalian organisms. Lipids are water-insoluble biomolecules and have a variety of biological roles: as energy stores and fuel molecules, and as signal molecules and structural components of membranes. Phospholipids, triglycerides, glycolipids and sterols are major types of lipids. Phospholipids are composed of glycerol or a more complex alcohol, connected to two fatty acid chains and a phosphorylated alcohol. Triglycerides represent the storage form, mainly present in adipocytes, and transport vehiculum of fatty acids and are composed of a glycerol backbone and three fatty acid molecules. Glycolipids are sugar-containing lipids. Cholesterol is one of the most important sterols and is a structural component of membranes as well as the precursor for bile acids and steroid hormones.

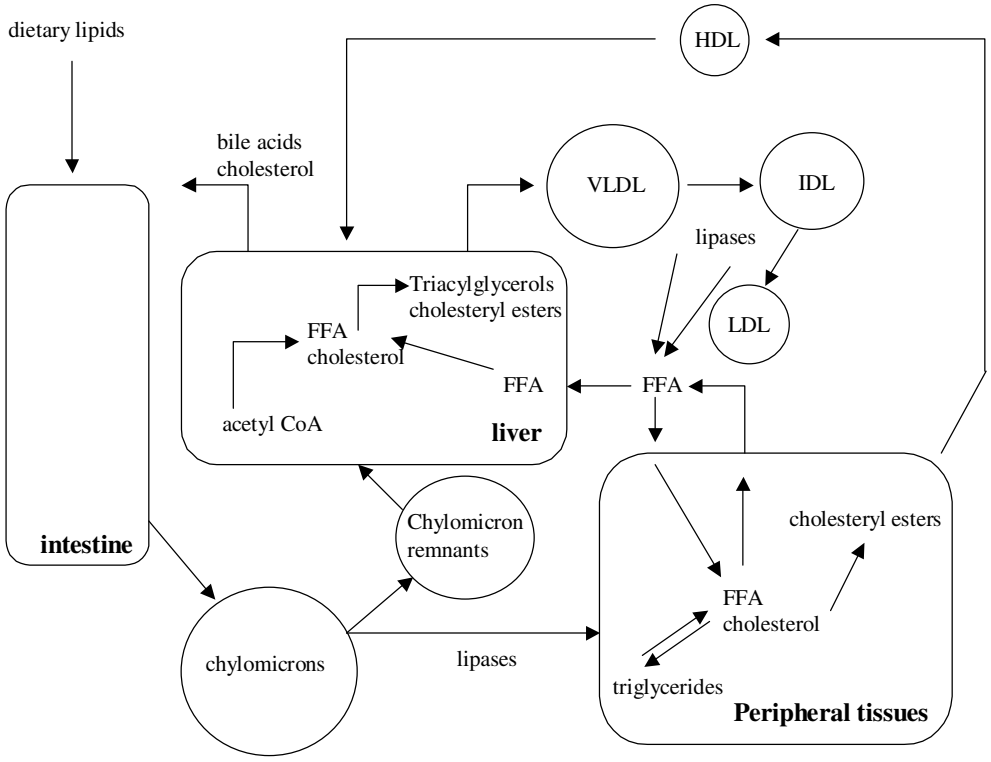
Fatty acids can both be taken up from the diet or synthesized in the body. The liver, intestine and adipose tissue have the capacity to synthesize fatty acids. The physiological importance of this metabolic route, which is also known as *de novo* lipogenesis, remains a matter of debate. Hellerstein and co-workers have provided data excluding a major quantitative role for hepatic *de novo* lipogenesis in adult life in western societies.[14] Only massive carbohydrate overfeeding has been shown to substantially induce lipogenesis in vivo.[15,16] They argued that hepatic *de novo* lipogenesis might be a rudimentary process or important only during fetal life. During the last trimester of pregnancy, the amount of adipose tissue increases to about 500g at birth.[17] Placental transfer of fatty acids or extrahepatic lipogenesis, *i.e.*, inside adipocytes, might be important in this respect. In addition, it is not known whether hepatic *de novo* lipogenesis is of quantitative importance during fetal development. Lipogenesis is tightly controlled by transcription factors, which will be discussed in paragraph 4.

Dietary intake is the main source of fatty acids in the body and their efficient uptake is essential, particularly in the neonatal period to provide energy required for rapid growth.

Triglycerides are hydrolyzed into free fatty acids and mono-acylglycerols by a process called lipolysis. Lipolysis involves multiple lipases produced by lingual and gastric mucosa and by pancreatic cells.[18] Bile is also important for efficient and high-capacity uptake of lipids, since fatty acids and cholesterol have a low solubility in aqueous solutions. Biliary bile acids have the ability to solubilize lipids thereby facilitating adequate intestinal lipid absorption. After uptake by intestinal cells, free fatty acids and monoacylglycerols are re-esterified into triglycerides. Inside the intestinal cells these lipids are assembled into chylomicrons. Chylomicrons are particles containing a hydrophobic core of triglycerides and cholesteryl esters surrounded by a monolayer of phospholipids and cholesterol in which apoproteins are embedded. Chylomicrons contain two major apoproteins important for intestinal secretion and subsequent hepatic uptake, apolipoprotein B48 and E. The lipids in these particles, *i.e.*, triglycerides and cholesterol, are taken up by hepatic and peripheral tissues, mainly muscle and adipose tissue, by the action of lipases. Excellent reviews are available that describe the process of intestinal lipid absorption in more detail.[18,19] The liver is not only able to take up triglycerides derived from chylomicrons but also from other lipoproteins, mainly very-low density lipoprotein (VLDL) remnants. Furthermore, hepatic lipid uptake occurs in the form of free fatty acids (FFA). FFA can be released from adipose tissue after lipolysis of stored triglycerides which is mediated by hormone sensitive lipase and then transported to the liver and muscle.

Lipids are also secreted by the liver in the form of lipoproteins. Apolipoprotein B (ApoB), a large protein (4536 amino acids, 520 kDa) is the most important apoprotein with respect to hepatic lipoprotein secretion. The major form of the secreted lipoproteins is as VLDL, containing a single apoB molecule per lipoprotein particle. VLDL production can be divided into two steps. First, lipid is transferred to apoB during its translation by the actions of microsomal transfer protein (MTP). MTP might be an important factor that determines the rate of VLDL production. The second step is fusion of triglyceride droplets with the apo B-containing precursor particles. Hepatic apoB content in itself is also regulated, not by inducing changes in apoB mRNA levels, but by modulating its degradation.[20] In the absence of adequate core lipids, apoB is rapidly degraded, although debate exists on the mechanisms involved.[20-22] The processes of VLDL secretion and apoB degradation were recently reviewed.[20,23] Insulin is a primary hormone involved in regulating VLDL secretion as is explained in paragraph 4. After secretion by the liver, the VLDL particles gradually lose their triglyceride component under the influence of lipases, mainly lipoprotein lipase (LPL). VLDL will subsequently become an intermediate density lipoprotein particle (IDL) and low density lipoprotein particle (LDL). The LDL particle itself can be taken up again by the liver, peripheral cells and macrophages[24], through receptor mediated uptake, *i.e.*, by the LDL receptor, LDLR-related protein (LRP) and the VLDL receptor (VLDLR). A schematic outline of these processes is shown in Figure 3.

Figure 3. Overview of lipoprotein metabolism. *VLDL*, very low-density lipoprotein; *IDL*, intermediate-density lipoprotein; *LDL*, low-density lipoprotein; *HDL*, high-density lipoprotein; *FFA*, free fatty acids.



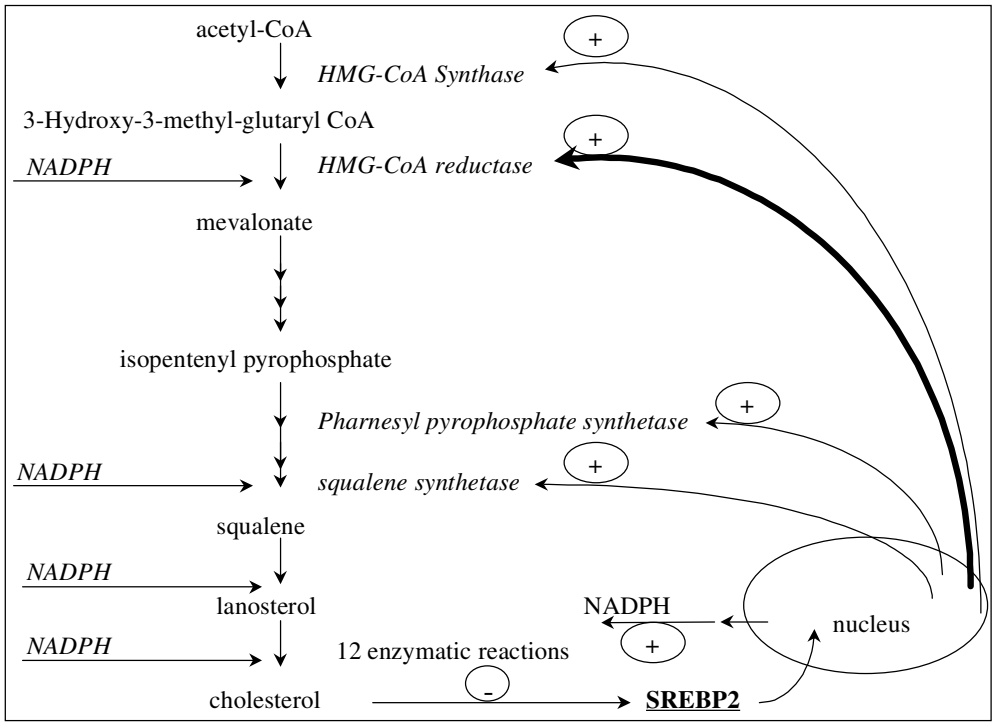
Cholesterol metabolism

Cholesterol is a sterol with special functions in various tissues and organs. First of all, it is a structural component of all cell membranes. Furthermore, it is the precursor molecule of steroid hormones, such as progesterone, testosterone and cortisol. Cholesterol can also be converted into bile acids. Cholesterol can enter the body through the diet and uptake by the intestine or it can be synthesized from acetyl-CoA. The central organ in cholesterol metabolism is the liver. Hepatic cholesterol can enter three metabolic routes apart from being stored as cholesterol ester. It can be secreted as lipoprotein particles, mainly as VLDL. Biliary cholesterol will enter the intestine, after which about 40 % is taken up again, although this efficiency declines when dietary cholesterol intake increases.[25] The remainder is excreted through the feces and thus is the route for removal of cholesterol. Finally, it can be used as precursor for bile acids. These processes are shown in Figure 3.

De novo synthesized cholesterol comprises more than 50 % of the cholesterol secreted by the liver[26,27] in the form of lipoproteins. Cholesterol is synthesized from acetyl-Co enzyme A (acetyl-CoA) by a process that mainly takes place in the endoplasmic reticulum.

A major enzyme in the cholesterol synthetic pathway is 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase. The rate of cholesterol synthesis is under tight control. Our understanding of the molecular events involved in the regulation of cholesterol biosynthesis has greatly expanded in the past few years, due to the identification of key regulatory proteins and the characterisation of their genes. Sterol regulatory element binding protein 2 is a transcription factor involved in control of cholesterol homeostasis.[28] SREBP cleavage-activating protein (SCAP) is an additional protein and is responsive to intracellular sterol depletion, leading to translocation to the Golgi network after forming a complex with SREBP. The SREBP is cleaved by site-1 and site-2 protease directing it to the nucleus, where it can activate gene transcription (see Brown *et al.*[29] for review). SREBP-2 is able to bind to specific sites in the promoter regions of genes encoding HMG-CoA reductase and other enzymes involved in the cholesterogenic pathway, as is shown in Figure 4.[29,30]

Figure 4. SREBP2 mediated regulation of cholesterol synthesis.



Plasma cholesterol can be derived from hepatic secretion, from the diet or from peripheral tissues. Peripheral efflux is the first step in the so-called reverse cholesterol transport, which is the major route for the body to get rid of excess cholesterol. High density

lipoprotein particles are able to take up cholesterol from peripheral cells, including macrophages, and transport it back to the liver.[31-33] Since it is not a major aspect of this thesis, the regulation of the reverse cholesterol pathway will not be explained in further detail. The pathway is schematically shown in Figure 3.

Physiological interaction between hepatic carbohydrate and lipid metabolism

Carbohydrate metabolism and lipid metabolism are linked in many ways. First of all, mammals are capable of turning glucose into fat. Glucose is degraded, through glycolysis, into acetyl-CoA, which is the precursor for fatty acid synthesis. On the other hand, however, fat cannot be turned into glucose by mammals, because the enzyme system for this conversion is lacking.

Evidence was generated in the sixties by Randle *et al.* that fat oxidation inhibits glucose oxidation, by interference at multiple levels.[34] Key enzyme in this inhibitory process is pyruvate dehydrogenase, which catalyzes the oxidative decarboxylation of pyruvate leading to the formation of acetyl-CoA. Randle and his group found that FFA increase concentrations of acetyl-CoA as well as of citrate, important in the citric acid cycle. Acetyl-CoA was found to decrease pyruvate dehydrogenase allosterically and citrate was found to inhibit phosphofructokinase 1, an enzyme involved in glycolysis. This whole process came to be known as the glucose-fatty acid cycle or Randle cycle. More recently, the group of Robert Wolfe provided data to indicate the opposite phenomenon.[35] Using a hyperinsulinemic-hyperglycemic clamp technique they found that elevated glucose concentrations inhibited fatty acid oxidation. This effect might be due to increased intracellular malonyl-CoA levels. Malonyl-CoA is produced from acetyl-CoA and is the first step in fatty acid synthesis, *i.e. de novo* lipogenesis. Increased glycolysis produces more pyruvate leading to increased acetyl-CoA production, which in turn will lead to more malonyl-CoA. Malonyl-CoA is known for its inhibitory effect on carnitine-palmitoyl transferase 1, an enzyme catalyzing the binding of carnitine to long-chain fatty acids, a necessary step for entry into mitochondria and subsequent oxidation.

Lipids and carbohydrates do not only influence each other in terms of oxidation but also in their synthetic processes. It has been known for some time that glucose is capable of promoting *de novo* lipogenesis (see reviews[36,37]). However, a high glucose intake probably does not promote hepatic synthesis of quantitatively important amounts of fatty acids in humans with a western dietary lifestyle.[14] Whether this is different in intra-uterine life or in prematurely born infants with a high glucose intake is not known. Very recently, it was found that the regulation of hepatic *de novo* lipogenesis is, at least partly, controlled by specific transcription factors. Multiple transcription factors are involved in regulation of *de novo* lipogenesis as summarized in Figure 4. Sterol regulatory element binding protein (SREBP) 1a and 1c induce the expression of acetyl-CoA carboxylase and fatty acid synthase, two important enzymes in the lipogenic pathway. SREBP's form a group of

transcription factors involved in control of both carbohydrate and lipid metabolism.[28] SREBP-1a and 1c are derived from a single gene through the use of alternative promoters, giving rise to alternate first exons.[29] As was explained previously, SREBP-2 is involved in regulation of cholesterol homeostasis. Recent evidence however shows that SREBP-1 and 2 can partially compensate each other, as SREBP-1 knockout mice showed elevated levels of SREBP-2 and increased cholesterol synthesis rates.[38]

Glucose is able to induce lipogenesis indirectly by inducing insulin secretion. Insulin has long been known for its lipogenic activity.[39] Recently, two groups separately found that insulin has an additional effect by enhancing SREBP-1c gene expression and the abundance of the protein in the endoplasmic reticulum.[40-42]

The carbohydrate responsive element binding protein (ChREBP)[43], which was reviewed recently[44], is also involved in transcriptional regulation of lipogenesis. ChREBP is induced in situations characterized by high glucose concentrations[43,45,46] ChREBP itself was found to activate gene expression of both pyruvate kinase and acetyl-CoA carboxylase.[43,45-47] No specific ligand for ChREBP has been found as of yet. Furthermore, the Liver X-receptor (LXR) has been found to play a role in control of lipogenesis, either directly or indirectly through induction of SREBP-1c.[48-52] LXR belongs to a subclass of nuclear hormone receptors that form an obligate heterodimer with the retinoid X receptor (RXR), a general partner for a variety of nuclear hormone receptors. LXR itself is activated by oxysterols and has been thought to act as a "cholesterol-sensing protein".

Hepatic VLDL secretion to plasma is also a process in which insulin is a primary factor. Insulin, after secretion in response to a rise in plasma glucose concentration, regulates VLDL-triglyceride secretion, either directly by influencing the rate of apoB synthesis, or indirectly via its effect on the supply of FFA to the liver.[53-55] The acute effects of insulin on regulation of VLDL secretion differ from its chronic effects. Acutely, insulin inhibits hepatic VLDL secretion[55-57], whereas chronic exposure to insulin has an stimulatory effect.[58-60]

In addition to the regulation of lipid synthesis and secretion by carbohydrates and insulin, lipids might also promote gluconeogenesis. FFA stimulate hepatic glucose production. However, fasting, a situation with increased FFA availability, is well-known to inhibit HGP mainly by a decrease in glycogenolysis with unaffected GNG.[2,61] Decreasing FFA levels by administration of antilipolytic agents such as acipimox, has produced differential results with respect to hepatic glucose production.[62-69] Some groups[65,69] found no changes in glucose production whereas others found a decrease.[63] Antilipolysis, however, unmistakably blunts the effects of FFA administration on GNG.[63-65,69] The association between FFA and gluconeogenesis might be related to the increase in acetyl-CoA, and formation of ATP and NADPH, upon lipid oxidation. This might facilitate GNG instead of lipogenesis, especially since NADPH stimulates the synthesis of glyceraldehyde-3-phosphate from 1,3-diphosphoglycerate and acetyl-CoA stimulates the formation of oxaloacetate through pyruvate carboxylase.

Another level of metabolic regulation by FFA might be related to the transcription factor PPARα. Peroxisome proliferator-activated receptor alpha (PPARα) is a nuclear

receptor that is activated by fatty acids and that promotes expression of various genes involved in fatty acid oxidation. PPARα has also been suggested to induce PEPCK gene expression.[70] PPARα knockout mice suffer from fasting induced hypoglycemia, indicating a possible role in control of hepatic glucose production.[70]

Apart from PPARα, evidence exists that other transcription factors are involved in regulation of glucose metabolism. Glucokinase expression is activated by hepatic nuclear factor 4alpha (HNF-4alpha).[71] Glucose, through activation phosphorylation/ dephosphorylation of ChREBP, influences transcription of pyruvate kinase.[47] Glucose-6-phosphatase expression is also found to be mediated by transcriptional mechanisms as well as by breakdown of mRNA[72], although the exact mechanisms remain unclear. In summary, transcriptional regulation is a form of metabolic regulation that is important for all metabolic routes of glucose. One must realize that it is likely that more transcription factors playing an important role in carbohydrate metabolism will be found in the future.

Pathophysiology of lipid and carbohydrate metabolism

Many metabolic diseases involve disturbances in carbohydrate and/or lipid metabolism. In fact, since such tight links exist between the two, it is almost impossible to have disturbances in one metabolic pathway without involvement of the other. This section will particularly focus on two diseases that clearly demonstrate the strong interactions between carbohydrate and lipid metabolism in metabolic disorders.

Interactions between lipid and carbohydrate metabolism in Glycogen Storage Disease

Glycogen Storage Disease type 1 (GSD-1) is caused by deficiency of the glucose-6-phosphatase (G6Pase) enzyme complex. G6Pase catalyzes the conversion of glucose-6-phosphate (G6P) into glucose and represents the final step in glucose production from either glycogen breakdown or gluconeogenesis. The enzyme complex is mainly active in liver but is also expressed in kidney and intestine and might be present in other tissues.[73] GSD-1 has been separated into at least two distinct types of diseases, *i.e.*, types 1a and 1b, on the basis of the underlying gene defects. The catalytic subunit of the G6Pase complex is deficient in GSD-1a[74], whereas the G6P translocase, responsible for transport of G6P from cytosol into the lumen of the endoplasmic reticulum, is deficient in GSD-1b.[75,76] Apart from the abnormalities found in carbohydrate metabolism (severe hypoglycemia, hyperlactacidemia, hepatic glycogen deposition), GSD-1 is also associated with distinct hyperlipidemia. Both plasma triglyceride and cholesterol concentrations are usually increased in GSD-1[77-79] and only partially respond to therapeutic interventions.[80-83] Furthermore, severe lipid accumulation in the liver is a characteristic hallmark of GSD-1.[78] A knockout mouse model for GSD-1a was generated by Lei *et al.*[84] GSD1a -/- were found to die postnatally from severe hypoglycemia and GSD +/- mice did not show any phenotype, limiting the possibilities to use this mouse for studying the mechanisms behind the metabolic disturbances in GSD-1.

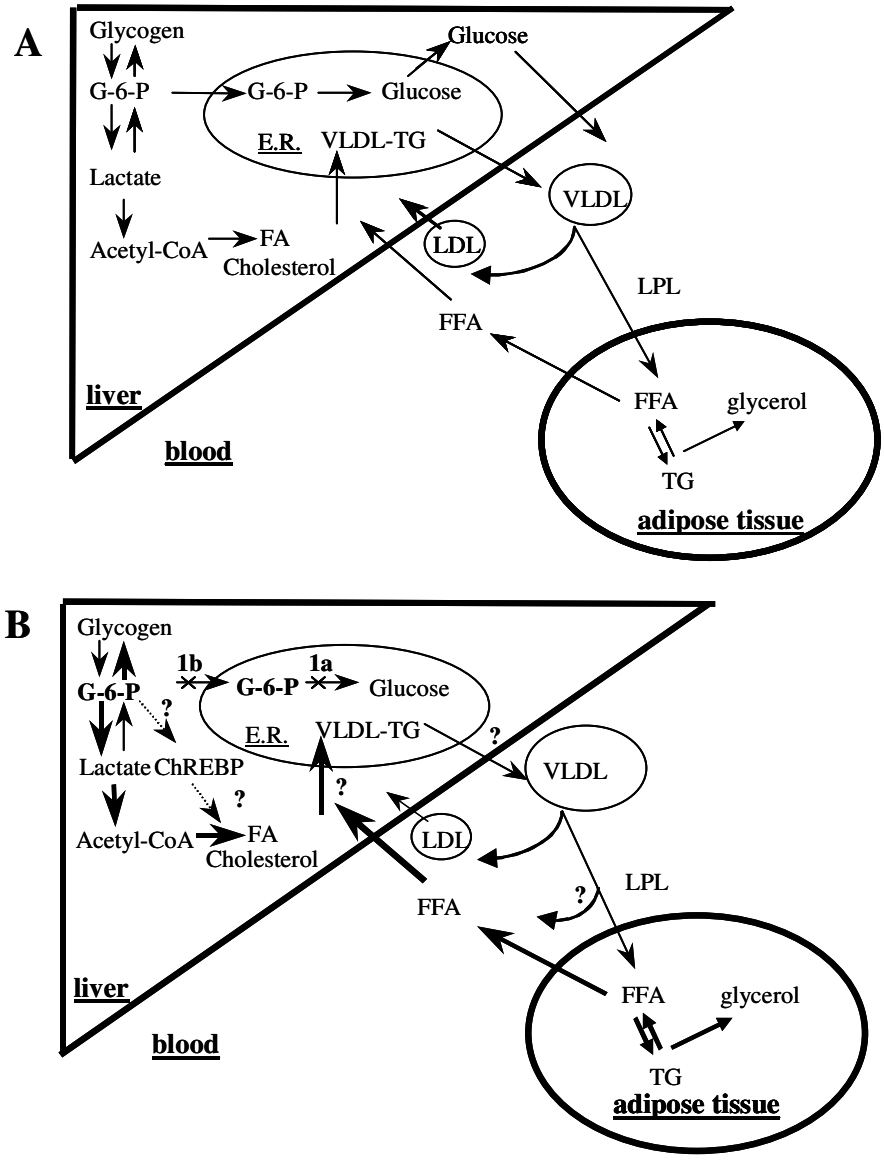
Hyperlipidemia and Glycogen Storage Disease type 1

Hyperlipidemia is present in both GSD-1a and GSD-1b[77-79] but GSD-1a is usually associated with much more severe lipid abnormalities than GSD-1b.[85] Hyperlipidemia in GSD-1 is characterized by a combined hypercholesterolemia and hypertriglyceridemia.[78] Increased concentrations of cholesterol are found in VLDL and LDL fractions whereas HDL cholesterol and apolipoprotein A-I concentrations are usually decreased.[80,81,86,87] VLDL and LDL particles are not only increased in numbers, as is evident from increased levels of apoliporotein B[80;81], but also in their sizes due to the accumulation of triglycerides in these fractions.[80] The introduction of nocturnal gastric drip feeding and resistant cornstarch for maintenance of normoglycemia at night time was found to lower plasma cholesterol and triglyceride levels[51,80,82,87-90] but generally not to normal values, as shown by Fernandes *et al.*[83] and others.[80,87-89,91] Treatment with fibrates[82,82,92] and/or fish oil [92,92,93] has also been shown to improve hyperlipidemia, although the effects of these therapies were found to diminish again over time in a number of patients.[92,93]

In GSD-1, evidence for increased synthesis and release of lipids into the blood compartment as well as decreased lipid clearance from the blood have been reported. Both processes may contribute to the development of hyperlipidemia. SREBP-1c expression is induced by insulin [41,94] and very recently it has been reported that both glucose and insulin are separately able to stimulate *de novo* lipogenesis through activation of ChREBP and SREBP-1c, respectively.[43] Whether these transcription factors play a role in the hyperlipidemia in GSD-1 is not known. An alternative option is that one or more of the glycolytic intermediates possesses metabolic regulatory functions, for example G6P, whose levels are increased in GSD-1 patients, as has been shown using phosphorus magnetic resonance spectroscopy.[95] Insulin is a well-known inhibitor of VLDL secretion[96], especially of triglyceride-rich VLDL1 particles. Lipogenesis and cholesterogenesis have also been implicated in regulation of VLDL secretion[97-99] and in GSD-1 patients, with generally low insulin concentrations. One might therefore expect increased hepatic secretion of triglyceride-rich particles. Lipolysis of circulating lipoproteins has been found to be impaired in GSD-1[80,87,100] Forget *et al.*[100] reported a two-fold decrease of lipoprotein lipase (LPL) activity in children with GSD-1 leading to a decreased triglyceride clearance from the blood compartment when compared to control children. Havel *et al.*[101] also reported a decrease in lipolytic activity which was confirmed by Levy *et al.*[80], describing a four-fold decrease in LPL activity as well as a ten-fold decrease in hepatic lipase (HL) activity in patients with GSD-1. Levy *et al.*[102] also showed a decreased uptake of LDL particles in vitro by fibroblasts from GSD-1 patients. Decreased LDL uptake might thus contribute to the hypercholesterolemia observed in these patients. However, it must be realized that measurements mentioned in the studies above were performed during fasting with low insulin and glucose concentrations. It is well known that insulin stimulates LPL activity. Increases in plasma free fatty acid levels, which are present in GSD-1 patients, indicate increased lipolysis in adipose tissue, which is a normal response during fasting and is probably more pronounced in GSD-1. However, in order for lipids to be released from adipose tissue, it must first be taken up from the blood compartment. This means that

although plasma lipolytic activity is probably decreased over a longer period of time due to a prolonged 'fasting' state in GSD-1 patients, sufficient lipolysis and uptake by the adipose tissue must be present during the absorptive period. An overview of the mechanisms involved in the development of hyperlipidemia in GSD-1 is shown in Figure 5.

Figure 5. Overview of lipid metabolism in healthy humans (A) and patients with GSD-1 (B).



Steatosis and Glycogen Storage Disease type 1

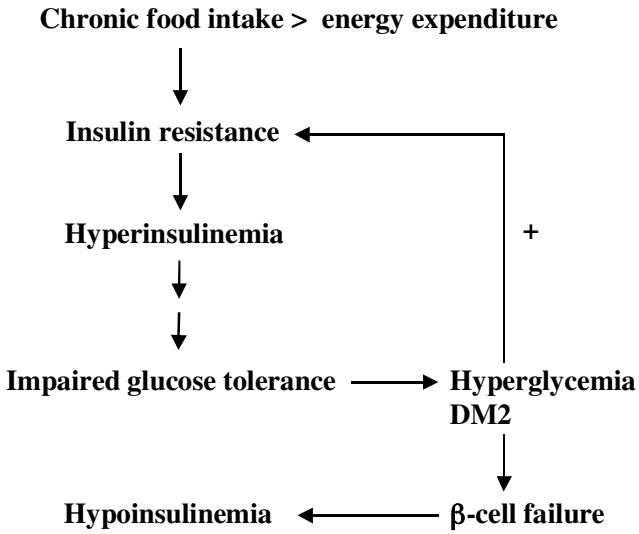
GSD-1 is associated with massive storage of neutral lipids in the liver.[103] Steatosis is an often-described phenomenon in many diseases, including diabetes, but the underlying mechanisms are often not clear and may be different in various disease states. Generally speaking, steatosis is the result of either increased hepatic uptake, increased synthesis, decreased secretion, impaired oxidation of fat, or a combination hereof.

It is assumed that, because of the elevated plasma free fatty acid levels, more fatty acids are taken up by the liver and converted to triglycerides and cholesterylester in GSD-1 patients. Decreased ketone body concentrations have been reported[104], indicating decreased fatty acid oxidation although one study did not confirm this finding.[101] The fact that lower ketone body concentrations are usually found in GSD-1 patients does not imply decreased ketogenesis by definition. In fact, it may reflect an increased ketone body flux through more rapid uptake by the brain. Furthermore, although hepatic fatty acid oxidation might be inhibited, fatty acid oxidation is probably very active in muscle. Indeed, data available so far indicate that elevated free fatty acid flux is probably the major contributor to development of hepatic steatosis in GSD-1. The possible mechanisms behind the steatosis in GSD-1 are shown in Figure 5.

Pathophysiology of carbohydrate and lipid metabolism in diabetes

Diabetes means “excessive urination”. The name diabetes mellitus was given to patients with excessive urine production in combination with a honey-flavored taste of the urine, caused by urinary glucose excretion. Diabetes mellitus today comprises a group of metabolic disorders characterized by chronic hyperglycemia. Currently, three types of diabetes mellitus are known: diabetes mellitus type 1, caused by an autoimmune-driven destruction of pancreatic β-cells; diabetes mellitus type 2 (DM2), or non-insulin dependent diabetes mellitus as it mistakenly is also known. The third group is called maturity-onset diabetes of the young (MODY), which is a group of genetic diseases caused by mutations in numerous genes such as glucokinase and insulin promoter factor 1.[10] DM2 is the most common disorder, accounting for more than 90 percent of cases, whose incidence is still growing in the western world even in children. The development of DM2 is in almost all cases caused by an overconsumption of food in relation to the energy expenditure and has become an epidemic disease in western societies. The primary event leading to full-blown DM2 is the development of insulin resistance, although discussion remains. Fat accumulation in muscle, liver and other tissues have been thought to induce insulin resistance.[105] Some researchers consider defective insulin secretion by the pancreas, instead of insulin resistance, to be primary in the development of DM2.[106] It is, however, clear that insulin resistance can precede clinically detectable DM2 by more than ten years[107], underscoring the importance of insulin resistance in the etiology of this disease. DM2 is associated with hyperglycemia and hyperlipidemia. Hyperinsulinemia occurs in the early stages of the disease when the pancreatic β-cells try to compensate for the insulin resistance by increasing insulin secretion. As the disease progresses, pancreatic β-cell failure develops

Figure 6. Mechanism of the development of diabetes mellitus type 2 (DM2).



giving rise to the full-blown DM2 phenotype. This process is illustrated in Figure 6.

Much is known about the mechanisms behind the development of hyperglycemia in DM2. First of all, basal hepatic glucose production is increased in DM2. In fact, a strong correlation exists between the rate of glucose production and degree of fasting hyperglycemia in DM2. Increased hepatic glucose production can, in theory, be caused by increased GNG and/or increased glycogenolysis. The increased production of glucose in DM2 arises from both increased glycogenolysis and gluconeogenesis, but differences in results remain with respect to the relative contribution of these two pathways.[108-116] Furthermore, evidence suggests that increased hepatic glucose production cannot be compensated by similar increases in peripheral glucose uptake.[117,118]

Increased hepatic glucose production in DM2 might be related to the increased hepatic lipid content as hepatic steatosis is also a feature in many patients with DM2.[119,120] In general, situations characterized by increased supply of FFA to the liver, *i.e.* during increased lipolysis or lipid infusions, are generally associated with increased HGP. Increased lipolysis is associated with increased GNG and lowering of FFA levels improve insulin resistance in DM2.[121] It has been shown that increased plasma FFA levels can predict the development of DM2.[122] Recent observations suggest that FFA cause mainly a decrease in the insulin-mediated suppression of glycogenolysis, leading to increased HGP in healthy human subjects.[123] In any event, it is evident that interaction between lipids and glucose is important in understanding the dysregulation of hepatic glucose production in DM2.

DM2 is also characterized by hyperlipidemia, including hypercholesterolemia and hypertriglyceridemia.[124] Increased levels of VLDL particles and small, dense LDL particles

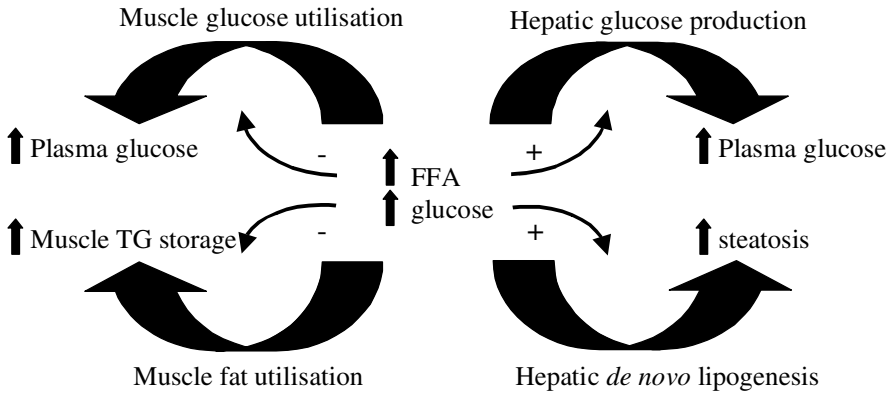
and decreased levels of HDL particles are commonly found[125], giving rise to an atherogenic lipid profile. The hyperlipidemia can in theory be caused by increased hepatic VLDL secretion into the blood, increased FFA release from adipose tissue or decreased triglyceride clearance from the blood. Of course, much research has been focused on the regulation of VLDL secretion by the liver and triglyceride clearance in healthy subjects and DM2 patients. Evidence for both processes to contribute to hyperlipidemia have been found in DM2 patients[126-132] (see[124,133] for reviews).

Increased VLDL secretion might result from the decreased sensitivity to the inhibitory effects on this process of insulin directly as studies in animal models of diabetes and diabetic humans have shown.[96,134,135] A mechanistic explanation might involve MTP, since an insulin response element was discovered on its promoter.[136] Increased VLDL secretion in DM2 might also be caused by insulin indirectly through modulation of the supply of FFA to the liver. Increased FFA flux by modulation of hormone sensitive lipase, which is observed in insulin resistant states has been suggested to enhance VLDL secretion by the liver. A number of studies have shown a diminished ability of insulin to suppress FFA rate of appearance in DM2 patients, which was reviewed by Lewis *et al.*[137] There is ample evidence that elevated FFA levels are associated with increased VLDL production in healthy humans.[138,139] However, some ex vivo studies found no effects of fatty acids on apoB secretion under basal conditions. [140,141] Interestingly, one study in Pima Indians with DM2 showed unaffected VLDL production[142], which might have been related to the absence of increased levels of FFA in these patients. Overall, consensus practically exists that increased FFA flux to the liver is an important cause of overproduction of VLDL-triglycerides by the liver in DM2.

Decreased clearance of triglycerides from the blood in DM2 patients is related to impaired lipolysis of VLDL-triglycerides. Since this process is mediated by lipoprotein lipase, which is an insulin-senstivie enzyme, insulin resistance can lead to decreased levels of lipoprotein lipase. Multiple studies have shown decreased triglyceride clearance[126,142], although this has not been conclusive.[143,144] In addition, studies have shown a reduced ability of skeletal muscle to oxidize fatty acids.[145,146] The combined processes of increased lipolysis from adipose tissue with reduced FFA uptake by skeletal muscle, might lead to re-direction of FFA from adipose tissue and skeletal muscle towards the liver. The interaction of glucose and fat occuring in insulin resistance in skeletal muscle and the liver is illustrated in Figure 7.

Multiple animal models have been used to study metabolism in diabetes mellitus, of which the leptin-deficient *ob/ob* mouse is perhaps best known. The leptin protein is produced by adipose tissue and is involved in regulation of food intake, thermogenesis and activity.[147-150] Leptin has also been implicated to directly influence hepatic glucose metabolism, by inhibiting glycogen phosphorylase and stimulating GNG and HGP.[151,152] *Ob/ob* mice develop severe obesity and insulin resistance and provide an excellent model to study the mechanisms behind the alterations in hepatic carbohydrate and lipid metabolism in DM2.

Figure 7. Glucose and free fatty acid interaction in diabetes mellitus type 2.



Stable isotopes technologies

Regulation of metabolic pathways can be studied at multiple levels. One can study the effects of an intervention on a certain metabolic route by focusing on the molecular level, *i.e.*, by determining effects on gene expression ("genomics"). One can also determine levels of intermediate metabolites of a metabolic pathway ("metabolomics") or levels of proteins ("proteomics"). One can study enzyme activity using in vitro techniques. Finally, one can study actual metabolic fluxes in the in vivo situation ("fluxomics"). Quantitative flux measurements can be performed in isolated cells, perfused organs or whole organisms. Knowledge about the effects of metabolic interventions on changes in fluxes can help to determine with a much higher degree of certainty than other procedures, whether this intervention is actually biologically relevant.

Fluxes can be determined using various techniques, but the most common is by isotopic labeling. The system under investigation is set at a metabolic steady state and one or more isotopically labeled materials are introduced into the system (*e.g.*, ^{13}C or ^{14}C for ^{12}C , ^{2}H or ^{3}H for ^{1}H). The assumption is that the labeled molecules are metabolized at the same rate as the natural compounds. Fluxes can be measured by determining the degree of labeling of the metabolites or endproducts of the pathways under investigation over time. In the past, radioactively labeled compounds were predominantly used, but in recent years, due to the development of detection technology, stably labeled compounds are being used for obvious reasons. The technology involved is mass-spectrometry, which is based on the principle that the path of an ionized molecule can be changed by electric of magnetic fields. This is dependent on the mass of the molecules injected into the mass spectrometer. The molecules under investigation are injected into the mass spectrometer, ionized, and detected based on mass differences. Since labeled molecules have a higher mass, mass spectrometry allows to

differentiate between unlabeled versus labeled molecules. For further reading, the excellent book by Wolfe on stable isotope methodologies is highly recommended.[153]

Glucose production or glucose rate of appearance (Ra) is preferentially determined with 1-^{13}C, 6-^{13}C, or U-^{13}C glucose, since there is no loss of carbon in the process of glycolysis. Hydrogen losses do occur making ^{2}H-labeled compounds less suitable. The principle is that a constant infusion of labeled glucose is given and the dilution of labeled versus unlabeled glucose is determined. The assumption is that the system and the degree of labeling is in a steady state.

At this time the Ra glucose is calculated according to:

$$Ra(glc) = MPE(glc)_{infusate}/MPE(glc)_{plasma} x \text{ infusion(glc)},$$

in wich MPE is the molar percent enrichment either in plasma or the infusate and the infusion(glc), the infusion rate of labeled glucose. With respect to glucose when using this technique, there is one confounding factor in the form of recycling of label. Consider labeled glucose that is broken down to pyruvate. The labeled pyruvate is converted into lactate which is a gluconeogenic precursor, allowing reappearance of label into newly form glucose. This recycling will then underestimate total production rates and is a limiting factor with all labels used.

Gluconeogenesis is traditionally determined using an infusion of a ^{13}C-labeled precursor (*e.g.*, alanine or lactate) and measurement of the precursor enrichment and enrichment of glucose at isotopic equilibrium. The fraction of glucose formed by gluconeogenesis instead of glycogenolysis is then calculated as:

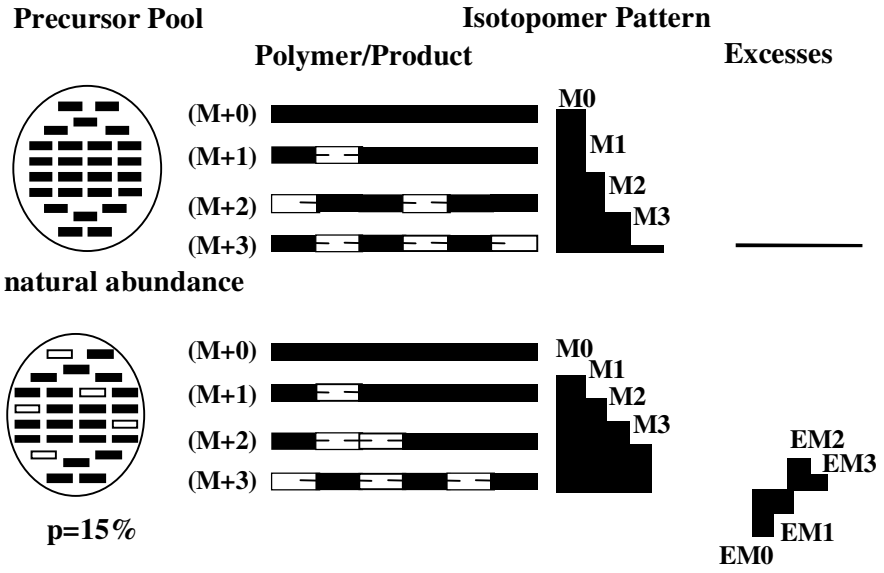
$$F \text{ glucose } Ra = E_{glucose}/E_{precursor},$$

in which E represents the isotopic enrichment either from glucose or the labeled precursor. The disadvantages of this technique are threefold. First, an isotope infusion period of at least 10 hours is needed to achieve isotopic equilibrium for glucose. Shorter infusion periods will underestimate greatly the fraction of gluconeogenesis. Secondly, determination of the enrichment of the precursor can be difficult, especially since one wants to sample the intrahepatic precursor pool but determines the enrichment in the blood compartments. Thirdly, the metabolic pathway from pyruvate goes through mitochondrial oxaloacetate which is exposed to metabolic sources of carbon dilution during an isotopic infusion.

An alternative approach was taken by development of a novel technique, called mass isotopomer distribution analysis (MIDA).[154] MIDA is a general technique for measurement of synthesis of biological polymers in vivo. It involves use of probability logic to calculate the isotopic enrichment of the real precursors from which the polymer was synthesized. The basic principles of MIDA are shown in Figure 8. Imagine a polymer that is synthesized out of 5 repetitive units, *i.e.*, the precursors. Every cell contains a certain amount of these precursors and the total of precursors in the whole organism represents the pool of this

precursor. By infusion of stable isotopically labeled precursors this pool will become enriched, to 15 % in the case of Figure 8. However, a certain percent of these precursors is already naturally enriched, for example 1 %. The polymer that is synthesized can be made from only unlabeled or completely labeled precursor or from a mixture of both. Depending on the degree of enrichment of the precursor pool, the polymers synthesized from this pool will be of a specific mixture. This mixture is reflected in an isotopomer pattern and will be unique for every degree of precursor pool labeling. By substracting the natural isotopomer pattern from the pattern after infusion of the isotopic precursor, it becomes possible to determine the fraction of newly synthesized molecules in a mixture. This method has been

Figure 8. Basic principles of MIDA (used with kind permission from Dr. Hellerstein).



used to determine gluconeogenesis[61,155-157], by infusion of [2-^{13}C]glycerol and determine the enrichment in plasma glucose. However, this technique can also be applied to measure cholesterol[27], fatty acid[158,159], protein[160] and DNA synthesis[161] as will be described below.

Glycogen fluxes have been proven difficult to determine, since it was impossible to sample the glycogen pool directly. Earliest measurements were made by taking glycogen biopsies[1], but ^{13}C NMR spectroscopy provided a new tool to determine glycogen content in humans.[2] However, using this technique it is not possible to determine simultaneous changes in glycogen synthesis or breakdown, since only changes in newly synthesized glycogen are measured. Hellerstein *et al.*[162-164] developed a technique by determining enrichments in glucuronated acetaminophen. Acetaminophen or paracetamol is

glucuronated in the liver by uridine diphosphate (UDP-glc) and its glucuronate is excreted into the urine. UDP-glc is an intermediate in glycogen metabolism. By labeling the glucose used for UDP-glc production and sampling the urine, one can determine the UDP-glc enrichment and the flux from glucose to glycogen. [1-$^{2}H_{1}$]galactose is taken up exclusively by the liver and can also be used to label the UDP-glucose pool and determine total glycogen synthesis, similar to the technique used for calculating Ra glucose. The glucuronate technique can be used in combination with the MIDA technique in order to determine the fraction of gluconeogenesis directed toward hepatic glycogen stores.

Lipogenesis and cholesterol synthesis can in theory be easily determined using labeling techniques. One should merely introduce a labeled substrate that enters the obligatory precursor pool of acetyl coenzyme A and quantify the incorporation of labeled acetate units into cholesterol or palmitate, or other lipid products produced from acetyl coenzyme A. However, the problem is the inaccessibility of the acetate precursor pool, since this is a subcellular pool which cannot be measured directly. It is not known whether subcellular compartmentalization is taking place and it is not known from which subcellular pool each lipid molecule is synthesized. An answer to this problem was provided with the introduction of the MIDA technique for determination of cholesterol synthesis and lipogenesis.[158,165] The technique is basically the same as for measuring gluconeogenesis, but instead of labeled glycerol, [1-^{13}C]acetate is infused to label the acetate pool. The advantage of the MIDA technique lies in the fact that each isotopomer pattern is unique for a degree of labeling of the acetate pool. By measuring the isotopomer pattern, the enrichment of the acetate pool can be back calculated. Subcellular compartmentalization or other confounding factors do not represent problems when using this approach.

Outline of this thesis

Glucose and lipid metabolism comprise a series of complex, tightly regulated processes that interact at various levels. Any problem arising in either one of these processes almost invariably results in serious changes in all other pathways of lipid and carbohydrate metabolism. The thesis revolves around the regulation of the synthetic pathways of carbohydrates and lipids, *i.e.*, hepatic *de novo* lipogenesis, cholesterogenesis and gluconeogenesis. In this thesis attention is focused on G6P as a potential regulating factor in both glucose and lipid homeostasis. Different human and animal models have been used to provide insight in the "lipid regulation" of glucose and glycogen synthesis and the "carbohydrate regulation" of cholesterol and fatty acid synthesis. *Chapter 2* deals with cholesterogenesis and *de novo* lipogenesis in premature infants. Infants born prematurely transcend from mainly carbohydrate-based nutrition to mainly lipid-based nutrition too early in their development. Furthermore, they usually receive parenteral nutrition for long periods of time, containing no cholesterol. Two questions will be dealt with. Is hepatic *de novo* lipogenesis an important pathway in premature infants with a high carbohydrate nutritional intake? Can premature infants produce quantitatively significant amounts of

cholesterol needed for growth and development in the absence of dietary cholesterol? In *Chapter 3*, the mechanisms behind hyperlipidemia in Glycogen Storage Disease type 1a (GSD-1a) patients, *i.e.*, deficient in hepatic production of glucose, are further addressed. More precisely, we deal with the question whether GSD1a patients have elevated rates of cholesterogenesis and *de novo* lipogenesis. Furthermore, this study provides possible explanations for the apparent protection against premature atherosclerosis in these patients. In *Chapter 4*, a rat model of GSD-1b is generated by pharmacological inhibition of G6P translocase. In this study the effects of acute G6P translocase inhibition on hepatic lipid metabolism in rats were addressed. Again, research was focused on cholesterogenesis and *de novo* lipogenesis as well as lipoprotein secretion. The metabolic changes occuring in this acute model could be compared with the metabolic changes occurring in "chronic" GSD-1 patients.

We then addressed the issue of hyperlipidemia in an animal model of insulin resistance, *i.e.*, the *ob/ob* mouse, which is leptin-deficient (*Chapter 5*). Is insulin resistance in the *ob/ob* mouse associated with increased cholesterogenesis and *de novo* lipogenesis and VLDL secretion, and what is the mechanism hereof?

Since discrepancies exist in literature on the exact mechanisms of hyperglycemia in type 2 diabetes, the same model was used to define the role of GNG and glycogenolysis in the overproduction of glucose in *ob/ob* mice (*Chapter 6*). In *Chapter 7* the relation of fatty acid oxidation and hepatic glucose metabolism was addressed. In this study PPARα-deficient mice were used that have a defect in fatty acid oxidation. In PPARα-deficient mice, the effect of impaired fatty acid oxidation on gluconeogenesis, glycogen metabolism and hepatic glucose production was studied The work presented in this thesis provides mechanistic insight in the complex interactions between glucose and lipid metabolism in physiology and in the pathophysiology of GSD-1 and DM2.

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Wie dan door bloed beneveld wordt
en jaren lang naar binnen stort
in krimpend maar steeds feller licht
vindt nog een wereld, even kort
even dichtbij maar even dicht
als het vergeten vergezicht

2

Cholesterol synthesis and *de novo* lipogenesis in premature infants determined by mass isotopomer distribution analysis

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Pediatric Research, in press

Abstract

Premature infants change from placental supply of mainly carbohydrates to an enteral supply of mainly lipids earlier in their development than term infants. The metabolic consequences hereof are not known but might have long-lasting health effects. In fact, knowledge of lipid metabolism in premature infants is very limited. We have quantified *de novo* lipogenesis and cholesterogenesis on the third day of life in seven premature infants (birth weight: 1319 ± 417 g; gestational age: 30 ± 2 wk). For comparison, five healthy adult subjects were also studied. All subjects received a 12 hours- [1-^{13}C] acetate infusion, followed by mass isotopomer distribution analysis (MIDA) on lipoprotein-palmitate and plasma *unesterified* cholesterol. The fraction of lipoprotein-palmitate synthesized at the end of the infusion period was 5.4 ± 3.9 % in infants, which was in the same range as found in adult subjects on a normal diet suggesting that hepatic *de novo* lipogenesis is not a major contributor to fat accumulation in these premature neonates. The fractional contribution of newly synthesized cholesterol to plasma unesterified cholesterol was 7.4 ± 1.3 % after a 12 h infusion. The calculated rate of endogenous cholesterol synthesis was 31 ± 7 mg/kg/day, a value ~ 3 times higher than that found in adult subjects (10 ± 6 mg/kg/day). These results indicate that the cholesterol-synthesizing machinery is well-developed in premature infants.

Introduction

The highest growth rate in a human life span occurs during the fetal period. During this rapid growth, the fetus requires significant amounts of cholesterol and fatty acids that may be acquired from maternal sources or synthesized *de novo* by the fetus itself. The relative contribution of either source is not known. Cholesterol is required for structural functions and as a precursor of various metabolically active compounds such as hormones, oxysterols and bile salts. Inborn errors in cholesterogenesis lead to severe congenital defects, such as the Smith-Lemli-Opitz syndrome (SLO), indicating that maternal supply cannot fully account for the demands during fetal development. Recently it was demonstrated that newly synthesized cholesterol activates Sonic hedgehog (Shh)[1], a signalling protein essential for normal embryonic development, which delineates the importance of this metabolic pathway.

Fatty acids are needed as structural components of membrane phospholipids, as a source of energy and, if metabolic regulation in the fetus is similar to that in the adult, as activators of specific transcription factors.[2, 3] Most observations suggest that in early gestation these fatty acids are derived from maternal sources after crossing the placenta, followed by a gradual shift to *de novo* synthesis in fetal tissues with advancing gestation.[4-7] Hepatic lipogenesis is quantitatively not important in adult human life in industrialised societies. However, it has been suggested to be of importance in the fetus.[8] No experimental data exists to indicate that hepatic *de novo* lipogenesis is a major contributor to the fetal lipid accumulation normally observed in the third trimester of pregnancy.

Because fat deposition and accretion of specific lipids occurs mainly during the last trimester of intrauterine development, the prematurely born neonate is prone to be deficient in specific metabolites (*e.g.*, brain docosahexaenoic acid and carnitine) as well as in the enzymes needed for fat digestion and metabolism. Furthermore, one of the most important differences in fat metabolism between premature and term neonates is that the very low birth weight neonate is often maintained on total parenteral nutrition for substantial periods of time after birth. Most parenteral nutrition solutions do not contain cholesterol. Breast milk does contain significant quantities of cholesterol. However, it is evident that many human infants thrive on cholesterol-poor formulas. It is not clear whether or to what extent cholesterol neosynthesis compensates for variations in dietary cholesterol intake in premature infants. There are indications to suggest relationships between the developmental stage at birth, the type and duration of infant feeding and susceptibility to develop chronic diseases in adulthood, such as atherosclerosis and insulin-dependent diabetes mellitus.[9,10] The mechanism underlying these long-lasting effects of early nutrition are not known but may be related to the amount of cholesterol ingested and/or the fatty acid quality and quantity during specific, critical periods of development.

The aim of this study was to gain insight in the contribution of hepatic *de novo* lipogenesis to overall lipid accumulation in premature infants. In addition, we set out to determine the rate of cholesterogenesis in premature infants without a substantial dietary cholesterol intake. We quantified both processes in vivo using stable isotope labeling of

plasma cholesterol and lipoprotein-palmitate with [1-^{13}C]acetate, followed by mass isotopomer distribution analysis (MIDA). These results provide the background information that is essential for studies on the metabolic consequences of composition of enteral and parenteral nutrition in this specific group of patients.

Methods

Patients and study design

Seven premature infants were studied (birth weight: 1319 ± 417 g; gestational age: 30 ± 2 wk) (Table 1), starting two days after birth. All infants required mechanical ventilation and were admitted to the neonatal intensive care unit of the Beatrix Children's Hospital in Groningen immediately after birth. The infants received parenteral feeding, which was initiated at 24 h after birth with a glucose intake of about 6 to 7 mg/kg/min during the time of the experiments. Parenteral feeding also included a lipid emulsion (Lipofundin 20 %) that was infused at a rate of 0.8 g/kg/day at the second day of life. A very limited enteral feeding schedule (7.0 ± 2.7 ml/day) was initiated on the day of the [1-^{13}C] acetate infusion. The ingested amount of cholesterol via the enteral route was less than 3 mg/day and the amount of carbohydrates via the enteral route was less than 0.5 mg/kg/min during the time of the experiment. All mothers except one (patient 5) had received antenatal steroids. The experimental protocol did not interfere with standard treatment for this group of patients. Exclusion criteria were: congenital infection, metabolic disorders, maternal endocrine disorders (diabetes and thyroid disorders) and chromosomal abnormalities. Patients received a constant intravenous infusion of [1-^{13}C] acetate (Isotec, Miamisburg, OH, U.S.A.) at a rate of 0.20 mmol/kg/h for 12 h. The start of the study (t=0) was defined by the start of [1-^{13}C] acetate infusion. Blood samples of 0.5 ml arterial blood were taken before and 8, 10, 11, 12 h after start of the infusion (incorporation phase), and subsequently at 2, 4, 6, 10, 21, 30 and 45 h after termination of the infusion (decay phase). The study protocol was approved by the Central Committee on Research Involving Human Subjects, the Netherlands, and written informed consent was obtained from the parents after the protocol had been fully explained.

For comparison 5 healthy adult control subjects were studied (mean age 32 ± 15 years, 64 ± 7 kg). All subjects were instructed to consume their regular diet until 22.00 h of the evening of the start of the study, at which time they started their fast till 09.00 h. At this time they received an oral liquid diet replacement (Nutridrink, Nutricia BV, The Netherlands) containing 39 percent of total energy as fat with no cholesterol, 13 percent as protein and 48 percent as carbohydrates, at an ingestion rate of 7 mg/kg/min of carbohydrates. At midnight, a [1-^{13}C] acetate infusion (0.20 mmol/kg/h) was started for 12 hours. Venous blood samples were taken before and 8, 10, 11, and 12 h after start of the infusion (incorporation phase), and subsequently at 1, 2, 4, 6, 18, 26, 42, 50, 66 and 74 h after termination of the infusion (decay phase). After cessation of the infusion, subjects were allowed their normal dietary intake.

Table 1. Characteristics of premature infants.

Patient	Gender	g (wk)	b (g)	p (%)	a (h)	c (mmol/l)	TG (mmol/l)
1	M	30	1240	P10-25	50	1.82	0.51
2	M	30	1435	P25-50	49	2.43	0.82
3	M	30	1315	P10-25	49	2.01	0.73
4	F	29	845	P5-10	43	0.89	1.00
5	F	34	1785	P25-50	64	1.28	1.27
6	F	30	1845	P50-90	42	1.32	0.81
7	M	29	765	<P5	53	1.16	0.65
Mean		30.4	1319		50	1.56	0.83
SD		1.5	417		7	0.54	0.25

Abbreviations used: g, gestational age; b, birth weight; p, percentile; a, age at start of study; c, cholesterol; TG, triglycerides.

Analytical procedure

Blood was collected from the patients and volunteers in EDTA tubes and was directly centrifuged to separate plasma and cells. The plasma was stored at –80 °C until further processing. Cholesterol for gas chromatography/mass spectrometry (GC/MS) analysis was extracted from 100 µl of plasma with 2 ml of 95 % ethanol/acetone (1:1, v/v) according to Neese *et al.*[11] Unesterified cholesterol was subsequently derivatized using N,O-bis-(trimethyl)trifluoroacetamide with 1 % trimethylchlorosilane at room temperature. Samples were dried under nitrogen and dissolved in 500 µl of hexane. Triacylglycerol-rich lipoproteins were isolated from plasma by ultracentrifugation as described earlier.[12] VLDL separated using this method does also include intermediate density lipoproteins (IDL) and potentially chylomicrons. Lipids were extracted from lipoprotein fractions with 2 ml methanol/hexane (4:1, v/v) and transmethylated to fatty acid-methyl esters using acetylchloride. Samples were dried under nitrogen and dissolved in 100µl hexane. Lipoprotein particle size was calculated according to Beil *et al.*[13], using the formula D = (0.211*([TG]/[PL]) + 0.38) / 0.0213, in which D is the diameter in Å. Assay kits for determination of plasma and lipoprotein triglyceride and cholesterol concentrations were obtained from Hoffmann-La Roche Ltd (Basel, Switzerland) and for determination of lipoprotein phospholipid concentrations from Waco Chemical (Marburg, Germany).

Enrichments of lipoprotein-palmitate and of cholesterol were measured by gas chromatography-mass spectrometry. Cholesterol TMS derivatives were separated with a HP 5890 Plus gas chromatograph (Hewlett-Packard, Palo Alto, CA, USA), using a 30 m x 0.25 mm (0.2 µm film thickness) DB5 ms column (J&W Scientific, Falson, CA, USA) directly inserted into the ion source of a Quadrupole mass spectrometer, model SSQ 7000 (Finnigan Matt, San Jose, CA, USA). The oven temperature was programmed from 150 to 320 °C at 30 °C/min. A splitless injection was applied. The mass fragments m/z 368, 369,

370 and 371 were monitored by selected ion recording using chemical ionization. For analysis of the methyl esters of lipoprotein-palmitate the same GC/MS mode as described above was used, equipped with a 20 m x 0.18 mm i.d. AT1701 column (0.4 μm film thickness, Alltech Associates Inc, Deerfield, USA). GC oven temperature increased from 100 ^{o}C to 280 ^{o}C at 20 ^{o}C/min. They were analyzed at mass-to-charge ratio (m/z) 271, 272 and 273 (mass isotopomers M_0, M_1 and M_2) using chemical ionization and selected ion recording, with methane as moderating gas.

Calculations

The theoretical background of the MIDA technique is described in detail elsewhere.[14, 15] MIDA is a method for calculating the fraction of newly synthesized polymers and the isotope enrichments of their monomer precursor subunits in vivo during a stable isotope labeling experiment. By applying probability logic with mass spectrometric analysis of polymers, the enrichment of the precursor pool (acetyl-CoA) and the fractional biosynthetic rates of the polymer can be calculated. This technique allows us to determine the fraction of newly synthesized cholesterol and palmitate molecules in the plasma pool. After cessation of label incorporation, the rate constant of decay of cholesterol is determined by analyzing enrichments of the higher isotopomers to exclude the chance of continuing label incorporation. It then becomes possible to calculate absolute cholesterol synthesis rates, using an estimate of body unesterified cholesterol pool size. An estimation of 130 mg/kg body weight for the total body unesterified cholesterol pool was used, as proposed by Neese and Hellerstein[11], to calculate absolute synthesis rates. The 'rapidly miscible' cholesterol pool supposedly includes liver, intestine and plasma. The following non-steady-state equation is used: $K = k \times f \times \text{pool size}/(1-e^{-kt})$, where K (mg/kg/h) is the absolute rate of cholesterol synthesis, f (in %) is the fraction of newly synthesized cholesterol molecules and k (h^{-1}) is the rate constant of isotopic cholesterol removal from plasma. Curves for the excess of isotopomer M+3 in premature infants and control subjects are shown in Figure 1. The 12 hour bloodsample was used for calculation of fractional synthesis rates of both cholesterol and lipoprotein-palmitate. Blood samples taken 1 to 72 hours after cessation of the infusion, the so-called decay phase, were used to calculate k.

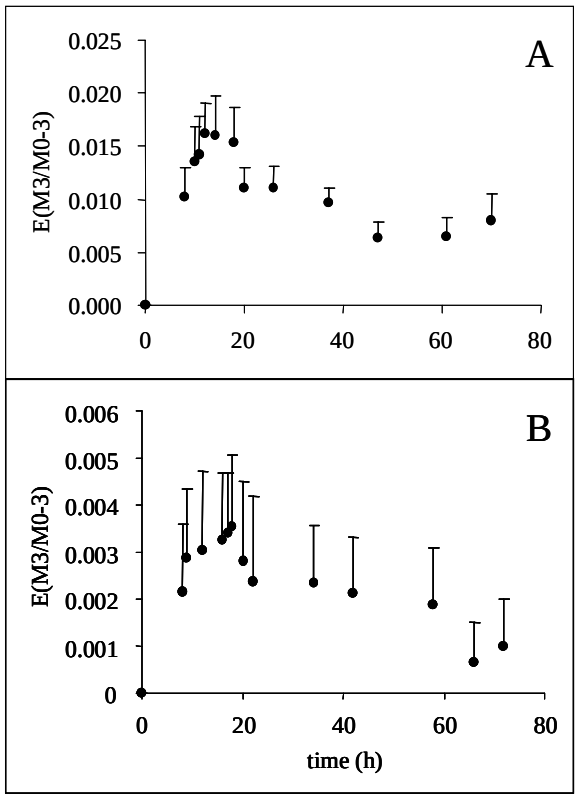
Statistical analysis

All values are expressed as means ± SD. Significance of differences was calculated using the two-tailed Student's t-test for normally distributed unpaired data or a Mann-Whitney U test for data that were not normally distributed. $P < 0.05$ was considered significant.

Results

The clinical characteristics of the infants are shown in Table 1. All the infants had an appropriate size for their gestational age (AGA), except for patient 4 and 7 that were small

Figure 1. Excess of isotopomer M+3 after start of the infusion with [1-^{13}C]acetate for premature infants (A) and control subjects (B).



for their gestational age (SGA). The mean age of the infants at start of the study was 50 ± 7 hours.

The fractions of circulating unesterified cholesterol (f) attributable to endogenous synthesis are shown in Table 2. After a 12-h infusion of ^{13}C-labeled acetate, 7.4 ± 1.3 % of plasma unesterified cholesterol was derived from acetyl-CoA in the premature infants and 3.2 ± 1.5 % in the adults. The fractional rate constant of decay (k) of endogenously labeled plasma cholesterol was 0.08 ± 0.02 h^{-1}. This was not significantly different from the rate constant calculated for adults: 0.05 ± 0.04 h^{-1}. Endogenous cholesterol synthesis was 39.9 ± 10.8 mg/day or 31.2 ± 7.2 mg/kg/day in infants. Cholesterol synthesis rates were 9.8 ± 6.2 mg/kg/day in adults. Thus, when normalized for body weight, cholesterol synthesis in premature infants was more than 3 times higher than in adults.

The enrichments of the hepatic acetyl-CoA precursor pools (p) and the fractional contribution (f) of *de novo* lipogenesis to lipoprotein-palmitate are summarized in Table 3.

Table 2. Cholesterol synthesis rates in premature infants.

Patient	p (%)	f (%)	K (h^{-1})	K (mg/day)	K (mg/kg/day)
1	16.7	8.4	0.06	45.5	36.0
2	16.7	8.8	0.07	50.9	39.0
3	15.0	6.6	0.06	36.0	27.3
4	10.7	8.6	0.12	34.7	41.0
5	13.7	7.1	0.07	50.9	28.5
6	13.3	5.6	0.07	40.8	22.1
7	12.7	6.4	0.07	20.4	26.7
Mean ± SD	14.1 ± 2.2	7.4 ± 1.3	0.08 ± 0.02	39.9 ± 10.8	31.2 ± 7.3
Mean ± SD	10.3 ± 3.8 *	3.2 ± 1.5 *	0.05 ± 0.04	593 ± 287	9.8 ± 6.2 *

Infusions of [1-^{13}C] acetate and analysis of plasma unesterified cholesterol by GC-MS were performed as described in the text. Abbreviations used: p, isotopic enrichment of precursor pool; f, fraction of cholesterol derived from endogenous synthesis; k, rate constant for exit of labeled cholesterol from plasma; K, absolute synthesis rate of plasma cholesterol. Values are expressed as mean ± SD. * Denotes significant difference ($P < 0.05$) from premature infants.

Table 3. *De novo* lipogenesis in lipoprotein-palmitate in premature infants.

Patient	Lipoprotein	
	p (%)	f (%/12 h)
1	15.6	2.5
2	10.8	2.8
3	18.3	0.9
4	11.9	11.6
5	10.0	6.6
6	11.6	9.3
7	16.7	3.9
Mean ± SD infants	13.6 ± 3.2	5.4 ± 3.9
Mean ± SD adults	9.1 ± 3.3 *	2.3 ± 2.9

Stable isotope infusions and GC-MS analysis of palmitate-methyl ester from plasma lipoproteins were performed as described in the text. Abbreviations: p, enrichment of precursor acetyl-CoA subunits that entered newly synthesized lipoprotein-palmitate molecules; f, fraction of circulating lipoprotein-palmitate derived from endogenous synthesis. * Denotes significant difference ($P < 0.05$) from premature infants.

The fraction of *de novo* lipogenesis was 5.4 ± 3.9 % in the premature infants and 2.3 ± 2.9 % in the healthy adult subjects after a 12 h infusion. There were no differences between calculated cholesterol and lipoprotein-palmitate precursor acetyl-CoA enrichments in both subject groups (compare Tables 2 and 3). However, acetyl-CoA enrichments were significantly higher in infants than in adults that received [1-^{13}C]acetate at similar infusion rates.

The isolated lipoprotein fractions contained on average 58.0 ± 13.6 % triglycerides, 27.9 ± 10.6 % phospholipids and 13.7 ± 7.1 % total cholesterol (Table 4). The two premature SGA infants (patient 4 and 7) showed higher triglyceride contents of their lipoprotein particles than the AGA infants, and consequently, lower phospholipid and cholesterol contents. The calculated lipoprotein particle size of the two SGA infants was larger than those of the AGA infants (Table 4).

Table 4. Composition and particle size of lipoproteins in premature infants.

Patient	TG	PL	TC	particle size
	%	%	%	nm
1	56.1	36.5	7.4	33.1
2	51.7	35.3	13.0	32.4
3	38.3	43.8	17.9	26.5
4	77.5	18.5	4.1	59.4
5	56.7	21.4	21.8	44.1
6	51.8	23.5	21.8	39.7
7	74.1	16.2	9.7	63.2
Mean	58.0	27.9	13.7	42.6
SD	13.6	10.6	7.1	14.0

Patient 4 and 7 are small for their gestatio nal age (SGA). Abbreviations used:
TG, triglycerides; PL, phospholipids; TC, total cholesterol.

Discussion

This study was designed to quantify the rates of cholesterogenesis and *de novo* lipogenesis in premature infants. The results of this study show that cholesterol synthesis in premature infants is relatively high, *i.e.*, 3 times higher than in adults, during the first days of life. Hepatic *de novo* lipogenesis in lipoprotein-palmitate was relatively low in premature infants and similar to values reported in adults in this study and by others.[15, 16]

The MIDA method has several advantages for the specific purposes of this study. First, precursor pool enrichments (acetyl-CoA) are calculated from the labeling pattern of the end

products, *i.e.*, cholesterol and fatty acids, which provides a highly accurate basis for calculation of actual synthesis rates. For instance, [U-^{13}C]glucose has been used to calculate fractional synthetic rates of palmitate in humans[17], which may underestimate the rate of *de novo* lipogenesis due to isotopic dilution in the precursor pool between plasma glucose and the precursor acetyl CoA pool. Another advantage of MIDA is that it allows the use of small blood samples (0.2 - 0.5 ml). Only for determination of the lipoprotein-palmitate enrichments, two samples of 1.0 ml blood were needed. For comparison, the deuterium incorporation method that also allows for quantification of fractional synthesis rate of cholesterol and palmitate requires blood samples of 8 ml.[18-21] Di Buono *et al.*[21] demonstrated that the deuterium incorporation technique and MIDA for measurement of cholesterol synthesis in humans yielded comparable results for fractional and absolute cholesterol synthesis rates. It should be realized, that MIDA calculates the amount of cholesterol in liver, erythrocytes and plasma[11] that is derived from endogenous synthesis over the course of the infusion period. The total body cholesterol synthesis is not measured, because tissue cholesterol synthesis that does not leave the cell or equilibrates with the plasma compartment will not be measured. This may lead to a certain degree of underestimation. The estimate of the unesterified cholesterol pool size, which is derived from a value calculated for adults[11], may lead to underestimation of absolute cholesterogenesis in premature infants. For practical reasons, we selected a 130 mg/kg pool size for all subjects (infants and adults), although plasma cholesterol concentrations were slightly lower in the infants. On the other hand, larger liver and blood volumes per body weight are present in infants compared to adults.[22] Liver cholesterol contributes twice as much to the rapidly miscible cholesterol pool size as plasma cholesterol does. If anything, we therefore expect the rapidly miscible cholesterol pool size in preterm infants to be larger. Choice of larger or smaller pool sizes would lead to proportionally higher or lower estimates of cholesterol synthesis, respectively.

To our knowledge, four studies examining cholesterol synthesis in human infants as a function of dietary cholesterol intake have been reported.[18-20,23] Using deuterium incorporation techniques, it has been estimated that fractional synthesis rate of cholesterol ranges from 2 to 11 %/day in infants, strongly depending on the type of feeding at 4 months of age. The differences in fractional synthesis rates have been attributed to the quantity of dietary cholesterol, which was 3-fold higher for breast-fed infants than for those fed regular formulas. As expected, the quantity of cholesterol intake was found to be negatively associated with the fractional synthesis rate. Several studies in animals and humans already indicated that the rates of sterol synthesis are much higher in the fetus than in the adult when presented on a per-gram tissue basis (reviewed[24]). The high rates of fetal sterol synthesis may be related to high expression levels or activities of transcription factors, such as sterol regulatory element binding proteins (SREBPs), although to the best of our knowledge no information has been published on their function during development .[25]
Fat accumulates during the last trimester of intra-uterine life and accounts for about 16 % of the total weight at birth.[26] Most fat is present in the form of white adipose tissue. Definite data on the contribution of *de novo* lipogenesis are not known. Studies indicate that the rate

of hepatic fatty acid synthesis is substantial in the fetus.[27-29] The fraction of *de novo* lipogenesis measured in this study in lipoprotein-palmitate in the premature infants varied between 0.9 and 11.9 %/12h. In adults on a normal Western diet the fractional contribution to VLDL-palmitate equaled less than 5 %, a value that may increase to nearly 30 % during high carbohydrate intake.[30] Our results in adult subjects are in line with these data. The fractional lipoprotein-palmitate synthesis found in premature infants in this small study was not related to birth weight, gestational age or gender. The lack of a significant difference between the two groups might be related to the high variability. We performed a post hoc power calculation that showed that a sample size of 24 should be sufficient to test whether a significant difference ($P < 0.05$, power 90 %) is different between premature infants and adult controls. Hellerstein *et al.* suggested that lipogenesis in humans is a rudimentary metabolic process, possibly only of importance in the fetus[31]; our data show that newly synthesized palmitate by the liver makes up a large part of the lipids that are present in the blood compartment.

Total parenteral nutrition (TPN) was initiated at 24 hours after birth and consisted of an amino acid mixture with glucose and a lipid emulsion. Hamilton *et al.*[32] showed that intravenous lipid emulsions in premature infants result in a marked increase in cholesterol synthesis. Furthermore, it is well known that infusing lipids downregulates *de novo* lipogenesis. However, fatty acid synthesis and cholesterol synthesis rates were measured at a mean age of 50 ± 7 hours when exogenous lipid supply was still low (0.8 g/kg/day), making a large influence of TPN on these parameters unlikely. Furthermore, TPN did not equilibrate in the VLDL fraction after ultracentrifugation (data not shown). On the other hand, carbohydrates have a well-known stimulatory effect on *de novo* lipogenesis and subjects received mostly glucose during the course of the experiment. Even in this relatively stimulatory situation, hepatic *de novo* lipogenesis was low in both study groups, underscoring the conclusion that this pathway is not of major importance in premature infants.

De novo synthesis of fatty acids depends on the substrate availability of precursors of acetyl-CoA and on the activity of the acetyl-CoA carboxylase and the fatty acid synthase complex. The enrichments of the acetyl-CoA pool found in the premature infants was higher than in the adult subjects that received the [1-^{13}C]acetate infusion at the same rate, indicating a decreased acetyl-CoA pool size or an increased flux through the acetyl-CoA pool that supplied the precursors for cholesterogenesis and lipogenesis. It is therefore unlikely that a relative deficiency in lipogenic precursors was present in the premature infants. The identity of acetyl-CoA pools for cholesterogenesis and lipogenesis, however, cannot be relied upon. Separate cytosolic acetyl-CoA could, in theory, have a similar enrichment in distinct cholesterogenic and lipogenic precursor pools. Enriching the acetyl-CoA pool might theoretically influence synthesis rates if they are precursor driven. However, in this study we found no correlation between acetyl-CoA pool enrichments and synthesis rates. This does not rule out some effect of label infusion, but does make it rather unlikely.

In this study we used adult subjects as a control group. Of course, this is not ideal, since cholesterol pool sizes and VLDL turnover rates might be different in adults and premature infants. Furthermore, dietary intake was not completely similar with a higher amount of lipids given to the adult subjects. However, since it was impossible to find parents of healthy newborn infants willing to let their baby be subjected to intravenous infusions and blood sampling, this experimental setup proved to be not feasible. Furthermore, comparing premature infants with adult subjects does provide information on whether hepatic *de novo* lipogenesis and cholesterogenesis are important in pre-term infants, since values in adults have been validated for their importance in lipid accumulation and cholesterol metabolism.

All mothers, except one, received antenatal corticosteroid therapy to promote fetal maturation of especially the lungs. This treatment has been shown to reduce mortality, respiratory distress syndrome, and intraventricular haemorrhage in premature infants. Several animal and human studies have indicated that the effect of glucocorticoids on lipogenesis may be organ-specific, in favor of lipogenesis in the lung in comparison to that in the liver. [32, 33, 34]

In summary, the present data show that cholesterogenesis is relatively high in premature infants. Furthermore, our data suggest that hepatic *de novo* lipogenesis is not a major contributor to fat accumulation in the premature infant shortly after birth. We speculate that extra-hepatic lipogenesis might be of more importance for the lipid accumulation in the fetus during the last trimester of pregnancy. It has been postulated, on the basis of animal and human studies[35, 36], that long-lasting effects of early nutrition may lead to a disturbed lipid metabolism later in life and development of chronic diseases. These effects may be caused by persistent changes in lipid metabolism that are induced by early feeding. With MIDA it has become possible to study short-term effects of dietary cholesterol and fatty acid composition in formulas and in human milk on synthesis and turnover of lipids, thus providing a clue on how early nutrition may affect these parameters.

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als het vergeten heuvelland
tenzij het golvend in de hand
binnen het gapen van de dood
ontdekt en hopeloos ontbloot
schreeuwt als vol vissen aan de kant
van een gegiste sloot.

3

Increased lipogenesis and resistance of lipoproteins to oxidative modification in two patients with Glycogen Storage Disease type 1a

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Abstract

We describe two Glycogen Storage Disease 1a (GSD-1a) patients with severe hyperlipidemia without premature atherosclerosis. Susceptibility of low-density lipoproteins (LDL) to oxidation was decreased, possibly related to the ~40 fold increase in palmitate synthesis altering lipoprotein saturated fatty acid contents. These findings are potentially relevant for anti-hyperlipidemic treatment in GSD-1a patients.

Introduction

Glycogen storage disease type 1a (von Gierke disease) is an inborn error of metabolism caused by deficiency of G6Pase, the enzyme catalyzing the conversion of G6P to glucose. The disease is characterized by hypoglycemia and hepatic glycogen and fat accumulation as well as severe hypertriglyceridemia, hypercholesterolemia and hyperuricemia.[1-5] The mechanistic relation between the primary abnormalities in glucose metabolism and hyperlipidemia are still speculative. Triglycerides and cholesterol are normally synthesized in the liver, incorporated into very-low density lipoprotein particles and secreted into the plasma. After lipolysis of the triglycerides, the fatty acids are removed from the blood and taken up by extrahepatic tissues, *i.e.*, fat and muscle predominantly. Indications for decreased plasma lipid clearance as well as for increased lipid production have been reported in GSD-1 patients.[4,6,7]

As the result of improved dietary management, patients with GSD-1a commonly reach adult age so that the potential contribution of hyperlipidemia to development of atherosclerosis becomes important. Conflicting reports have appeared on development of atherosclerosis in GSD-1 and the use of lipid lowering treatment.[8,9]

We describe two young adult brothers with GSD-1a with severe hyperlipidemia without clinical signs of atherosclerosis. Susceptibility of low-density lipoproteins to oxidative modification, one of the primary steps in atherogenesis, was decreased in the patient compared to controls, related to an increased lipoprotein saturated fatty acid content. We hypothesized this might be related to increased production rates of (mainly saturated) fatty acids. By measuring the incorpororation of ^{13}C labeled precursors into cholesterol and palmitate strongly elevated synthesis of cholesterol and fatty acids was found. These observations indicate that lipid-lowering treatment in GSD-1a patients might not be beneficial.

Methods

Subjects

Two GSD-1a patients and 6 healthy volunteers (mean age: 27 years, range: 22-39 years; mean body mass index: 22.6 kg/m^2, range: 19.5-25.5 kg/m^2) participated in this study. The patients were 25-year old, non-identical twin brothers A and B. All participants were non-smokers, had no familial history of hyperlipidemia or premature heart disease and none was taking any medication or special diet. Subjects were instructed to consume their regular diet until 22.00 h of the evening of the start of the study. Informed written consent was obtained in accordance with the University Hospital Groningen Ethical Committee.

In patient A the diagnosis was made by mutation analysis. At the age of 19 years, patient A was referred to our hospital, when physical examination showed a mildly mentally retarded boy, with stunted height (158 cm, -3,5 SD), normal weight (52 kg) and severe hepatomegaly. Numerous xantholasmata were present. Plasma cholesterol and

triglyceride concentrations were 26.2 mmol/l (1013 mg/dl) and 36.6 mmol/l (3242 mg/dl), respectively. Apolipoprotein A-I levels were 1.1 g/l (normal range: 1.35-2.35 g/l), apolipoprotein B levels were 2.1 g/l (normal range: 0.4-1.0 g/l) and the patient had an apo E phenotype E4/4. Dietary treatment was intensified and fat- (8 energy %), lactose-, and sodium restricted (protein: 13 energy %, carbohydrate: 78 energy %), with dietary triglycerides containing 27 % SFA, 15 % MUFA and 58 % PUFA. However, severe hyperlipidemia remained.

In patient B the diagnosis GSD-1a was also confirmed by mutation analysis. At the age of 23 years, patient B was referred to our hospital, when physical examination showed a mentally normal young man, with normal height (176 cm, -1.8 SD) and weight (68kg), and a mild hepatomegaly. Total cholesterol was 7.9 mmol/l (305 mg/dl), triglycerides 13.5 mmol/l (1196 mg/dl), apolipoprotein A-I 1.1 g/l and apolipoprotein B 1.1 g/l. Dietary treatment was adjusted by increasing the amount of slowly releasing carbohydrates and was fat-restricted (16,8 energy %; protein: 10 energy %, carbohydrate: 73 energy %) with a fatty acid composition of 26 % SFA, 26 % MUFA and 48 % PUFA.

Measurement of lipogenesis, cholesterogenesis and lipoprotein oxidation

Two healthy volunteers were studied without treatment and a second time after taking 8 g/day of cholestyramine for two weeks to also compare cholesterogenesis in controls to the patients after strong induction. They fasted from 22.00 h the day before the experiment till 10.00 h when they received an oral liquid diet replacement (Nutridrink, Nutricia BV, The Netherlands) at a rate of about 7 mg/kg/min of carbohydrates. This rate was similar to the amount of carbohydrates the two patients received through a nasogastric tube from 22.00 h until the end of the experiment to maintain normoglycemia (glucose levels 3-6 mmol/l). At midnight an infusion of [1-^{13}C]acetate (Isotec, Miamisburg, OH, U.S.A) was started in volunteers and patients through a nasogastric tube at a rate of 0.12 mmol/kg/h for 16 hours. Blood samples were taken before, throughout and after the infusion. After 16 h the infusion was stopped and subjects were allowed to return to their regular diet.

Cholesterol was extracted from total plasma and derivatized according to Neese *et al.*[10] VLDL from plasma samples was isolated[11] and palmitate from VLDL fractions was methylated as described elsewhere.[11] Lipids were analyzed by gas chromatography/mass spectrometry.[11,12] *De novo* synthesis of cholesterol and palmitate in plasma and VLDL, respectively, were measured by MIDA, as described in detail previously.[9-11] To obtain a semi-quantitative value for palmitate synthesis, we multiplied fractional synthesis *de novo* by the total amount of palmitate in VLDL at the end of the experiment. This reveals the total amount of newly synthesized palmitate present in VLDL after 16 hours of ^{13}C-acetate infusion.

The oxidation of LDL and VLDL was measured according to the Esterbauer method with some modifications.[13] Tocopherols (α and γ) and β-carotene were determined by high-performance liquid chromatography [14] and ubiquinol levels were analyzed as described.[15]

Results

At the time of the experiment, plasma triglyceride concentrations in patient A (18.2 mmol/l, 1612 mg/dl) and patient B (11.9 mmol/l, 1054 mg/dl) were more than ten times higher than in the control subjects (0.8 ± 0.4 mmol/l, 71 mg/dl). Likewise, plasma cholesterol concentrations in the patients were 15.0 mmol/l (580 mg/dl) and 10.8 mmol/l (418 mg/dl), respectively, which was markedly higher than in controls, *i.e.*, 4.2 ± 0.4 mmol/l (162 ± 15 mg/dl). Increased lipid concentrations were almost solely due to increases in the VLDL fraction as determined by fast performance liquid chromatography (data not shown). Uric acid concentrations were normal with 0.26 mmol/l and 0.35 mmol/l in patient A and B, respectively. Mean glucose concentrations during the experiment

Table 1. Oxidation characteristics of VLDL and LDL particles, fasting plasma antioxidant concentrations and VLDL and LDL fatty acid composition.

		Patient A	Patient B	Controls *
Plasma	α-tocopherol (µmol/l)	57.5	81.7	21.7 ± 4.9
	Relative (µmol/mmol)	3.1	2.7	4.6 ± 0.7
	β-carotene (µmol/l)	0.17	0.76	0.74 ± 0.39
	Relative (µmol/mmol)	0.01	0.03	0.16 ± 0.10
	ubiquinol (µmol/l)	1.21	1.44	0.92 ± 0.37
	Relative (µmol/mmol)	0.06	0.05	0.20 ± 0.07
VLDL	Lag time (min)	359	372	135 ± 12
	Propagation speed (nmol/mg/min)	4.5	3.5	13.1 ± 2.3
	SFA (%)	51.8	50.0	45.6 ± 4.6
	MUFA (%)	28.5	36.8	26.1 ± 2.2
	PUFA (%)	19.7	13.2	28.3 ± 3.2
LDL	Lag time (min)	97	107	84 ± 6
	Propagation speed (nmol/mg/min)	7.0	6.7	10.9 ± 0.9
	SFA (%)	43.7	40.8	30.8 ± 1.0
	MUFA (%)	28.4	25.4	20.1 ± 3.3
	PUFA (%)	27.9	33.8	49.1 ± 4.2

Lag time denotes the time after administration of copper until oxidation starts and propagation speed the actual rate of oxidation. *SFA* denotes saturated fatty acid, *MUFA* monounsaturated fatty acid, and *PUFA* polyunsaturated fatty acid. * Values were obtained from five control subjects and are displayed as means ± SD. 'Relative' values for α-tocopherol, β-carotene, and ubiquinol concentrations refer to antioxidant concentrations divided by cholesterol plus triglyceride concentrations.

were 4,45 mmol/l in patient A and 4,50 mmol/l in patient B and mean lactate levels were 3,2 and 3,4 mmol/liter (normal upper value: 2,2 mmol/l), respectively .

Susceptibility of both LDL and VLDL to oxidative modification was markedly lower in the GSD patients (Table 1), as indicated by an increased lag time and a decreased propagation rate. Plasma concentrations of α-tocopherol, β-carotene and ubiquinol showed no major differences when expressed relative to total lipid content: if anything, the relative content of β-carotene and ubiquinol appeared to be decreased (Table 1).

Fatty acid composition of LDL particles and, to a lesser extent, of VLDL particles showed increased SFA contents in the patients. The high relative amount of SFA was markedly different from the composition of their dietary fat intake, which consisted mainly of PUFA. In contrast, the lipoprotein fatty acid composition of the healthy volunteers matched the estimated fatty acid composition of their diet, which, based on a recent regional survey, contained 41 %, 21 % and 38 % SFA, MUFA and PUFA, respectively. Significant correlations were observed between propagation speed in LDL and SFA, MUFA and PUFA content (Figure 1). Significant, but less pronounced, correlations were also found for VLDL lag time and propagation speed and lipoprotein fatty acid composition.

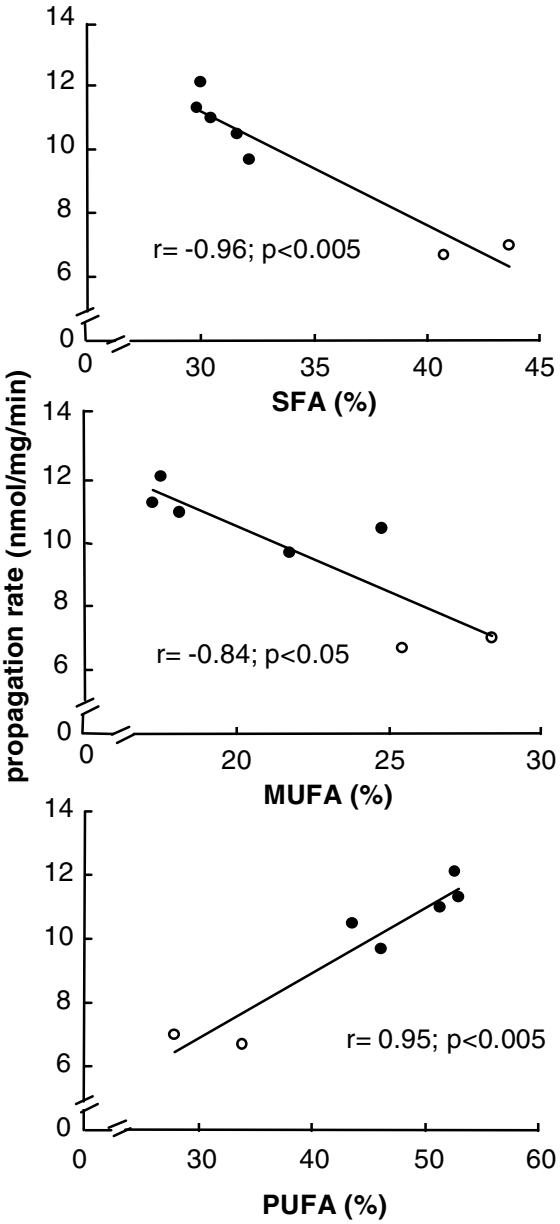
A more than 40-fold increase in the amount of newly synthesized VLDL-palmitate in the two GSD patients compared to controls was calculated (Table 2). Calculation of absolute cholesterol synthesis rates revealed a 7-fold increase in the two patients which was even higher than in the volunteers after cholestyramine treatment to induce this process. Calculation of precursor pool enrichments, which were at steady state after 6 hours of [1-^{13}C]acetate infusion, revealed a much lower acetyl-CoA pool enrichment in the patients compared to the control subjects.

Table 2. Cholesterol and palmitate synthesis in two GSD-1a patients and control subjects, the latter before and after cholestyramine treatment.

Subject	Absolute cholesterol production (mg/day)	Newly synthesized VLDL palmitate (mmol)	Precursor pool enrichment † (%)
Patient A	4419	119.7	8
Patient B	2212	209.0	9
Subject A	505	7.5	16
Subject B	426	0.3	15
Subject A*	1256	25.4	16
Subject B*	1477	2.6	15

* Values after 2-week treatment with cholestyramine. † Calculated from isotopomer distribution of plasma cholesterol at 12 and 16 hours after start of the ^{13}C-acetate infusion.

Figure 1. Correlation between SFA, MUFA and PUFA content of LDL particles and the oxidation propagation speed, in healthy volunteers (closed symbols) (n = 5) and in patients (open symbols).



Discussion

A crucial and at present unanswered question is whether the severe hyperlipidemia in GSD-1a patients will ultimately lead to an increased risk for atherosclerosis, especially since patients have a better life expectancy due to improved dietary treatment. A few GSD-1 patients who were in their thirties have been described with atherosclerosis.[8] However, Lee *et al.*,[9] using ultrasound techniques to investigate endothelial function, found no indication for premature atherosclerosis in GSD-1 patients. We chose to study two adult GSD-1a patients with severe hyperlipidemia and atherogenic lipid profile who were expected to show signs of premature atherosclerosis. Surprisingly, determination of ankle-brachial indices, aortic distensibility and intima-media thickness of the carotid and femoral arteries showed no signs of atherosclerotic lesions (data not shown). Ex vivo Cu-induced oxidation of lipoprotein particles, an important indicator of their atherogenicity[16], revealed a much lower oxidation susceptibility of patient LDL compared to controls. Antioxidants and fatty acid composition are known to influence the oxidizability of lipoprotein particles.[16] Our data do not support a role for antioxidants in decreasing lipoprotein oxidizability in GSD-1a, since Tocopherols, β-carotene and uric acid concentrations were not increased, although hyperuricemia is a common phenomenon in GSD-1a patients. It is well known that PUFA display a higher susceptibility to oxidation than MUFA and SFA.[16] Although we have only obtained data from two patients, our data suggests that the relatively high lipoprotein SFA content in GSD-1a patients plays a role in protection of plasma lipoproteins against oxidative modification. An important question is why patients have a relatively high SFA lipoprotein content compared to healthy control subjects, since patients have a high relative PUFA intake. Application of stable isotopes revealed that synthesis of saturated fatty acids, *i.e.* palmitate, as well as cholesterogenesis were severely increased compared to healthy controls. Control values found in this study were similar to values previously reported.[17] With respect to the values found for *de novo* lipogenesis a number of factors must be taken into account. Similar amounts of carbohydrates were given to patients and controls, which led, however, to higher insulin concentrations in control subjects compared to the patients (data not shown). This might be partly attributable to hepatic glucose uptake, which is transformed to G6P and then unable to be released again as glucose. Lockwood *et al.* have shown decreased insulin secretion to a carbohydrate load in adult GSD-1 patients.[18] Insulin and glucose are both separate stimulators of *de novo* lipogenesis.[19] Differences in lipogenesis between control subjects and patients are therefore probably underestimated. A second factor potentially influencing the calculated synthesis values is the possible decreased clearance of VLDL triglycerides.[4] The values for lipogenesis are a combination of formation and clearance. Decreased clearance is expected to lower the fraction of newly synthesized palmitate found at the end of the experiment, since it increases palmitate pool size leading to a higher dilution with unenriched palmitate molecules. Finally, the increases in lipid synthesis found in the patients studied here might be more pronounced than in GSD patients in better metabolic control and with less severe hyperlipidemia.

Decreased acetyl-CoA pool enrichments observed in the GSD-1a patients indicates that labeled acetyl-CoA is diluted to a larger extent with endogenous acetyl-CoA, reflecting a higher glycolytic flux towards the acetyl-CoA pool. This increased flux may contribute to higher fatty acid synthesis in GSD-1a patients by stimulating acetyl CoA carboxylase. Furthermore, data suggests that G6P itself might act as a mediator of carbohydrate-induced lipogenic activity.[20]

In conclusion we hypothesize that absence of G6Pase activity together with a low fat diet increases lipogenesis, somewhat paradoxically in view of the well-known association of dietary SFA intake with atherosclerosis incidence, decreases the degree of oxidative modification of LDL by altering lipoprotein fatty acid profile. The use of fish oil might not be helpful to prevent premature atherosclerosis in GSD-1a patients, since normolipidemia is usually not achieved and fish oil could lead to increased lipoprotein oxidizability by increasing the lipoprotein PUFA content.

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Ik daalde tussen de cellen neer
en sinds ik in het plasma drijf
zie ik die bron dat hele lijf
niet helder meer niet meer

4

Acute inhibition of glucose-6-phosphate translocator activity leads to increased *de novo* lipogenesis and development of hepatic steatosis without affecting VLDL production in rats

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Abstract

Glucose-6-phosphatase (G6Pase) is a key enzyme in hepatic glucose metabolism. Altered G6Pase activity in Glycogen Storage Disease and diabetic states is associated with disturbances in lipid metabolism. We studied the effects of acute inhibition of G6Pase activity on hepatic lipid metabolism in unanaesthesized rats. Rats were infused with an inhibitor of the glucose-6-phosphate (G6P) translocator (S4048, 30 mg/kg/h) for 8 hours. Simultaneously, [1-^{13}C]acetate was administered for determination of *de novo* lipogenesis and fractional cholesterol synthesis rates by mass isotopomer distribution analysis. In a separate group of rats, Triton WR 1339 was injected for determination of hepatic VLDL-triglyceride production. S4048 infusion significantly decreased plasma glucose (-11 %) and insulin (-48 %) levels and increased hepatic G6P (+201 %) and glycogen (+182 %) contents. Hepatic triglyceride contents increased from 5.8 ± 1.4 µmol/g liver in controls to 20.6 ± 5.5 µmol/g liver in S4048-treated animals. *De novo* lipogenesis was increased more than 10-fold in S4048-treated rats, without changes in cholesterol synthesis rates. Hepatic mRNA levels of acetyl CoA carboxylase and fatty acid synthase were markedly induced. Plasma triglyceride levels increased four-fold but no differences in plasma cholesterol levels were seen. Surprisingly, hepatic VLDL-triglyceride secretion was not increased in S4048-treated rats. These studies demonstrate that inhibition of the G6Pase system leads to acute stimulation of fat synthesis and development of hepatic steatosis without affecting hepatic cholesterol synthesis and VLDL secretion. Results emphasize the strong interactions that exist between hepatic carbohydrate and fat metabolism.

Introduction

Phosphorylation and dephosporylation of glucose by glucokinase and glucose-6-phosphatase (G6Pase), respectively, are key steps in hepatic glucose uptake and release. The balance between the activities of these enzymes represents an important site for control of hepatic glucose production.[1,2] G6Pase is located in the endoplasmic reticulum (ER) of liver, kidney and, as recently shown, intestinal cells.[3] The glucose-6-phosphate (G6P) metabolizing machinery consists of a putative translocator[4,5], responsible for transporting G6P from the cytosol into the ER lumen, and a catalytic subunit that converts G6P to glucose and inorganic phosphate.[6] The catalytic subunit is localized to the inner ER membrane. Interestingly, there are several indications to suggest that this site of regulation of glucose metabolism is linked to that of hepatic lipid metabolism. G6Pase activity is increased in patients and animal models of Diabetes Mellitus (DM) [2,7,8], probably contributing to increased hepatic glucose production in these conditions. Diabetes in general is associated with hyperlipidemia, which has been found to be mainly due to overproduction of very low density lipoprotein-triglycerides (VLDL-triglycerides) in DM type 2 (DM2).[9-11] Deficiency of G6Pase activity, the metabolic basis of Glycogen Storage Disease type I (GSD-1), also leads to abnormalities in lipid metabolism, characterized by severe hypertriglyceridemia and hypercholesterolemia.[12-15] GSD is caused by mutations in the genes encoding either the putative translocator (type non-1a)[4,5] or the catalytic subunit (type 1a) [6,16,17] of the G6Pase system. Overexpression of hepatic glucokinase also leads to hyperlipidemia in fed rats.[18] Brown *et al.* showed that the phosphorylation process is important for regulation of assembly and secretion of triglyceride-containing very low density lipoproteins by hepatocytes.[19] Little is known about the mechanisms underlying the apparent paradox that hyperlipidemia develops in conditions associated with high as well as low G6Pase activity, *i.e.*, diabetes and GSD.

A class of chlorogenic acid derivatives has recently been developed, eventually aimed at treatment of hyperglycemic conditions in DM2, that potently and specifically inhibit G6Pase activity by blocking the translocase of the G6Pase complex.[20-22] Infusion of members of this class of compounds in anaesthetized rats resulted in reduction of blood glucose levels and increased concentrations of intrahepatic G6P and glycogen.[21,22] Recently, it has been reported that acute inhibition G6Pase activity also increases hepatic triglyceride concentrations.[23] Due to their mode of action, chlorogenic acid derivatives induce a situation resembling GSD-1 and provide excellent tools to unravel the interactions between carbohydrate and lipid metabolism.

In the present study, we acutely inhibited G6Pase activity by infusion of the chlorogenic acid derivative S4048 in vivo in conscious, unrestrained non-diabetic rats. We questioned whether acute increases in hepatic G6P concentrations would lead to increased hepatic *de novo* lipogenesis, cholesterogenesis and VLDL-triglyceride secretion.

Methods

Animals

Male Wistar rats (Harlan Laboratories, Zeist, The Netherlands) weighing between 290-350 g (mean ± SD: 318 ± 25 g) were used to study the in vivo effects of S4048. To allow infusion and blood collection in freely moving, unrestrained animals, rats were equipped with two permanent heart catheters via the right jugular vein as described by Kuipers *et al.*[24] After surgery, animals recovered for a period of 7 days in individual cages in a temperature- controlled room (20 ºC) with food and water available ad libitum. At 8h before the start of the experiment, cages were cleaned and food was removed.

Experimental procedures

Animals received an i.v. infusion of the G6P translocator inhibitor (S4048, 30 mg/kg/h, infusion rate 3 ml/h) or of the solvent, phosphate-buffered saline (PBS) with dimethylsulfoxyde (DMSO), for 8 hours. Rats were allowed to move freely throughout the experiment and the animals did not show signs of stress. The S4048 compound was synthesized by the chemical department of Aventis Pharma (Frankfurt, Germany).[21] At the infusion rate employed, S4048 is expected to partially inhibit G6P translocase activity.[21] Simultaneously, all animals received an infusion of [1-^{13}C]acetate (0.8 mmol/kg/hr). The infusates were freshly prepared before each experiment. S4048 was dissolved in 20 % DMSO in PBS (vol/vol) and PBS was added to reach a final concentration of 6.1 % DMSO (vol/vol). The solution was then immediately adjusted to pH 7.4. Before the infusion, a basal blood sample (~300 µl) was taken to determine baseline values of the metabolites studied. During the infusion blood samples (~200 µl) were taken after 30, 60, 120, 240, 360, 420, and 480 minutes. Samples were heparinized and immediately placed on ice and centrifuged at 5000 rpm for 10 minutes at 4 ºC. Plasma was stored at -20 ºC until analysis. Despite the relatively high infusion rate, hematocrit levels did not fall significantly during the experiment. At the end of the infusion period, animals were anaesthetized with sodium-pentobarbital and a large bloodsample was taken by heart puncture. The abdomen was opened and the liver was rapidly exposed, excised and stored in parts at -80 ºC for measurement of G6P and glycogen content, lipid analysis and RNA isolation or rapidly frozen in liquid isopentane for histological analysis (see below).

In vivo VLDL-triglyceride production

The effects of S4048 on hepatic VLDL production were studied in a separate experiment. After the surgical procedures and recovery as described above, rats were infused with S4048 or its solvent and, after 3 hours of infusion, received an intravenous Triton WR 1339 injection (Tyloxapol, Sigma Chemical Co., St. Louis, MO) as a (12 % $^{wt}/_{wt}$) solution dissolved in saline in a dose of 5 ml/kg body weight. Triton WR 1339 blocks lipolysis of lipoproteins and their accumulation in plasma over time allows calculation of hepatic VLDL-triglyceride production rates.[25] To exclude any interference of the solvent containing DMSO on VLDL-triglyceride secretion, a separate group of rats received a

saline infusion. After Triton WR 1339 injection, blood samples were taken after 30, 60, 120 and 180 minutes for measurement of triglyceride concentrations. VLDL production rates were calculated from the slope of the linear triglyceride accumulation curves in time. At t = 180 min, a large blood sample was taken for isolation of VLDL/LDL particles and animals were sacrificed. Lipoproteins were isolated according to Pietzsch *et al.*[26] using a solution of 15.3 % NaCl and 35.4 % KBr in saline with a density < 1.019 g/ml. Plasma (0.5 ml) was overlayed with 0.6 ml of the NaCl-KBr solution, centrifuged for 100 minutes at 120.000 rpm and 4 ºC in a Beckman Optima TLX Ultracentrifuge (Beckman Instruments, Palo Alto, CA) and the VLDL-fraction was collected by tube slicing and frozen until analysis. VLDL particle size was calculated as described by Beil *et al.*[27]

Analytical procedures

Hepatic lipids were extracted using the Bligh and Dyer method.[28] Assay kits for determination of plasma and hepatic triglyceride and cholesterol concentrations were obtained from Hoffmann-La Roche Ltd (Basel, Switzerland) and for determination of plasma and hepatic phospholipid and plasma free fatty acid concentrations from Waco Chemical (Marburg, Germany). Plasma β-hydroxybutyrate was measured using a commercially available kit from Sigma Diagnostics (St Louis, MO). Total protein content of tissue homogenates was determined according to Lowry *et al.*[29] Plasma insulin was determined by a radio immunoassay (RIA) RI-13K (Linco Research, Inc., St. Charles, MO). Plasma glucose concentration was determined enzymatically by use of the Beckmann glucose analyzer II (Beckmann Instruments, Palo Alto, CA). Hepatic glycogen was determined after extraction with a 1 mol/l KOH solution by sonication. The extract was incubated for 30 minutes at 90 ºC, cooled and brought to pH 4.5 by addition of 3 mol/l acetic acid. Precipitated protein was removed by rapid centrifugation (10.000 rpm, 1min). Glycogen was converted to glucose by treating the samples with amyloglucosidase, followed by assay of glucose at pH 7.4 with ATP, NADP+, hexokinase and G6P dehydrogenase.[30] Liver samples for the determination of G6P were treated by sonification in a 5 % (w/v) $HClO_4$ solution. Precipitated protein was removed by rapid centrifugation at 10.000 rpm for 1 min in a cold microcentrifuge and the supernatant was neutralized to pH 7 by addition of small amounts of a mixture of 2 mol/l KOH and 0.3 mol/l MOPS. G6P was determined fluorimetrically with NADP+ and G6P dehydrogenase.[31] Activites of liver enzymes, *i.e.*, alanine aminotransferase (ALAT) and aspartate aminotransferase (ASAT) were determined by routine chemical chemistry procedures.

Liver histology

To visualize fat deposition in the liver staining with Oil-red-O was performed on 4 μm frozen liver slices and counterstaining with hematoxylin, according to standard procedures.

GC-MS analysis

For gas chromatography/mass spectrometry (GC/MS) analysis, plasma cholesterol was extracted and derivatized as described elsewhere.[32] Palmitate from isolated VLDL fractions was trans-methylated according to Lepage *et al.*[33]

Cholesterol and fatty acid derivatives were analyzed on a magnetic sector mass spectrometer (70-250S; VG, Manchester, U.K.) using a Chrompack CP-Sil 19 column (Middelburg, the Netherlands) for assessment of isotopomer distribution patterns. For cholesterol samples, the oven temperature increased from 120 to 260 ^{o}C at a rate of 20 ^{o}C/min, from 260 to 280 ^{o}C at 2.5 ^{o}C/min and finally from 280 to 300 ^{o}C at 20 ^{o}C/min. The ions at m/z 368 to 371 were measured under selected ion recording. For fatty acid samples the oven temperature increased from 100 to 300 ^{o}C at a rate of 12.5 ^{o}C/min. The ions of the palmitate derivative were measured at m/z 270 to 272 under selected ion recording.

Calculations

The principle of the MIDA technique is described in detail elsewhere.[32,35] Briefly, MIDA allows to determine the enrichment of the pool of acetyl-CoA precursor units that has entered newly synthesized cholesterol or palmitate molecules during the course of a [1-^{13}C]acetate infusion, by analyzing the isotopomer pattern of the molecules of interest. This isotopomer pattern is compared with a theoretical table generated using binomial expansion and known isotope frequencies of the atomic isotopes. When the enrichment of the acetyl-CoA pool is known it becomes possible to calculate the fraction (f) of newly synthesized cholesterol and palmitate molecules in plasma or VLDL and in liver homogenates. For determination of the absolute amount of newly synthesized hepatic palmitate we multiplied f by the total amount of hepatic palmitate at the end of the experiment.

Gene expression studies

Liver samples of approximately 30 mg were used for total RNA isolation with the Trizol method (GIBCO, Paisley, United Kingdom) and the SV Total RNA Isolation System (Promega, Madison, WI). Single stranded cDNA was synthesized using materials from Boehringer Mannheim (Mannheim, Germany), according to manufacturer's instructions. PCR was performed in 50 µl preparations using 3 µl cDNA, 0.25 Taq polymerase, 5 µl tenfold buffer, 0.75 µl dNTP-mix (10 mM) (all from Hoffmann-La Roche), 2 µl DMSO, and 1 µl of each primer (25 pmol, GIBCO). The following primer sets were used: for acetyl-CoA carboxylase (*ACC*, GenBank accession number J03808), sense primer was GGG ACT TCA TGA ATT TGC TGA TTC TCA GTT and anti-sense primer was GCT ATT ACC CAT TTC ATT ACC TCA ATC TC[34]; for fatty acid synthase (*FAS*, GenBank X13415), sense primer was GGC TTT GGC CTG GAA CTG GCC CGG TGG CT and anti-sense primer was TCG AAG GCT ACA CAA GCT CCA AAA GAA TA[34]; for sterol regulatory element binding protein-1 (*SREBP-1a* and *1c*, GenBank L16995), sense primer was CCT GTG TGT ACT GGT CTT CCT G and anti-sense primer was ACA AGA TGG CCT CCT GTG TAC T; for *SREBP-2* (GenBank U02031), sense primer was CAA TGG

CAC GCT GCA GAC CCT TG and anti-sense primer was ATG GCC TTC CTC AGA ACG CCA G; for 3-hydroxy-3-methylglutaryl-CoA reductase (*HMG-CoA reductase*, GenBank M29249), the sense primer was GAC ACT TAC AAT CTG TAT GAT G and anti-sense primer was CTT GGA GAG GTA AAA CTG CCA; for *HMG-CoA synthase* (GenBank X52625), sense primer was TAC GAT GGT GTA GAT GCT GG and anti-sense primer was AGT TCT TCT GTG CTT TTC ATC CAC; for apolipoprotein B (*apoB*, GenBank M14952), sense primer was GAC ATG GTG AAT GGA ATC ATG and anti-sense primer was TGA AGA CTC CAG ATG AGG AC[34]; for *β-actin* (GenBank M12481), sense primer was AAC ACC CCA GCC ATG TAC G and anti-sense primer was ATG TCA CGC ACG ATT TCC C; for microsomal triglyceride transfer protein (*MTP*, GenBank LA7970)), sense primer was ATC TGA TGT GGA CGT TGT GT and anti-sense primer was CCT CTA TCT TGT AGG TAG TG; for carnitine palmitoyltransferase I (*CPT-I*, GenBank L07736), sense primer was GCA TCA TCA CTG GTG TGT TC and anti-sense primer was TCT CCA TGG CGT AGT AGT TG. For each primer set, an increasing number of PCR-cycles with otherwise fixed conditions was performed to determine to optimal number of cycles, which was chosen as the number halfway through the exponential phase. The PCR products were separated on 2.5 % agarose gels. Images were made using a CCD video camera (Image Master VDS system, Pharmacia, Upsalla, Sweden). Intensities of the bands were quantified using the program Image Master 1D Elite 3.0.

Statistical analysis

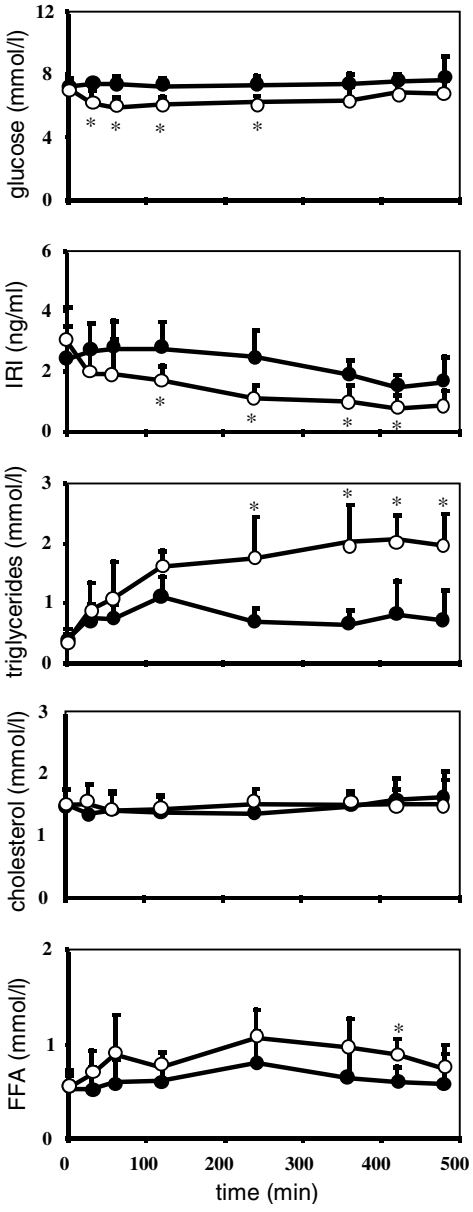
All values reported are mean ± SD. Significance was determined using the non-parametric Mann Whitney test for unpaired data. Differences were considered significant at $P < 0.05$.

Results

Effects of S4048 on plasma parameters

Figure 1 shows the effects of S4048 infusion on plasma glucose, insulin, cholesterol, triglyceride and free fatty acid concentrations. Infusion of S4048 modestly decreased plasma glucose concentrations ($P < 0.05$), especially during the first two hours of the experiment, with a subsequent significant decrease ($P < 0.05$) in plasma insulin concentrations. Both plasma glucose and insulin concentrations reached values approaching those in control rats at the end of the experiment. Triglyceride concentrations increased significantly during the course of the experiment from 0.4 ± 0.1 to 1.9 ± 0.6 mmol/l in S4048-treated rats. Cholesterol levels did not change during the course of the experiment, while free fatty acid levels displayed a modest increase during S4048 infusion.

Figure 1. Concentrations of plasma glucose, insulin (IRI), triglycerides, cholesterol and free fatty acids (FFA) in rats during an 8 h infusion with S4048 (open symbols) or the solvent (closed symbols) (n = 6 in both groups).



Data are means ± SD. * Indicates significant differences between the two groups.

The ketone body β-hydroxybutyrate concentration was 0.54 ± 0.23 mmol/l in the control and 0.75 ± 0.71 mmol/l in the S4048-treated animals. No effects of DMSO, S4048 or Triton WR1339 administration on ASAT on ALAT activities in plasma were found, indicating absence of direct hepatotoxic actions of these compounds. A modest increase was found in ASAT and ALAT activities in the animals receiving all three compounds simultaneously.

Table 1. Intrahepatic content of G6P, glycogen, triglycerides and cholesterol.

	Control (µmol / g liver)	S4048 (µmol / g liver)
n	8	8
G6P	0.23 ± 0.11	0.70 ± 0.30 *
Glycogen	94.0 ± 64.9	265.4 ± 76.2 *
Triglycerides	5.8 ± 1.4	20.6 ± 5.5 *
Cholesterol	5.3 ± 1.3	5.4 ± 1.6
Cholesteryl esters	0.5 ± 0.2	1.1 ± 0.4 *

Data are means ± SD. * Significantly different from contr ol rats.

Effect of S4048 on hepatic parameters

Infusion of S4048 clearly affected hepatic carbohydrate and lipid contents (Table 1). Liver weight, expressed as percentage of body weight, was 3.0 ± 0.4 % and 3.3 ± 0.2 % (NS) in the control and S4048 group, respectively. Hepatic G6P and glycogen contents both increased almost threefold after S4048 infusion. Total cholesterol content was not affected, although a higher relative cholesteryl ester content was found. Triglyceride content was markedly increased in the S4048 group, *i.e.*, S4048 induced massive steatosis within the 8 h time frame of the experiment.

Figure 2 shows representative sections of livers from solvent-treated and S4048-treated rats, stained for neutral fat by Oil-red-O. Massive amounts of neutral fat were present in livers of S4048-treated rats, mainly in periportal areas of the hepatic lobuli. In contrast, the relatively small amounts of neutral fat present in the control liver were concentrated around the central veins, *i.e.*, in perivenous areas of the lobuli.

Effect of S4048 on *de novo* lipogenesis and cholesterogenesis

In Table 2 the effects of S4048 on palmitate and cholesterol synthesis rates are summarized. Plasma fractional cholesterol synthesis rates were similar in both groups of rats. Fractional *de novo* lipogenesis in plasma VLDL and liver were increased almost three-fold in the treated animals. When the increased hepatic palmitate content was taken into account, the absolute amount of newly synthesized palmitate was increased > 10-fold

Figure 2. Oil-red-O stained liver sections of a control rat (A) and an S4048-treated rat (B). S4048 treatment results in increased fat deposition in the liver with a preferential localization in the periportal area of the liver lobulus. PP=periportal area, surrounding the portal triad; PV=perivenous area, surrounding the hepatic vein.

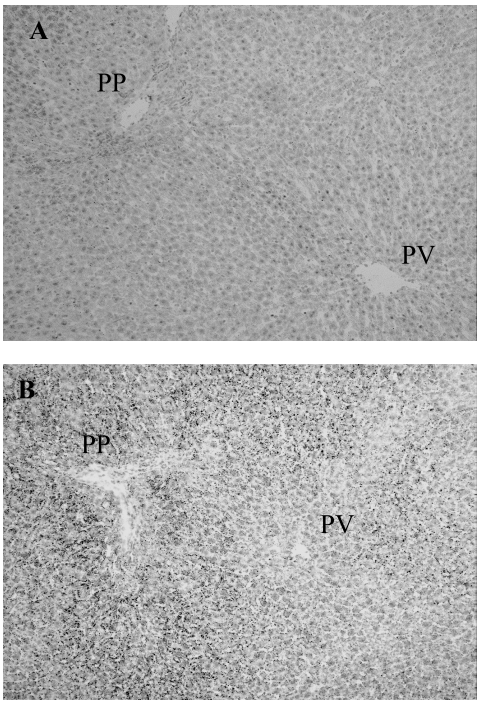
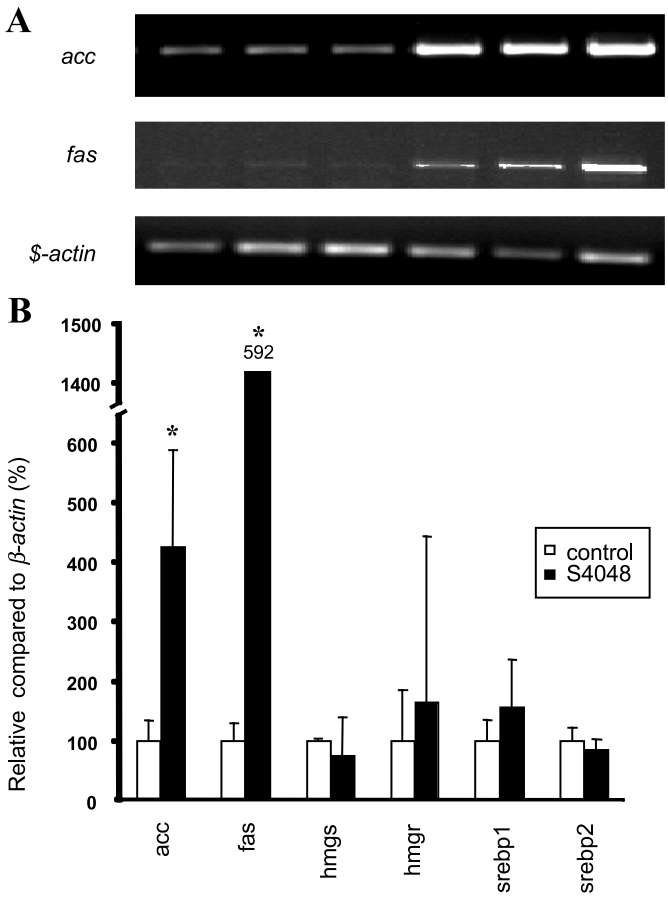


Table 2. Plasma and hepatic fractional cholesterol and palmitate synthesis values, absolute amount of hepatic newly synthesized palmitate and acetyl-CoA pool enirchments in S4048-treated and control rats.

	Control	S4048
Fraction plasma cholesterol (%)	4.0 ± 1.3	5.1 ± 1.2
Fraction of VLDL palmitate (%)	3.0 ± 1.9	7.1 ± 3.8
Fraction of liver palmitate (%)	3.3 ± 1.2	8.7 ± 3.8 *
Newly synthesized hepatic palmitate (nmol/mg	0.2 ± 0.1	2.6 ± 1.2 *
Acetyl-CoA pool enrichments (%)	8.2 ± 1.3	4.4 ± 2.0 *

Data are means ± SD. Samples were obtained from 5 S4048-treated and 6 control rats for all analyses, except for fractional VLDL palmitate synthesis (n = 3 for both groups).
* Indicates significantly different from values in control rats.

Figure 3. Image of PCR products of genes upregulated by S4048, *i.e.*, *ACC* and *FAS* in comparison with *β-actin* (A). The mRNA levels determined by reversed transcriptase PCR (n = 3 in both groups) and normalized to *β-actin* mRNA, expressed as mean percent compared to control values are depicted in B. Levels are shown for *ACC*, *FAS*, *HMG-CoA synthase*, *HMG-CoA reductase* and *SREBP-1* and *2*. * Significantly different from control values.

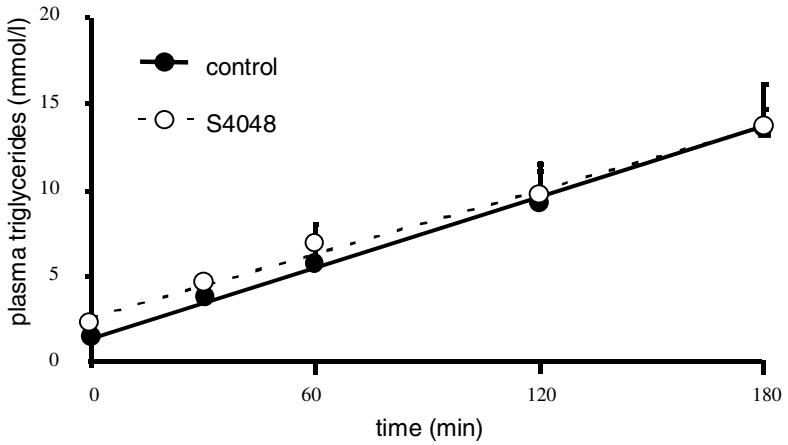


in the S4048-treated group in comparison to the control group. Calculated enrichments of the acetyl-CoA pools showed significantly decreased values in the S4048-treated rats.

S4048 induces expression of lipogenic genes

Figure 3 shows the mRNA levels of *ACC*, *FAS*, *HMGCoA-reductase*, *HMGCoA-synthase* and *SREBP-1* and *2* in control and S4048-treated rats, as determined by a semi-quantitative RT-PCR approach. Intensities of bands were normalized to those of *β-actin* (Figure 3B). Results clearly show upregulation of mRNA levels of *ACC* (~4-fold) and *FAS*

Figure 4. Determination of very low density lipoprotein-triglyceride production rates. Plasma samples were taken for a period of three hours after Triton WR 1339 injection at t = 0 min in three S4048-treated (open symbols) and four control rats (closed symbols) and assayed for triglycerides. Infusion of S4048 or solvent was started three hours before injection of Triton WR 1339. No significant difference was found between the two groups.



(~14-fold) in S4048-treated rats, but no effects on mRNA levels of *HMGCoA-reductase* and *HMGCoA-synthase*. Furthermore, steady state mRNA levels of transcription factors controlling *de novo* lipogenesis and cholesterol synthesis, *i.e.*, *SREBP-1* and *2*, were not affected by S4048 infusion. As expected on the basis of unaltered plasma β-hydroxybutyrate concentrations, S4048 did not induce changes in *CPT-I* mRNA levels (data not shown).

Effect of S4048 on VLDL production

In spite of the significant upregulation of hepatic lipogenesis and hepatic lipid content, S4048 infusion did not affect hepatic VLDL-triglyceride production rate (Figure 4): values of 151 ± 33 and 137 ± 14 µmol/kg/h were calculated for control and S4048-treated rats, respectively. Values obtained in the animals infused with DMSO solution alone were similar to those found in rats infused with saline for the same period of time (154 ± 29 µmol/kg/h), indicating no effects of the solvent at this dose on VLDL-triglyceride production. The size of the VLDL particles produced by rats treated with the solvent or with S4048 was also similar, with average diameters of 40.7 ± 6.7 nm and 45.9 ± 9.6 nm in control and S4048-treated animals, respectively. As expected, no differences were found in hepatic expression of *apoB* or *MTP* genes (data not shown).

Discussion

Acute inhibition of the G6Pase system by S4048 stimulated hepatic *de novo* lipogenesis more than 10-fold in rats, despite decreased circulating levels of insulin, a well-known stimulator of lipogenesis.[37] Increased *de novo* lipogenesis was associated with an increased flux through the acetyl-CoA pool, since lower ^{13}C-acetyl-CoA enrichments were calculated for rats treated with S4048. Thus, more substrate for fatty acid synthesis was produced in the S4048-treated rats than in control rats, presumably due to an increased glycolytic flux. Fatty acid synthesis was not only stimulated by increased precursor supply. Concomitantly, hepatic mRNA levels of two key enzymes in *de novo* lipogenesis, *ACC* and *FAS* were markedly induced within the 8 h time frame of the experiment.

Pathways of *de novo* lipogenesis are under transcriptional control of sterol regulatory element binding proteins (SREBP's)[38,39], a group of transcription factors (SREBP1a, SREBP1c and SREBP2) that regulate the expression of genes involved in cholesterol, fatty acid and glucose metabolism. SREBP-1 gene knockout mice show a very low basal expression of *ACC* and *FAS* and hardly possess the ability to upregulate *de novo* lipogenesis.[38] In contrast, overexpression of the nuclear form of SREBP-1a leads to massive steatosis and increased *de novo* lipogenesis, albeit in absence of hypertriglyceridemia.[40] In S4048-treated rats *SREBP-1* mRNA expression was not significantly induced, but this obviously does not exclude the possibility of direct SREBP1 - mediated activation of gene expression, particularly in view of very recent results by Foretz *et al.*[41] These authors have shown that enhancement of mRNA expression of *ACC* and *FAS* by SREBP-1c in isolated hepatocytes critically depends on the presence of glucose in the medium. Furthermore, it is well established that glucose exerts stimulatory effects of lipogenic gene expression only after being metabolized to either G6P[42] or xylose-5-phosphate.[43] In a recent study it was shown that overexpression of glucokinase in fed rats, leading to increased G6P concentrations, resulted in a marked increase in plasma triglyceride levels.[18] In light of the three-fold increase in G6P concentration in the liver of rats infused with S4048, potentiation of transcriptional activity of SREBP-1 by G6P is highly likely to occur in our model.

Despite increased production of the obligatory precursor, *i.e.*, acetyl-CoA, our stable isotope study showed unaffected cholesterol synthesis rates during infusion of S4048 and unaffected hepatic mRNA levels *HMG-CoA synthase* and *HMG-CoA reductase*. In accordance, overexpression of glucokinase, resulting in an increased hepatic G6P content, did not increase plasma cholesterol levels either.[18] SREBP-2 is a strong regulator of cholesterol synthesis, having the ability to upregulate various genes involved in the cholesterogenic pathway, such as *HMG-CoA synthase* and *HMG-CoA reductase*.[44,45] *SREBP-2* mRNA levels were not affected in the S4048-treated animals. In combination, these results clearly demonstrate that in spite of common regulatory mechanisms involved, *i.e.* SREBP-modulated activation of gene expression, *de novo* lipogenesis and cholesterogenesis are differentially regulated under conditions associated with increased glycolytic flux in rat liver.

Partial inhibition of G6Pase for an 8 hour period was associated with massive steatosis. Fat accumulation was much more pronounced in hepatocytes located in the zone surrounding the portal vein and hepatic artery (periportal area), than in those surrounding the hepatic vein (perivenous area). This is probably related to the predominant periportal localization of G6Pase.[46] Increased *de novo* lipogenesis contributed to the development of steatosis, but the quantitative contribution of newly synthesized fatty acids to the steatosis appeared rather limited, *i.e.*, was less than 10 %. Furthermore, VLDL-triglyceride secretion was similar in both groups (see below) and thus did not differentially influence hepatic lipid stores. Increased fat uptake therefore must have contributed, either in the form of VLDL/LDL-triglycerides or as free fatty acids, although plasma free fatty acid concentrations were increased only moderately upon treatment. The latter, however, does not exclude an enhanced free fatty acid flux to the liver. Furthermore, fatty acid oxidation might have been impaired in the S4048-treated rats, although similar mRNA levels of *CPT-I* in livers and unaffected plasma β-hydroxybutyrate concentrations were found. Malonyl-CoA is produced during the course of fatty acid synthesis and is expected to be accumulate in the livers of the S4048-treated rats. Malonyl-CoA is a strong, allosterically acting, inhibitor of fatty acid oxidation[47], which can thus be achieved without changes in CPT-I levels. Furthermore, the increase in hepatic triglyceride content with similar β-hydroxybutyrate concentrations strongly suggests a shift in the balance between fatty acid oxidation and esterification in the liver.

Many factors are known to influence hepatic VLDL-triglyceride production and secretion. *De novo* lipogenesis has been suggested to be of regulatory importance for VLDL production.[48] Furthermore, many studies have shown that increases in fatty acid delivery to the liver, leading to increased triglyceride synthesis, are accompanied by increases in VLDL secretion.[49-51] The balance between apolipoprotein B synthesis and degradation is an important factor in controlling hepatic triglyceride secretion and inhibition of protein synthesis has been shown to reduce VLDL-triglyceride secretion.[52] Additionally, insulin is a well-known acute inhibitor of VLDL secretion[53-55], and insulin resistance is associated with increased VLDL-triglyceride and apoB secretion.[9-11] Furthermore, more recent data has shown that phosphorylation of glucose is also of importance in regulation of assembly and secretion of triglyceride-containing VLDL.[19] In our study, surprisingly, we did not find increased VLDL-triglyceride secretion in the S4048-treated rats. Moreover, neither number nor size of VLDL particles were affected by S4048 infusion. In our model, with induction of *de novo* lipogenesis, strongly increased hepatic triglyceride content in combination with increased hepatic G6P levels and decreased insulin concentrations, increased secretion of VLDL-triglycerides was expected to occur. However, a number of factors have to be taken into account. First of all, it is not known whether apoprotein synthesis was impaired in the S4048-treated rats. Furthermore, if glucose itself is also important in this process, lowered plasma glucose concentrations in the S4048-treated rats might cause an inability to adequately upregulate VLDL secretion. It should be stressed that, in our model, G6P content is increased in the cytoplasm but probably decreased in the endoplasmic reticulum. Compartmentalization of G6P could

potentially play a role in its capability to influence VLDL secretion, but further studies are needed to clarify this phenomenon.

The observation that VLDL-triglyceride secretion was not increased in rats after S4048 treatment indicates that the hyperlipidemia observed after G6Pase inhibition must have originated from decreased triglyceride clearance. Insulin is a well-known stimulator of adipocyte lipoprotein lipase activity. In the S4048 model with low insulin concentration, lipoprotein lipase activity was probably decreased, leading to decreased lipolysis of VLDL-triglycerides. Indeed, studies in GSD patients have shown low LPL activity in GSD patients.[56]

Data in the literature on the relationship between G6Pase activity and lipid metabolism is confusing. Based on our results, we postulate that G6P concentrations in the liver, specifically in certain compartments, play a pivotal role in determining triglyceride concentration in liver and plasma. Altered activation of SREBP-1 and/or changes in the intrahepatic concentration of G6P in itself are most likely of more importance than the plasma concentration of glucose or insulin to explain the apparently conflicting effects of G6Pase activity on hepatic lipid metabolism in diabetes and GSD.

In conclusion, acute inhibition of G6Pase activity in rats leads to increased *de novo* lipogenesis and massive steatosis within a relatively short time frame. Cholesterogenesis was not affected in our study, implying a dissociated regulation of cholesterol and fatty acid synthesis under the conditions employed. Increased *de novo* lipogenesis and hepatic lipid accumulation alone is not sufficient to stimulate VLDL-triglyceride secretion. This study underlines the important function of G6P in control of hepatic lipid metabolism.

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En toch de beelden van weleer
waarin ik van ons dromen blijf
kleden zelfs de witte cellen
met menselijke vellen

5

Hepatic VLDL production in *ob/ob* mice is not stimulated by massive *de novo* lipogenesis but is less sensitive to the suppressive effects of insulin.

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Abstract

Type 2 diabetes in humans is associated with increased *de novo* lipogenesis (DNL), increased fatty acid (FA)-fluxes, decreased FA oxidation and hepatic steatosis. In this condition, VLDL production is increased and resistant to suppressive effects of insulin. The relationships between hepatic FA metabolism, steatosis and VLDL production are incompletely understood. We investigated VLDL-triglyceride and apolipoprotein (apo)B production in relation to DNL and insulin sensitivity in female *ob/ob* mice. Hepatic triglyceride (5-fold) and cholesteryl ester (15-fold) contents were increased in *ob/ob* mice compared to lean controls. Hepatic DNL was increased ~10-fold in *ob/ob* mice whereas hepatic cholesterol synthesis was not affected. Basal rates of hepatic VLDL-triglyceride and apoB100 production were similar between the groups. Hyperinsulinemic clamping reduced VLDL-triglyceride and -apoB100 production rates by ~60 % and ~75 %, respectively, in lean mice but only by ~20 % and ~20 %, respectively, in *ob/ob* mice. No differences in hepatic expression of genes encoding apoB and microsomal triglyceride transfer protein were found. Hepatic expression and protein phosphorylation of insulin receptor and insulin receptor substrate-isoforms were reduced in *ob/ob* mice. Thus, strongly induced hepatic DNL is not associated with increased VLDL production in *ob/ob* mice, possibly related to differential hepatic zonation of apoB synthesis (periportal) and lipid accumulation (perivenous) and/or relatively low rates of cholesterogenesis. Insulin is unable to effectively suppress VLDL-triglyceride production in *ob/ob* mice, presumably due to impaired insulin signaling.

Introduction

Type 2 diabetes mellitus (DM2) is associated with increased *de novo* lipogenesis (DNL), decreased plasma fatty acid (FA) oxidation and an increased FA-flux from peripheral tissues to the liver.[1] These factors may all contribute to hepatic steatosis and increased hepatic VLDL production, two characteristic hallmarks of type 2 diabetes,[2] and are probably related to hepatic insulin resistance, i.e., an insensitivity of hepatic metabolic processes to the effects of insulin. The relative contribution of the various pathways in hepatic lipid metabolism to the development of a fatty liver and disturbances in VLDL production is unknown but may, at least in part, be related to the localization of these processes within the liver. Fatty acid synthesis and triglyceride (TG) accumulation occur predominantly in the perivenous areas (zone 3) of the liver whereas FA oxidation is more associated with the periportal areas (zone 1).[3,4] VLDL secretion has so far not been restricted to a specific hepatic zone.

Leptin-deficient *ob/ob* mice develop a fatty liver, insulin resistance and hyperlipidemia.[5,6] The contribution of hepatic lipoprotein production to the development of hyperlipidemia in these mice is not clear. Hyperglycemia together with an increased glycolytic activity in *ob/ob* mice may lead to an increased availability of acetylCoA residues for DNL,[7] as supported by increased hepatic expression levels and activities of glycolytic enzymes, *i.e.,* glucokinase,[8,9] phosphofructokinase[7] and pyruvate kinase.[7,8] Furthermore, elevated plasma FFA levels[9] and increased hepatic expression of fatty acid translocase (FAT or CD36) and plasma membrane-fatty acid binding protein (pmFABP) have been reported in this model.[10] Increased endoplasmic reticulum (ER)-associated acetylCoA synthase (ACS) activity may increase the FA availability for esterification rather than for oxidation,[10] which could contribute in increased TG and cholesteryl ester formation.

Insulin resistance seen in DM2 is associated with increased VLDL production.[2] Acute hyperinsulinemia reduces VLDL production in healthy volunteers[11,12] but not in DM2 patients[2] and obese individuals.[11] Despite the insulin resistant condition and an increased hepatic TG content in *ob/ob* mice, a decreased VLDL-TG production rate under basal fasted conditions has been reported in this model.[13,14] However, increased VLDL-TG secretion in *ob/ob* mice associated with enhanced expression and activity of the microsomal triglyceride transfer protein (MTP) has also been reported.[15] The reason for these discrepant observations is unknown. The impact of insulin on VLDL production in the *ob/ob* mouse model has not been reported previously. Therefore, we quantified hepatic DNL and cholesterol synthesis using mass isotopomer distribution analysis (MIDA) and related the synthesis rates to VLDL-TG and -apoB production rates determined under basal conditions and during hyperinsulinemic clamps in *ob/ob* mice and in lean littermates. Hepatic insulin signaling and expression levels of genes encoding transcription factors and important enzymes involved in fatty acid and cholesterol metabolism, VLDL formation and insulin signaling, were studied to provide a mechanistic basis for our findings.

Methods

Animals

Female *ob/ob* and lean littermates were purchased from Harlan (Zeist, The Netherlands) and housed in a light- and temperature controlled facility. Experimental protocols were approved by the local Experimental Ethical Committee for Animal Experiments.

Analytical kits

Plasma and hepatic triglyceride (TG), cholesterol and glucose levels were determined by commercially available kits (Roche, Mannheim, Germany). Plasma and hepatic phospholipid concentrations and plasma free fatty acids (FFA) concentrations were determined with Phospholipid-kit and NEFA-C kit, respectively (Wako Chemical GmbH, Neuss, Germany). Plasma insulin was determined by a radio-immunoassay (RIA) RI-13K (Linco Research, Inc., St. Charles).

Experimental procedures

Female *ob/ob* and lean mice, weighing between 51-63 gram and 24-28 gram respectively, were *ad libitum* fed normal chow diet (RMH-B 2181, Hope Farms BV, Woerden, The Netherlands) enriched with 2 % [1-^{13}C]-acetate (Isotec, Miamisburg, OH). After 11 days, mice were fasted for 4 h, anaesthetized with halotane and livers were excised. A portion of abdominal fat was also collected. Liver and fat tissue were immediately frozen in liquid nitrogen and stored at -80ºC. Blood was collected by heart puncture and immediately placed on ice in EDTA-containing tubes and centrifuged 10 minutes at 5,000 rpm at 4ºC.

Hyperinsulinemic clamp

To study effects of insulin on lipoprotein metabolism, a second group of lean and *ob/ob* mice received a hyperinsulinemic clamp or a saline infusion under anesthesia after a 9 hour fast. Based on euglycemic insulin clamps performed in rats and mice by Hawkins and Rossetti *et al.*,[16,17] in which insulin concentrations were fixed at ~25 ng/ml, we used a single infusate to establish hyperinsulinemia and euglycemia. The procedure was tested in pilot experiments. The infusate contained insulin (18 mU/kg/min; Novo Nordisk, Bagsvaerd, Denmark), somatostatin (1.5 µg/kg/min; UCB, Breda, The Netherlands) and glucose (25 mg/kg/hr; Merck, Darmstadt, Germany). All solutions were freshly prepared in saline containing 1.5 % BSA (Sigma, St. Louis, MO). Blood glucose concentration was determined with a GlucoTouch-glucose analyzer (LifeScan, Beerse, Belgium). The total infusion time was 2 hours. After 1 h mice received a Triton WR1339 injection (Sigma, St. Louis, MO) as a 12 % $^{wt}/_{wt}$ solution dissolved in saline, in a dose of 5 ml/kg lean BW.[19] Blood samples were taken before (t_0) and 30 and 60 minutes after Triton injection. At the end of the experiment a large blood sample was obtained by heart puncture for isolation of VLDL particles (see further). VLDL production rates were calculated from the slope of the linear TG *versus* time curves (Figure 4D). Since mouse liver secretes TG-rich lipoproteins as IDL-like and VLDL particles, we used a solution of 15.3 % NaCl and 35.4 % KBr (final

concentration 0.65 % and 1.52 %, respectively) in saline with a density < 1.019 g/ml to isolate VLDL/IDL. Plasma (0.2 ml) was mixed with 0.8 ml of the NaCl-KBr solution and centrifuged for 100 minutes at 120,000 rpm (627000 g) and 4ºC in an ultracentrifuge (rotor TLA 120.2 Beckman). Tubes were sliced at 1.5cm and the top-fraction, containing VLDL/IDL, was collected and frozen at -80ºC until composition analyses. VLDL/IDL particle size was determined using a Submicron Particle Sizer (Nicomp, Santa Barbara, CA, USA).

Liver lipid analysis

Liver lipids were extracted according to Bligh & Dyer[19] and determined using commercially available kits. Protein content of tissue homogenates was determined according Lowry *et al.*[20]

Histology

The localization of hepatic TG and apolipoprotein B (apoB) mRNA were visualized as indicators of TG deposition and VLDL formation, respectively. Hepatic morphology was visualized by standard Hematoxilin Eosin (HE)-staining and neutral lipids were visualized by "Oil-Red-O" (ORO). The apoB *in situ* hybridization technique was similar to that previously described for apoE.[21] The pGEM-3z vector containing apoB cDNA was a gift form dr. H.M. Princen (Gaubius Laboratory, TNO Prevention and Health, Leiden, The Netherlands).

Mass isotopomer distribution analysis

MIDA allows quantitation of the biosynthesis of polymers *in vivo* and is described in detail elsewhere.[22] The enrichment of the pool of acetylCoA precursor units (p) that have entered newly synthesized cholesterol and palmitate during feeding of a [1-^{13}C]-acetate-enriched diet can be calculated by comparison with a theoretical table generated using binomial expansion and known isotope frequencies of the atomic isotopes. When the enrichment of the acetylCoA pool is known, it becomes possible to calculate the fraction (f) of newly synthesized cholesterol and palmitate molecules in plasma or tissues. To determine the absolute amount of newly synthesized hepatic cholesterol and palmitate, we multiplied f by the total amount of hepatic free cholesterol and palmitate, respectively. Oral labelling of acetyl-CoA pools with ^{13}C-acetate was described by Jung *et al.*[23] By label-feeding for longer periods of time, pools with a slow turnover, such as connective tissue and arterial walls, are also being labelled. After 10 days of labelling, pools with a rapid turnover will have reached a steady state, but pools with a slow turnover might not have. One must realize therefore that this may lead to a certain underestimation in synthesis rates in *ob/ob* mice, mainly for palmitate, since pool sizes of palmitate are larger in these animals.

Gas chromatography/mass spectrometry (GC/MS) analysis

Plasma cholesterol was extracted and derivatized as described.[24] Cholesterol-TMS derivates were separated on a HP 5890 Plus gas chromatograph (Hewlett-Packard, Palo Alto, CA)

Table 1. List of sequences of primers and probes used.

Standard	Type	Sequence	GenBank no.
β-Actin	Forward	AGC CAT GTA CGT AGC CAT CCA	NM007393
	Reverse	TCT CCG GAG TCC ATC ACA ATG	
	Probe	TGT CCC TGT ATG CCT CTG GTC GTA CCAC	
Transcription factors			
Srebp-1c	Forward	GGA GCC ATG GAT TGC ACA TT	BI656094
	Reverse	CCT GTC TCA CCC CCA GCA TA	
	Probe	CAG CTC ATC AAC AAC CAA GAC AGT GAC TTC C	
Srebp-2	Forward	CTG CAG CCT CAA GTG CAA AG	AF374267
	Reverse	CAG TGT GCC ATT GGC TGT CT	
	Probe	CCA TCC AGC AGC AGG TGC AGA CG	
Ppar-α	Forward	TAT TCG GCT GAA GCT GGT GTA C	X57638
	Reverse	CTG GCA TTT GTT CCG GTT CT	
	Probe	CTG AAT CTT GCA GCT CCG ATC ACA CTT G	
Ppar-γ	Forward	CAC AAT GCC ATC AGG TTT GG	X57638
	Reverse	GCT GGT CGA TAT CAC TGG AGA TC	
	Probe	CCA ACA GCT TCT CCT TCT CGG CCT G	
Lxr	Forward	GCT CTG CTC ATT GCC ATC AG	AF085745
	Reverse	TGT TGC AGC CTC TCT ACT TGG A	
	Probe	TCT GCA GAC CGG CCC AAC GTG	
Chrebp	Forward	GAT GGT GCG AAC AGC TCT TCT	AF156604
	Reverse	CTG GGC TGT GTC ATG GTG AA	
	Probe	CCA GGC TCC TCC TCG GAG CCC	
DNL, cholesterogenisis and β-oxidation			
Fas	Forward	GGC ATC ATT GGG CAC TCC TT	AF127033
	Reverse	GCT GCA AGC ACA GCC TCT CT	
	Probe	CCA TCT GCA TAG CCA CAG GCA ACC TC	
Acc	Forward	GCC ATT GGT ATT GGG GCT TAC	AF374170
	Reverse	CCC GAC CAA GGA CTT TGT TG	
	Probe	CTC AAC CTG GAT GGT TCT TTG TCC CAG C	
Hmgr	Forward	CCG GCA ACA ACA AGA TCT GTG	BB664708
	Reverse	ATG TAC AGG ATG GCG ATG CA	
	Probe	TGT CGC TGC TCA GCA CGT CCT CTT C	

Table 1. Continued.

	Type	Sequence	GenBank no.
DNL, cholesterogenisis and β-oxidation			
CptIa	Forward	CTC AGT GGG AGC GAC TCT TCA	AF017175
	Reverse	GGC CTC TGT GGT ACA CGA CAA	
	Probe	CCT GGG GAG GAG ACA GAC ACC ATC CAA C	
Mcad	Forward	GCA GCC AAT GAT GTG TGC TTA C	NM007382
	Reverse	CAC CCT TCT TCT CTG CTT TGG T	
	Probe	CCC TCC GCA GGC TCT GAT GTG G	
Hmgs	Forward	TGG TGG ATG GGA AGC TGT CTA	U12790
	Reverse	TTC TTG CGG TAG GCT GCA TAG	
	Probe	CCA AGG CCC GCA GGT AGC ACT G	
VLDL metabolism			
Apob	Forward	GCC CAT TGT GGA CAA GTT GAT C	AW012827
	Reverse	CCA GGA CTT GGA GGT CTT GGA	
	Probe	AAG CCA GGG CCT ATC TCC GCA TCC	
Apobec-I	Forward	TCG TCC GAA CAC CAG ATG CT	NM031159
	Reverse	GGT GTC GGC TCA GAA ACT CTG T	
	Probe	CCT GGT TCC TGT CCT GGA GTC CCT G	
Apoe	Forward	CCT GAA CCG CTT CTG GGA TT	NM009696
	Reverse	GCT CTT CCT GGA CCT GGT CA	
	Probe	AAA GCG TCT GCA CCC AGC GCA GG	
Dgat-I	Forward	GGT GCC GTG ACA GAG CAG AT	NM010046
	Reverse	CAG TAA GGC CAC AGC TGC TG	
	Probe	CTG CTG CTA CAT GTG GTT AAC CTG GCC A	
Mttp	Forward	CAA GCT CAC GTA CTC CAC TGA AG	NM008642
	Reverse	TCA TCA TCA CCA TCA GGA TTC CT	
	Probe	ACG GCA AGA CAG CGT GGG CTA CA	
Insulin signaling			
Ir	Forward	TGA GTC AGC CAG TCT TCG AGA A	NM010568
	Reverse	ACT ACC AGC ATT GGC TGT CCT T	
	Probe	CTG CCA TCA TGT GGT CCH CCT TCT	
Irs-1	Forward	AGC ACC TGG TGG CTC TCT ACA	NM010570
	Reverse	CAG CTG CAG AAG AGC CTG GTA	
	Probe	CTC GCT ATC CGC GGC AAT GGC	
Irs-2	Forward	AGT CCC ACA TCG GGC TTG AAG	AF090738
	Reverse	GGT CTG CAC GGA TGA CCT TAG	
	Probe	CCT TCA AGT CAG CCA GCC CCC TG	

using a 30 m x 0.25 mm (0.2 μm film thickness) DB5 ms column (J&W Scientific, Falson, CA, USA) inserted into the ion source of a Quadrupole mass spectrometer, model SSQ 7000 (Finnigan Matt, San Jose, CA, USA). The mass fragments m/z 368, 369, 370 and 371 were monitored by selected ion recording.

For analysis of the methyl esters of palmitate[25] the same GC/MS mode described above was used, equipped with a 20 m x 0.18 mm AT1701 column (0.4 μm film thickness, Alltech Associates Inc, Deerfield, USA). They were analyzed at mass-to-charge ratio (*m/z*) 271, 272 and 273 (mass isotopomers M_0, M_1, and M_2) using chemical ionisation and selected ion recording.

ApoB quantification

ApoB100 concentrations were quantified by comparison to an IDL apoB100 standard isolated from healthy human subjects.[26] Since human LDL does not contain apoB48, we were not able to accurately quantify apoB48 levels by this procedure; these levels were estimated. Isolated VLDL samples (10 μl) were delipidated with methanol and diethylether and dried under nitrogen. Delipidated lipoproteins were reduced in SDS sample buffer (8 M urea, 10 mM Tris base, 2 % SDS, 10 % glycerol, 5 % β-mercaptoethanol) and separated by SDS-PAGE using 4-15 % gradient gels (Ready gels, Biorad, Hercules, CA). Gels were either subjected to silver-staining[26] or were used for Western blot analysis. Proteins were transferred onto nitrocellulose membranes (Hybond ECL, Amersham Pharmacia Biotech, Buckinghamshire, UK). Blots were stained with the primary polyclonal antibody against human apolipoprotein B, raised in sheep (dilution 1:100,000; Roche, Mannheim, Germany) and secondary IgG, anti-sheep antibody conjugated with horseradish-peroxidase activity (dilution 1:10,000; Calbiochem, San Diego, CA).

Hepatic gene expression

Total RNA was isolated from ~30 mg tissue using Trizol-methodology (GIBCO, Paisley, UK) followed by the SV Total RNA Isolation System (Promega, Madison, WI). RNA was converted to single-stranded cDNA by a reverse transcription procedure with M-Mulv-RT (Boehringer Mannheim, Mannheim, Germany) and mRNA levels were quantified by real-time PCR using the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). Procedures were optimized for the particular genes using appropriate forward and reverse primers (GIBCO, Paisley, UK) and a template-specific 3'-TAMRA, 5'-6-FAM labeled Double Dye Oligonucleotide probe (Eurogentec, Seraing, Belgium). Calibration curves were run on serial dilutions of a 8x concentrated cDNA solution, resulting in a series containing 8x, 4x, 2x, 1x, 0.5x, 0.125x, 0.062x, and 0.031x of the cDNA present in the assay incubation. Both assay and calibration incubations were done simultaneously. The fluorescence data obtained were processed using the software program ABI Sequence Detector v1.6.3 (System Applied Biosystems, Foster City, CA). All quantified expression levels were within the linear part of the calibration curves and calculated using these curves. The primers and probe sets used are listed in Table 1.

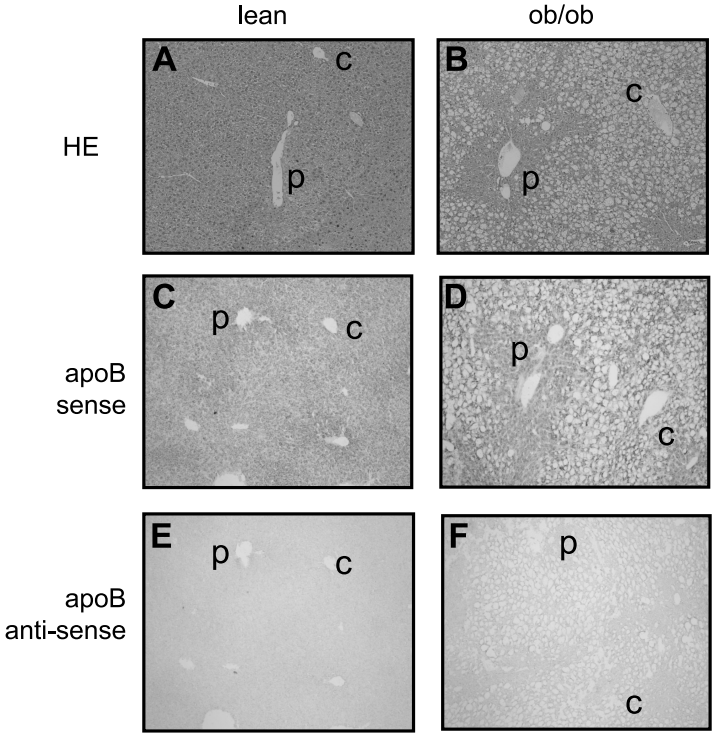
Hepatic insulin signaling

For analysis of IRβ and IRS-1,2,3 phosphorylation, liver tissue was homogenized in RIPA - buffer (30 mM Tris, pH 7.5, 1 mM EDTA, 150 mM NaCl, 0.5 % Triton X-100, 0.5 % deoxycholate, 1 mM sodium orthovanadate, 10 mM sodium fluoride, and protease inhibitors (Complete: Boehringer Mannheim)) using a Ultraturrax mixer followed by centrifugation (14 krpm; 15 min; 4ºC). Protein content of supernatants was determined using BCA-kit (Pierce, Rockford, IL). A total of 25 μg protein was analyzed by immunoblotting for expression of IRβ subunit (Transduction Laboratories, Lexington), IRS-1,[27] IRS-2[28] and IRS-3. Anti- IRS3 antibody was obtained from rabbits immunized with a recombinant His-tagged IRS3 fusion protein produced from pET16B-IRS3 (aa198-494 of rat IRS3) as described by Ouwens *et al.* [27]

Statistical analysis

All values reported are means ± SD; statistical significance implies $P < 0.05$. Because of small sample sizes, all statistical analyses involved non-parametrical Mann-Whitney *U* tests.

Figure 1. Hematoxilin Eosin (HE) staining (A, B) and apoB in situ hybridization in liver sections from lean (C, E) and *ob/ob* mice (D, F), respectively. p = portal area (zone 1), c = central area (zone 3).



Results

Animal characteristics

Mean body weight was 26 ± 1 *vs.* 58 ± 5 g in the lean and *ob/ob* mice, respectively ($P < 0.05$). Fasting plasma glucose, insulin, TG, cholesterol, and FFA concentrations were elevated in *ob/ob* mice (Table 2). Excess TG and cholesterol in *ob/ob* plasma was predominantly found in VLDL-sized fractions upon FPLC separation (data not shown). Liver weight (2-fold), total amount of hepatic TG (5-fold), total cholesterol (~2.6-fold), free cholesterol (~1.6-fold), cholesteryl ester (~15-fold) and glycogen levels (~1.8-fold) were all increased in *ob/ob* mice. No differences in hepatic phospholipid and glucose-6-phosphate (G6P) levels were detected between lean and *ob/ob* mice (Table 2).

Neutral fat deposition (not shown in Figure 1) in *ob/ob* mice was clearly associated with the perivenous (zone 3) area of liver lobules resulting in enlarged, fat-laden hepatocytes in these parts of the liver (Figure 1, A vs. B). To check whether localization of fat in *ob/ob* liver was compatible with that of apolipoprotein B gene expression, *Apob* mRNA was visualized by *in situ* hybridization in lean and *ob/ob* mouse liver (Figure 1, C and D, respectively). *Apob* mRNA was present in the entire liver lobe but a stronger signal was observed in the periportal zone of the liver both in lean and *ob/ob* mice, suggesting zonal differentiation between VLDL formation and TG deposition in *ob/ob* mouse liver.

Table 2. Plasma and hepatic parameters after a 4 hr fast in lean and *ob/ob* mice.

Plasma	Lean	*Ob/ob*
Glucose (mM)	8.6 ± 3.0	16.9 ± 4.6 *
Insulin (ng/ml)	1.3 ± 0.9	6.9 ± 0.6 *
Triglycerides (mM)	0.3 ± 0.03	0.7 ± 0.1 *
Cholesterol (mM)	1.7 ± 0.4	4.1 ± 0.3 *
Free fatty acids (mM)	0.7 ± 0.1	1.3 ± 0.1 *
Liver		
Liver weight (g)	1.3 ± 0.1	2.7 ± 0.3 *
Triglycerides (μmol/g liver)	1.2 ± 0.4	2.7 ± 0.5 *
Total cholesterol (μmol/g liver)	1.3 ± 0.4	1.6 ± 0.3 *
Free cholesterol (μmol/g liver)	0.8 ± 0.1	1.0 ± 0.1
Cholesteryl esters (μmol/g liver)	0.1 ± 0.01	0.5 ± 0.3 *
Phospholipids (μmol/g liver)	0.3 ± 0.1	0.2 ± 0.3
Glucose-6-phosphate (μmol/g liver)	0.2 ± 0.03	0.1 ± 0.1
Glycogen (μmol/g liver)	212 ± 11	183 ± 17

Data are means ± SD. $n = 5$ per group. * $P < 0.05$, Mann-Whitney *U* test.

***De novo* lipogenesis and cholesterol synthesis**

Palmitate and cholesterol synthesis rates in lean and *ob/ob* mice fed an [1-^{13}C]-acetate-enriched diet are summarized in Table 3. The enrichment of acetylCoA pool and fractional synthesis rate values could not be calculated in adipose tissue of *ob/ob* mice due to low isotopic enrichments. Enrichments of the hepatic actylCoA pools for DNL and cholesterogenesis were similar between lean and *ob/ob* mice. Hepatic fractional *de novo* lipogenesis (DNL) was increased 1.7-fold in *ob/ob* mice in comparison with lean controls. The absolute amount of newly synthesized hepatic palmitate was 10-fold higher in livers of *ob/ob* mice than in those from controls. DNL values in adipose tissue of lean controls indicate that adipocytes may significantly contribute to total DNL in mice. The absolute amounts of newly synthesized cholesterol were similar in livers of both groups.

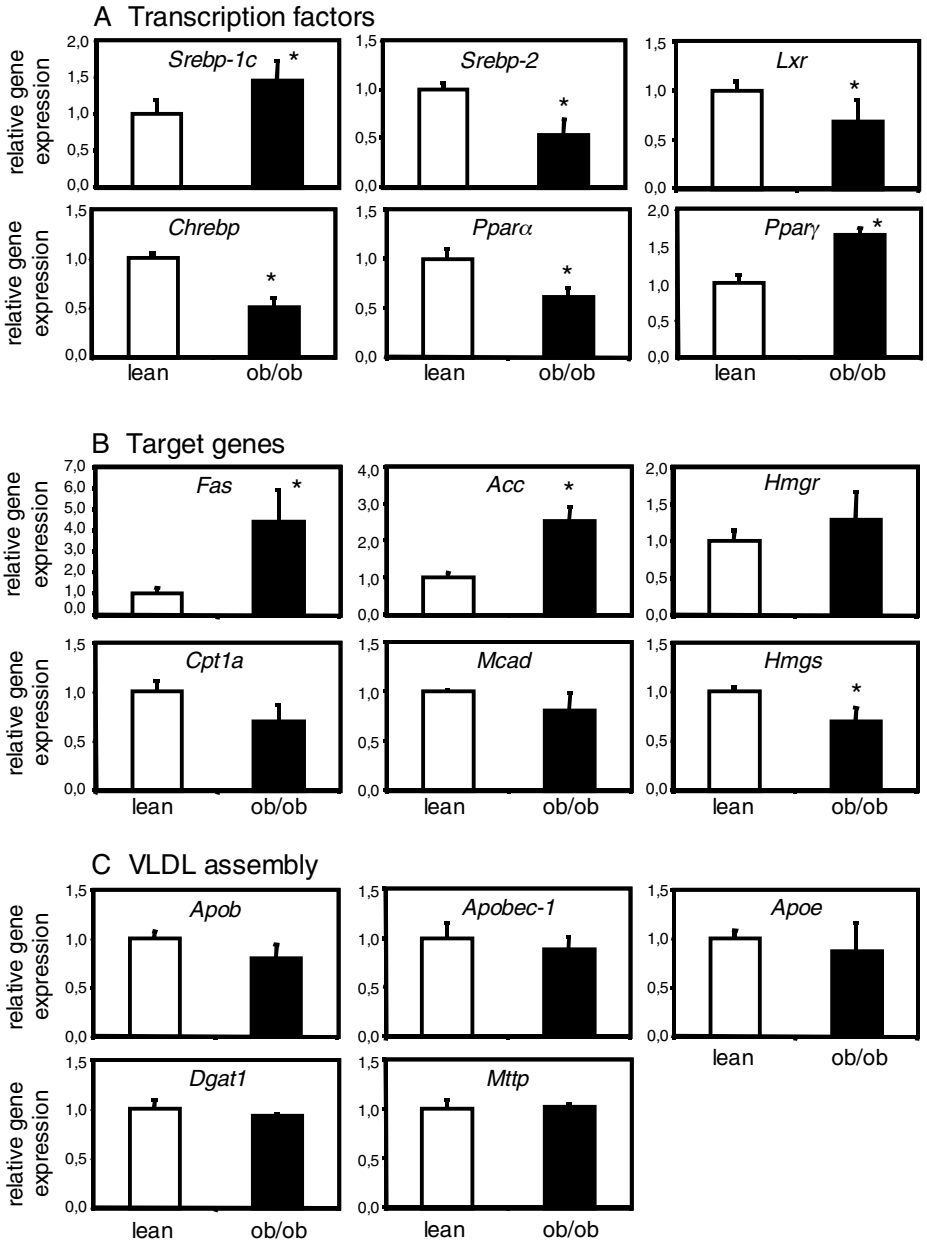
Expression levels of key genes involved in DNL, *i.e.*, fatty acid synthase (*Fas*), and acylCoA carboxylase (*Acc*) were clearly increased in livers of *ob/ob* mice. The mRNA levels of the transcription factor sterol regulatory element binding protein-1c (*Srebp-1c*) was also increased, but gene expression of liver X receptor (*Lxr*) and carbohydrate responsive element-binding protein (*Chrebp*), recently implicated in control of *Fas* expression, were significantly decreased. Expression level of the sterol regulatory binding

Table 3. Acetyl-CoA pool enrichment and fractional palmitate synthesis values of adipose tissue and liver and hepatic acetyl-CoA pool enrichment and fractional cholesterol synthesis values in [1-13C]-acetate-enriched diet fed lean and *ob/ob* mice.

	Lean	*ob/ob*
De novo lipogenesis		
Liver		
Acetyl-CoA pool enrichment (%)	6.3 ± 0.3	5.9 ± 0.8
Fractional hepatic palmitate (%)	31.0 ± 6.1	53.1 ± 4.7 *
Hepatic palmitate (μmol/liver)	1.1 ± 0.4	6.3 ± 0.9 *
Newly synthesized hepatic palmitate (μmol/liver)	0.3 ± 0.1	3.4 ± 0.6 *
Adipose tissue		
Acetyl-CoA pool enrichment (%)	1.6 ± 0.5	ND
Fractional synthesis adipose tissue palmitate (%)	21.5 ± 8.7	ND
Cholesterol synthesis		
Acetyl-CoA pool enrichment (%)	6.3 ± 0.1	5.5 ± 0.5
Fractional synthesis hepatic free cholesterol (%)	17.8 ± 4.0	12.6 ± 3.4 *
Hepatic free cholesterol (μmol/liver)	1.7 ± 0.1	2.7 ± 0.5 *
Newly synthesized hepatic cholesterol (μmol/liver)	0.3 ± 0.1	0.3 ± 0.2

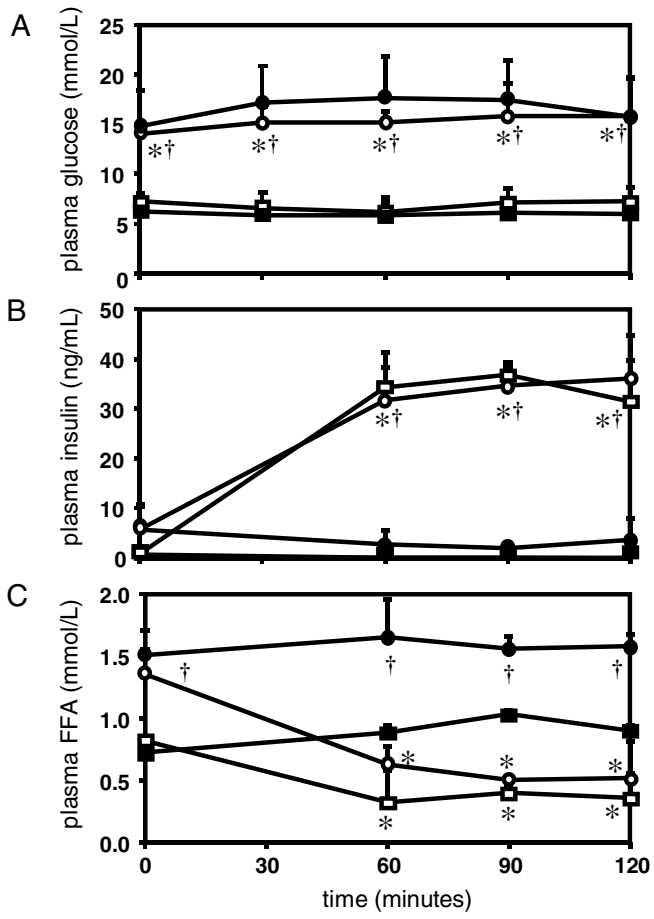
Data are means ± SD. n =5 lean and 5 *ob/ob* mice. * $P < 0.05$, Mann-Whitney U test.
ND = not detectable.

Figure 2. Relative hepatic expression levels of several transcription factors (A); their target genes in fatty acid synthesis, cholesterol synthesis and fatty acid oxidation (B); and genes involved in VLDL assembly (C) in lean (white bars) and *ob/ob* mice (black bars). Genes are relative to the hepatic β-Actin expression level. * $P < 0.05$, Mann-Whitney *U* test.



element protein-2 (*Srebp-2*), involved in control of cellular cholesterol homeostasis, was decreased in *ob/ob* mice liver but this did not result in reduced expression of its target gene HMGCoA reductase (*Hmgr*). Hepatic expression levels of the transcription factor peroxisomal proliferator-activated receptor (*PPAR*)-α was decreased but the expression level of PPAR-γ was increased in *ob/ob* mouse liver. Genes involved in ketogenesis and β-oxidation that are controlled by PPARα, *i.e.*, mitochondrial HMG-CoA-synthase (*Hmgs*), carnitine palmitoyl transferase-1a (*Cpt1a*) and medium chain acyl dehydrogenase (*Mcad*), tended to be decreased in *ob/ob* mice, suggesting a decreased β-oxidation in *ob/ob* mouse liver (Figure 2B).

Figure 3. Plasma glucose (A), insulin (B), and FFA (C) levels during a hyperinsulinemic clamp in lean mice receiving saline (closed squares) or insulin (open squares) and in *ob/ob* mice receiving saline (closed circles) or insulin (open circles). $n = 4$ per group. * $P < 0.05$ insulin effect, † $P < 0.05$ mouse strain effect, Mann-Whitney *U* test.



Hepatic VLDL production under basal conditions and hyperinsulinemic clamp
During the clamp, plasma glucose levels were fixed at fasting (9 h) plasma concentrations that were reached within 60 minutes. Average plasma glucose levels were 7 ± 1 mM and 15 ± 1 mM for lean and *ob/ob* mice, respectively (Figure 3A). Plasma insulin increased to stable levels of 34 ± 3 ng/ml and 34 ± 2 ng/ml in lean and *ob/ob* mice, respectively (Figure 3B). Saline-infused mice maintained their fasting insulin level during the clamp (0.7 ± 0.4 ng/ml and 5 ± 2 ng/ml in lean and *ob/ob* mice, respectively). Plasma FFA levels decreased in the insulin-infused mice only (Figure 3C). Although *ob/ob* mice showed higher basal plasma FFA concentrations, insulin reduced plasma FFA levels to a similar concentration as in lean mice within 60 minutes.

After 60 minutes of saline infusion or hyperinsulinemia, Triton WR1339 was injected to determine VLDL-TG and apoB100 production rates. Basal VLDL-TG production rates were similar in lean and *ob/ob* mice (64 ± 14 and 52 ± 7 µmol/kg/hr, respectively, Figure 4A). Acute hyperinsulinemia reduced VLDL-TG production rate to 27 ± 1 µmol/kg/hr (-58 %) in lean mice but only to 41 ± 1 µmol/kg/hr (-21 %) in *ob/ob* mice (Figure 4A). ApoB100 production showed a similar pattern as VLDL-TG production rates. Insulin suppressed apoB100 production much more pronounced in lean mice than in *ob/ob* mice (Figure 4B). The apoB100/B48 ratio in nascent VLDL particles, as determined by intensity scanning of Western blots (Figure 4E), was much higher in *ob/ob* mice than in lean controls: this ratio decreased upon insulin infusion.

Expression of genes encoding apolipoproteins involved in VLDL assembly and secretion, *i.e.*, apoB and apoE, were similar in liver of lean and *ob/ob* mice. In spite of the increased apoB100/B48 ratio in *ob/ob* mouse VLDL, expression of A*pobec-1*, encoding the *Apob* mRNA-editing protein, was not different between both groups indicating a posttranscriptional upregulation of editing activity in *ob/ob* mice. Expression of the genes encoding MTP and DGAT, essential for VLDL lipidation, did not differ between lean and *ob/ob* mice (Figure 2C).

Insulin signaling.
Hepatic mRNA levels of the insulin receptor (*Ir*) and insulin receptor substrate isoforms (*Irs1* and *Irs2*) were decreased in *ob/ob* mice (Figure 5A). Also, phosphorylation of IRβ, IRS-1 and IRS-2 proteins was reduced in *ob/ob* mice liver (Figure 5B), indicating decreased hepatic insulin signaling. IRS-3 phosphorylation was slightly increased in the *ob/ob* mouse liver (Figure 5B).

Discussion

The primary defect in the *ob/ob* mouse model is the absence of leptin, resulting in an obese and diabetic phenotype.[5] The intracellular signal transduction of leptin is similar to that of class 1 cytokine receptors and involves JAK-STAT signaling. Some of these receptors and possibly also leptin signaling can be linked to mitogen-activated protein kinase (MAPK)

Figure 4. VLDL-TG production rate (A), apoB100 production rate (B) and apoB100/B48 band-density-scan-ratio (C) during a hyperinsulinemic clamp in lean and *ob/ob* mice receiving saline (white bars) or insulin (black bars). Triglyceride accumulation curve (D) and a representative Western blot of apoB (E) during hyperinsulinemic clamp in lean and *ob/ob* mice are also shown. $n = 4$ per group. * $P < 0.05$ insulin effect, † $P < 0.05$ mouse strain effect, Mann-Whitney U test.

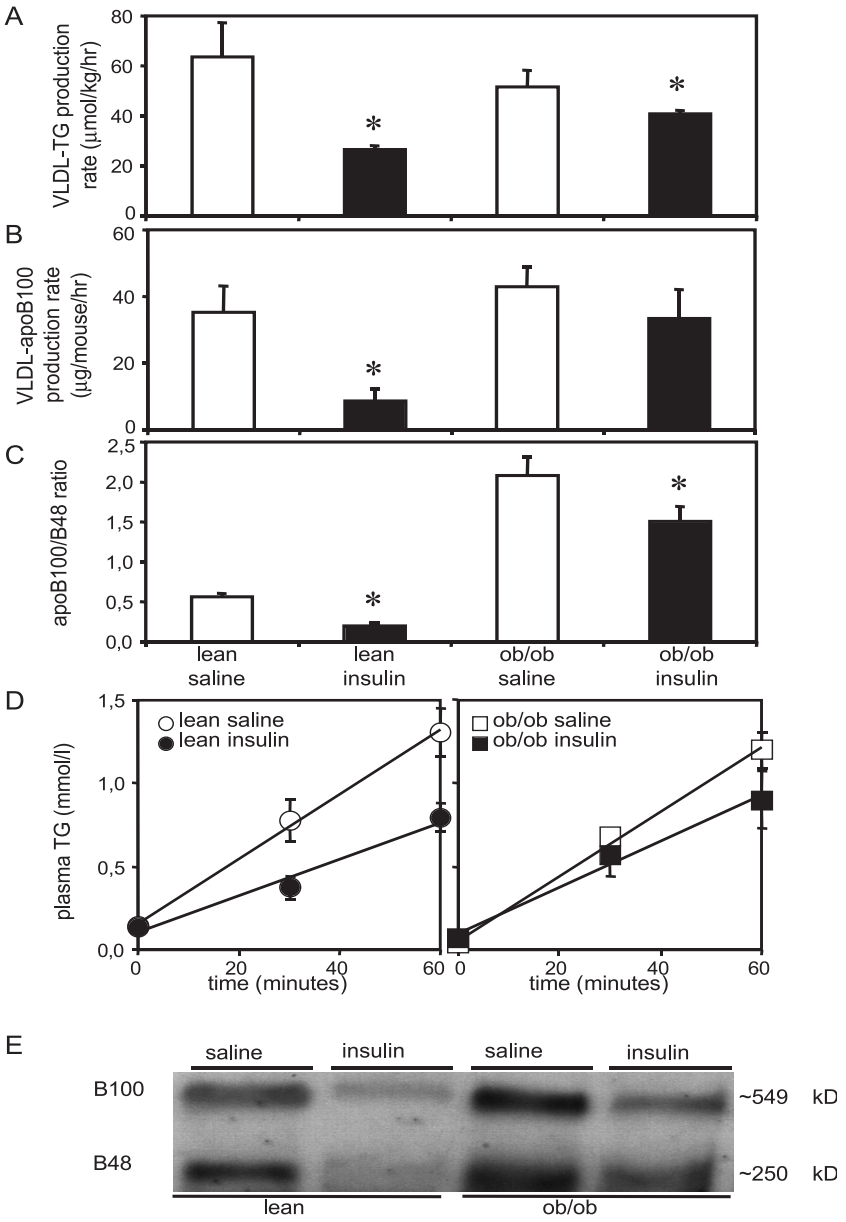
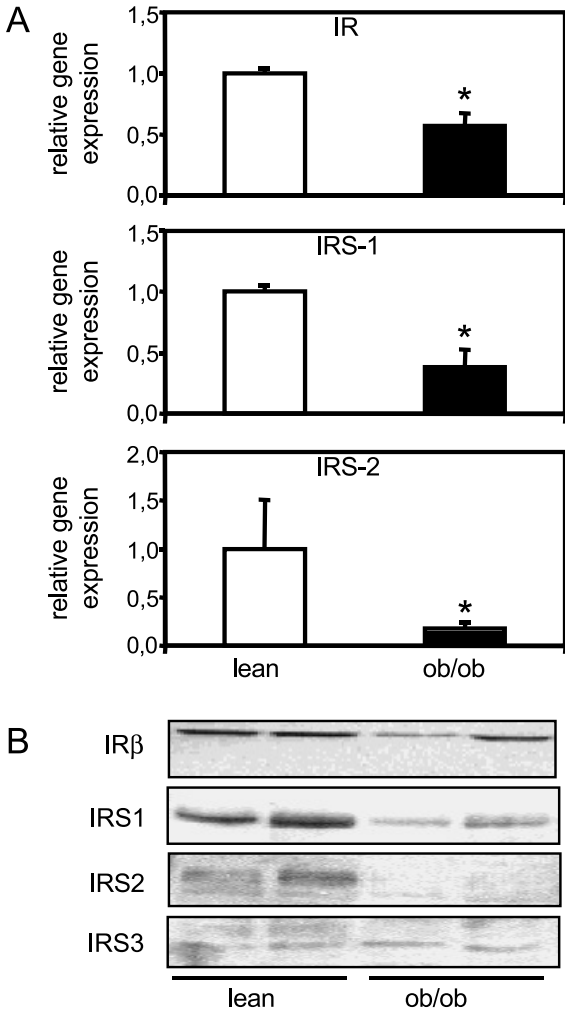


Figure 5. Hepatic gene expression levels (A) and phosphorylation (B) of the insulin receptor (IR) and insulin receptor substrate (IRS)-isoforms in lean and *ob/ob* mice. * $P < 0.05$, Mann-Whitney U test.



and phosphatidylinositol 3-kinase (PI3K) kinase pathways.[29,30] However, the metabolic relevance of leptin signaling via these pathways on hepatic fat and cholesterol metabolism and on VLDL production is currently not known. In this study, *ob/ob* mice showed increased plasma FFA levels, a 10-fold increase in hepatic *de novo* lipogenesis (DNL) and a severe, perivenously-localized, hepatic steatosis. Despite these diabetic characteristics, hepatic VLDL production was not increased under fasting conditions, as is the case in humans with insulin resistance or DM2.[31] The absence of a simultaneous up-

regulation of hepatic cholesterol synthesis, recognized as a crucial factor in control of VLDL production rates,[32-34] might contribute to this discordant phenotype. We further demonstrate that the VLDL production process in *ob/ob* mice was insensitive to the suppressive effects of insulin. This disturbance in the control of VLDL production is likely a result of impairment in the transduction pathway(s) of insulin: in livers of *ob/ob* mice IRβ, IRS-1 and IRS-2 gene expression levels and protein phosphorylation were clearly decreased. Similar decreases of hepatic and muscle IRS phosphorylation after insulin stimulation *in vivo* were observed in other studies.[35-37] It is well-established that insulin-mediated suppression of VLDL-apoB secretion in rodent liver cells requires PI3K activation.[38,39] Phosphatases might play a role in the sequence of events. The phosphotyrosine phosphatase-1B (PTP-1B) has been associated with insulin signaling in different models.[40,41] Whether this phosphatase is involved in defective insulin signaling in *ob/ob* mouse liver remains to be determined.

Female *ob/ob* mice have been used extensively in metabolic studies concerning hepatic (and muscle) insulin sensitivity.[8,42] To be able to compare our data with published work we chose to use female *ob/ob* mice for the current experiments. Importantly, studies by Li *et al.* indicate that there are no differences between male and female *ob/ob* mice regarding hepatic lipid deposition or VLDL triglyceride production. [13,18]

De novo lipogenesis (DNL), suggested as a regulator of VLDL production, was 10-fold increased in *ob/ob* liver. Expression of enzymes involved in lipogenesis are under control of at least three transcription factors, *i.e.*, SREBP-1c, LXR and CHREBP.[43-45] Interestingly, hepatic SREBP-1c expression levels were increased in *ob/ob* mice, whereas those of LXR and CHREBP were decreased, indicating that SREBP-1c is independently able to induce DNL. Since SREBP-1c expression is influenced by insulin[46] and insulin levels are elevated in *ob/ob* mouse, insulin may continuously induce expression of SREBP-1c and, thereby, of its target genes.[43] Thus, insulin resistance may not involve all branches of insulin signaling. Alternatively, leptin has been shown to be able to down-regulate SREBP-1c expression and protein levels and expression of its target gene (*Fas*) in *ob/ob* adipocytes[47] and in wildtype mouse liver.[48] $IRS2^{-/-}$ mice, like *ob/ob* mice, have increased hepatic SREBP-1c levels, which normalize upon leptin treatment.[49] Irrespective of the underlying mechanism, however, our results indicate that upregulated DNL per se is not a regulator of hepatic VLDL production by mouse liver.

In our clamp experiments, plasma insulin levels were similar in both groups. However, both groups were clamped at their basal glucose levels resulting in higher glucose levels in *ob/ob* mice. The question arises to which extent this may have influenced our results with respect to insulin sensitivity of VLDL-TG production. It could be argued that hyperglycemia may directly promote VLDL production. This effect might counteract inhibitory effects of insulin on VLDL-TG secretion. However, in the basal state VLDL production was not increased despite hyperglycemia in *ob/ob* mice, indicating that hyperglycemia is not an independent driving force for VLDL secretion in these animals. Therefore, it is rather unlikely that hyperglycemia per se underlies the profound insulin resistance of VLDL production.

Theoretically, it may be that TG and apoB, required for VLDL assembly, are functionally separated in the *ob/ob* liver. Using *in situ* hybridization, we found that *Apob* mRNA is present in all cells in the liver lobule, but with highest intensity in periportal hepatocytes of control mice. Funahashi *et al.*[50] reported a uniform distribution of *Apob* mRNA in rat liver, suggesting the existence of species-differences in this respect. In any case, our results suggest that perivenously localized TG in the *ob/ob* mouse liver may be less available for VLDL production.

Substrate availability has been proposed to regulate hepatic VLDL output.[32-34] Since the availability of plasma FFA, *de novo* synthesized FA and hepatic TG were all increased in *ob/ob* mice, it is unlikely that the supply of TG is rate-controlling in this respect. The availability of newly synthesized cholesterol may also influence VLDL formation, as has been shown in rats[32], rabbits[33], and humans[34]. Total hepatic cholesterol content in *ob/ob* mouse liver was increased but the absolute cholesterol synthesis rate, as determined by MIDA, was not different from that in lean mice. Cellular cholesterol homeostasis is controlled by SREBP-2. Sterol depletion induces cleavage of membrane-bound SREBP-2, allowing its translocation to the nucleus to induce expression levels of genes involved in cholesterol synthesis and uptake. Increased hepatic cholesterol levels were associated with decreased SREBP-2 expression in *ob/ob* mice, however, this did not lead to alterations in expression levels of HMGCoA reductase (Figure 2) or absolute cholesterol synthesis rates (Table 3). We are aware that our studies were performed under specific experimental conditions. Yet, we tend to hypothesize that limited availability of newly synthesized cholesterol may compromise the ability of the *ob/ob* mouse liver to remove excess TG in the form of VLDL under basal conditions. The resistance of the VLDL assembly/secretion process to the suppressive effects of insulin, however, must result from decreased insulin signaling. According to the current state knowledge,[38,39] it is highly likely that the PI3K pathway is defective in *ob/ob* mouse liver.

In conclusion, DNL is clearly increased in *ob/ob* mice, probably related to increased SREBP-1c expression levels and despite downregulation of LXR and CHREBP expression. Insufficient supply of newly synthesized cholesterol may become rate-controlling for VLDL production in *ob/ob* mice in a situation in which the supply of FA from plasma and DNL is excessive. Metabolic zonation of TG accumulation and apoB production may contribute in this respect. The inability to induce VLDL-production under these conditions, in combination with impaired hepatic β-oxidation, contributes to development of hepatic steatosis. Insulin signaling is clearly impaired in fatty livers of *ob/ob* mice, resulting in a decreased ability of insulin to suppress VLDL production.

Acknowledgements

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6

Enhanced glucose cycling and suppressed *de novo s*ynthesis of glucose-6-phosphate results in a net unchanged hepatic glucose output in *ob/ob* mice

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Diabetologia, in press

Abstract

Diabetes mellitus type 2 (DM2) is associated with insulin resistance and hyperglycemia. Leptin-deficient *ob/ob* mice are hyperinsulinemic and hyperglycemic and provide an appropiate model for DM2. The mechanisms behind the hyperglycemia in *ob/ob* mice has remained largely undefined sofar. To evaluate the effects of chronic leptin deficiency on glucose metabolism, *ob/ob* mice and lean littermates were fasted for 9 hours after which they were infused with [U-^{13}C]-glucose, [2-^{13}C]-glycerol, [1-^{2}H]-galactose and paracetamol for 6 h. Mass isotopomer distribution analysis was applied on blood glucose and urinary paracetamol-glucuronide. When expressed on the basis of body weight, endogenous glucose production (109 ± 23 vs. 152 ± 27 μmol/kg/min, obese vs. lean, $P <$ 0.01) and *de novo* synthesis of glucose-6-phosphate (122 ± 13 vs. 160 ± 6 μmol/kg/min, obese vs. lean, $P < 0.001$) were lower in *ob/ob* mice than in lean littermates. In contrast, glucose cycling was greatly increased in obese mice (56 ± 13 vs. 26 ± 4 μmol/kg/min, obese vs. lean, $P < 0.001$). As a result, total hepatic glucose output remained unaffected (165 ± 31 vs. 178 ± 28 μmol/kg/min, obese vs. lean, NS). Metabolic clearance rate was significantly lower in obese mice (8 ± 2 vs. 18 ± 2 ml/kg/min, obese vs. lean, $P < 0.001$). Hepatic mRNA levels of genes encoding for glucokinase and pyruvate kinase were markedly increased in *ob/ob* mice. Unaffected total hepatic glucose output in face of hyperinsulinemia reflects hepatic insulin resistance in *ob/ob* mice, which is associated with markedly increased rates of glucose cycling. However, hyperglycemia in *ob/ob* mice primary results from a decreased metabolic clearance rate of glucose.

Introduction

Hyperinsulinemia and fasting hyperglycemia are hallmarks of type 2 diabetes. Insulin resistance of peripheral organs, *i.e.,* muscle and adipocytes, as well as of the liver may contribute to fasting hyperglycemia. Peripheral insulin-resistance reduces the ability of peripheral organs to clear glucose from the circulation. Hepatic insulin-resistance develops in two stages. During early stages in the development of type 2 diabetes, characterized by hyperinsulinemia and normoglycemia, hepatic glucose production is still normal under fasting conditions. However, hepatic glucose production remains inappropriately high during absorptive phases when insulin concentrations are elevated. At later stages in the development of type 2 diabetes in humans, hepatic glucose production is increased also under fasting conditions.[1]

Both gluconeogenesis and glycogenolysis may contribute to an elevated hepatic glucose production. Furthermore, data indicates that cycling of glucose, *i.e.*, the process of sequential glucose phosphorylation by glucokinase (GK) and dephosphorylation by G6Pase, occurs at increased rates in humans with type 2 diabetes.[2,3] Little is known about the quantitative role of glucose cycling in the increased hepatic glucose production in type 2 diabetes. Depending on the methodologies used for quantification of hepatic glucose fluxes, increased glucose cycling may affect the reported rates of gluconeogenesis and glycogenolysis.

Ob/ob mice suffer from severe obesity and diabetes due to leptin-deficiency and provide a model for type 2 diabetes. These mice display age-dependent hyperglycemia and hyperinsulinemia. Quantitative data on the perturbations of glucose metabolism in these mice *in vivo* are scarce. *In vitro* studies in perfused isolated livers of *ob/ob* mouse showed that glycogen turnover was increased.[4] Lahtela *et al*. found greatly increased glucose cycling rates in hepatocytes isolated from 24-h fasted *ob/ob* mice.[5]

Novel methodologies using multiple stable isotopes *in vivo* now allow for determination of flux rates through the separate metabolic pathways involved in hepatic carbohydrate metabolism.[6-8] In the current study, we used these methods to evaluate the quantitative role of gluconeogenesis, glycogenolysis, and glucose cycling in hyperglycemia in modestly fasted *ob/ob* mice.

Methods

Animals

Female *ob/ob* mice (n = 7) and lean littermates (n = 7) were purchased from Harlan (Zeist, The Netherlands) and were housed in a temperature-controlled (21°C) room on a 12-hr dark, 12-hr light cycle. Experimental procedures were approved by the Ethics Committee for Animal Experiments of the State University Groningen. Body weights were 22.7 ± 1.2 g for the lean mice and 49.7 ± 3.0 g for the *ob/ob* mice. Mice were equipped with a permanent

catheter in the right atrium, via the right jugular vein as described previously.[9] Mice were allowed to recover from surgery for at least 4 days.

Materials

The following isotopes were used: [2-^{13}C]-glycerol (99% ^{13}C APE), [1-^{2}H]-galactose (98% ^{2}H APE) (Isotec Inc., Miamisburg, OH, USA), [U-^{13}C]-glucose (99% ^{13}C APE) (Cambridge Isotope Laboratories, Inc., Andover, MA, USA). All chemicals used were reagent pro analysis grade. Bloodspots and urine were collected on Schleicher en Schuell No. 2992 filter paper (Schleicher en Schuell, 's Hertogenbosch, The Netherlands). Infusates were freshly prepared and sterilized by the Hospital Pharmacy at the day before the experiment.

Animal experiments

Experiments were performed in awake, chronically-catheterized mice, essentially as described by Van Dijk *et al.*[10] Mice were fasted for 9 h after which they were placed in metabolic cages to allow frequent collection of bloodspots and urine. Mice were infused with a sterile solution, containing [U-^{13}C] glucose (13.9 μmol.ml^{-1}), [2-^{13}C]glycerol (160 μmol.ml^{-1}), [1-^{2}H]galactose (33 μmol.ml^{-1}) and paracetamol (1.0 mg.ml^{-1}) at a rate of 0.6 ml.hr^{-1}. During the experiment, blood glucose was measured using EuroFlash™ test strips (LifeScan Benelux, Beerse, Belgium). Bloodspots were collected on filterpaper before the start of the infusion and hourly afterwards until 6h after the start of the infusion. Blood spots were air-dried and stored at room temperature until analysis. Timed urine samples were collected at hourly intervals on filter paper strips. Strips were air-dried and stored at room temperature until analysis. At the end of the experiment animals were anesthetized with isofurane and a large blood sample was collected in heparin-containing tubes by heart puncture, centrifuged immediately and stored at –20 °C until analysis. The liver was quickly excised, weighed and frozen immediately in liquid N_2.

Metabolite concentrations

Plasma was isolated from blood by centrifugation and liver tissue was homogenized. Plasma β-hydroxybutyrate, lactate, free fatty acid were determined using commercially available kits (Roche Diagnostics, Mannheim, Germany and Wako Chemicals GmbH, Neuss, Germany). Plasma insulin levels were determined with a radioimmunoassay (RI-13K, Linco Research, St. Charles, MO). Total liver protein content was determined according to Lowry *et al.*[11] Hepatic glycogen was determined after extraction with a 1 mol/l KOH solution by sonication. The extract was incubated for 30 minutes at 90 ºC, cooled and brought to pH 4.5 by addition of 3 mol/l acetic acid. Precipitated protein was removed by centrifugation. Glycogen was converted to glucose by treating the samples with amyloglucosidase, followed by assay of glucose at pH 7.4 with ATP, $NADP^+$, hexokinase and G6P dehydrogenase. Liver samples for the determination of G6P were treated by sonification in a 5% (w/v) $HClO_4$ solution. Precipitated protein was removed by rapid centrifugation at 10.000 rpm for 1 min in a cold microcentrifuge and the supernatant

Table 1. List of sequences of primers and probes used in PCR measurements.

Gene	Sequence		GenBank no.
β-actin	Forward	ACC CAC ACT GTG CCC ATC TAC	NM007393
	Reverse	GCT CGG TCA GGA TCT TCA TGA	
	Probe	AGG GCT ATG CTC TCC CTC ACG CCA	
18S rRNA	Forward	CGG CTA CCA CAT CCA AGG A	X00686
	Reverse	CCA ATT ACA GGG CCT CGA AA	
	Probe	CGC GCA AAT TAC CCA CTC CCG A	
G6ph	Forward	CTG CAA GGG AGA ACT CAG CAA	NM008061
	Reverse	GAG GAC CAA GGA AGC CAC AAT	
	Probe	TGC TCC CAT TCC GCT TCG CCT	
G6pt	Forward	GAG GCC TTG TAG GAA GCA TTG	NM008063
	Reverse	CCA TCC CAG CCA TCA TGA GTA	
	Probe	CTC TGT ATG GGA ACC CTC GCC ACG	
Gk	Forward	CCT GGG CTT CAC CTT CTC CTT	NM010292
	Reverse	GAG GCC TTG AAG CCC TTG GT	
	Probe	CAC GAA GAC ATA GAC AAG GGC ATC CTG CTC	
Gp	Forward	GAA GGA GGC AAA CGG ATC AAC	NM133198
	Reverse	TCA CGA TGT CCG AGT GGA TCT	
	Probe	CCT CTG CAT CGT GGG CTG CCA	
Gs	Forward	GCT CTC CAG ACG ATT CTT GCA	NM145572
	Reverse	GTG CGG TTC CTC TGA ATG ATC	
	Probe	CCT CTA CGG GTT TTG TAA ACA GTC ACG CC	
Pk	Forward	CGT TTG TGC CAC ACA GAT GCT	NM013631
	Reverse	CAT TGG CCA CAT CGC TTG TCT	
	Probe	AGC ATG ATC ACT AAG GCT CGA CCA ACT CGG	
Pepck	Forward	GTG TCA TCC GCA AGC TGA AG	NM011044
	Reverse	CTT TCG ATC CTG GCC ACA TC	
	Probe	CAA CTG TTG GCT GGC TCT CAC TGA CCC	

was neutralized to pH 7 by addition of small amounts of a mixture of 2 mol/l KOH and 0.3 mol/l MOPS. G6P was determined fluorimetrically with $NADP^+$ and G6P dehydrogenase.

Hepatic mRNA levels

Total RNA was isolated from liver tissue using the Trizol method (Invitrogen, Paisley, United Kingdom). RNA was converted to cDNA with M-Mulv-RT (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol using random primers. cDNA levels of the genes of interest were measured by real-time PCR using the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). An

amount of cDNA corresponding to 20 ng of total RNA was amplified using the qPCR core kit (Eurogentec, Seraing, Belgium) according to the manufacturer's protocol using the appropiate forward and reverse primers (Invitrogen, Paisley, United Kingdom), and a template specific 3'-TAMRA, 5'-FAM labeled Double Dye Oligonucleotide probe (Eurogentec, Seraing, Belgium). Calibration curves were run on serial dilutions of pooled cDNA solutions as used in the assay. The data were processed using the ABI Sequence Detector v1.6.3 (System Applied Biosystems, Foster City, CA, USA). Quantified expression levels were within the linear part of the calibration curves. PCR results were normalized by S18-rRNA levels. The sequence of the primers and probes used in this study are listed in Table 1.

Measurement and Analysis of Mass Isotopomer Distribution by GC -MS

Analytical procedures for extraction of glucose and paracetamol-glucuronide (Par-GlcUA) from bloodspot and urine filterpaper strips, respectively, derivatization of the extracted compounds and GC-MS measurements of derivatives were essentially performed according to Van Dijk *et al.*[8,10] The measured fractional isotopomer distribution by GCMS was corrected for the fractional distribution due to natural abundance of ^{13}C, by multiple linear regression as described by Lee *et al.*[6] to obtain the excess mole fraction of mass isotopomers due to incorporation and dilution of infused labelled compounds, *i.e.,* [2-^{13}C]-glycerol, [U-^{13}C]-glucose and [1-^{2}H]-galactose. This distribution was used in mass isotopomer distribution analysis (MIDA) algorithms of isotope incorporation and dilution according to Hellerstein *et al.*[7] as described by Van Dijk *et al.*[8,10]

Statistical analysis

All values reported are mean ± SD. Levels of significance of difference of metabolite concentrations, gene expression and the values of the individual timepoints during isotope infusion experiments were determined using the non-parametric Mann Whitney test for unpaired data. Levels of significance of differences between the averages of the values of the fluxes at the individual timepoints between 3 and 6 h during the experiment were estimated using ANOVA with repeated measurements. Differences were considered significant at $P < 0.05$.

RESULTS

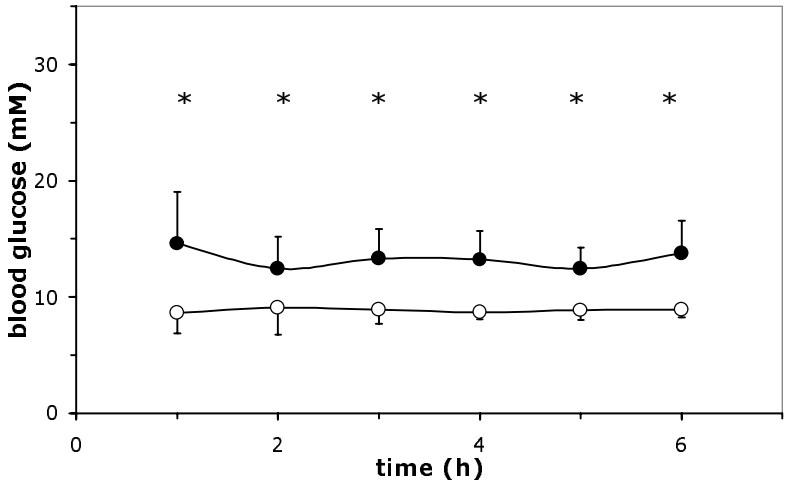
Body and liver weights of obese mice were more than double than those of lean mice (Table 2). Consequently, normalization of liver weight to body weight yielded no difference between obese and lean mice. Protein contents per gram of liver tissue were not significantly different between obese and lean mice. Hepatic glycogen content was mildly increased in the *ob/ob* mice, whereas glucose-6-phosphate (G6P) levels were not significantly different between *ob/ob* and lean mice. Plasma NEFA concentrations were almost two-fold higher in the *ob/ob* mice than in lean mice at the end of the experiment.

Plasma lactate and alanine concentrations were similar in the two groups but plasma β-hydroxybutyrate concentrations were elevated in *ob/ob* mice.

Table 2. Hepatic and plasma parameters in *ob/ob* mice and lean littermates (n = 7/group), * $P < 0.05$.

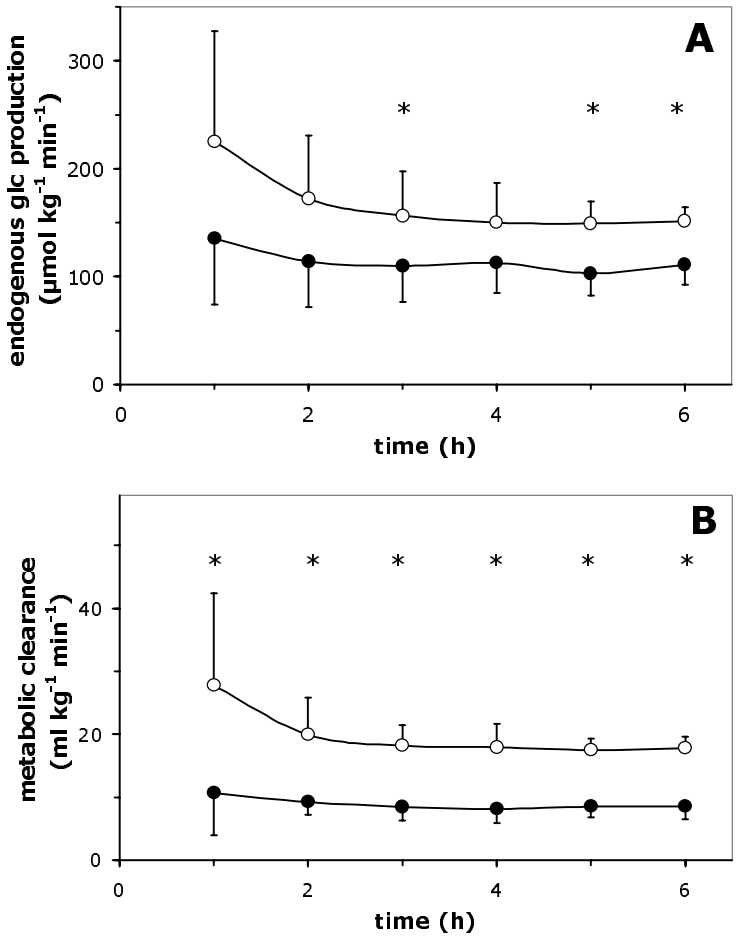
	lean	*ob/ob*
Liver		
Body weight (g)	23 ± 1	50 ± 3 *
Liver weight (g)	1.1 ± 0.0	2.6 ± 0.4 *
Relative liver weight (% body weight)	4.7 ± 0.3	5.0 ± 0.8
Total liver protein (mg)	156 ± 20	325 ± 63 *
Liver protein content (mg protein/g liver weight)	144 ± 18	127 ± 18
G6P (nmol/g liver weight)	118 ± 56	153 ± 34
Glycogen (μmol glucose/g liver weight)	179 ± 16	207 ± 11 *
Plasma		
NEFA (mM)	0.5 ± 0.1	0.9 ± 0.2 *
3-Hydroxybutyrate (mM)	0.8 ± 0.4	3.1 ± 1.4 *
Alanine (μM)	133 ± 113	176 ± 33
Lactate (mM)	3.5 ± 1.0	3.5 ± 1.0

Figure 1. Plasma glucose concentrations during experiments in female lean (open circles) and *ob/ob* mice (closed circles). Each value represents the mean ± SD; n = 7, * $P < 0.05$ compared to lean animals.



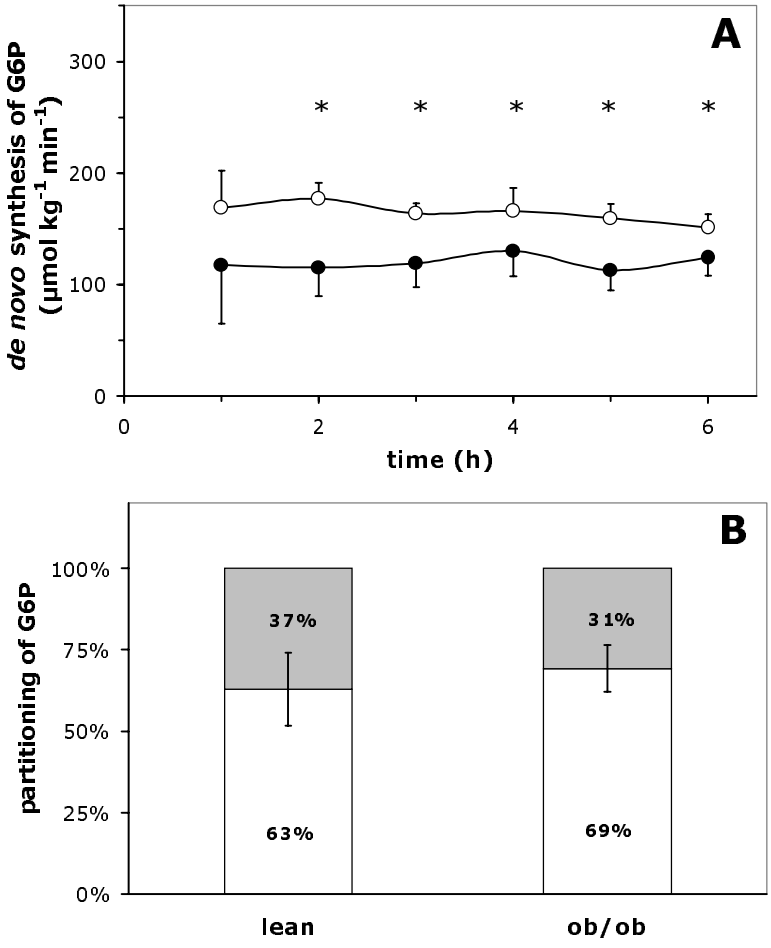
The time course of blood glucose concentrations during experiments are shown in Figure 1. Obese mice were clearly hyperglycemic (8.8 ± 0.5 *vs.* 13.2 ± 1.9 mM, lean *vs.* obese, $P < 0.05$). Glucose concentrations remained constant during the experiment in both groups. Furthermore, obese mice were clearly hyperinsulinemic. Insulin concentrations remained constant (45 ± 10 pM at t = 0 and 60 ± 45 pM at t = 6 h) during the experiment in lean mice but decreased from 900 ± 480 pM at t = 0 to 435 ± 270 pM at t = 6 h in *ob/ob* mice. In Figure 2, endogenous glucose production (2A) and metabolic clearance rates of glucose (2B) are shown. At isotopic steady-state, *i.e.,* between 3 and 6 h after the start of the infusion of labeled compounds, whole body and hepatic glucose metabolism was evaluated. As is clear from this Figure, endogenous glucose production was significantly

Figure 2. Endogenous glucose production (A) and metabolic clearance (B) during the infusion experiment in lean (open circles) and *ob/ob* (closed circles) mice. Each value represents the mean ± SD; $n = 7$. * $P < 0.05$ compared to lean animals.



decreased in *ob/ob* mice (152 ± 27 *vs.* 109 ± 23 µmol.kg^{-1}.min^{-1}, lean *vs.* obese, $P < 0.001$) as was the metabolic clearance rate of glucose (18 ± 2 *vs.* 8 ± 2 ml.kg^{-1}.min^{-1}, lean *vs.* obese, $P < 0.001$). The rate of *de novo* synthesis of G6P in obese mice was significantly decreased in comparison with lean control mice (160 ± 6 *vs.*122 ± 13 µmol.kg^{-1}.min^{-1}, lean *vs.* obese, $P < 0.001$, Fig 3A). Partitioning of newly synthesized G6P towards plasma glucose or glycogen was not affected in obese mice when compared to lean control mice (Fig 3B). In contrast, glucose cycling was greatly enhanced (Fig 4), *i.e.*, 56 ± 13 µmol.kg^{-1}.min^{-1} in obese mice and only 26 ± 4 µmol.kg^{-1}.min^{-1} in lean control animals ($P < 0.001$).

Figure 3. The total rate of *de novo* synthesis of G6P in lean (open circles) and *ob/ob* (closed circles) mice (A) and the partitioning of the rates of *de novo* synthesis of G6P to UDP-glucose (black), and to glucose (white) (B) during last 3 h of the infusion experiment in lean and *ob/ob* mice. Each value represents the mean ± SD; $n = 7$. * $P < 0.05$ compared to lean animals.



As a consequence, total hepatic glucose production, *i.e.* the sum of endogenous glucose production and glucose cycling, was similar in obese and lean mice (178 ± 28 *vs.* 165 ± 31 μmol.kg^{-1}.min^{-1}, lean *vs.* obese, NS , Fig 4B).

Figure 4. Rate of cycling between glucose and G6P during the infusion experiment in lean (open circles) and *ob/ob* (closed circles) mice (A) and the contributions of endogenous glucose production (white) and glucose cycling (black) to the total endogenous glucose production in lean and *ob/ob* mice, during the last 3 h of the infusion experiment. Each value represents the mean ± SD; $n = 7$. * $P < 0.05$ compared to lean animals.

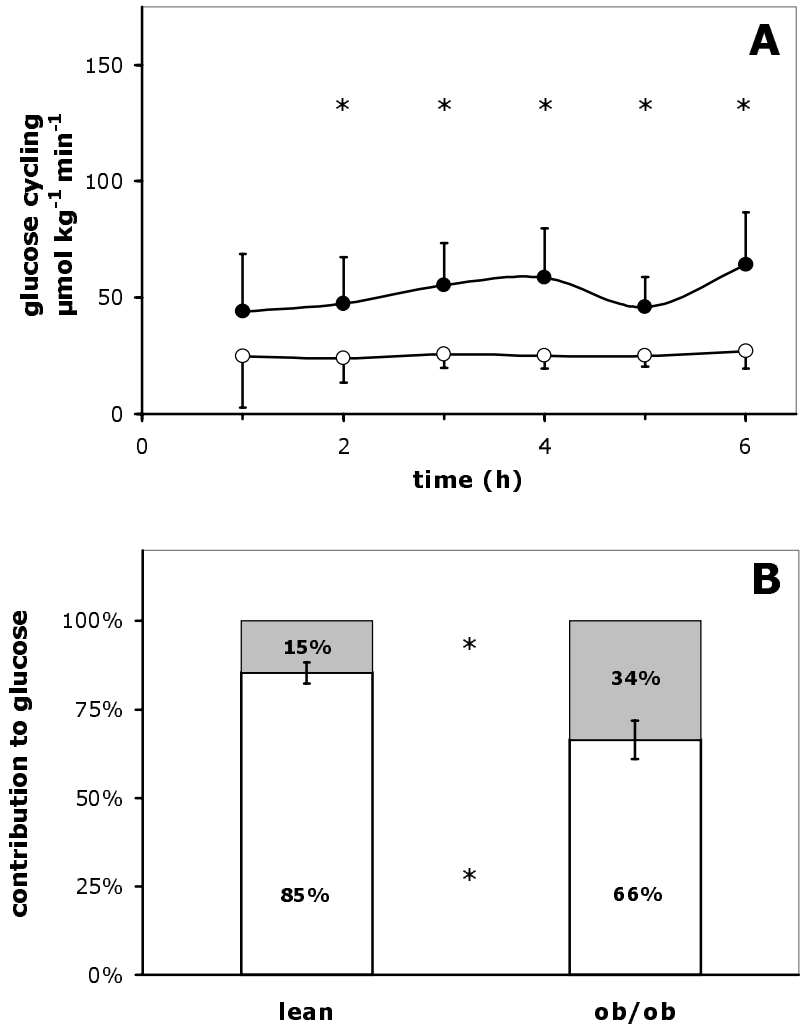
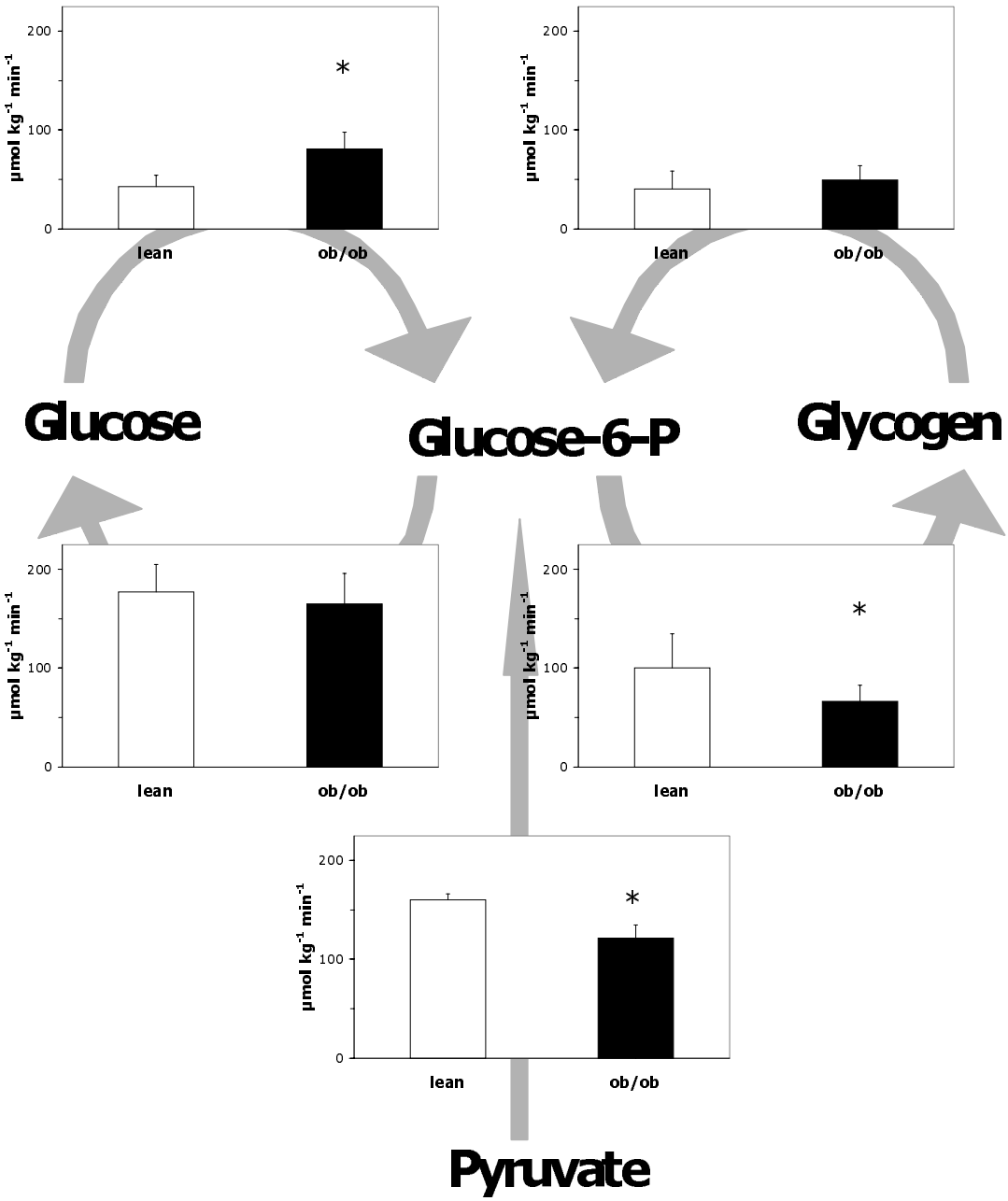


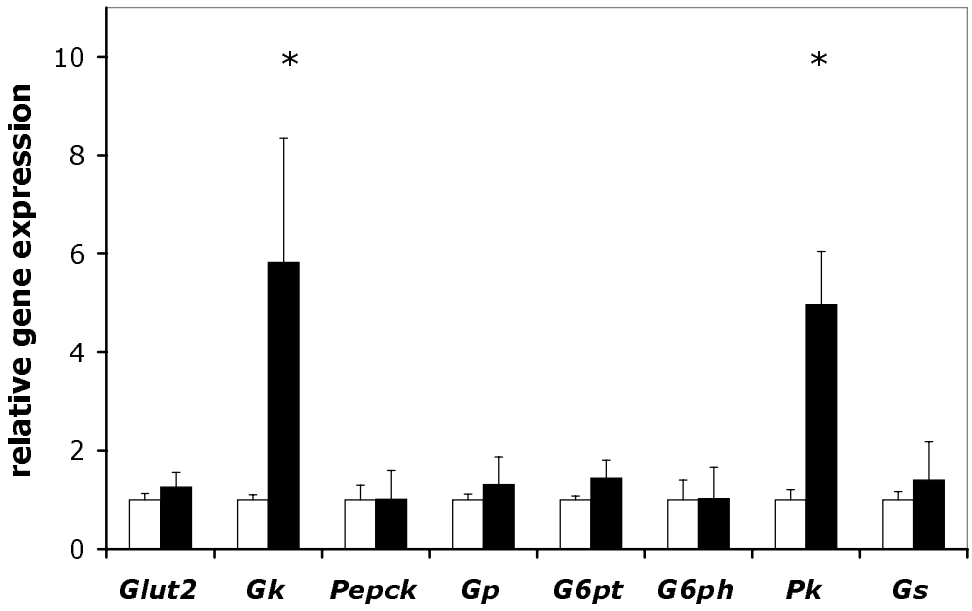
Figure 5. Separate fluxes through the relevant enzymic pathways in hepatic carbohydrate metabolism during the last 3 h of the infusion experiment in lean and *ob/ob* mice. Each value represents the mean ± SD; $n = 7$. * $P < 0.05$ compared to lean animals.



In Figure 5 the calculated mean values obtained at steady-state are shown for the individual fluxes through the various enzymic pathways of hepatic glucose metabolism. As anticipated based on the strong increase of glucose cycling, the calculated isotopic flux through GK was strongly increased in obese mice. The G6Pase flux, equivalent to the total hepatic glucose production, was unchanged. The flux through glycogen phosphorylase was not altered significantly whereas the glycogen synthase flux was significantly decreased in obese mice in comparison with lean littermates.

Expression of relevant genes in livers of lean and *ob/ob* mice are shown in Figure 6. Expression of the genes encoding *Gk* and *Pk* were strongly up-regulated in *ob/ob* livers.

Figure 6. Gene expression of enzymes involved in glucose metabolism at the end of the infusion expriment in lean and *ob/ob* mice, $n = 3$ per group. Levels of cDNA were measured by real-time PCR as described in the Methods section. Data are expressed relative to *18S* rRNA and the results of the lean animals are set equal to 1. * $P < 0.05$ compared to lean animals. *Glut 2,* glucose transporter 2; *Gk*, glucokinase; *Pepck,* phosphoenolpyruvate carboxykinase; *Gp*, glycogen phosphorylase; *G6pt*, glucose-6-phosphate translocase; *G6ph*, glucose-6-phosphate hydrolase; *Pk*, pyruvate kinase; *Gs*, glycogen synthase. Expression of genes were normalized by *18S*-rRNA, since the level of *β-actin* mRNA in livers of obese mice was increased by ~30% when normalized by liver weight. In contrast, *18S*-rRNA levels were similar in livers of obese and lean mice.



The mRNA levels of other key genes involved in carbohydrate metabolism, *i.e. Pepck*, *G6ph* and *G6pt* did not differ significantly betweem obese and lean mice. Expression levels of *Srebp-1c* and *Pppar*γ, transcription factors involved in control of hepatic glucose and fat metabolism, were significantly elevated in livers of *ob/ob* mice. As shown previously[12], mRNA levels of *Irs-1*, but particularly *Irs-2*, were strongly repressed in livers of obese mice in comparison with lean littermates.

DISCUSSION

The leptin-deficient *ob/ob* mouse is a commonly used mouse model of type 2 diabetes, but quantitative *in vivo* data on the disturbances that underly hyperglycemia in this model are sparse. In this study, we determined flux rates through various enzymic pathways, relevant in hepatic carbohydrate metabolism, in *ob/ob* and lean mice. When expressed on the basis of body weight, activity of hepatic glucose metabolism was, in general, supressed in obese mice in comparison with their lean littermates. One exception was glucose cycling. This flux was greatly increased in obese mice. Interestingly, the newly produced G6P was not preferentially directed towards plasma glucose in *ob/ob* mice but partitioned to glycogen stores to a similar extent as in lean mice. Furthermore, expression of genes of key enzymes involved in glucose metabolism was only different for *Gk* and *Pk*, indicating enhanced glycolysis in *ob/ob* mice.

Before discussing the results, some methodological issues have to be addressed. In this study, a multiple isotope infusion protocol according to Hellerstein *et al.*[7] was used to calculate the relevant fluxes of glucose metabolism. The validity of the isotope model, with the application of glycoconjugates, and the MIDA approach has been substantiated in various studies although some controversy still remains.[13,14] We have validated the application of MIDA in 9 h fasted C57Bl6/J mice in a separate study.[10] In the current study, metabolic fluxes were compared between groups of mice with strongly different body compositions. Flux rates can be either normalized to body weight or to lean body mass. Lean body mass is slightly less in *ob/ob* mice in comparison with lean littermates while fat mass is disproportionally increased.[15] Because of the following reasons, we have used the first approach for our calculations. Firstly, adipose tissue actively metabolises glucose. Therefore, it contributes to peripheral glucose clearance which is not accounted for when flux rates are normalized to lean body mass. Secondly, we noticed that relative liver weights *i.e.*, normalized to body weight, in obese and lean mice did not differ significantly. Furthermore, similar protein contents were measured in livers of obese and lean mice. This indicates, to our opinion, that irrespective of the increased fat content of livers of *ob/ob* mice, the increase in liver weight is not associated with an increase in metabolically active cell mass. Based on these considerations we felt that, in this particular case, comparison between *ob/ob* mice and lean littermates should be done on the basis of flux rates normalized to body weight.

We found that the flux through G6Pase, reflecting total hepatic glucose output, was not affected in *ob/ob* mice in comparison to lean mice. In the calculation of total endogenous glucose production the isotopic model considers 3 contributions *i.e.*, (1) *de novo* synthesis of G6P, partitioned to plasma glucose, (2) glycogen breakdown and (3) glucose recycling. The unaffected G6Pase flux was observed in the face of hyperinsulinemia and hyperglycemia. Probably as a consequence of hepatic insulin resistance, *de novo* synthesis of G6P was diminished by only 30% in *ob/ob* mice when compared to lean littermates. Furthermore, it is important to note that autoregulation of hepatic glucose output, that is normally observed in the presence of high glucose concentrations in plasma, apparently malfunctions in *ob/ob* mice because normal rates of endogenous glucose production were observed. In livers of *ob/ob* mice a major contribution to the G6Pase flux could be assigned to glucose cycling, the process of concomitant glucose phosphorylation by GK and dephosphorylation by G6Pase. Glucose cycling was increased by a factor of ~2.5 in livers of *ob/ob* mice. Lahtela *et al*.[5] also observed a high rate of glucose cycling in hepatocytes isolated of livers from fasted *ob/ob* mice. Blood glucose concentrations in *ob/ob* mice during the experiment were 13.2 ± 1.9 M compared to 8.8 ± 0.5 mM in lean littermates, an increase by a factor of 1.5, *i.e.*, well below the relative increase in glucose recycling.

Glycogen metabolism in livers of *ob/ob* mice did not differ much from that observed in lean littermates, with the exception of a relatively small decrease in the flux through GS. This represents another hallmark of hepatic insulin resistance in *ob/ob* mice. The role of leptin in hepatic glycogen metabolism is still controversial. *In vivo* infusion of leptin into fasted Wistar rats suppressed the contribution of glycogenolysis to hepatic glucose production.[16] Hyperleptinemia in Wistar rats, brought about by recombinant adenovirus gene delivery, had a glycogen sparing effect.[17]

Besides hepatic insulin resistance, peripheral organs were also found to be insulin resistant in *ob/ob* mice. Metabolic clearance of plasma glucose was found to be decreased by a factor of ~2, at blood glucose concentrations that was almost double that in lean mice. This indicates that net glucose uptake by peripheral tissue was similar in *ob/ob* and lean mice, irrespective of the elevated insulin concentrations in the first group. As a consequence, it can be concluded that hyperglycemia in *ob/ob* mice is mainly related to peripheral insulin resistance. These considerations are in agreement with an earlier publication showing peripheral insulin resistance in *ob/*ob mice by different means: uptake of 2-deoxyglucose was severely inhibited in isolated skeletal muscle of obese mice in comparison to muscle from lean mice.[18]

As discussed, we observed “normal” rates of total glucose output and high rates of glucose cycling which could not be explained by hyperglycemia *per se*. In accordance with these observations, “normal” mRNA levels of the genes encoding the gluconeogenic enzymes *G6ph* and *Pepck* were observed, while mRNA levels of *Gk* were significantly increased in livers of obese mice when compared to lean littermates. Similarly, in an earlier publication GK activity was found to be increased in isolated livers of fasted *ob/ob* mice.[19] Concommitantly, we observed a significant increase in mRNA levels of *Srebp-1c*

and its target genes in liponeogenesis, *i.e.*, *Fas* and *Acc1* in livers of fasted *ob/ob* mice, in accordance with Wiegman *et al.*[12] and Shimomura *et al.*[20] In view of the observed hepatic insulin resistance in *ob/ob* mice, it is of interest to note that recently data became available indicating that hyperglycemia *per se* was able to induce increased expression of *Srebp-1c* and the glycolytic enzyme *Pk* in an insulin-independent way.[21]

It is tempting to speculate that high rates of glucose cycling observed in livers of *ob/ob* mice reflect a consequence of an inappropriate co-localization of GK and G6Pase activities in hepatocytes. The liver exhibits a marked metabolic heterogeneity along the radius of the hepatic lobule. Activities of enzymes involved in hepatic glucose metabolism are differentially distributed along the acinar porto-central axis. During fasting and refeeding these gradients changes reciprocally. During fasting the gradients of PEPCK and G6Pase extent from the periportal into the perivenous zone while GK activity gradient is largely confined to the perivenously located hepatocytes only. During refeeding the opposite takes place.[22] In this way, co-localization of concurrent enzymic reactions in hepatocytes is minimized. In livers of fasted *ob/ob* mice this mechanism appeared to be perturbed. Levels of mRNA of *Gk* remained high during fasting which, to our opinion, can be considered an indication for a persistent perivenous-periportal activity gradient of GK. On the other hand, mRNA levels of *Pepck* and *G6ph* in livers of fasting *ob/ob* mice were similar to those observed in lean littermates, pointing to a periportal-perivenous activity gradient of these enzymes in livers of *ob/ob* mice as normally observed in livers of fasting wild type mice. As a result considerable overlap occurs of GK and G6Pase activities in hepatocytes of *ob/ob* mice. The mechanism by which *Gk* mRNA is maintained at high levels during fasting in *ob/ob* mouse liver remains elusive, but is likely related to the constitutively high expression of SREBP-1c.

In humans with diabetes mellitus type 2, evidence for contributions of both induced gluconeogenesis and glycogenolysis to fasting hyperglycemia has been found.[23-28] However, untill now only very few studies have explicitely considered the quantitative role of glucose cycling in hepatic glucose production. There are indications to suggest that hepatic cycling of glucose is elevated in humans with type 2 diabetes.[2,3] If glucose cycling is indeed a major contributor to the elevated G6Pase flux in type 2 diabetes, as we observed in *ob/ob* mice, this would lead to an overestimation of both gluconeogenic flux, measured by $^{2}H_2O$ method and glycogenolytic flux, measured by ^{13}C-MRS in these patients. Furthermore, in most studies with (often obese) diabetic subjects data was normalized to lean body mass instead of bodyweight, leading to inappropriately elevated values for gluconeogenic and glycogenolytic fluxes when compared to non-diabetic subjects.

In conclusion, this study demonstrates that in *ob/ob* mice *de novo* synthesis of glucose-6-phosphate was diminished while glucose cycling was increased, resulting in a “normal” total glucose output by the liver. However, these normal values were observed in face of hyperglycemia and hyperinsulinemia. This points to a co-existence of hepatic and peripheral insulin resistance with peripheral insulin resistance as the major cause of hyperglycemia.

Acknowledgements

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Hepatic *de novo* synthesis of glucose-6-phosphate is not affected in PPARα-deficient mice but is preferentially directed towards hepatic glycogen stores after a short-term fast

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Abstract

Apart from impaired β-oxidation, Pparα-deficient (Pparα$^{-/-}$) mice suffer from hypoglycemia during prolonged fasting, suggesting alterations in hepatic glucose metabolism. We compared hepatic glucose metabolism *in vivo* in wild-type (WT) and *Pparα*$^{-/-}$ mice after a short-term fast, applying novel isotopic methods. After a 9h fast mice were infused with [U-^{13}C] glucose, [2-^{13}C]glycerol, [1-^{2}H]galactose and paracetamol for 6 h and blood and urine was collected in timed intervals. Plasma glucose concentrations remained constant and were not different between the groups. Hepatic glycogen content was 69 ± 11 and 90 ± 31 µmol/g liver after 15 hr of fasting in WT and *Pparα*$^{-/-}$ mice, respectively. The gluconeogenic flux towards glucose-6-phosphate was not different between the groups, *i.e.*, 157 ± 9 and 153 ± 9 µmol/kg/min in WT and *Pparα*$^{-/-}$ mice, respectively. The gluconeogenic flux towards plasma glucose, however, was decreased in *Pparα*$^{-/-}$ mice, *i.e.*, 142 ± 9 vs 124 ± 13 µmol/kg/min ($P < 0.05$), accounting for the observed decrease (-15 %) in hepatic glucose production in *Pparα*$^{-/-}$ mice. Expression of the gene encoding glucose-6-phosphate hydrolase (G6ph) was lower in the *Pparα*$^{-/-}$ mice compared to WT mice. In conclusion, *Pparα*$^{-/-}$ mice were able to maintain a normal total gluconeogenic flux to glucose-6-phosphate during moderate fasting, despite their inability to upregulate β-oxidation. However, this gluconeogenic flux was directed more towards glycogen leading to a decreased hepatic glucose output. This was associated with a downregulation of the expression of G6ph in *Pparα*-deficient mice.

Introduction

Fuel selection to meet the body's energy demand is of crucial importance during feeding-fasting transitions. The liver plays a central role in this switch. It changes from glucose uptake and glycogen synthesis during feeding to glucose production by gluconeogenesis (GNG) and glycogenolysis during fasting. The origin of hepatic glucose production (HGP) shifts from mainly glycogenolysis to GNG as fasting prolongs. These changes in hepatic glucose metabolism are accompanied by adaptation of hepatic fatty acid metabolism, *i.e.*, from fatty acid synthesis to fatty acid oxidation. This adaptation allows for the optimization of fuel substrate utilization. These metabolic changes are, at least in part, affected by the reciprocal action of insulin and glucagon. However, the discovery of nuclear hormone receptors and the (partial) elucidation of their mode of action as ligand-activated regulators of gene expression has considerably complicated the picture. One member of the nuclear hormone receptors is of particular importance in mediating the adaptive response to fasting, *i.e.,* peroxisome proliferator-activated receptor alpha (PPARα). PPARα is a fatty acid-activated transcription factor that upregulates the expression of a variety of genes that encode proteins involved in β-oxidation and lipoprotein metabolism.[1] Lack of this receptor in *Pparα*$^{-/-}$ mice results in the inability to upregulate hepatic fatty acid oxidation and ketogenesis upon fasting in face of increased concentrations of free fatty acids in the circulation.[2] It also became apparent that mice lacking PPARα develop hypoglycemia after a prolonged fast.[2]

The etiology of hypoglycemia in fasting *Pparα*$^{-/-}$ mice is still unclear. It has been hypothesized that it reflects decreased GNG secondary to impaired hepatic fatty acid β-oxidation.[2] Surprisingly, a recent study suggested increased HGP in long-term (24 h) fasted *Pparα*$^{-/-}$ mice despite the development of hypoglycemia.[3] Furthermore, glycogen content of the liver in *Pparα*$^{-/-}$ mice after a prolonged fast was not reduced in comparison to wildtype (WT) mice but, unexpectedly, tended to be higher in the *Pparα*$^{-/-}$ *mice*. In contrast, glycogen content of the liver did not increase in *Pparα*$^{-/-}$ mice upon refeeding, whereas in WT mice glycogen content strongly increased. These data suggests that the balance between HGP, glycogen synthesis and GNG at the level of glucose-6-phosphate (G6P) is perturbed in *Pparα*$^{-/-}$ mice.

Partitioning of G6P can be studied *in vivo* using the glycoconjugate probe technique and mass isotopomer distribution analysis (MIDA) as described by Hellerstein and coworkers.[4] Recently, we applied these stable isotope techniques to study partitioning of newly synthesized G6P while glucose-6-phosphatase (G6Pase) activity was partially inhibited in 24 h fasted rats.[5] We were able to show that when HGP was diminished by partial inhibition of G6Pase activity, quite surprisingly, *de novo* synthesis of G6P was unaffected. It appeared that newly synthesized G6P was repartitioned away from plasma glucose to glycogen synthesis and, as a consequence, glycogen content of livers of treated rats increased several fold. The importance of these observations, substantiating data from other groups[6], is that partitioning of G6P independent of its *de novo* synthesis represents an additional mechanism of regulation of hepatic glucose production. We now have

miniaturized these methods for application in mice.[7] In the present study we addressed the following questions: 1) Is there a role of PPARα in the control of *de novo* synthesis of G6P, and 2) What are the effects of PPARα-deficiency on partitioning of newly synthesized G6P during fasting? We approached these questions experimentally in short-term fasted *Pparα*$^{-/-}$ mice by infusion of [U-^{13}C]-glucose [2-^{13}C]-glycerol, [1-^{2}H]-galactose and paracetamol, collecting serial blood and urine spots on filter paper and measuring the mass istopomer distribution in glucose and paracetamol-glucuronide. The fluxes were subsequently compared with expression of genes encoding enzymes involved in hepatic glucose metabolism and fatty acid oxidation, enabling us to delineate functional consequences of PPARα-deficiency-induced changes in gene expression.

Methods

Animals

Male *Pparα*$^{-/-}$ mice and WT mice on a SV129 background were housed in a temperature-controlled (21°C) room on a 10 h dark, 14 h light cycle. Experimental procedures were approved by the Ethics Committee for Animal Experiments of the State University Groningen. Mice were equipped with a permanent heart catheter that was attached to the skull with acrylic glue.[8] Mice were allowed to recover from surgery for at least 4 days.

Fasting experiments

The adaptive response to fasting in *Pparα*$^{-/-}$ and WT mice was compared first. Mice were fasted up to 24 h and blood samples were taken at t = 0 (fed), 15 and 24 h by tail bleeding and livers were removed at t = 0.15 and 24 h for lipid analysis and RNA isolation. Hepatic in vivo carbohydrate metabolism was measured according to the protocol described by Van Dijk *et al.*[7] On the day of the experiment, mice were placed in individual metabolic cages. Filter paper was placed under the wired floor of the cage to collect urine samples. Food was removed 9 h before start of the experiments. Body weight was 25.0 ± 1.9 g for WT mice and 24.1 ± 1.1 g for *Pparα* $^{-/-}$ mice at the time of the experiments. Mice received an infusion at a rate of 0.6 ml h^{-1} during 6 h of a solution consisting of [U-^{13}C]-glucose (13 μmol ml^{-1}), [2-^{13}C]-glycerol (160 μmol ml^{-1}), [1-^{2}H]-galactose (33 μmol ml^{-1}), and paracetamol (1 mg ml^{-1}). Blood glucose during the experiment was measured using EuroFlash™ test strips (LifeScan Benelux, Beerse, Belgium) and bloodspots obtained by tail bleeding for gas chromatopraghy-mass spectrometry (GC-MS) measurements were collected before the start of the infusion and hourly afterwards until 6 h after the start of the infusion. The filterpaper placed under the wired floor of the cage was replaced at hourly intervals. A large bloodsample was taken by heart puncture under halothane anaesthesia at the end of the experiment and the liver was quickly excised and frozen immediately in liquid N_2 for lipid analysis and RNA isolation.

Metabolite concentrations and enzyme activities

Plasma was isolated by centrifugation and liver tissue was homogenized and lipids were extracted using a modified Bligh & Dyer method.[9] Plasma βhydroxybutyrate, lactate, free fatty acid and liver triglyceride content were determined using commercially available kits (Roche Diagnostics, Mannheim, Germany and Wako Chemicals GmbH, Neuss, Germany). Alanine concentrations were analyzed by ion exchange column chromatography followed by post-column ninhydrine derivatization[10,11] on a Biochrome 20® automated amino acid analyser (Pharmacia, Roosendaal, The Netherlands). Glycerol concentrations were measured by GC-MS using [1,1,2,3,3-d_5]glycerol as an internal standard. Samples were derivatised to their tri-acetate by adding 100 µl pyridine and 200 µl acetic acid-anhydride to 50 µl plasma and incubating the solution at 80 ºC for 30 minutes. Samples were measured on a Trace-GC-MS (Finnigan Matt, San Jose, CA, USA). Plasma insulin levels were determined by radioimmunoassay (RI-13K, Linco Research, St. Charles, MO). Total hepatic protein content was determined according to Lowry *et al.*[12] Hepatic glycogen content was determined in freeze clamped liver tissue after extraction in 1 M KOH solution by sonication. The extract was incubated for 30 minutes at 90 ºC, cooled and brought to pH 4.5 by addition of 3 M acetic acid. Precipitated protein was removed by centrifugation. Glycogen was converted to glucose by treating the samples with amyloglucosidase, followed by assay of glucose at pH 7.4 with ATP, NADP+, hexokinase and G6P dehydrogenase.[13] Liver samples for the determination of G6P were treated by sonification in a 5 % (w/v) $HClO_4$ solution, centrifuged and supernatant neutralized to pH 7 by addition of small amounts of a mixture of 2 M KOH and 0.3 M MOPS. G6P was determined fluorimetrically with NADP+ and G6P dehydrogenase.[13] Hepatic ATP levels were measured using a bioluminescence assay kit (Roche Diagnostics).

Hepatic mRNA levels

Total RNA was isolated from liver tissue using the Trizol method (GIBCO, Paisley, United Kingdom). RNA was converted to cDNA with M-Mulv-RT (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol using random primers. cDNA levels of the genes of interest were measured by real-time PCR using the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). An amount of cDNA corresponding to 20 ng of total RNA was amplified using the qPCR core kit (Eurogentec, Seraing, Beldium) according to the manufacturer's protocol using the appropiate forward and reverse primers (GIBCO), and a template specific 3'-TAMRA, 5'-FAM labeled Double Dye Oligonucleotide probe (Eurogentec). Calibration curves were run on serial dilutions of pooled cDNA solutions as used in the assay. The data were processed using the ABI Sequence Detector 6.3 (System Applied Biosystems, Foster City, CA, USA). Quantified expression levels were within the linear part of the calibration curves. PCR results were normalized to β-actin mRNA levels. The sequence of the primers and probes used in this study are listed in Table1.

Table 1. Sequences of primers and probes used.

Glucose metabolism		Sequence	GenBank no.
G6ph	Forward	CTG CCA GGG AGA ACT CAG CAA	NM008061
	Reverse	GAG GAC CAA GGA AGC CAC AAT	
	Probe	TCG TTC CCA TTC CGC TTC GCC T	
G6pt	Forward	GAG GCC TTG TAG GAA GCA TTG	NM008063
	Reverse	CCA TCC CAG CCA TCA TGA GTA	
	Probe	CTC TGT ATG GGA ACC CTC GCC ACG	
Gk	Forward	CCT GGG CTT CAC CTT CTC CTT	L38990
	Reverse	GAG GCC TTG AAG CCC TTG GT	
	Probe	CAC GAA GAC ATA GAC AAG GGC ATC CTC CTC	
Gp	Forward	GAA GGA GGC AAA CGG ATC AAC	BC013636
	Reverse	TCA CGA TGT CCG AGT GGA TCT	
	Probe	CCT CTG CAT CGT GGG CTG CCA	
Gs	Forward	GCT CTC CAG ACG ATT CTT GCA	AA537291
	Reverse	GTG CGG TTC CTC TGA ATG ATC	
	Probe	CCT CTA CGG GTT TTG TAA ACA GTC ACG CC	
Pk	Forward	CGT TTG TGC CAC ACA GAT GCT	NM013631
	Reverse	CAT TGG CCA CAT CGC TTG TCT	
	Probe	AGC ATG ATC ACT AAG GCT CGA CCA ACT CGG	
Pepck	Forward	GTG TCA TCC GCA AGC TGA AGA	NM011044
	Reverse	CTT TCG ATC CTG GCC ACA TCT	
	Probe	CAA CTG TTG GCT GGC TCT CAC TGA CCC	
Chrebp	Forward	GAT GGT GCG AAC AGC TCT TCT	AF156604
	Reverse	CTG GGC TGT GTC ATG GTG AA	
	Probe	CCA GGC TCC TCC TCG GAG CCC	
Fatty acid oxidation			
Pparα	Forward	TAT TCG GCT GAA GCT GGT GTA C	X57638
	Reverse	CTG GCA TTT GTT CCG GTT CT	
	Probe	CTG AAT CTT GCA GCT CCG ATC ACA CTT G	
Pparγ	Forward	CAC AAT GCC ATC AGG TTT GG	NM011146
	Reverse	GCT GGT CGA TAT CAC TGG AGA TC	
	Probe	CCA ACA GCT TCT CCT TCT CGG CCT G	

Table 1. Continued.

Fatty acid oxidation		Sequence	GenBank no.
CPT1α	Forward	CTC AGT GGG AGC GAC TCT TCA	AF017175
	Reverse	GGC CTC TGT GGT ACA CGA CAA	
	Probe	CCT GGG GAG GAG ACA GAC ACC ATC CAA C	
Mcad	Forward	GCA GCC AAT GAT GTG TGC TTA C	NM007382
	Reverse	CAC CCT TCT TCT CTG CTT TGG T	
	Probe	CCC TCC GCA GGC TCT GAT GTG G	
Hmgs	Forward	TGG TGG ATG GGA AGC TGT CTA	U12790
	Reverse	TTC TTG CGG TAG GCT GCA TAG	
	Probe	CCA AGG CCC GCA GGT AGC ACT G	

Measurement of mass isotope distribution by GC-MS

Analytical procedures for extraction of glucose and paracetamol-glucuronide from bloodspot and urine filterpaper strips, respectively, derivatization of the extracted compounds and GC-MS measurements of derivatives were essentially according to Van Dijk *et al*.[5,7] In short, glucose was extracted by incubating a disk (6.5 mm) punched out of a bloodspot with ethanol/water (10/1 v/v) mixture. After drying the sample under a stream of N_2, glucose was derivatized to its pentaacetate-ester and aldonitril-pentaacetate-ester. The final derivatives were dissolved in 100 µl ethylacetate for injection. Paracetamol-glucorionide (Par-GlcUA) was exctracted from filter paper with methanol/water (3/1 v/v) and subsequently isolated by a Milton Roy HPLC system (Interscience, Breda, The Netherlands) on a Nucleosil 7C18 SP250/10 column (Bester, Amstelveen, The Netherlands) eluted with a gradient of 0.2 % (v/v) ammonium formate in water (pH 4.8) and 40 % (v/v) acetonitril in water. The fraction containing the isolated compound was dried under a stream of N_2 and subsequently derivatized to its trimethylsilyl-ethyl-ester or oxidized to saccharic acid by nitrite in nitric acid and derivatized to its tetraacetate-diethyl-ester. After drying of the samples under a stream of N_2, the dry residues were dissolved in 200 µl ethylacetate for injection. All samples were analyzed by GC-MS (SSQ7000; ThermoFinnigan, San Jose, CA, USA) on an AT-5MS 30 m x 0.25 mm ID (0.25 µm film thickness) capillary column (Alltech, Breda, the Netherlands). For all calculations of mass isotopomer distribution, Excaliber software (ThermoFinnigan, San Jose, CA, USA) was used. Mass spectrometric analyses of glucose pentaacetate, glucose aldonitril pentaacetate and saccharic acid diethyl-, tetraacetate-ester were performed by positive ion chemical ionization with methane. Ions monitored for glucose pentaacetate were m/z 331-337, for glucose aldonitril pentaacetate m/z 328-334, and for saccharic acid diethyl-ester tetraacetate m/z 375-381, all corresponding to the m_0-m_6 mass isotopomers. Mass spectrometric analyses for Par-GlcUA ethyl-, tetra-(trimethylsilyl)-ester were performed by electron impact ionization. The ions monitored were m/z 331-337 corresponding to the m_0-m_6 mass

isotopomers. Series of measurements were composed of experimental samples, control samples and a dilution series obtained from a mixture of the last, most enriched, samples taken at the end of an experiment.

A series of measurements was accepted for further calculations when 2 conditions were met as described by Van Dijk *et al.*[7] Firstly, for each derivative the coeficient of variance of the fractional contribution of m0, m1 and m2 to total ion abundance in control samples must be smaller than 1 % for m0, and 2 % for m1 and m2. Secondly, for each derivative the fractional contribution of m1, m2 and m6 to total ion abundance measured in experimental samples must be within the range of constant response of the GCMS as estimated from the values of the fractional contribution of m1, m2 and m6 to total ion abundance of the inserted dilution series.

Mass Isotopomer Distribution Analysis (MIDA)

The measured fractional isotopomer distribution by GC-MS (m_0-m_6) was corrected for the fractional distribution due to natural abundance of ^{13}C. This was done by multiple linear regression as described by Lee *et al.*[14] to obtain the excess fractional distribution of mass isotopomers (M_0-M_6) due to incorporation of infused labeled compounds, *i.e.,* [2-^{13}C]-glycerol, [U-^{13}C]-glucose and [1-^{2}H]-galactose. This distribution was used in MIDA algorithms of isotope incorporation and dilution according to Hellerstein *et al.*[15] as described by Van Dijk *et al.*[5] Total rate of appearance of glucose into plasma (Ra(glc;whole body)) was calculated by isotope dilution as follows:

$$Ra(glc;whole\ body) = MPE(glc;M_6)_{infusate} / MPE(glc;M_6)_{plasma} \times infusion(glc;M_6) \qquad (Eq.\ 1)$$

in which $MPE(glc;M_6)_{infusate}$ is the mole percent enrichment of infused [U-^{13}C]-glucose, $MPE(glc;M_6)_{plasma}$ is the mole percent enrichment of plasma [U-^{13}C]-glucose, and $infusion(glc;M_6)$ is the infusion rate of uniformly labeled [U-^{13}C]-glucose.

Total rate of appearance of UDPglc (Ra(UDPglc;whole body)) was calculated according to:

$$Ra(UDPglc;whole\ body) = MPE(gal;M_1)_{infusate} / MPE(pGlcUA;M_1)_{urine} \times infusion(gal;M_1) \qquad (Eq.\ 2)$$

in which $MPE(gal;M_1)_{infusate}$ is the mole percent enrichments of infused [1-^{2}H]-galactose, $(pGlcUA;M_1)_{urine}$ is the mole percent enrichments of hepatic UDP-glucose as measured in Par-GlcUA, and $infusion(gal;M_1)$ is the infusion rate of [1-^{2}H]-galactose.

Ra(UDPglc;whole body) was calculated with the assumption of a constant and complete entry of infused galactose into the hepatic UDP-glucose pool. Furthermore, it was assumed that the fractional isotopomer distribution observed in urinary Par-GlcUA reflects the fractional isotopomer distribution in hepatic UDPglc.

Rates of endogenous plasma glucose (Ra(glc;endo)) and UDP-glucose (Ra(UDPglc;endo)) appearance were calculated as follows:

$$Ra(glc;endo) = Ra(glc;whole\ body) - infusion(glc;M_6) \text{ and} \quad \text{(Eq. 3)}$$

$$Ra(UDPglc;endo) = Ra(UDPglc;whole\ body) - infusion(gal;M_1) \quad \text{(Eq. 4)}$$

Fractional gluconeogenic contribution to both plasma glucose (f(glc) and hepatic UDP-glucose (f(UDPglc)) were calculated using MIDA of glucose and Par-GlcUA derivatives, respectively, as described in detail elsewhere.[16,17] The absolute gluconeogenic flux into both plasma glucose (GNG(glc) and hepatic UDP-glucose (GNG(UDPglc)) were calculated as follows:

$$GNG(glc) = f(glc) \times Ra(glc;whole\ body) \text{ and} \quad \text{(Eq. 5)}$$

$$GNG(UDPglc) = f(UDPglc) \times Ra(UDPglc;whole\ body) \quad \text{(Eq. 6)}$$

The total absolute gluconeogenic flux is the sum of both components corrected for recycling:

$$Total\ GNG = (1-c(glc)) \times GNG(glc) + (1-c(UDPglc)) \times GNG(UDPglc) \quad \text{(Eq. 7)}$$

$$c(glc) = MPE(pGlcUA;m_6)_{urine}/MPE(glc;m_6)_{plasma} \quad \text{(Eq. 8)}$$

in which $MPE(pGlcUA;m_6)_{urine}$ and $MPE(glc;m_6)_{plasma}$ are the mole percent enrichments of urinary p-GlcUA and plasma glucose, respectively, during an infusion of [U-^{13}C]-glucose.

$$c(UDPglc) = MPE(glc;m_1)_{plasma}/\ MPE(pGlcUA;m_1)_{urine} \quad \text{(Eq. 9)}$$

in which $MPE(glc;m_1)_{plasma}$ and $MPE(pGlcUA;m_1)_{urine}$ are the mole percent enrichments of plasma glucose and urinary p-GlcUA, respectively, during an infusion of [1-^{2}H]-galactose.

Statistical analysis.

All values reported are means ± SD. Significance for metabolite and activity levels was determined using the non-parametric Mann Whitney test for unpaired data. Significance for fluxes calculated over time was determined using multiple measurements ANOVA. Differences were considered significant at $P < 0.05$.

Results

The adaptive response of *Pparα*$^{-/-}$ mice to fasting

In Table 2 the effects of fasting are shown in *Pparα*$^{-/-}$ and WT mice of various blood-born compounds. Glucose concentration tended to be lower at 15 h of fasting, but became significantly lower after 24h fasting in *Pparα*$^{-/-}$ mice compared to WT mice. No differences were observed between WT and *Pparα*$^{-/-}$ mice with respect to plasma lactate concentration: lactate concentrations decreased to a similar extent in both groups of mice. Lactate/pyruvate ratio's after 24 h fasting were 21.4 ± 4.3 and 22.3 ± 5.0 in WT and *Pparα*$^{-/-}$ mice, respectively. Free fatty acid concentrations were significantly higher at 15 h of fasting in *Pparα*$^{-/-}$ mice and remained elevated upto 24 h of fasting. In contrast, the ketotic fasting response was diminished in *Pparα*$^{-/-}$ mice. After an intial rise in the concentration of 3β-hydroxybutyrate in both groups of mice, the 3β-hydroxybutyrate concentration increased further in WT but not in *Pparα*$^{-/-}$ mice. The changes in plasma glycerol concentration did not differ between *Pparα*$^{-/-}$ or WT mice. After 24 h of fasting a decrease in glycerol concentration was observed in both groups of mice. Alanine concentrations were 393 ± 61 µmol/l in WT mice and 212 ± 27 µmol/l ($P < 0.05$) in the *Pparα*$^{-/-}$ mice at the end of the fasting period.

Table 2. Plasma glucose, lactate, free fatty acid, 3-hydroxybutyrate and glycerol concentrations in wild-type (WT) and *Pparα* $^{-/-}$ (-/-) mice, either fed or fasted for 15 h and 24 h. Each value represents the mean ± SD; $n = 5$ or 6 per group. * $P < 0.05$ between *Pparα* $^{-/-}$ compared to WT mice.

	Fed	Fasted 15 h	Fasted 24 h
Glucose			
WT	6.3 ± 1.0	3.5 ± 0.3	3.3 ± 0.4
-/-	6.2 ± 0.6	2.6 ± 0.5	2.1 ± 0.3 *
Lactate			
WT	ND	3.0 ± 0.7	1.7 ± 0.4
-/-	ND	2.5 ± 0.5	1.4 ± 0.6
Free fatty acid			
WT	0.3 ± 0.1	0.4 ± 0.1	0.4 ± 0.1
-/-	0.4 ± 0.1	1.0 ± 0.4 *	0.9 ± 0.3 *
3β-Hydroxybutyrate			
WT	0.2 ± 0.0	0.4 ± 0.2	2.4 ± 0.4
-/-	0.1 ± 0.0	0.5 ± 0.1	0.7 ± 0.1 *
Glycerol			
WT	0.3 ± 0.0	0.5 ± 0.2	0.3 ± 0.0
-/-	0.3 ± 0.0	0.5 ± 0.1	0.4 ± 0.2

Figure 1. Hepatic triglyceride (A) and glycogen (B) content in wild-type (WT) and *Pparα*$^{-/-}$ mice fed or fasted for 15 h and 24 h. Contents were determined as described under "Experimental Procedures". Each value represents the mean ± SD; n = 5 or 6 per group, * $P < 0.05$ compared to WT mice.

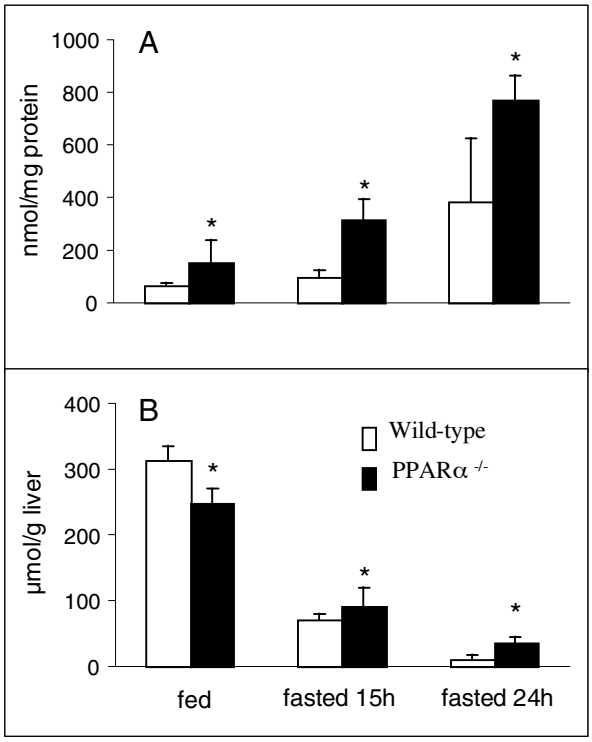
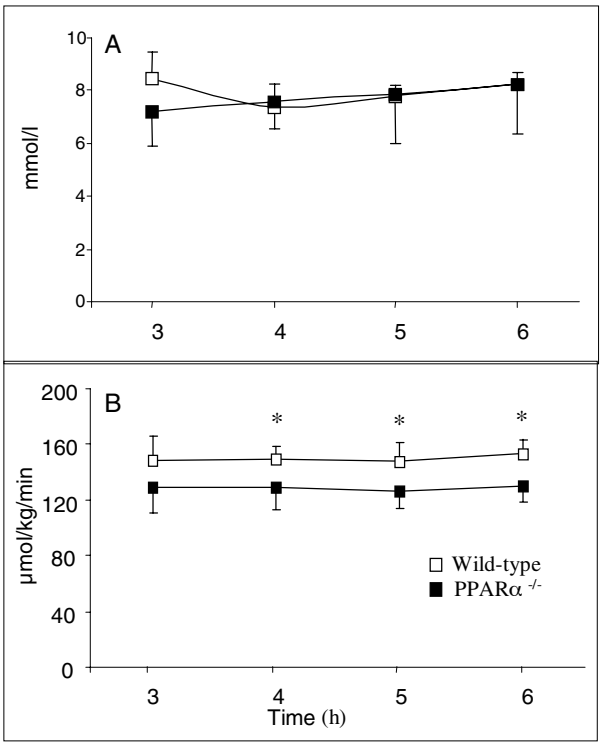


Figure 1 shows the changes in hepatic glycogen and triglycerides content in *Pparα*$^{-/-}$ and WT mice upon fasting. Liver weight was not different between groups, *i.e.*, 0.9 ± 0.1 g and 1.0 ± 0.1 g in WT and *Pparα* $^{-/-}$ mice, respectively. When fed, hepatic glycogen content was significantly lower in *Pparα*$^{-/-}$ mice than in WT mice. Upon fasting, glycogen decreased more strongely in WT than in knock-out mice. After 15 h fasting, hepatic glycogen content in *Pparα*$^{-/-}$ mice was 90 ± 28 µmol/g liver, compared to 69 ± 10 µmol/g liver in WT mice. This difference in response of the hepatic glycogen content was apparent also after 24 h fasting, *i.e.*, 9 ± 9 µmol/g liver in WT mice and 36 ± 10 µmol/g liver in *Pparα*$^{-/-}$ mice. Hepatic G6P levels, determined after 15 h of fasting, were not significantly different, *i.e.* 322 ± 57 and 397 ± 51 µmol/g liver in the WT and *Pparα*$^{-/-}$ mice, respectively. During fasting hepatic triglyceride content increased in both group of mice. However, in *Pparα*$^{-/-}$ mice the increase in hepatic triglyceride content was significantly more pronounced than in WT mice.

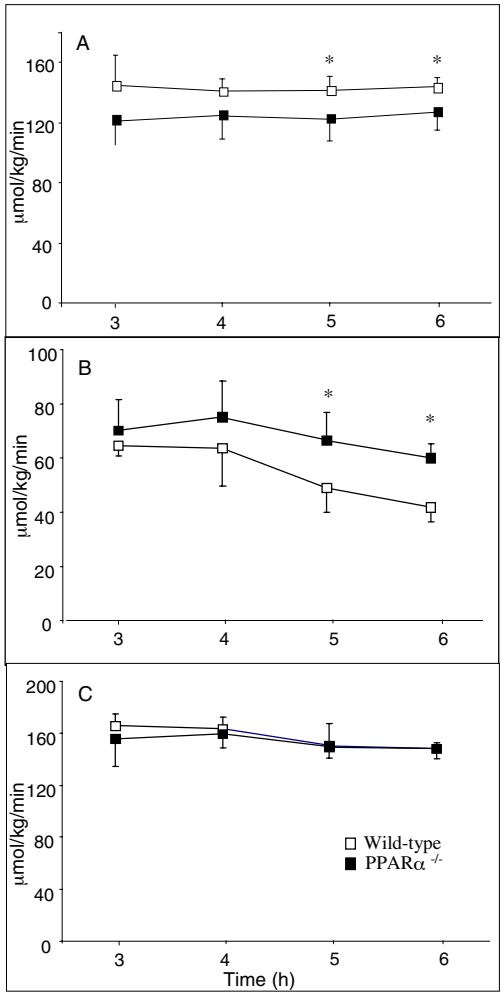
Figure 2. Plasma glucose concentrations (A) and total endogenous glucose production (B) during the last 3 h of the experiment in WT and *Pparα*$^{-/-}$ mice. Fluxes were determined as described under "Experimental Procedures". Each value represents the mean ± SD; $n = 6$ per group, * $P < 0.05$ compared to WT mice.



Hepatic glucose metabolism in *Pparα*$^{-/-}$ mice

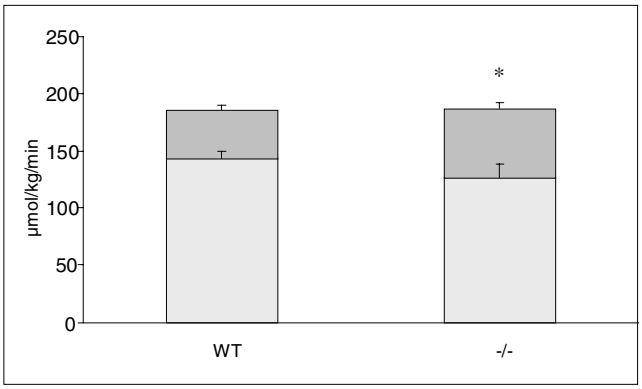
In Figure 2 the time courses are shown of plasma glucose concentration and endogenous glucose production rates during the last 3 h of the label infusion experiment. The end of the experiment corresponds to 15 h of fasting. Mice were fasted for 9 h before the label infusion started. Plasma insulin concentrations showed no difference between both groups at the end of the experiment, *i.e.*, 0.23 ± 0.04 ng/ml in WT mice and 0.24 ± 0.02 ng/ml in *Pparα* $^{-/-}$ mice. These values were not different from those measured after 24 h of fasting *i.e.*, 0.25 ± 0.05 ng/ml and 0.25 ± 0.03 ng/ml in WT and *Pparα* $^{-/-}$ mice, respectively. As is clear from fig 2A, plasma glucose concentrations remained constant and the values were very similar in both groups of animals. Glucose concentration was 8.0 ± 0.4 mM and 7.7 ± 1.5 mM in WT and *Pparα*$^{-/-}$ mice, respectively. During the last 3 h of the experiment, a constant endogenous glucose production could be documented. The data clearly show a significantly decreased endogenous glucose production in *Pparα*$^{-/-}$ mice (129 ± 13 µmolkg^{-1}min^{-1}) in comparison with WT mice (149 ± 11 µmolkg^{-1}min^{-1}, $P < 0.05$).

Figure 3. Gluconeogenic flux to glucose (A), to UDP-glucose (B), and total gluconeogenic flux to G6P (C) during the last 3 h of the experiment in wild-type and *Pparα*$^{-/-}$ mice. Fluxes were determined as described under "Experimental Procedures". Each value represents the mean ± SD; $n = 6$ per group, * $P < 0.05$ compared to WT mice.



In Figure 3 the rates of *de novo* synthesis of G6P into plasma glucose (3A), into UDPglucose (3B) and the total *de novo* synthesis of G6P (3C) are shown during the final 3 h of the label infusion experiment. *De novo* synthesis of G6P into plasma glucose was constant during the experiment. In *Pparα*$^{-/-}$ mice the absolute rate of GNG towards plasma glucose was significantly diminished compared to WT mice, *i.e.*, 124 ± 13 µmolkg^{-1}min^{-1} vs. 142 ± 9 µmolkg^{-1}min^{-1} ($P < 0.05$), respectively. A different observation was made with regard to the absolute rate of appearance of newly synthesized G6P into UDPglucose pool.

Figure 4. Absolute (A) and fractional (B) partitioning of G6P to glucose (light grey) and to UDP-glucose (dark grey) during the last 3 h of the experiment in WT and *Pparα*$^{-/-}$ mice. Fluxes were determined as described under "Experimental Procedures". Each value represents the mean ± SD; $n = 6$ per group, * $P < 0.05$ compared to WT mice.



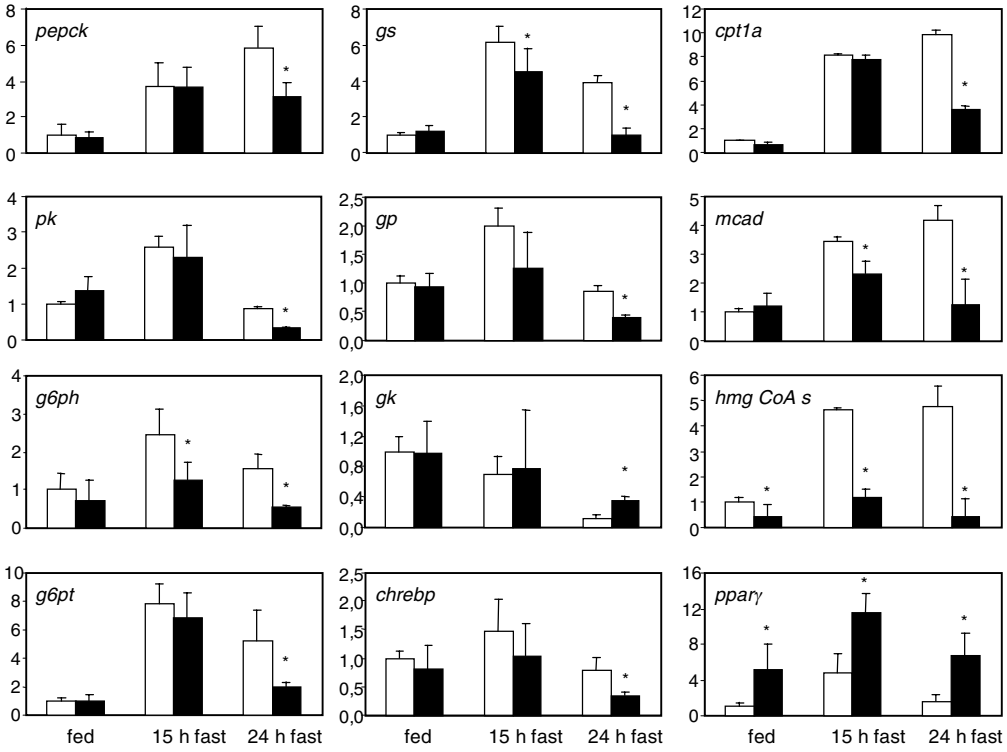
In the course of the experiment a slow but significant decline was observed in the rate of appearance of newly formed UDPglucose. In *Pparα*$^{-/-}$ mice the decline, from 70 ± 4 µmolkg^{-1}min^{-1} at 3 h to 60 ± 5 µmolkg^{-1}min^{-1} at 6 h after the start of the label infusion ($P < 0.05$), was less pronounced than in WT mice, in which it declined from 65 ± 11 µmolkg^{-1}min^{-1} at 3 h to 42 ± 5 µmolkg^{-1}min^{-1} at 6 h after the start of the label infusion ($P < 0.05$). At the end of the experiment the rate of appearance of newly formed UDPglucose in *Pparα*$^{-/-}$ mice was therefore significantly higher than in WT mice. Interestingly, total rate of *de novo* synthesis of G6P remained constant during the second half of the experiment and was not different between the two groups, *i.e.*, 153 ± 9 µmolkg^{-1}min^{-1} and 157 ± 9 µmolkg^{-1} min^{-1} in *Pparα*$^{-/-}$ and WT mice, respectively.

In Figure 4 the the partitioning of newly synthesized G6P into plasma glucose and UDPglucose is given at 6 h after the start of the label infusion. As is clear from this figure, there was a significant larger part of newly synthesized G6P diverted to UDPglucose in *Pparα*$^{-/-}$ mice than in WT mice. At the end of the experiment the fractional contribution of newly formed G6P to UDPglucose synthesis was 0.40 ± 0.04 in *Pparα*$^{-/-}$ mice and 0.29 ± 0.03 in WT mice, a significant increase of 32 % in *Pparα*$^{-/-}$ mice as compared to WT mice.

Hepatic Gene Expresion Profiles

Hepatic expression of genes involved in glucose and fat metabolism in fed and 15 h and 24 h fasted mice are shown in Figure 5. The expected response was observed in expression of genes involved in hepatic glucose en fatty acid oxidation during fasting in WT mice. Expression of *Pepck, G6ph, G6pt, Gp* and *Cpt1a*, *Mcad* and *Hmgs* all increased upon fasting. *Pepck* expression was similarly increased after the 15 h fast compared to the fed situation in both groups, but significantly less in the *Pparα*$^{-/-}$ than in WT mice after a 24 h

Figure 5. Gene expression of enzymes involved in fatty acid β-oxidation and glucose metabolism in the fed state or after 15 and 24 h of fasting in WT and *Pparα*$^{-/-}$ mice. Levels of cDNA were measured by real-time PCR as described under "Experimental Procedures". Data are expressed as relative to β-actin and the results of the fed wild-type animals are defined as one. Each value represents the mean ± SD; $n = 5$ or 6 per group, * $P < 0.05$ compared to WT mice. *pepck*, phosphoenolpyruvate carboxykinase; *gs*, glycogen synthase; *cpt1a*, carnitine palmitoyltransferase 1a; *pk*, pyruvate kinase; *gp*, glycogen phosphorylase; *mcad*, medium chain acylCoA dehydrogenase; *g6ph*, glucose-6-phosphate hydrolase; *gk*, glucokinase; *hmgs*, 3-hydroxy-3-methylglutaryl-CoA synthase (mitochondrial); *g6pt*, glucose-6-phosphate translocase; *chrebp*, carbohydrate responsive element-binding protein; *pparγ,* peroxisome proliferator-activated receptor gamma.



fast. Only the expression of *G6ph* and *Gs* were significantly affected at 15 h of fasting in *Pparα*$^{-/-}$ mice compared to WT mice. Hepatic expression of the other genes involved in glucose metabolism *i.e. G6pt*, *Gp*, *Gk* and the transcription factor Chrebp were not different in *Pparα*$^{-/-}$ mice when compared with WT mice after 15 h fasting. Expression of genes involved in fatty acid oxidation were differently affected. Expression of *Cpt1a* did not differ between *Pparα*$^{-/-}$ and WT mice at 15 h of fasting, but expression of Mcad and

particularly of *Hmgs* was significantly decreased in *Pparα*$^{-/-}$ mice when compared to WT mice. The hepatic expression of the *Pparγ* gene was increased under all circumstances tested in *Pparα*$^{-/-}$ mice in comparison to WT mice. Thus, after 24 h fast expression of most genes studied was decreased compared to the fed situation and more severely so in *Pparα*$^{-/-}$ mice. This raised the question whether this effect of 24 h fasting could be aspecific and simply to due a severe energy shortage in livers of these animals. To try to clarify this, we measured hepatic ATP levels. Hepatic ATP levels showed a trend, albeit not significant, to a decrease after 24 h of fasting in the *Pparα*$^{-/-}$ mice compared to WT mice, *i.e.*, 0.56 ± 0.13 nmol ATP/mg liver vs. 0.93 ± 0.39 nmol ATP/mg liver.

Discussion

In the present study we addressed the role of PPARα in the control of hepatic glucose metabolism, *i.e.*, *de novo* synthesis of G6P and its partitioning. The results show that after a short term fast of 15 h *de novo* synthesis of G6P was not affected by PPARα-deficiency. However, newly synthesized G6P was partitioned away from plasma glucose to glycogen synthesis. Furthermore, deficiency of PPARα resulted in a reduced hepatic expression of *G6ph*, *Gs* and to a lesser extent, of *Gp*.

The validity of the isotope model, with the application of glycoconjugates, and the MIDA approach has been substantiated in various studies although some controversy still remains.[18] We have validated the application of MIDA in mice in a separate study.[7] In that study, we observed major adaptations in whole body and hepatic glucose metabolism in 24 h fasted mice but not in 9 h fasted mice during the course of 6 h infusion of stable isotopically-labeled compounds.

The alterations in the adaptive response to fasting of *Pparα*$^{-/-}$ mice that we observed were in line with those reported by other investigators.[2,3] The hypoglycemia in *Pparα*$^{-/-}$ mice did not appear to be due to an enhanced glucose consumption by peripheral tissues, *i.e.*, metabolic clearance of glucose was similar in WT and *Pparα*$^{-/-}$ mice. Differences exist with respect to the reported time-course of development of hypoglycemia in fasted *Pparα*$^{-/-}$ mice. Kersten *et al.*[2] and Xu *et al.*[3] reported significantly lower plasma glucose concentrations after, respectively, 15 h and 17 h of fasting in *Pparα*$^{-/-}$ mice compared to WT mice. In our hands, blood glucose concentrations decreased to a larger extent in WT mice than reported by Kersten *et al.*[2] As yet, no explanation can be given for this discrepancy.

During infusion of stable isotopically-labeled compounds no decrease was observed in plasma glucose concentration of either *Pparα*$^{-/-}$ or WT mice. This was different from the observations made in non-infused mice during the adaptive response. Differences in plasma insulin concentrations do not offer an explanation; they were low as would have been expected for fasting mice and did not differ from the values observed in 24 h fasted, non-infused WT and *Pparα*$^{-/-}$ mice. In our opinion, the absence of a decrease in plasma glucose concentration upon prolonged fasting in infused mice might have been due to the delivery

of gluconeogenic substrates by the infusion of stable isotopically-labeled compounds. The rates of infusion were considerable, particularly that of [2-^{13}C]-glycerol at ~60 μmol kg^{-1} min^{-1}. When all the infused glycerol would have been utilized for GNG to plasma glucose, however, only 15-20 % of the amount of glucose produced through GNG can be accounted for by the glycerol infusion. Furthermore, Previs *et al.*[18] showed that infusion of this amount of glycerol did not increase endogenous glucose production in 30 h fasted BALBc mice. Their experiments, however, consisted of short-term infusion (3 h) of labeled glycerol instead of 6 h as in our experiments and were performed under conditions in which blood glucose concentrations were normal. On the other hand, Xu *et al.*[3] concluded from their experiments that in *Pparα*$^{-/-}$ mice glycerol appeared to be the preferred gluconeogenic substrate at the expense of lactate. Our results imply that *de novo* synthesis of G6P exhibits a high elasticity towards the supply of gluconeogenic substrates.

PPARα-deficiency resulted in a lower endogenous rate of appearance of glucose than observed in WT mice. When GNG would have been calculated based on its fractional contribution to plasma glucose alone, our results would have led us to infer that total gluconeogenic flux was inhibited in parallel with inhibition of glucose production. By analyzing both plasma glucose and urinary paracetamol-glucoronic acid, however, we were able to show that the decrease in hepatic glucose production was not associated with a decrease in the rate of *de novo* synthesis of G6P but with a more predominant partitioning of newly synthesized G6P into glycogen. In line with this observation, glycogen content of liver of *Pparα*$^{-/-}$ mice remained higher during fasting when compared to WT mice. The metabolic fate of newly synthesized G6P therefore seems to be without consequence for the rate of its *de novo* synthesis. Similar lack of feedback inhibition of *de novo* synthesis of G6P by its product has been documented by us[5] as well as by others.[6] In contrast to our conclusions, Xu *et al.*[3] reported an increase in endogenous glucose production in *Pparα*$^{-/-}$ mice. However, they did not study partitioning of newly synthesized G6P. It is therefore not clear whether the reported increase in glucose production resided in an increased rate of *de novo* synthesis of G6P or in a altered partitioning of newly synthesized G6P. Another potentially important aspect is that these authors performed their experiments in *Pparα*$^{-/-}$ mice bred on to a C57Bl/6 background, while we studied *Pparα*$^{-/-}$ mice bred on to a SV 129 background.

Our study brings into focus another mechanism by means of which impairment of fatty acid oxidation influences GNG. It is widely accepted that inhibition of β-oxidation impaires GNG by diminished delivery of energy and/or reducing equivalents. However, early data already indicated that inhibiting β-oxidation elicited different effects on GNG, depending on the substrate available. When livers of fasted guinea pigs were perfused with glycerol or propionate as gluconeogenic substrates, inhibition of fatty acid oxidation by CPT I blockade did not perturb GNG from these substrates.[19] Only when lactate and pyruvate were used as substrate, inhibition of fatty acid oxidation at CPT I resulted in inhibition of GNG. A similar mechanism might have been active in the experiments of Xu *et al.* on substrate selection for gluconeogenesis in *Pparα*$^{-/-}$ mice.[3] The data presented in this study show that, in addition, impairment of β-oxidation of fatty acids can affect partitioning of

newly synthesized G6P between plasma glucose and glycogen without affecting the rate of *de novo* synthesis of G6P. As dicussed earlier by us[5] and others[6] the reponse of the hepatocyte to maintain homeostasis of intracellular G6P might be central in this partitioning.

Our experiments allow us only to speculate how PPARα-deficiency brings about the preferential partitioning of newly synthesized G6P to glycogen. The mRNA levels of the *G6ph* gene were decreased, while expression of the *G6pt* gene was unaffected in the knockout mice. Gene expression of *Pepck* was not affected in short term fasted *Pparα*$^{-/-}$ mice. This would indicate that reduced G6ph gene expression might be a way by which PPARα influences endogenous glucose production. In the promotors of either gene, no PPRE's have been reported so far. The effects of PPARα might therefore be indirect. Changes in intracellular concentration of long-chain acyl-CoA thioesters, ligands for HNF4α, might be of importance. In the G6ph promotor a region has been identified which binds HNF4α.[20] Furthermore, binding of fatty acyl CoA's to HNF4α modulates its DNA binding activity and thereby the expression of the G6ph gene.[21] We should realize, however, that expression of the genes involved in glycogen metabolism, *i.e.*, *Gs* and *Gp*, were also depressed while glycogen metabolism measured by isotopic means, appeared to be enhanced.

In conclusion, PPARα-deficiency results in partioning of newly synthesized G6P away from plasma glucose resulting in a decreased endogenous glucose production during short-term fasting. Decreased expression of G6ph might have been a mechanism by which this effect of PPARα-deficiency is mediated.

Acknowledgements

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8

General discussion

Part of this general discussion has been submitted

The economics of science

The incidence of obesity and type 2 diabetes both in adults and children has grown to pandemic proportions in recent years.[1-3] More than one billion people is overweight[4] and more than half of black children and one third of white children are overweight by the age of 19 in the United States.[3] Obesity is associated with development of type 2 diabetes and atherosclerosis. Over-consumption of (energy-rich) foods, in addition to lack of physical activity, especially in industrialized societies, has created an ever-growing number of people suffering from diseases such as diabetes and atherosclerosis. There is great irony in the rise in incidence and handling of obesity, diabetes and atherosclerosis on the one hand and the worldwide poverty-related malnutrition on the other hand. Millions of people worldwide still lack adequate nutrition, causing high death rates among infants and high malnutrition-associated morbidity. The World Health Organization recently reported that 27% of all children under the age of 5 are underweight.[2]

One can postulate that market economies stimulate the prevalence of obesity, since most industries benefit from over-consumption of food, from agriculture to drug industries. in the U.S. more than 3800 kilocalories per person per day is produced for national consumption, which is far more than is necessary for most adults. With the obvious relationship between the amount food consumption and the incidence of obesity, health measures to promote lower food intakes are of pivotal importance, next to stimulation of physical exercise. Despite the fact that an increased morbidity of especially the working population has a negative economic impact, a lack of economic incentive still might exist for preventive policies for obesity.

The economic aspects of food consumption and obesity might also influence research in two ways. First of all, available research funds related to obesity are mostly allocated to projects on possible treatments of obesity, type 2 diabetes and atherosclerosis. Although this will benefit scientific knowledge on metabolism greatly and health to some degree, focusing research funding more on prevention might be more sensible with respect to general health benefits. Secondly, since such large amounts of funding are being directed to obesity-related research, this might provide conflicts of interests for research institutes.[6] Promising hypotheses for treatment of obesity or type 2 diabetes are likely to increase levels of funding related to such hypotheses. Research institutes might be tempted to go along with such hypotheses even when they are no longer valid. It might also stimulate researchers to overreport positive data and underreport negative data. One therefore must remain critical to the “why” and “how” of obesity-related research and to available research data and interpretation of this data.

The increasing need for fluxomics in metabolic research

“Metabolism” comprises a complex network of events, with direct and regulatory links between many metabolic routes in protein, carbohydrate and lipid metabolism. Regulation

of these processes has been the point of interest for many researchers in the past and present. Questions such as: "What controls the rate of glucose production?" have been prominent. During the last years, impressive progress in molecular biological techniques and, particularly, in the possibilities for "functional genomics" have provided tools for novel approaches in metabolic research. Functional genomics is currently widely used in biochemical and biomedical literature to describe relationships between genome and phenotype of cells or whole organisms and encompasses the various "omics" approaches, *i.e.*, transcriptomics (mRNA or gene expression profiling), proteomics (describing the protein complement) and metabolomics (describing the organic metabolic complement). The systematic analysis of these classes of molecules in cells or organs (by "bioinformatics") is often presented as a description of a complex metabolic process or even as an explanation of disturbances herein. As eloquently pointed out by Hellerstein in two excellent reviews[7,8], these approaches are based on the assumption that the molecular parameters that are measured by these omics approaches actually represent, and therefore may reveal, "real" sites of metabolic control. However, a wealth of information is available to indicate that this may not be the case: even a complete picture of gene expression and proteins in a living system will not reveal in a predictable or reliable manner the activity of the integrated metabolic pathways that they comprise. One must realize that metabolic regulation does not only take place at a molecular level which means that a priori transcriptomics are limited in the sense that they cannot account for all metabolic changes taking place. Regulation taking place at a time scale of seconds to minutes is performed by reversible binding of metabolites to enzymes and activation and deactivation of enzymes through covalent modification. Protein levels can be regulated, not only by their rate of synthesis, but also by modulation of the rate of degradation. For example, secretion of Apolipoprotein B, a structural component of very-low-density lipoproteins, by liver cells appears to be regulated exclusively by changing the rate of its intracellular degradation.[9] Another limitation of studying transcriptional regulation of one enzyme, is that multiple enzymes are usually involved in a specific metabolic pathway. It has long been thought that there must be rate-controlling enzymes in metabolic pathways, visualized as a chain with the weakest link controlling its strength. However, in recent years, evidence has grown towards the idea that the rate-limiting enzyme theory is not sufficient to explain the regulation of metabolism. Rather, it has been proposed that quantitative scales for the influence of an enzyme exist in metabolic flux regulation.[10] All enzymes have an influence on the rate of a certain metabolic flux and they all might have multiple possibilities for regulation and interaction with feedback and feedforward systems. Regulatory mechanisms for metabolic pathways are therefore often extremely complex with many points of interaction and integration. It is therefore virtually impossible to accurately predict the effect of alteration of one protein in that pathway on whole body kinetics. Studying protein levels or intermediates or endproducts of a metabolic cascade, *i.e.*, proteomics and metabolomics, respectively, have the shortcoming that they do not explain why or how a protein or product levels are decreased or increased and, furthermore, these levels represent static parameters that are dependent not only on production but also on uptake and/or

breakdown of that product or protein. One continuously has to realize that changes in RNA and or proteins will only in a limited way predict the changes for a metabolic pathway as a whole. Therefore, over-interpretation of data acquired through a reductionist approach, *i.e.*, by analyzing separate elements of a metabolic pathway, can often lead to incorrect conclusions about the pathway as a whole.

Therefore, although it is evident that important new information is being generated by the current "omics" approaches, it is important to realize that full understanding of control of metabolic pathways and how they are affected by genetic and acquired diseases or drugs, requires additional experimental strategies. Measurement of molecular fluxes through stringently defined metabolic fluxes in vivo, which can be referred to as "fluxomics", provides such a strategy. This advantage of fluxomics using either radioactive, but more preferably stable isotopes, is that it is dynamic in nature and, if desired, can encompass the entire metabolic pathway that one is interested in. By labeling a precursor and measuring the incorporation into its endproduct in vivo, one is able to accurately calculate the rate of the flux of that metabolic pathway. Data obtained through fluxomics represents the sum of all transcriptional and posttranscriptional points of regulation in an in vivo situation. During the last decades, various approaches for metabolic flux measurements in humans and in experimental animals have been developed for this purpose. Particularly in combination with techniques and/or novel drugs that specifically alter expression of individual genes or series of genes involved in certain pathways, these approaches have and will contribute to our understanding of metabolic control in health and disease. In the following, some recent examples from our laboratory will be presented to delineate the usefulness of this combined genomics-fluxomics approach in metabolic research.

Gluconeogenesis, i.e, *de novo* synthesis of glucose by liver and kidney, is a tightly controlled process in which insulin plays a pivotal role as a powerful suppressor of this pathway. Recent data suggests that certain transcription factors are also involved in the regulation of the gluconeogenic pathway.[11] Peroxisome proliferator-activated receptor alpha (PPARα, NR1C1) is a nuclear receptor that is activated by fatty acids and fibrates and that promotes expression of various genes involved in fatty acid oxidation among other genes. PPARα has also been suggested to be involved in transcriptional regulation of gluconeogenesis by inducing gene expression of phosphoenolpyruvate carboxykinase (PEPCK), an enzyme in the gluconeogenic pathway[11], although evidence for direct transcriptional control of the PEPCK gene is lacking. Indeed, PPARα knockout (*Pparα*$^{-/-}$) mice were shown to suffer from fasting-induced hypoglycemia supporting this concept.[11] The liver X receptor (LXRα, NR1H3; LXRβ, NR1H2) is another nuclear receptor involved in the regulation of gluconeogenesis and also belongs to the superfamily of nuclear hormone receptors. LXR is activated by physiological concentrations of oxidized derivatives of cholesterol.[12] LXR itself has been shown to suppress PEPCK gene expression supposedly by direct inhibition of transcription.[13,14] To make matters even more complex, recent data indicates that PPARα inhibits LXR signalling.[15] If one takes all available molecular data together, one would expect decreased gluconeogenesis in *Pparα*$^{-/-}$ mice. In a recent study we infused stably labeled glucose and a gluconeogenic precursor (glycerol) for

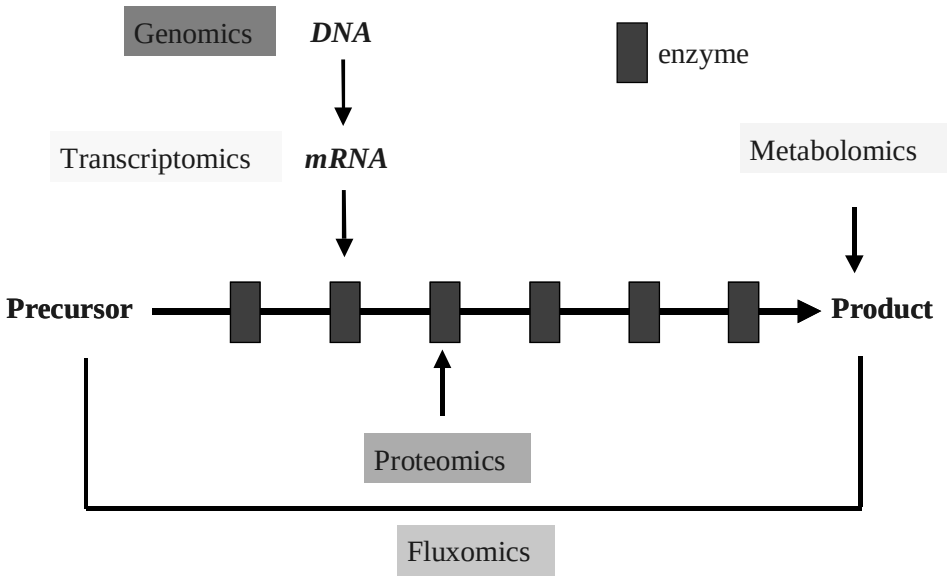
6 hours and determined its incorporation into glucose throughout the infusion period at regular intervals in moderately fasted *Pparα*$^{-/-}$ and control mice.[16] Through analysis of the incorporation of both labels over time it was possible to calculate rate of gluconeogenesis. This study showed that PPARα-deficiency was not associated with decreased but rather with similar rates of gluconeogenesis upto glucose-6-phosphatee (G6P).[16] Using a similar technique, Xu *et al.*[17] determined endogenous glucose production in *Pparα*$^{-/-}$ mice and found this to be even increased compared to control mice. Stimulating LXR by pharmacological means, did also not lead to changes in the gluconeogenic flux in mice although mRNA levels of PEPCK were slightly decreased (unpublished data). Taken together, these data imply that the transcription factors LXR and PPARα are not of pivotal importance to maintain a normal gluconeogenic flux or able to solely inhibit this flux, although this was expected based on molecular data alone. Compensatory mechanisms must be present or, alternatively, the known molecular modes of regulation are not quantitatively important for the metabolic flux as a whole.

A second example addresses control of bile salt metabolism, which forms an important component of cholesterol homeostasis in the body. Bile salts are synthesized in the liver from cholesterol, after which they are excreted into bile. Bile salt synthesis and metabolism is controlled by at least two transcription factors, *i.e.*, the nuclear receptor LXR and the Farnesoid X receptor (FXR, NR1H4).[18,19] A primary enzyme in the bile salt synthetic cascade is cholesterol 7alpha-hydroxylase. Molecular research has shown that LXR is involved in stimulation of transcription of cholesterol 7alpha-hydroxylase (Cyp7a1) in rodents, but not in humans.[20,21] FXR appears to be essential in downregulating Cyp7a1 transcription that is mediated through a mechanism involving two nuclear hormone receptors, *i.e.*, liver receptor homolog 1 (LRH-1, NR5A2) and small heterodimer partner (SHP, NROB2).[18,22-24] FXR is activated by bile salts that return to the liver during their enterohepatic circulation and acts by activating transcription of SHP. SHP subsequently binds and inactivates LRH-1, thereby repressing transcription of Cyp7al.[24] Furthermore, FXR stimulates transcription of the Ileal bile acid-binding protein transporter (IBABP), which is a small soluble protein and is expressed in the terminal ileum.[25,26] IBABP is thought to be involved in facilitating uptake of bile salts and their intracellular trafficking in the small intestine[27], thereby assisting in preserving the bile acid pool. Based on transcriptional data, one might expect strongly upregulated bile salt synthesis and decreased intestinal bile salt uptake in Fxr$^{-/-}$ mice. However, a recent study showed that the effects of FXR deficiency on the basal expression of Cyp7a1 (+150%) and on bile salt synthesis (+67%) were relatively modest.[28] Furthermore, intestinal bile salt uptake was even increased in Fxr$^{-/-}$ mice in this study. These results showed that although FXR has important regulatory functions, the effects of activation of this transcription factor on bile salt metabolism cannot be predicted on transcriptional data alone.

Transcriptional research suffers from apparent limitations as the data on transcriptional regulation of gluconeogenesis and bile salt synthesis and uptake showed. Apart from fluxomics all other “omics” research is static and results clearly cannot be directly extrapolated to a dynamic in vivo situation. Numerous fluxomics techniques have been

applied sofar, including labeled water techniques and mass isotopomer distribution analysis or MIDA[29], which involves use of probability logic to ultimately calculate synthetic rates. Molecular changes that do not lead to changes in the overall rate of production along a certain metabolic pathway, due to counter regulatory mechanisms or lack of regulatory importance in an in vivo situation, will not be detected using fluxomics. Fluxomics is therefore of pivotal importance for verifying the predictions that are made based on results obtained from transcriptomics, proteomics and metabolomics. A schematic representation of fluxomics and other levels of metabolic research are schematically depicted in Figure 1. The examples of transcriptional regulation of gluconeogenesis and bile salt synthesis underscore the fact that combined approaches are needed to be able to fully comprehend the complex regulatory mechanisms involved in a metabolic pathway. Fluxomics is an indispensable tool for integrating data obtained through other levels of metabolic research and to reach conclusions on metabolic regulation that represent true physiological in vivo systems.

Figure 1. Levels of metabolic research



The effects of glucose on lipid metabolism

This thesis comprises studies on metabolic regulation of carbohydrate and lipid metabolism. In these studies physiological and pathophysiological regulation is examined, when possible, at the transcriptional and post-transcriptional level combined with measuring actual fluxes.

Two central questions guided the research presented in this thesis:

1. How are cholesterogenesis and *de novo* lipogenesis regulated, and what is the role of carbohydrates herein?
2. How are gluconeogenesis and hepatic glucose production regulated, and what is the role of fatty acids herein?

Better insight in these regulatory processes will be helpful in understanding the metabolic changes occurring in diseases as type 2 diabetes, glycogen storage disease type 1 and other metabolic disorders. Multiple animal models and patient groups were used in trying to answer these questions. For measuring metabolic fluxes novel stable isotope methodologies were developed and used.

A long-standing discussion remains on whether hepatic *de novo* lipogenesis is of quantitative importance in human life. Hepatic *de novo* lipogenesis was studied under three conditions in this thesis to add to the discussion on this issue, *i.e.*, prematurity, glycogen storage disease and type 2 diabetes. One unanswered question is whether hepatic *de novo* lipogenesis is important in late intrauterine and early postnatal life, since it has been shown to be of little quantitative importance in adults on a western diet. Our data showed that hepatic *de novo* lipogenesis was present in pre-term infants, but at very low levels. This is in a sense remarkable, since at the time of the study, infants received almost solely carbohydrates as nutritional intake, which is supposed to increase lipogenic rates. It is known that fatty acid synthesis rates are regulated by various transcription factors, *i.e.*, SREBP1a, 1c[30-32], LXR[33] and ChREBP[34,35], with the latter one activated by glucose or one of its metabolic derivatives. Whether developmental aspects of this transcriptional pathways account for the lack of high rates of hepatic *de novo* lipogenesis remains to be seen. This finding also raises questions about how lipid accumulation in the fetus during the third trimester of pregnancy takes place. Some reservations must be included when extrapolating data from early postnatal, prematurely born, life to late intrauterine life. Taken into account these reservations, our data mig ht suggest that extrahepatic lipogenesis contributes to accumulation of lipids during late fetal life. Lipogenesis has been shown to occur in adipocytes[36], although probably at very low rates in adult humans[37], but could theoretically be more active during certain early periods in life. On the molecular regulation of lipogenesis in adipocytes, insight is starting to evolve. Recent studies suggest that PPARγ and LXR, which are both expressed in adipocytes, are involved in the regulation of lipogenesis in these cells.[38,39] Whether these transcription factors are important in the lipid accumulation during fetal life remains to be elucidated but provides a promising hypothesis. Studies using nuclear magnetic imaging techniques might help to elucidate the quantitative role of adipocyte *de novo* lipogenesis early in human life.

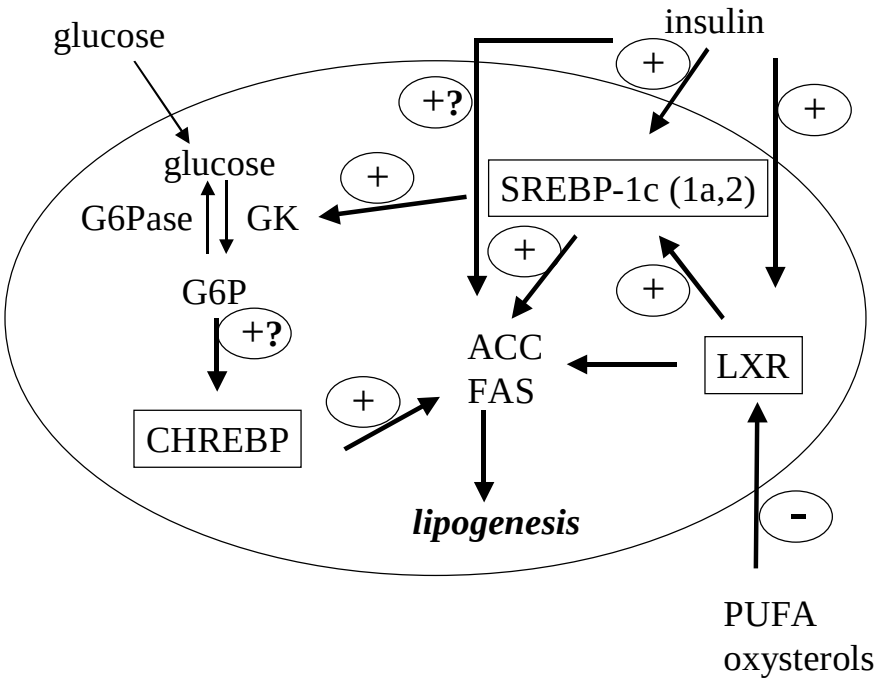
The metabolic state of patients with glycogen storage disease type 1 (GSD-1) is different from that of pre-term infants in many respects, although both states are associated with a high net hepatic glucose uptake. In GSD-1 patients, glucose entering the liver is in a sense trapped and must be metabolized either to glycogen or to acetyl-CoA. In both the

GSD-1 patients and the rat model of GSD-1, we did find strongly elevated levels of hepatic *de novo* lipogenesis. The transcription factor ChREBP might play a role in the induction of hepatic *de novo* lipogenesis in GSD-1, since ChREBP responds to changes in intracellular carbohydrate contents. However, in GSD-1, intrahepatic glucose concentrations are lower, because deficient glucose production is an intrinsic part of the disease. An intriguing hypothesis might therefore be that G6P is involved in transcriptional activation of lipogenesis. During the last years several studies have indicated a strong metabolic regulatory function for glucose-6-phosphate (G6P).[40-42] However, an alternative hypothesis is that another intermediate in hepatic glucose metabolism, *i.e.*, xylulose-5-phosphate (X5P) plays a role as metabolic regulator.[43] Future research is needed to assess if and how G6P might be to perform this regulatory role. Furthermore, it remains to be determined whether G6P/X5P is a specific ligand for ChREBP and what the role is of ChREBP in the development of increased hepatic *de novo* lipogenesis in GSD-1.

Strongly induced hepatic *de novo* lipogenesis was found in a type 2 diabetic model, *i.e.*, the *ob/ob* mouse, which could be attributed to an induced expression of at least one transcription factor, namely SREBP-1c. The expression of the genes encoding for LXR and ChREBP were not induced, although this does not exclude activation of the proteins. Using specific inhibiting systems it should be possible to distillate the relative importance of the various transcription factors in the regulation of *de novo* lipogenesis in the physiological and diabetic state. Hepatic *de novo* lipogenesis might not be an important pathway in healthy adults on a western diet, but can be induced substantially in various disease states. An overview of the molecular mechanisms involved in regulation of hepatic *de novo* lipogenesis are shown in Figure 2.

Cholesterogenesis was studied in the same metabolic situations as hepatic *de novo* lipogenesis. Cholesterogenesis is transcriptionally regulated almost entirely by SREBP2.[44] In pre-term infants increased rates of cholesterogenesis were found, when compared to adult subjects. Whether preferential development of transcriptional activation of the cholesterogenic pathway is involved in attaining the high rates of cholesterogenesis is unknown. With respect to the situation of GSD-1, only in the GSD-1a patients and not in the animal model of GSD-1b, evidence for increased cholesterogenesis was found. Why cholesterogenesis was upregulated in the GSD-1a patients and not in the rat GSD-1b model remains unclear. Is it related to differences between the acute model in the rats and the chronic patients? In the GSD-1 patients, a “diabetic” plasma lipid profile is present , with hypertriglyceridemia, low HDL-cholesterol, high LDL-cholesterol and decreased VLDL clearance.[45] In addition, indications exist that these patients are insulin resistant[46], of course without the hyperglycemia normally present in DM2. Since it is known that in DM2 patients cholesterogenesis is elevated[47], the high cholesterol synthesis rates in GSD-1a patients might be related to the insulin resistance. However, in the animal model used for DM2, *i.e.*, *ob/ob* mice, no increased rates of cholesterogenesis were found, although plasma cholesterol concentrations were increased. No clear explanation for the lack of increased cholesterogenesis is present at the moment. Since, other proteins (SCAP, S1P, S2P) are required for SREBP processing, overactivation of SREBP1c or SREBP1a to a higher extent

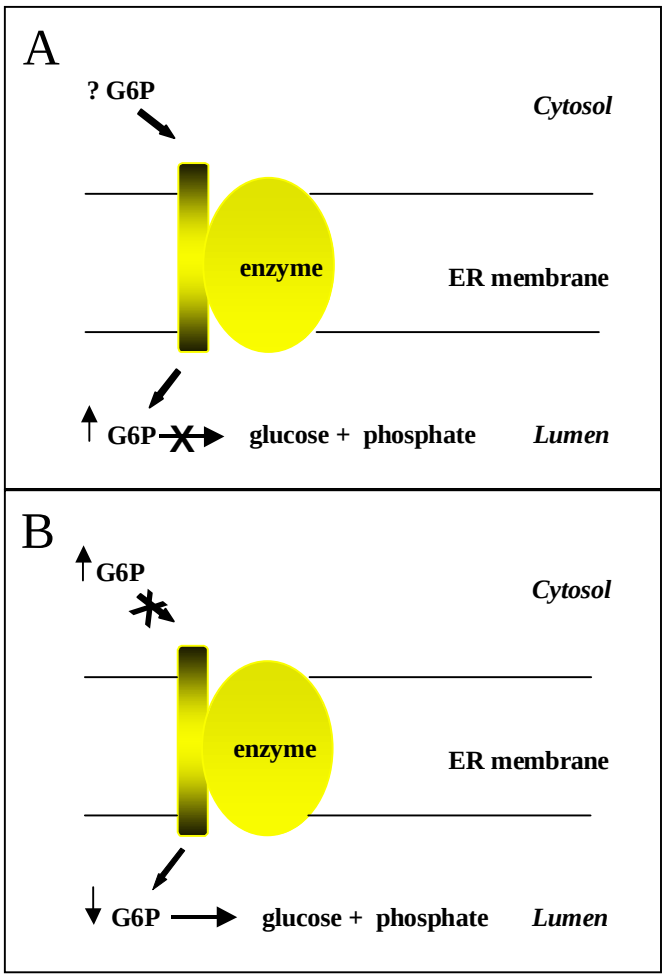
Figure 2. Overview of molecular regulation of hepatic *de novo* lipogenesis.



than SREBP2, might prevent upregulation of SREBP2 translocation to the nucleus, due to a relative shortage of SCAP, S1P or S2P.

Much debate remains on whether hepatic cholesterogenesis and *de novo* lipogenesis have a regulatory role in VLDL production and thereby development of hyperlipidemia. The increases in synthesis rates of cholesterol and fatty acids could only partially explain the hyperlipidemia in GSD-1a patients. We hypothesize that increased lipolysis and increased VLDL production account for the hyperlipidemic phenotype in GSD-1a. G6P might also be play a role in this respect, since Brown *et al.* found that phosphorylation of glucose is essential for upregulation of VLDL secretion by glucose.[48] However, we found no indication of increased hepatic VLDL secretion in an acute animal model of GSD-1b. There are some considerations with respect to this finding. First of all, the studies were performed in an acute animal model, whereas GSD-1 patients of course suffer from the disease chronically. It might be that the experiments were not long enough to observe changes in VLDL secretion. Furthermore, rats used in this study were fasted, causing elevation of FFA flux to the liver and perhaps already a maximal stimulation of VLDL secretion. An alternative hypothesis is that compartmentalization of G6P is responsible for the effects of G6Ptranslocase inhibition as is schematically shown in Figure 3. In GSD-1a,

Figure 3. Schematic overview of the G6Pase enzyme complex in GSD-1a (A) and GSD-1b (B).



G6P is able to enter the luminal side of the endoplasmic reticulum via interaction with G6P translocase. However, the formation of glucose is inhibited due to deficiency of the G6Pase catalytic subunit. Theoretically, this situation will lead to elevated levels of G6P inside the ER. In GSD-1b, G6P is unable to enter the ER, due to deficiency of G6P translocase, causing elevated levels of G6P inside the cytosol, but absence of G6P inside the ER. Since at least the early steps in VLDL formation take place inside the ER, fluctuations in G6P content in this compartment might influence VLDL formation. Determining VLDL secretion under the circumstance of specific inhibition of G6Ptranslocase or G6Pase might

help to unravel the importance of G6P compartmentalization for lipoprotein assembly and/or secretion.

Recently, a mouse model for GSD-1a was created.[49] Homozygous *G6Pase* $^{-/-}$ mice suffer from severe hypoglycemia and newborn pups die soon after birth without continuous exogenous supply of glucose. The severity of the disease makes in vivo studies in adult *G6Pase* $^{-/-}$ mice practically impossible. Heterozygous *G6Pase* $^{+/-}$ mice have no apparent phenotype of hypoglycemia or hyperlipidemia. However, when metabolically challenged, a phenotype might become apparent. It would be of interest to determine insulin sensitivity in these mice during, for example, a high-fat diet, which is known to cause insulin resistance in normal mice. In the same line of reasoning, it would also be of interest to study (heterozygous) parents of GSD-1 patients to determine insulin resistance, either by performing glucose tolerance tests, or better, by performing hyperinsulinemic euglycemic clamps together with carbohydrate flux measurements. If indeed this group of individuals shows a relative insulin insensitivity, it would indicate that even small increases in intrahepatic G6P content is enough to produce insulin resistance. This would perhaps provide more insight in the subtle regulation of carbohydrate metabolism and the primary features occurring in the development of DM2.

Although *de novo* lipogenesis was increased, basal VLDL secretion was not increased in *ob/ob* mice with hepatic insulin resistance. This finding underscores the complexity of the mechanisms behind the regulation of hepatic VLDL secretion. Carbohydrates, cholesterol and free fatty acids, all have been found to regulate VLDL secretion. Also SREBP's have been shown to negatively regulate transcription of an important protein involved in VLDL formation, *i.e.*, microsomal triglyceride transfer protein.[50] SREBP-1c mRNA levels were higher in the *ob/ob* mice, although SREBP-2 mRNA levels were lower compared to control mice. It would be worthwhile to separate these individual components in an in vivo system, to be able to understand what components are quantitatively important in regulation of VLDL secretion and how they are interrelated. Considering the type of hyperlipidemia observed in GSD-1 and the possible role of ChREBP herein, it is tempting to speculate on a possible role of ChREBP in the phenotype of DM2. Since the functions and ligand(s) of ChREBP have remained largely unknown up to now, future research should be aimed at trying to gain more knowledge on the basic properties of ChREBP.

The effects of lipids on glucose metabolism

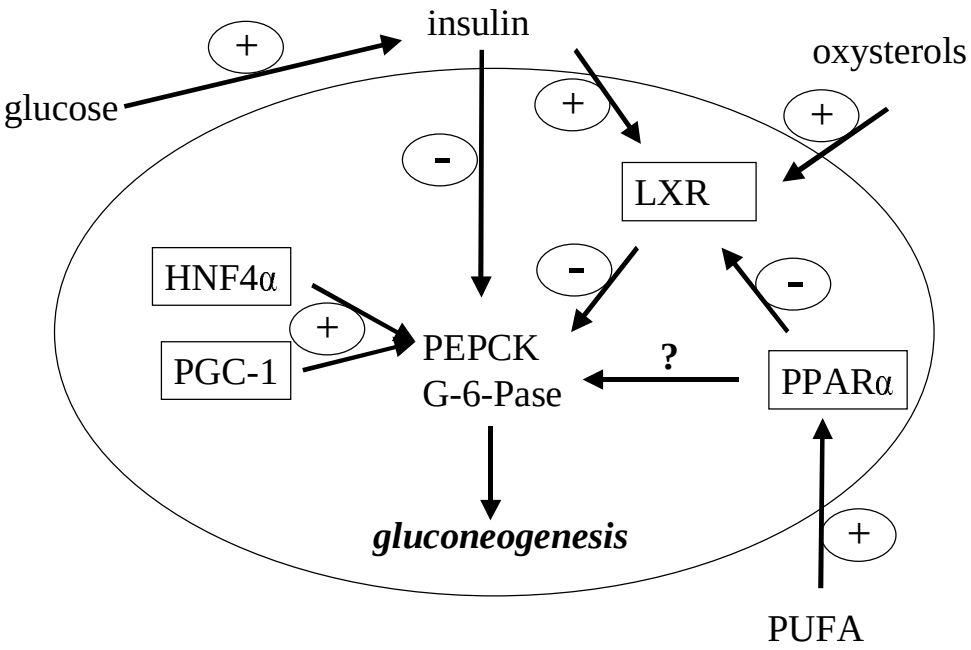
The second question that was addressed in this thesis concerned the regulation of gluconeogenesis and hepatic glucose production (HGP) and, especially, the role of fatty acids in this regulation. DM2 is characterized by insulin resistance, leading to fasting hyperglycemia. In addition, hepatic steatosis is a common feature of this disease. The hyperglycemia is related to elevated HGP in DM2. In theory, elevated HGP can be caused by an increase in GNG and/or glycogenolysis. Furthermore, if one defines HGP as the total flux from G6P to glucose, cycling between glucose and G6P could in theory also increase

HGP. We found evidence for highly induced glucose cycling, defined as the cycling between hepatic G6P and glucose, in *ob/ob* mice. Furthermore, total GNG, defined as the new synthesis of G6P, was significantly decreased in *ob/ob* mice in comparison with lean control mice, compared to controls. As a consequence, total hepatic glucose production was similar in both mice groups, despite higher insulin concentrations in the *ob/ob* mice. Insulin is a well-known inhibitor of GNG and a recent study provided molecular evidence for the inhibitory function of insulin on PEPCK.[51] Insulin resistance could therefore lead to an upregulation of Pepck gene transcription, although this was not observed in the study in the *ob/ob* mice. Recently, it was discovered that a co-activator of nuclear receptors, PGC-1, and HNF-4α are both responsible for transcriptional activation of Pepck and glucose-6-phosphatase (G6pase).[52] Activation of PGC-1 has been shown to be present in *ob/ob* mice[52], although we found similar PGC-1 mRNA levels in this study. Although many possible explanations for relatively increased HGP in *ob/ob* mice are apparent, no conclusive factors can be appointed as of yet.

PPARα is a transcription factor controlling genes involved in fatty acid oxidation, but it has also been hypothesized that PPARα is involved in regulation of hepatic glucose metabolism.[11] PPARα might provide a molecular link between fatty acid oxidation and glucose production, which could be important for understanding of the pathophysiology of DM2. HGP was decreased in *Pparα* $^{-/-}$ mice compared to control mice. One can speculate as what caused the specific effect of PPARα deficiency on HGP. It might be that PPARα transcriptionally regulates the G6Pase enzyme complex, although, again, no direct evidence has been produced sofar. An alternative hypothesis is that decreases in HGP are related to alterations in glycogen metabolism. Decreased HGP could also be secondary to decreased peripheral glucose utilization. PPARα might be be involved in thermogenesis, either directly or through interaction with leptin. Future studies to study the specific role of PPARα in hepatic G6P partitioning and peripheral glu cose utilization are required.

Interestingly, PPARα-deficiency had no effect on total GNG during fasting as already mentioned earlier. Furthermore, an earlier study where the G6Pase flux was pharmacologically inhibited in rats also showed unaffected GNG.[53] These data suggest that adequate GNG can be maintained under many circumstances, even in the presence of high intrahepatic G6P contents or decreased β-oxidation and that these processes are not directly linked during fasting. Instead of altering the rate of GNG, the liver rather redirects newly formed G6P towards either glucose or glycogen depending on the hormonal status of the organism, which might be related to the regulatory processes mentioned above. The combination of these experiments indicates that other factors are influencing the gluconeogenic flux toward glucose than the molecular regulatory factors known at present. An overview of the molecular mechanisms involved in regulation of GNG and HGP are shown in Figure 4.

Figure 4. Overview of molecular regulation of hepatic gluconeogenesis and glucose production.



Closing remarks

Metabolic disorders such as DM2 and GSD-1 represent, in some ways, opposite extremes of a metabolic state, one associated with overexpression of the G6Pase enzyme system (DM2), one with absence of this system (GSD-1). Obtaining a metabolic state associated with limited pathological consequences has been proven difficult in both diseases. Pharmaceutical research in the area of DM2 during the last years has been focused on partial inhibition of the G6Pase system. However, pharmaceutical solutions might not exist with respect to the treatment of obesity and DM2. A study presented in this thesis provided data on the overall metabolic effects of inhibition of the G6Pase system (chapter 4). A drug inhibiting glucose-6-phosphate translocase was developed for treatment of hyperglycemia in DM2. This study showed that partial inhibition of G6Pase caused severe hepatic steatosis in a 6-hour time frame in rats. In a study by Desai *et al.*[54] adenovirus-mediated glucokinase overexpression was achieved to study its effect in type 2 diabetic mice. Although whole body insulin resistance improved in these mice, hepatic triglyceride content did not decrease and hepatic glycogen content increased strongly in comparison to untreated mice. This new phenotype is expected to cause problems of hepatic steatosis and liver fibrosis. These studies underscore the inherent limitations of potential drugs stimulating or inhibiting specific enzymes in treating DM2. Since the various pathways in carbohydrate

and lipid metabolism are linked, it is expected that when one pathway is partially blocked by pharmaceutical inhibition, accumulation of an intermediate metabolic product will take place, especially in the case of continuous elevated caloric intake. Pharmaceutical intervention for diseases associated with a western life style, such as obesity and DM2, are therefore limited and perhaps only palliative in nature and should always be used in combination with dietary measures and exercise. This does not remove the relevance of this kind of research for society as a whole, and diseases such as DM2 and GSD-1 specifically, but scientific modesty in this respect is warranted.

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Zoals een mug nadat ik web
na webstructuur doorvlogen heb
hang ik gewurgd in deze bouw
van logica zo dik als touw
dat niet verbogen wil.
Laat me nou terug.

Leo Vroman

Summary

Carbohydrates and fat represent the most important sources of energy for the human body. Through a fine intricate system of regulation, the body is able to select to what extent it will use either fatty acids or carbohydrates for generation of energy in the form of adenosine-triphosphate (ATP). Breakdown of glucose through the process of glycolysis produces potential precursors for the synthesis of fat, *i.e.*, *de novo* lipogenesis, and of cholesterol. On the other hand oxidation of fat results nearly exclusively in acetyl-CoA, which is not a precursor for the synthesis of glucose, a process that is called gluconeogenesis. However, more and more data indicates that fat are involved in regulation of glucose metabolism and *vice versa*. This thesis is focused on the interactions between carbohydrate and lipid metabolism and especially on the regulation of *de novo* lipogenesis and cholesterogenesis by carbohydrates and the regulation of gluconeogenesis by fat. A special regulatory role for glucose-6-phosphate, an intermediate in the gluconeogenic and glycolytic pathway, in the regulation of *de novo* lipogenesis is suggested. Novel stable isotope techniques have been developed and used to determine the lipogenic, cholesterogenic and gluconeogenic rates in animal models as well as in humans.

In chapter 2 the importance of the hepatic *de novo* lipogenesis and cholesterogenesis is determined in prematurely born infants. Hepatic *de novo* lipogenesis has been found to be an unimportant pathway in adult humans on a western diet, but might be quantitatively important during the fetal period, since 16% of a full-term baby consists of fat at the time of birth. During this period the unborn infant receives its nutrition primarily through placental delivery of carbohydrates. Preterm infants were infused with a stable isotopically-labeled precursor of cholesterol and fatty acids (^{13}C-actetate) during the first 48 hours after birth when dietary intake consisted almost entirely of glucose. The distribution of label incorporation was measured and analysis of the mass isotopomer distribution (MIDA) was performed to determine hepatic *de novo* lipogenesis and cholesterogenesis. Hepatic *de novo* lipogenesis represented only 5% of fatty acids in secreted very low-density lipoprotein particles, indicating that this pathway is not quantitatively important during in preterm infants. Extrahepatic lipogenesis, possibly in adipocytes, might contribute to the fat accumulation during gestation. Hepatic cholesterogenesis, however, was about three times the rate per kilogram bodyweight as found in healthy adult subjects. These results might suggest that cholesterogenesis is a quantitatively important pathway in late fetal life.

Glycogen storage disease type 1 is a disease caused by deficiency of the glucose-6-phosphatase (G6Pase) enzyme complex. G6Pase catalyzes the conversion of glucose-6-phosphate (G6P) into glucose and represents the final step in glucose production from either glycogen breakdown or gluconeogenesis. GSD-1 can be separated into at least two distinct types of diseases, *i.e.*, types 1a and 1b, on the basis of the underlying gene defects. The catalytic subunit of the G6Pase complex is deficient in GSD-1a, whereas the G6P translocase, responsible for transport of G6P from cytosol into the lumen of the endoplasmic reticulum, is deficient in GSD-1b. GSD 1 patients do not only suffer from hypoglycemia, but also develop severe hypercholesterolemia and hypertriglyceridemia. We

chose to investigate the origin of the abnormalities in lipid homeostasis in GSD-1 patients (chapter 3). By using the same methodology as used in chapter 2, it was shown that GSD-1 patients had strongly upregulated rates of hepatic cholesterogenesis and *de novo* lipogenesis, compared to healthy adult subjects. However, hepatic *de novo* lipogenesis could not fully account for the observed hypertriglyceridemia in these patients. Furthermore, despite their severe atherogenic lipid profile most studies indicate that GSD-1 patients are not prone to early development of atherosclerosis. An additional finding in our study was that lipoproteins from GSD-1 patients have a lower oxidizability rate than lipoproteins from healthy adult subjects. Oxidation of lipoproteins is a key step in the formation of atherosclerotic lesions and its rate is related to the fatty acid composition. In our study GSD-1 patients were found to have strongly elevated synthesis rates of palmitate, a saturated fatty acid. We found an inverse correlation between the relative amount of saturated fatty acid of lipoproteins and their oxidizability. Therefore this study provides data indicating that GSD-1 patients are protected from early development of atherosclerosis by high rates of hepatic *de novo* lipogenesis.

In chapter 4, an animal model of GSD-1 was used to further clarify the origin of the hyperlipidemia in this disease. A class of chlorogenic acid derivatives had recently been developed for the treatment of hyperglycemic conditions in DM2. These compounds specifically inhibit G6Pase activity by blocking the translocase of the G6Pase complex and thereby increasing hepatic G6P levels. One of these compounds, S4048, was infused in moderately fasted rats to address the question whether acute increases in hepatic G6P levels were associated with increased rates of *de novo* lipogenesis and cholesterogenesis and whether this would affect hepatic VLDL secretion. Acute inhibition of G6P translocase indeed led to strongly increased rates of *de novo* lipogenesis as determined by MIDA, *i.e.*, more than ten times compared to untreated rats. However, cholesterol synthesis rates were not affected by inhibition of G6P translocase. Finally, although studies indicated that high rates of *de novo* lipogenesis are associated with increased rates of hepatic VLDL secretion, these rates were similar between our two study groups, contributing to the observed hepatic steatosis in the S4048-treated rats. From this study we concluded that intrahepatic G6P elevation can lead to a stimulation of *de novo* lipogenesis within a short-time frame, but that the increase in *de novo* lipogenesis is not sufficient to stimulate VLDL secretion.

Diabetes Mellitus type 2 (DM2) is associated with increases in cholesterol, and triglyceride concentrations. A model for DM2 is the *ob/ob* mouse, which is characterized by leptin-deficiency. These mice suffer from fasting hyperglycemia and hyperinsulinemia as well as from hypercholesterolemia and hypertriglyceridemia. In chapter 5 this model was used to further clarify the interactions between glucose and lipid metabolism. It was found that hepatic *de novo* lipogenesis was strongly increased in *ob/ob* mice compared to lean littermates and contributed to the hypertriglyceridemia. However, cholesterogenesis was similar between the two groups despite increases in plasma concentrations and hepatic content. Basal hepatic VLDL secretion was also similar in *ob/ob* mice and lean littermates. Only under influence of acute hyperinsulinemia VLDL secretion was less suppressed in *ob/ob* mice compared to lean littermates. As found under circumstances of acute elevation

of hepatic G6P content, increases in hepatic *de novo* lipogenesis are not sufficient to upregulate VLDL secretion. Whether a lack of increase in cholesterogenesis limits the amount of VLDL particles secreted remains speculative.

In chapter 6 different labelling techniques were used to answer the question to what extent hepatic gluconeogenesis, glucose cycling and peripheral clearance of glucose contribute to hyperglycemia in an animal model of DM2, *i.e.* the *ob/ob* mouse. Mice were fasted for 9 hours after which they were infused with a sterile solution, containing [U-^{13}C] glucose, [2-^{13}C]glycerol, [1-^{2}H]galactose and paracetamol. By analysis of glucose and paracetamol-glucuronide from respectively bloodspots and urine samples taken throughout the infusion period metabolic flux rates could be calculated. The rate of *de novo* synthesis of G6P in obese mice was significantly decreased in comparison with lean control mice. Partitioning of newly synthesized G6P towards plasma glucose or glycogen was not affected in obese mice when compared to lean control mice. In contrast, glucose cycling was greatly enhanced in obese mice. As a consequence, total hepatic glucose production, *i.e.*, the sum of endogenous glucose production and glucose cycling, was similar in obese and lean mice. Furthermore, metabolic clearance rate of glucose was strongly decreased in *ob/ob* mice compared to control mice. In conclusion, this study demonstrated that in *ob/ob* mice *de novo* synthesis of glucose-6-phosphate was diminished while glucose cycling was increased, resulting in a "normal" total glucose output by the liver. However, these normal values were observed in face of hyperglycemia and hyperinsulinemia pointing to a co-existence of hepatic and peripheral insulin resistance with peripheral insulin resistance as the major cause of hyperglycemia.

Peroxisome proliferator activated receptors (PPARs) are a group of proteins that regulate expression of genes and are activated by fatty acids and fibrates. PPARα regulates the expression of a variety of proteins involved in β-oxidation and lipoprotein metabolism. Interestingly, mice deficient in PPARα (*Pparα*$^{-/-}$) suffer from severe hypoglycemia during fasting. In a study in *Pparα*$^{-/-}$ mice it was tried to clarify the exact disturbances in carbohydrate metabolism in order to gain insight in the relation between β-oxidation and hepatic glucose production (chapter 7). The same methodology as in chapter 6 was used for this study. Hepatic glucose production was lower in *Pparα*$^{-/-}$ compared to *Pparα*$^{+/-}$ mice after a moderate fast. However, total *de novo* synthesis of G6P was similar between the two groups. Altered partitioning of G6P, which was preferentially directed towards hepatic glycogen stores, was responsible for the observed decrease in hepatic glucose production. This study indicates that β-oxidation and hepatic *de novo* production of G6P are not directly metabolically linked processes.

In conclusion, the studies described in this thesis have given additional insight in the complex interactions that exist between carbohydrate and lipid metabolism. Hepatic *de novo* lipogenesis is not a major pathway in early and adult human life even under circumstances of high carbohydrate intake and might only be a quantitatively significant in specific metabolic disorders, such as GSD-1 and DM2. G6P, an intermediate in the gluconeogenic pathway, might be an important factor in the regulation of hepatic *de novo* lipogenesis by carbohydrates. *De novo* synthesis of G6P is probably not transcriptionally

regulated by fat through PPARα, but PPARα does play a role in the partitioning of G6P. Since data on transcriptional regulation not necessarily corresponded with kinetic data, these studies understate the need to combine molecular with kinetic research to come to any conclusions regarding metabolic regulation.

Samenvatting

Onderzoek naar ziektebeelden die veroorzaakt worden of gepaard gaan met stoornissen in de stofwisseling, ook wel metabolisme genoemd, is van groot belang gezien de sterke toename van stofwisselingsziekten zoals diabetes mellitus type 2 (DM2) en aandoeningen als atherosclerose. Een beter inzicht in het mechanisme achter het ontstaan van deze welvaartsziekten is relevant voor zowel preventie als de behandeling ervan. Overgewicht, met daaraan gerelateerde veranderingen in suiker- en vetstofwisseling, is een belangrijke factor in het ontstaan van DM2 en naar schatting lijden wereldwijd meer dan een miljard mensen aan overgewicht. Overgewicht is niet alleen een probleem van de volwassen bevolking maar neemt ook sterk toe in kinderen. Meer dan de helft van alle zwarte kinderen en eenderde van alle blanke kinderen in de Verenigde Staten hebben last van overgewicht op de leeftijd van 19 jaar. Hiermee gepaard gaande is ook de incidentie van DM2 in kinderen de laatste jaren sterk toegenomen. Stoornissen in de vetstofwisseling die zijn geassocieerd met DM2, die o.a. tot uiting komen in een verhoogde hoeveelheid schadelijke vetten in het bloed, zoals cholesterol, zijn aanleiding tot een sterk verhoogde kans op het onstaan van atherosclerose.

Inzicht in de (dys)regulatie van specifieke metabole processen leidt mogelijk tot nieuwe mogelijkheden tot therapeutisch ingrijpen in ziektebeelden zoals DM2. Het onderzoek gepresenteerd in dit proefschrift tracht meer inzicht te geven in de complexe interacties die plaatsvinden tussen suiker- en vetstofwisseling in (patho)fysiologische condities. Suikers en vetten vormen de belangrijkste energiebronnen voor het menselijk lichaam. Cellen in het lichaam kunnen met behulp van een complex regulatiesysteem bepalen in welke mate zij vetten danwel suikers verbranden. Tijdens de verbranding van suikers worden, via een proces dat glycolyse wordt genoemd, potentiële bouwstenen voor de produktie van vetten geproduceerd. Aan de andere kant leidt verbranding van vetten niet tot de vorming van bouwstenen voor de produktie van de belangrijkste suiker, glucose. De produktie van glucose wordt gluconeogenese genoemd. Veel onderzoek laat echter zien dat vetten wel betrokken zijn bij de regulatie van glucose metabolisme en *vice versa*. Onderzoek beschreven in dit proefschrift richt zich met name op de regulatie van de produktie van vetten door glucose en de regulatie van gluconeogenese door vetten. Nieuwe stabiele isotopen technieken zijn ontwikkeld en gebruikt om de produktie van vetten en de gluconeogenese door de lever te kunnen meten.

In hoofdstuk 2 is getracht te bepalen hoe belangrijk de produktie van vetten door de lever zijn voor de te vroeg geboren baby. Het is aangetoond dat de aanmaak van vetten geen belangrijke metabole route is in volwassen mensen met een westers dieet, maar zou kwantitatief belangrijk kunnen zijn gedurende de foetale periode, aangezien 16% van het lichaamsgewicht van een voldragen baby bestaat uit vet. Gedurende deze periode bestaan de voedingsstoffen met name uit glucose. Te vroeg geboren babies werden ongeveer 48 uur na de geboorte geinfundeerd met gemerkte bouwstoffen, zogeheten stabiele isotopen (^{13}C-acetaat), van cholesterol en vetzuren. Op dat moment bestond de voedingsinname bijna uitsluitend uit glucose. De verdeling van de ^{13}C-acetaat inbouw in cholesterol en vetzuren

werd gemeten en analyse werd verricht, via de methode van massa isotopomeer verdeling (MIDA), om de produktie van vetzuren en cholesterol te bepalen. De door de lever nieuw geproduceerde vetzuren maakten slechts voor 5% deel uit van de door de lever uitgescheiden vet deeltjes, zogeheten very low-density lipoproteinen. Wel waren de te vroeg geboren babies in staat tot het produceren van grote hoeveelheden cholesterol, hetgeen meer was per kilogram lichaamsgewicht dan volwassenen produceren. Deze data suggereren dat in de periode voor de geboorte de lever waarschijnlijk niet veel bijdraagt aan de vetopbouw in het lichaam. Vetcellen zijn mogelijk zelf in staat zijn tot het aanmaken van grote hoeveelheden vet voor de geboorte.

Glycogeen stapelingsziekte type 1 (GSD-1) is een ziekte die veroorzaakt wordt door een ontbreken (deficiëntie) van het glucose-6-fosfatase (G6Pase) enzym complex. G6Pase katalyseert de omzetting van glucose-6-fosfaat (G6P) naar glucose en vormt de laatste stap in de glucose productie via zowel glycogeen afbraak als de gluconeogenese. Glycogeen is de opslagvorm van glucose, en wordt vooral gevormd in de lever. GSD-1 kan opgesplitst worden in tenminste twee ziektebeelden, type 1a en 1b, op basis van het onderliggende gen defect. Het enzym complex G6Pase bestaat uit een G6P transporter, die G6P transporteert van het ene cellulaire compartiment, het cytosol, naar het andere, te weten de binnenkant van het endoplasmatisch reticulum. Daarnaast bestaat G6Pase uit een onderdeel dat de omzetting van G6P naar glucose katalyseert, de zogeheten katalytische subeenheid. Deficiëntie van de transporter leidt tot GSD-1b en van de kataliserende subeenheid tot GSD-1a. GSD-1 patiënten hebben niet alleen lage concentraties glucose in het bloed, hypoglycemie, maar hebben tevens hoge vet- (hypertriglyceridemie) en cholesterol (hypercholesterolemie) concentraties in het bloed. In de studie beschreven in hoofdstuk 3 werden de afwijkingen in de vetstofwisseling in patiënten met GSD-1 onderzocht. Met behulp van dezelfde technieken als gebruikt in hoofdstuk 2 werd gevonden dat GSD-1 patiënten sterk opgereguleerde verzadigde vetzuur en cholesterol produktiesnelheden hadden vergeleken met gezonde vrijwilligers. De vetzuur produktie door de lever kon niet volledig de hypertriglyceridemie verklaren, dus verminderde verwijdering van vet uit het bloed moet eveneens een rol spelen. Ondanks de hoge vetconcentraties in het plasma, die naar verwachting tot vroegtijdige atherosclerose zouden leiden, suggereren meerdere studies dat dit niet het geval is. Een bijkomende bevinding was dat de transportdeeltjes van vetten door het bloed, zogeheten lipoproteinen, minder makkelijk tot oxidatie te stimuleren waren dan lipoproteinen van gezonde vrijwilligers. De oxidatie van lipoproteinen wordt gezien als een belangrijke stap in de ontwikkeling van atherosclerose. De oxidatie van lipoproteinen is gerelateerd aan de samenstelling van de vetzuren, dat wil zeggen verzadigde versus onverzadigde vetzuren. Een omgekeerde relatie werd gevonden tussen de relatieve hoeveelheid verzadigde vetzuren in de lipoproteinen en hun oxideerbaarheid in GSD patienten en gezonde vrijwilligers. Deze studie levert dan ook aanwijzingen dat GSD-1 patiënten beschermd zijn tegen vroegtijdige atherosclerose door hoge (verzadigde) vetzuur produktiesnelheden.

In hoofdstuk 4 werd een diermodel van GSD-1 gebruikt om verder het mechanisme van het ontstaan van de hypertriglyceridemie te onderzoeken. Een groep van derivaten van

chlorogeenzuur is ontwikkeld met als doel hyperglycemie (hoge concentraties suiker in het bloed) in DM2 te behandelen. Deze groep van middelen remt specifiek de G6Pase activiteit door de translocase van het G6Pase complex te blokkeren en daardoor G6P concentraties te verhogen. Een van deze stoffen, S4048, werd continue toegediend aan gevaste ratten om te bestuderen of acute stijgingen in G6P concentraties in de lever geassocieerd waren met verhoogde produktiesnelheden van vetzuren en cholesterol door de lever. Tevens werd onderzocht of dit effect had op de uitscheiding van transportdeeltjes van vetten, namelijk very-low-density lipoproteins (VLDL) door de lever. Acute remming van G6P translocase leidde inderdaad tot sterk verhoogde vetzuur produktiesnelheden, meer dan tien maal vergeleken met onbehandelde ratten. Opmerkelijk was dat de cholesterol produktiesnelheden niet werden beïnvloed door remming van het G6Ptranslocase enzym. Een aantal studies geven aan dat verhoogde vetzuur produktie geassocieerd is met verhoogde VLDL uitscheiding door de lever. Echter in de huidige studie met sterk verhoogde vetzuur produktie in de met S4048 behandelde ratten, was de VLDL uitscheiding gelijk ten opzichte van onbehandelde ratten. Hierbij ontwikkelden de S4048 behandelde ratten tevens een ernstige vette lever. Uit de studie werd geconcludeerd dat verhoging van de G6P concentratie in de lever kan leiden tot acute stimulering van de vetzuur produktie, maar dat verhoogde produktie van vetzuren op zich niet voldoende is om VLDL uitscheiding te verhogen.

Diabetes mellitus type 2 is geassocieerd met hypertriglyceridemie en hypercholesterolemie. Een model voor DM2 is de *ob/ob* muis, die gekarakteriseerd wordt door een ontbreken van het leptine eiwit. Deze muizen hebben, naast extreem overgewicht, hyperglycemieen tijdens vasten en een te hoge insuline concentratie. De dieren zijn relatief ongevoelig voor insuline dat onder meer een belangrijke rol speelt in het onderdrukken van de glucose productie door de lever. In hoofdstuk 5 werd dit model gebruikt om de interacties tussen suiker- en vetstofwisseling verder te bestuderen in de context van DM2. De vetzuur produktie door de lever was sterk verhoogd in de *ob/ob* muis vergeleken met controle muizen. De cholesterol produktiesnelheid was echter even groot in beide groepen muizen, ondanks verhoogde cholesterol concentraties in het bloed in de lever. Basale VLDL uitscheiding was ook gelijk in *ob/ob* muizen en controle muizen. Onder invloed van acute verhogingen van insuline concentraties in het bloed was de VLDL uitscheiding echter minder onderdrukt in de *ob/ob* muizen ten opzichte van de controle muizen. Evenals in de studie waar het G6Ptranslocase enzym werd geremd, leidde ook in deze studie een verhoogde vetzuur produktie niet tot een stimulering van de VLDL uitscheiding door de lever. Mogelijk blijft door het afwezig zijn van een stijging van de cholesterol produktie in de lever de hoeveelheid uitgescheiden VLDL deeltjes beperkt.

In hoofdstuk 6 werden verschillende stabiele isotopen technieken gebruikt om het mechanisme van de hyperglycemie in *ob/ob* muizen te achterhalen. *Ob/ob* muizen werden geinfundeerd met gelabeld glucose ([U-^{13}C] glucose) en bouwstoffen van glucose ([2-^{13}C]glycerol en [1-^{2}H]galactose). Frequente bloedmonsters en urinemonsters werden tijdens het infuus afgenomen en door onder andere analyse van ^{13}C gelabelde glucose kon de nieuwe glucose produktie (gluconeogenese) en glycogeen afbraak (glycogenolyse) worden

bepaald. Tevens kon zo worden berekend hoeveel glucose door de lever werd opgenomen en direct weer uitgescheiden (glucose cycling). Zowel glucose als insuline concentraties waren sterk verhoogd in *ob/ob* muizen vergeleken met slanke controle muizen. De gluconeogenese was uitgedrukt per kilogram lichaamsgewicht lager in *ob/ob* muizen vergeleken met slanke controle muizen. De hoeveelheid glucose cycling was echter duidelijk toegenomen in *ob/ob* muizen. Hierdoor bleef de totale glucose uitscheiding door de lever gelijk in *ob/ob* muizen en controle muizen. Hierbij was de hoeveelheid glucose die per tijdseenheid uit het bloed verwijderd kon worden duidelijk lager in *ob/ob* muizen. Uit deze studie concludeerden we dat met name glucose cycling verantwoordelijk is voor een te hoge glucose productie bij een verhoogde insuline concentratie. Een verlaagde mogelijkheid van het lichaam om glucose uit het bloed te verwijderen is met name verantwoordelijk voor de hyperglycemie in *ob/ob* muizen.

Peroxisome proliferator-activated receptors (PPARs) zijn een groep van eiwitten die de expressie van genen reguleren en die zelf geactiveerd worden door vetzuren. PPARα is een eiwit uit deze groep dat de expressie van een uitgebreid aantal genen, betrokken bij de vetzuur verbranding en lipoproteine stofwisseling, reguleert. Muizen die geen PPARα bezitten (*Pparα*$^{-/-}$) zijn niet goed in staat om vetzuren te verbranden en leiden aan ernstige hypoglycemieen tijdens vasten. In een studie met *Pparα*$^{-/-}$ muizen werd getracht te onderzoeken of een stoornis in de vetzuur verbranding leidt tot een verminderde gluconeogenese (hoofdstuk 7). Hiervoor werd dezelfde methodologie gebruikt als in hoofdstuk 6. Glucose produktie in de lever was lager in de *Pparα*$^{-/-}$ muizen in vergelijking met controle muizen na een relatief korte periode van vasten. De nieuw produktie van G6P, een belangrijk regulerend tussenproduct in de glucose productie, was echter wel gelijk in beide groepen muizen. Een veranderde verdeling van G6P, waarbij G6P preferentieel werd omgezet in glycogeen, was verantwoordelijk voor de verminderde glucose produktie door de lever. Deze studie toont aan dat vetzuur verbranding en de produktie van G6P niet direct aan elkaar gerelateerde processen zijn.

De studies uit dit proefschrift vergroten het inzicht in de complexe interacties tussen suiker- en vetstofwisseling. Vetzuur produktie in de lever is een kwantitatief onbelangrijk proces in het vroege en volwassen leven en is mogelijk alleen kwantitatief van belang in specifieke stofwisselingsstoornissen, zoals GSD-1 en DM2. Toegenomen intrahepatische concentraties van G6P, een intermediair in de gluconeogenese, zijn mogelijk van belang in de opregulatie van de vetzuur produktie in patienten met GSD-1 en DM2. De snelheid van gluconeogenese door de lever tot het nivo van G6P wordt waarschijnlijk niet op gen nivo gereguleerd door vetten via PPARα, maar PPARα speelt mogelijk wel een rol in de verdeling van gevormd G6P. Aangezien data met betrekking tot transcriptionele regulatie niet noodzakelijkerwijs overeenkwamen met kinetische data in bovenstaande studies, onderschrijven deze studies de noodzaak om moleculair en kinetisch onderzoek met elkaar te kombineren om conclusies te kunnen trekken over regulatie van stofwisseling.

List of abbreviations

ACC	acetyl-CoA carboxylase
AGA	appropriate size for gestational age
CE	cholesteryl ester
ChREBP	carbohydrate responsive element binding protein
CPT1A	carnitine palmitoyltransferase 1a
DM2	diabetes mellitus type 2
FAS	fatty acid synthase
FFA	free fatty acid
G6P	glucose-6-phosphate
G6Pase	glucose-6-phosphatase
G6PH	glucose-6-phosphate hydrolase
G6PT	glucose-6-phosphate translocase
GC-MS	gas chromatography-mass spectrometry
GK	glucokinase
GNG	gluconeogenesis
GP	glycogen phosphorylase
GS	glycogen synthase
GSD	glycogen storage disease
HDL	high-density lipoprotein
HGP	hepatic glucose production
HMGS	3-hydroxy-3-methylglutaryl-CoA synthase (mitochondrial)
HNF	hepatic nuclear factor
IDL	intermediate-density lipoprotein
IR	insulin receptor
IRS	insulin receptor substrate
LDL	low-density lipoprotein
LXR	liver X receptor
MCAD	medium-chain acylCoA dehydrogenase
MIDA	mass isotopomer distribution analysis
MODY	maturity-onset diabetes of the young
MTP	microsomal triglyceride transfer protein
MUFA	mono-unsaturated fatty acids

PEPCK	phosphoenolpyruvate carboxykinase
PI3K	phosphatidyl-inositol 3-kinase
PK	liver-type pyruvate kinase
PPAR	peroxisome proliferator-activated receptor
PUFA	polyunsaturated fatty acids
SCAP	SREBP cleavage activating protein
SFA	saturated fatty acids
SGA	small for gestational age
SREBP	sterol regulatory element binding protein
TG	triglyceride
VLDL	very low-density lipoprotein
WT	wild-type
X5P	xylose-5-phosphate

De hoofdrolspelers in willekeurige volgorde:

Anke ter Harmsel:	De godin van de chemie en beschermheilige van de man met teveel delegase, zoals Folkert het ooit noemde. Oh Anke, hoe moet het nu verder zonder jou?
Theo van Dijk:	Heerser over MIDA land en als deel van Theo en Theo de Statler en Waldorf van het lab.
Theo Boer:	GC deskundige en MS held. Droog als gort. Eveneens deel van het komisch duo Theo en Theo
Folkert Kuipers:	Waakt over alle AIO's, analisten en de rest van de onderzoeks bende. Tevens het brein achter de meeste geniale ideeën. Het kan slechter qua baas.
Frans Stellaard:	Ex-hippie, nu tovenaar met isotopen. Deelt het roddelhok met Henkjan en Folkert.
Anniek Werner:	Snoepnimf, paranimf, prinses van de woordgrapjes, barones van de neologismen.
Dirk-Jan Reijngoud:	De Panoramix van het lab. Bovennatuurlijke chemische kennis. Stiekem onmisbaar.
Pieter Sauer:	En hij legde geduldig uit hoe dat ook alweer moest, dat nadenken over de beste behandeling. Op zijn Gronings: 't kan slechter qua baas.
Henk Jan Verkade:	Als hij diep nadenkt barsten de ruiten. Helpt tevens regelmatig een verdwaalde AIO.
Deanna Minich:	Een Sirene in een sprookjesbos. Niemand kon haar gezang weerstaan.
Rick Havinga:	De spin in het web van het lab. Zonder hem geen experimenten. Zonder hem geen filosofische roddels. Alle vrouwen zijn stiekem een beetje verliefd op hem.
Mini Kalivianakis:	Bij elke ondergaande zon denkt hij met weemoed aan deze ZonneAIO.
Renze Boverhof:	Overziet het lab en zegt dat het goed is. Weet chemische orde te creëren uit chaos, tevens wereldreiziger.
Feike van der Leij:	Mr. Molecular
Fjodor van der Sluijs:	PCR koning. Praktiseert humor voor gevorderden.
Anja Hafkamp:	Bilirubine AIO en kamergenoot van Robert, Aldo, Jeanine en the Mediterranean girls. Een lieve preciezerd, met wie het heerlijk dansen was.
Henk Elzinga:	Alwetende IRMS'er.
Trijnie Bos:	Vriendelijke stofwisselaarster.
Antonella Pellicoro:	Pasta Princess, die nog eens zou koken voor een hongerige AIO.

Han Moshage:	Seppuku kenner met humor. Wat zou het saai zijn zonder Dr. Moshage.
Guido Hooiveld:	De gepromoveerde bier brabo
Christiaan Hulzebos:	Mr. Sympathico. Gatverdamme wat is die jongen aardig en attent. Schijnt tevens geweldige vader en dokter te zijn.
Juul Baller:	Histologie equals Juul. Ook al zo'n vriendelijke jongen.
Aldo Grefhorst:	Mag het een onsje droger voor deze AIO?
Baukje Elzinga:	Ex-AIO, nu Miss Ireland.
Pim Modderman:	Relaxte metabool.
Jeanine Kruit:	Juffrouw Vrolijk. Ze is blij, blij en blij.
Torsten Plösch:	Voortrekker van technische moleculaire innovaties. Voelt zich op het lab als een vis in demiwater.
Mariska Geuken:	Oogverblindend elegant en brood droog. Weet alles van hippe kleding en hippe muziek.
Klaas-Nico Faber:	Een slimme begeleider van dat "andere" lab. Een onvervalste bioloog.
Thera Vos:	Publiceerde alleen in Gastro en Hepatology.
Manon Buist-Homan:	Godin van de levende cellen. Heeft nog nooit haar serene kalmte verloren.
Marieke de Wiljes:	Voor een praatje en een regeldingetje.
Jenny Ros:	Goochelde met MRP, jongleerde met MDR en NF-kappaB.
Gijs Nagel:	Sportieve super GC-MS'er en piekjeskenner.
Albert Gerding:	Paté master en metabole analytico.
Jan-Peter Rake:	Een gezellige metabool. Weet alles van stapelen en tegenwoordig ook van house parties.
Peter Smit:	De man die nog meer dan alles weet van stapelen en ook verbranden.
Vincent Bloks:	De webmaster en alleskunner.
Peter Voshol:	Oom Losbol. Was lid van de gezelligste AIO kamer van Nederland. Wist bijna meer van bier dan van MDR2. Verliezer van een weddenschap.
Marieke Schoemaker:	Een gesprek met Marieke is als een kerstavond bij haardvuur.
Lena Westerdijk:	Hoedster over ons allen.
Lisette Bok:	De analiste met de eeuwige kuiltjes.
Janette Terpstra-Heegsma:	Naast analiste, dromer van het wijde westen en lid van het brood droog team.
Henk Wolters:	Analytisch kunstenaar.
Hilde Haaksema:	Geen boekje zonder Hilde.
Anette van Assen-Bolt:	Vriendelijke metaboolse

Jannie Tjassing: Voor al uw geestelijke en praktische ondersteuning. Weet alles van Friesland en fietsen door wijde landschappen.

Han Marra: Voor al uw geestelijke en praktische ondersteuning, of even voor de gezelligheid.

Coen Wiegman: De man met het paarse haar. Ook ex-lid van de gezelligste AIO kamer van Nederland.

Renate Hagedoorn: Sugarqueen AIO.

Arjen Mensenkamp: klassieke VLDL wizard.

Laura Conde de la Rosa: Miss Hola Ole.

Edmund Rings: Lid van de gastro boys. Droomt nog altijd een beetje van Philly.

Nienke Koopen: Ook zij was lid van de meest gezellige AIO kamer van Nederland.

Jenny Janssens-Puister: lid van het metabole dream team

Janneke van der Molen: Goedlachse juffrouw van het metabole clubje

Frank Bodewes: Lid van de gastro boys. Blanke yu di Korsou. Tevens tipgever over hoeveel melk er ook alweer in zo'n krijsende baby gekieperd moet worden.

Hans Blokzijl: ADHD^2. Als hij niet zo gezellig was, zouden we hem dagelijks in de Ritalin dompelen.

Klary Niezen: Waar zouden enzymen zijn zonder Klary?

Marion Priebe: Altijd aan het dietisten voor Roel.

Willie van Waarde: Waarom zijn niet alle slimme dokters en onderzoekers zo lief? Weet veel van hoge en lage suikers.

Lorraine Renfurm: Babystofwisselingskenster.

Klaas Bijsterveld: Het onmisbare lid van het metabole team.

Hermi Kingma: Rustig ronddolend tussen de stofwisselingsziekten.

Jacqueline Plass: ex-AIO en droomster van het hoge noorden. Zit nu ergens knackerbrod te eten.

Nicolette Huykman: The knockout girl.

Ekkerhard Sturm: Lid van de gastro boys. Weet in al zijn bescheidenheid best heel veel, vooral van Kupffer en Connecticut.

Han Roelofsen: De saxofonerende proteomic.

Janny Takens: Janny is Janny is Janny en gezellig lid van het Feike team.

Marius Heiner: De meest coole analytische rocker.

Greet Nieborg: Ze metaboolt rustig door.

Ingrid Schippers: De goedlachse ex-postdoccer en bridgster.

Hans Sauerwein: Eén van de drie wijzen en hij kwam uit het westen.

Roel Vonk: De man die mij binnenliet in de wereld van de wetenschap en mij regelmatig de weg wees.

Professor Wolffenbuttel:	Eén van de drie wijzen en hij kwam uit het noorden.
Marc Hellerstein:	God of MIDA, sciencing with unstoppable enthusiasm and cleverness.
Rich Neese:	MIDA hippy, who made life and research fun in Berkeley.
Paul Kluytmans:	Movieing through life.
Heiko Laarman:	Grondlegger van de biologen humor en paranimf.
Henk van Renssen:	Weet alles van het meeste. Vriend en criticus van wazige stellingen. Tevens Sokurov's grootste fan.
Tineke Kok:	Ongemerkt de slimste en meest avontuurlijke van ons allemaal. Helemaal niet ongemerkt de allermooiste.
Jan Bandsma:	Superbro.
Maya Schroevers:	De liefste layoutster van de hele wereld met overweldigende levenslustigheid.
Remco Zegers:	Maintaining the vision on joy.

Ode aan Anke

Drentse winden waaien
en laten moleculen draaien.
De puzzel valt meteen
in stukjes ineen.

Dansend langs pipetten
En puks en petterfletten.
Draaiend tot de waarheid
drijft in de verborgenheid.

Zonnestraal op gel en cel.
Licht schijnt in waarheidswel,
Wanneer ze werkt in extase
voor 't heertje Delegase.

Curriculum Vitae

The author Robert Bandsma (1972), got his high school diploma (Gymnasium) at the Veluws College, Apeldoorn in 1990. He studied medicine at the University of Groningen, where he also started his scientific career under supervision of professor Folkert Kuipers. In 1995 he attended the University of Calfornia at Berkeley, where he worked on the development of novel stable isotope methodologies under supervision of professor Marc Hellerstein. In 1999 he attended the Children's Hospital of Philadelphia, for additional training of clinical and scientific skills. After his graduation he started his training in Pediatrics at the Beatrix Children's Hospital, University Hospital Groningen, The Netherlands. Upon receiving the prestigous AGIKO grant from the Dutch Organization of Scientific Research in 1999, he focused his research on the interactions between hepatic carbohydrate and lipid metabolism in health and metabolic diseases.

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