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PERSPECTIVE AND DIRECTIONS FOR FUTURE STUDIES

Tahira Farooqui[1] and Akhlaq A. Farooqui[2]

[1]*Department of Entomology/Center of Molecular Neurobiology, The Ohio State University, Columbus, Ohio, USA*
[2]*Department of Molecular and Cellular Biochemistry, The Ohio State University, Columbus, Ohio, USA*

27.1 INTRODUCTION

Oxidative stress, an unavoidable consequence of life in an aerobic oxygen-enriched atmosphere, is a cytotoxic process that occurs in cells when antioxidant mechanisms are overwhelmed by reactive oxygen species (ROS). This imbalance causes damage to important biomolecules and cells, with potential impact on the whole organism [1]. ROS are atoms or molecules possessing one or more unpaired electrons in the outer orbit and, therefore, are prone to react chemically [2]. ROS include superoxide anions ($O_2^{\bullet -}$), hydroxyl ($^{\bullet}OH$), alkoxyl ($RO^{\bullet -}$), and peroxyl radicals ($ROO^{\bullet}$), and hydrogen peroxide (H_2O_2). These radicals are common products of life in an aerobic environment, and they are responsible for oxygen toxicity. The "dark side" of oxygen is related to the fact that each oxygen atom has one unpaired electron in its outer valence shell, and molecular oxygen has two unpaired electrons. Thus atomic oxygen is a free radical, and molecular oxygen is a (free) biradical. Concerted tetravalent reduction of oxygen by the mitochondrial electron transport chain, to form water, is considered a relatively safe process; however, the univalent reduction of oxygen generates reactive intermediates. Thus the "oxygen paradox" is dangerous to all forms of life for whom it is an essential component of energy production [3]. The major sources of ROS include mitochondrial respiratory chain, xanthine/xanthine oxidase, myeloperoxidase in cytoplasm, uncontrolled arachidonic acid (ARA) cascade, and NADPH oxidase (Fig. 27.1). Over 90% of ROS production occurs "accidentally" in mitochondria during metabolism of oxygen when some of electrons passing "down" the electron transport chain leak away from the main path and go directly to reduce oxygen molecules to the superoxide anion [4]. NADPH oxidase generates superoxide radical by the one-electron reduction of oxygen, using NADPH as the electron donor [5, 6]. The ability of NADPH oxidase inhibitors to retard ROS-mediated cytotoxicity provides strong support for the idea that ROS are generated through the activation of NADPH oxidase. In the presence of metal ions such as Fe^{2+} and Cu^{2+}, H_2O_2 is also transformed into hydroxyl radical ($^{\bullet}OH$) through the Fenton reaction (Fig. 27.1). Hydroxyl radicals can attack polyunsaturated fatty acids in neural membrane phospholipids, forming the peroxyl radical ($ROO^{\bullet}$), and then propagate the chain reaction of lipid peroxidation. ROS production plays an important role in cell signaling. ROS modulate the transcription factor NF-κB through the activation of kinases that phosphorylate the inhibitory subunit of NF-κB/I-κB, causing its ubiquitination and release of NF-κB from the NF-κB complex. Free NF-κB migrates from cytosol to the nucleus, where it binds to the κB domain of the target gene promoter, leading to transcriptional activation of many proinflammatory enzymes, cytokines (TNF-α, IL-1β, and IL-10), chemokines, immune receptors, and cell surface adhesion molecules (Fig. 27.1) [7], which are

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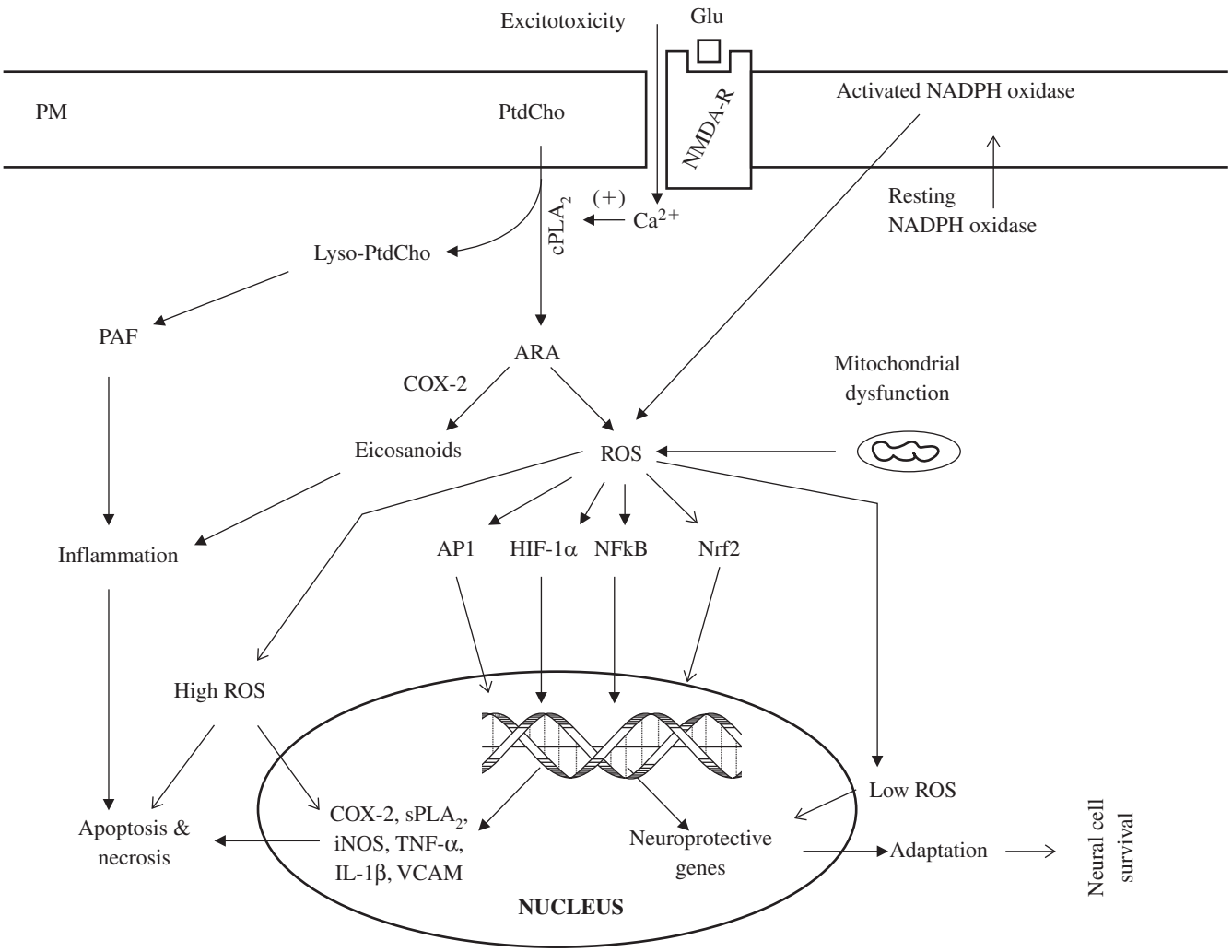

Fig. 27.1 Hypothetical model showing generation of reactive oxygen species (ROS) from various sources and effect of ROS on various transcription factors. PM, plasma membrane; NMDA-R, *N*-methyl-D-aspartate receptor; Glu, glutamate; PtdCho, phosphatidylcholine; lyso-PtdCho, lyso-phosphatidylcholine; $cPLA_2$, cytosolic phospholipase A_2; $sPLA_2$, secretory phospholipase A_2; COX-2, cyclooxygenase; ARA, arachidonic acid; NF-κB, nuclear factor κB; iNOS, inducible nitric oxide synthase; PAF, platelet-activating factor; VCAM-1, vascular cell adhesion molecule-1; AP-1, activator protein 1; HIF-1α, hypoxia-inducible transcription factor-1α; Nrf2, NF-E2-related factor 2.

involved in different cellular processes such as cell proliferation, survival, stress responses, cellular immunity, and inflammation. Dysregulation of the NF-κB pathway leads to diseases such as autoimmunity and cancer.

Although chemical and biochemical reactions for the production of ROS in vertebrates and invertebrates systems are similar, cellular heterogeneity in various tissues and organs makes the intensity of oxidative stress responses very different. The consequences of oxidative stress depend not only on cell type and nature of ROS but also on endogenous antioxidant status and cooperation among various antioxidant systems [3]. In general, the preservation of redox status is a major factor for cell survival. Brains from vertebrate and invertebrate phyla may respond differently in terms of intensity of oxidative stress compared to visceral organs of vertebrates (liver, kidneys, and lung) and similar systems in invertebrates. The mammalian brain demands high energy and possess highly active mitochondrial metabolism with high oxygen utilization (20% of the total oxygen inspired). This high utilization of oxygen comes at a heavy biological price. As important as oxygen is for the survival of neurons and glia, it also indirectly contributes to their destruction and death over time. The reason for this is that a small percentage (an estimated 1–4%) of the oxygen that enters cells is metabolized to derivatives that gradually erode and destroy essential molecules [8]. These destructive derivatives of oxygen are often referred to

as free radicals or ROS. Brain not only possesses high levels of polyunsaturated fatty acids but also contains transition metals such as iron and copper, which are capable of generating hydroxyl radical [9]. In addition, brain contains low levels of cytosolic antioxidants compared to liver and other visceral tissues [10, 11]. Thus brain is unable to protect itself from toxic effects of high levels of ROS. Superoxide radical reacts with nitric oxide ($NO^{\bullet}$) to produce the peroxynitrite anion ($ONOO^{-}$), a nonradical product, which is as toxic as $^{\bullet}OH$ in terms of its ability to oxidize and destroy by standard molecules. $NO^{\bullet}$ and $ONOO^{-}$ are often referred to as reactive nitrogen species (RNS). Like ROS, RNS oxidize lipids and protein components. Studies on ROS and RNS indicate that ROS/RNS are highly reactive and short-lived species that do not accumulate to significant levels, and it is not possible to measure them directly; rather, one must measure either the accumulation of biomolecules or the exogenously added indicators that are modified by ROS and RNS. In other words, generation of ROS and RNS leaves its footprint in the cell in the form of different oxidatively modified components.

27.2 ENDOGENOUS ANTIOXIDANT DEFENSE MECHANISMS IN VERTEBRATES AND INVERTEBRATES

Under physiological conditions, the antioxidant defense system within vertebrate and invertebrate bodies can easily neutralize the amount of ROS produced through ROS generating systems [2]. The antioxidant systems include low-molecular-weight antioxidants like glutathione and vitamin C, antioxidant enzymes such as superoxide dismutase, catalase, transferrin, and glutathione peroxide, and the ROS defense system involving the participation of enzymes associated with DNA and membrane repair. These enzymes repair ROS-mediated oxidative damage to cellular structures [2]. Thus during normal aerobic metabolism ROS generation remains under tight control through the activities of the above-listed antioxidant defense systems. Low levels of ROS are needed for fundamental cellular functions such as growth and adaptation responses and for optimal functioning of the immune system. Oxidative stress induces a number of biochemical changes in vertebrate and invertebrate systems. The extent of these changes depends on the severity of the oxidative stress. In vertebrate and invertebrate systems, low levels of ROS promote cell proliferation, while intermediate ROS levels produce growth arrests [2, 8]. Under a sustained environmental stress, ROS are produced over a long time, and thus significant damage may occur to cell structure and functions. This damage has been implicated in a wide variety of chronic diseases, including neurodegenerative diseases (Alzheimer disease, Parkinson disease, amyotrophic lateral sclerosis); arteriosclerosis and heart disease; strokes and ischemia/reperfusion injury; chronic inflammatory diseases (rheumatoid arthritis, lupus erythematosus, and psoriatic arthritis); and mutagenesis, cell transformation, and cancer [12, 13].

To counteract ROS-mediated damage, vertebrate and invertebrate systems have evolved several mechanisms. These mechanisms include (a) termination of the ROS using free radical scavengers and antioxidant enzymes, (b) induction of endogenous signaling systems that protect cells from toxic effects of ROS, and (c) repair of damaged cellular components. These mechanisms may also involve the activation of redox-sensitive transcription factors and increased expression of antioxidant enzymes and antiapoptotic proteins. One of the major cellular antioxidant responses in vertebrate and invertebrate systems is the induction of detoxification enzymes through the cytoplasmic oxidative stress system (Nrf2-Keap1). It is activated by ROS (Fig. 27.1). The transcription factor Nrf2 is constitutively expressed in all tissues of vertebrates and invertebrates, although amounts may vary among various cells and organs [14, 15]. The detoxification organs (kidney and liver) have the highest levels of Nrf2. Nrf2 may be further induced by cellular stressors including endogenous ROS or exogenous electrophiles [16]. Under normal conditions, Keap1 forms a complex with Nrf2 and keeps it within the cytoplasm, targeting it for ubiquitination and proteasomal degradation. This results in the maintenance of low levels of Nrf2 that mediate the constitutive expression of Nrf2 downstream genes. When vertebrate and invertebrate cells are exposed to oxidative stress, a signal involving phosphorylation and/or redox modification of critical cysteine residues in Keap1 inhibits the enzymic activity of the Keap1-Cul3-Rbx1 E3 ubiquitin ligase complex, leading to reduction in Nrf2 ubiquitination and degradation. As a result, free Nrf2 translocates from the cytosol into nucleus, where it forms a heterodimer with members of the small musculo-aponeurotic fibrosarcoma (Maf) transcription factor family [15]. These Nrf2/Maf heterodimers bind to antioxidant response elements present in the promoters of numerous antioxidant genes, including NQO-1, glutathione *S*-transferase, glutathione peroxidase (GPx), catalase, and HO-1 [14, 17, 18]. Upon recovery of cellular redox homeostasis, Keap1 travels into the nucleus to dissociate Nrf2 from the ARE. Subsequently, the Nrf2-Keap1 complex is exported out of the nucleus by the nuclear export sequence (NES) in Keap1. Once in the cytoplasm, the Nrf2-Keap1 complex associates with the Cul3-Rbx1 core ubiquitin machinery, resulting in degradation of Nrf2 and termination of the Nrf2/ARE signaling

pathway [16, 19]. The Nrf2 signaling pathway mediates multiple avenues of cytoprotection by activating the transcription of more than 200 genes that are not only crucial in metabolism of drugs and toxins, protection against oxidative stress, and inflammation but also play an integral role in stability of proteins and in the removal of damaged proteins via proteasomal degradation or autophagy [20]. Nrf2 interacts with other important cell regulators such as tumor suppressor protein 53 (p53) and nuclear factor-κB (NF-κB) and through their combined interactions act as the guardian of a healthy life span, protecting against many age-related diseases such as cancer and neurodegeneration. It is hypothesized that this signaling pathway plays a critical role in the determination of species longevity and that this pathway may indeed be the master regulator of the aging process [21]. Since Nrf2-mediated cellular defense response protects multiple organs or multiple tissues, activation of Nrf2 has been implicated in conferring protection against many human diseases, including cancer, neurodegenerative diseases, cardiovascular diseases, acute and chronic lung injury, autoimmune diseases, and inflammation [18].

Oxidative stress also promotes both the transcriptional activity and protein stability of FoxOs, forkhead transcript factors that are expressed abundantly throughout the body of vertebrates and invertebrates [22–24]. FoxOs are major targets for insulin signaling. FoxO1 belongs to a nuclear protein subfamily that includes FoxO3a, FoxO4, and FoxO6 in mammals and its ortholog DAF-16 in *Caenorhabditis elegans* [25]. These proteins, characterized by a highly conserved central DNA binding domain and a carboxyl *trans*-activation domain, play important roles in mediating the effect of insulin or IGF on metabolism, growth, survival, differentiation of cells, oxidative stress response, DNA repair, cell cycle, and apoptosis [26]. Among the FoxO isoforms, FoxO1 preserves redox balance by promoting protein synthesis and subsequently inhibiting cell cycle arrest. This evidence indicates that FoxO1 integrates and orchestrates responses to different stress signals to maintain cellular function [22, 23, 26].

In addition, vitagenes play an important role in conferring protection against oxidative stress. These genes include genes for heat shock proteins (Hsps), thioredoxin/thioredoxin reductase system, and heme oxygenase-1 [27, 28]. Heat shock response contributes to establish a cytoprotective state in a wide variety of oxidative stress-mediated diseases, including inflammation, cancer, aging, and neurodegenerative disorders. When appropriately activated, heat shock response not only initiates and restores cellular homeostasis but rebalances redox equilibrium. Activation of this pathway is particularly important for neural cells with relatively weak endogenous antioxidant defenses [28, 29].

27.3 BIOMARKERS OF OXIDATIVE STRESS IN VERTEBRATES AND INVERTEBRATES

As stated above, direct measurement of ROS in vivo is difficult because of the highly reactive nature of these compounds and their minute concentrations in biological fluids. Instead, one relies on measurement of stable end products of oxidation of different molecules. ROS-mediated damage may occur to DNA, proteins, and lipids in vertebrate and invertebrate systems. Several markers of ROS-mediated damage have been described in the literature. An ideal biomarker for the detection of oxidative stress in chronic neurodegenerative and visceral diseases should be precise and reliable. It should be easy to quantify and be reproducible. It should not be subjected to wide variation in the general population and not affected by comorbid factors. To evaluate the effect of medication, the biomarker should change linearly with disease progression. Among biomarkers for oxidative stress, F_2-isoprostanes, 4-hydroxynonenal (4-HNE), and 8-hydroxy-2-deoxyguanosine have attracted considerable attention. F_2-isoprostanes and 4-HNE are compounds derived from arachidonic acid (ARA) via a free radical-catalyzed mechanism (Fig. 27.2). Several F_2-isoprostanes have been described in the vertebrate system, but it is becoming increasingly evident that 15-F_{2t}-isoprostane (15-F_{2t}-IsoP) is a good biomarker for oxidative stress [30]. Isoprostanes are generated from cell membrane-bound ARA by free radical attack. They are cleaved from the sites of their origin by phospholipase A_2 and then circulate in plasma and are excreted in urine [31, 32]. F_2-isoprostanes can be detected in biological fluids, such as urine, blood plasma, bronchoalveolar lavage, or cerebrospinal fluid (CSF), as well as in tissues. The main advantage of urinary measurements of F_2-isoprostanes is that the compounds are very stable and are not formed ex vivo [33]. On the other hand, in blood plasma and tissues, autooxidation may occur. Several methods are available for F_2-isoprostane detection including gas chromatography-mass spectroscopy and liquid chromatography-mass spectroscopy. In addition, a reliable ELISA technique suitable for analysis of large numbers of samples has recently been developed [32, 34, 35]. No information is available on the determination of isoprostanes in invertebrate systems. In future studies, attempts should be made to determine levels of isoprostanes in invertebrate systems by gas chromatography-mass spectroscopy and liquid chromatography-mass spectroscopy.

4-HNE is the most prevalent toxic lipid peroxidation product formed during oxidative stress. It is derived from nonenzymic oxidation of ARA. It not only modifies amino acid residues of proteins (sulhhydryl groups of cysteine, imidazole moiety of histidine, and the ε-amino

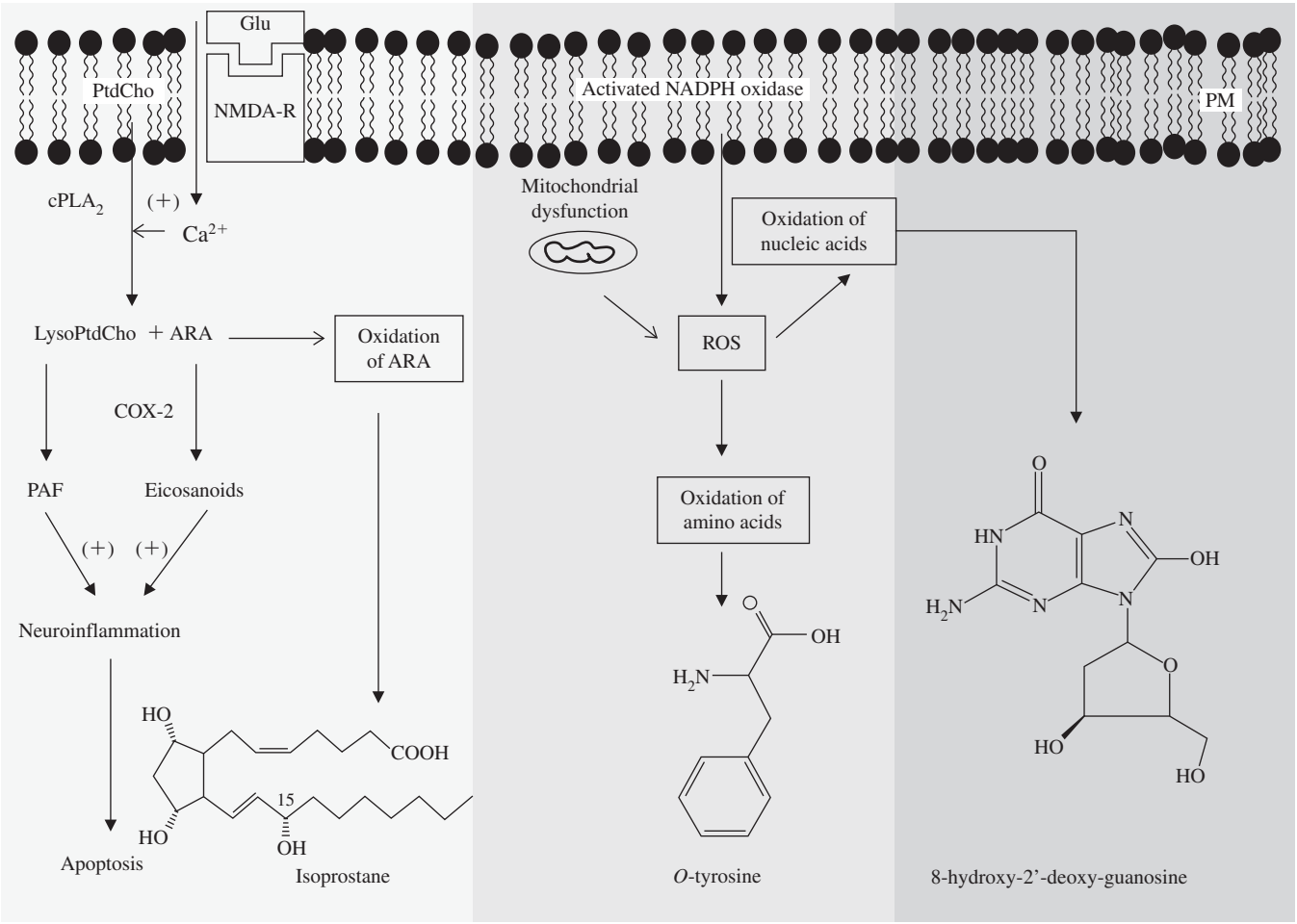

Fig. 27.2 Generation of biomarkers for oxidative stress in vertebrates. PM, plasma membrane; NMDA-R, *N*-methyl-D-aspartate receptor; Glu, glutamate; PtdCho, phosphatidylcholine; lyso-PtdCho, lyso-phosphatidylcholine; $cPLA_2$, cytosolic phospholipase A_2; COX-2, cyclooxygenase; ARA; arachidonic acid; PAF, platelet-activating factor; ROS, reactive oxygen species. *(See color insert.)*

group of lysine) but also inhibits DNA and protein synthesis and inactivates enzymes, modifies low-density lipoproteins, and modulates gene expression [36]. Mitochondrial proteins are targets of 4-HNE adduct formation following oxidative stress in vivo and in vitro. In addition, 4-HNE also inactivates the 2-oxoglutarate dehydrogenase and pyruvate dehydrogenase complexes, cytochrome *c* oxidase, and NADH-linked respiration in isolated mitochondria [37, 38]. 4-HNE produces a variety of cytotoxic effects such as the inhibition of DNA, RNA, and protein synthesis, cell cycle arrest, mitochondrial dysfunction, and apoptosis in vertebrate and invertebrate systems [39]. Thus 4-HNE is a good biomarker for oxidative stress. 4-HNE has been detected by gas chromatography-mass spectroscopy and liquid chromatography-mass spectroscopy in vertebrate systems, and studies on the use of 4-HNE as a biomarker for oxidative stress in invertebrates are beginning to appear [25].

8-Oxodeoxyguanosine (8-oxodG) is another important biomarker for oxidative stress in vertebrate and invertebrate systems [40–42]. It is synthesized in vivo by the attack of ROS on DNA. It is highly mutagenic, resulting in GC to TA transversions. After cleavage from DNA as a result of DNA repair, 8-oxodG is excreted in body fluids including blood, urine, and CSF of vertebrates. Another significant source of extracellular 8-oxodG may be oxidation of the nucleotide pool [43]. Urinary 8-oxodG levels are therefore considered a general biomarker of oxidative stress. Methods used for 8-oxodG detection include high-performance liquid chromatography, tandem mass spectroscopy, and a recently developed competitive ELISA [44]. These methods can be used for the determination of oxidative stress-mediated DNA damage in invertebrate systems.

Proteomics can be used for determining levels of not only oxidative/nitrosative protein modifications but also

protein-bound methionine oxidation [45, 46]. It is controversial whether ROS- and RNS-mediated protein modifications and oxidized methionines have a significant direct physiological and pathological impact on cellular injury. The generation of these proteins may be a secondary phenomenon. A clear delineation of the causal connections cannot be provided at present, but it is becoming increasingly evident that high levels of ROS/RNS produce distinct pathological cell consequences that greatly amplify and propagate injury, leading to irreversible cell and tissue degeneration [45, 46]. It is tempting to speculate that redox proteomics can be used to define redox molecular mechanisms associated with oxidative stress in vertebrate and invertebrate systems.

27.4 OXIDATIVE STRESS AND AGING

Although information on the effect of oxidative stress in vertebrate and invertebrate systems has been discussed by Rizvi and Pandey in this book, it is important to emphasize that production of ROS has a major impact on cell aging and tissue damage [47, 48], particularly in cardiovascular and nervous systems [49]. Levels of ROS are elevated with age, as biomarkers of lipid peroxidation (isoprostanes) are increased and antioxidant activity is decreased. However, in elderly individuals who are still healthy, the ROS level has been reported to be similar to that of young adults [50], or at least comparable to antioxidant defenses [51], supporting the view that markers of oxidative stress are not influenced by old age when good health and nutritional status are preserved. In addition, psychological stress and lifestyle factors such as smoking, lack of exercise, and status of n-3 fatty acids in the body have an impact on the level of ROS [52–54]. ROS are not only responsible for whole body accelerated aging but also for decline in cognitive functional. Thus in an elderly population (>80 years old), free radicals are involved in poorer cognitive function, loss of autonomy, loss of ability to perform daily activities, and institutionalization, as well as depressive symptoms [55].

27.5 CONCLUSION

Oxidative stress is defined as an imbalance between ROS production and their removal by antioxidant systems with increased accumulation of free radicals. Major production of ROS occurs in mitochondria during oxygen utilization. In addition, ROS may be synthesized in phagocytic cells, as well as in vascular wall and various other tissues by enzymes such as NADPH oxidase, myeloperoxidase, xanthine oxidase, cyclooxygenase, and lipoxygenase. At low concentrations, ROS are associated with a vast array of physiological functions, such as gene expression and immune responses. At high levels, ROS react with lipids, carbohydrates, proteins, and DNA, altering their structure and function and resulting in inflammation, apoptosis, and mutagenesis.

These days, we have been empowered by lipidomics, proteomics, and genomics. These procedures can be used not only for identifying and determining levels of biomarkers for oxidative stress in various tissues of vertebrate animals and invertebrate organisms but also for detecting biomarkers levels in biological fluid samples. Identification of biomarkers for oxidative stress may lead not only to early diagnosis and follow-up of the progression of neurodegenerative and chronic visceral diseases but also to monitoring of therapeutic responses. Identification of ROS/RNS-modified proteins by proteomics in vertebrates and invertebrates can provide important information on cellular function of modified proteins. Another important challenge for future studies will be to incorporate this knowledge into a framework whereby these complex functional and regulatory alterations in the cellular proteome can be used to increase our understanding of and improve the treatment of diseases with the component of oxidative stress in vertebrate and invertebrate systems.

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