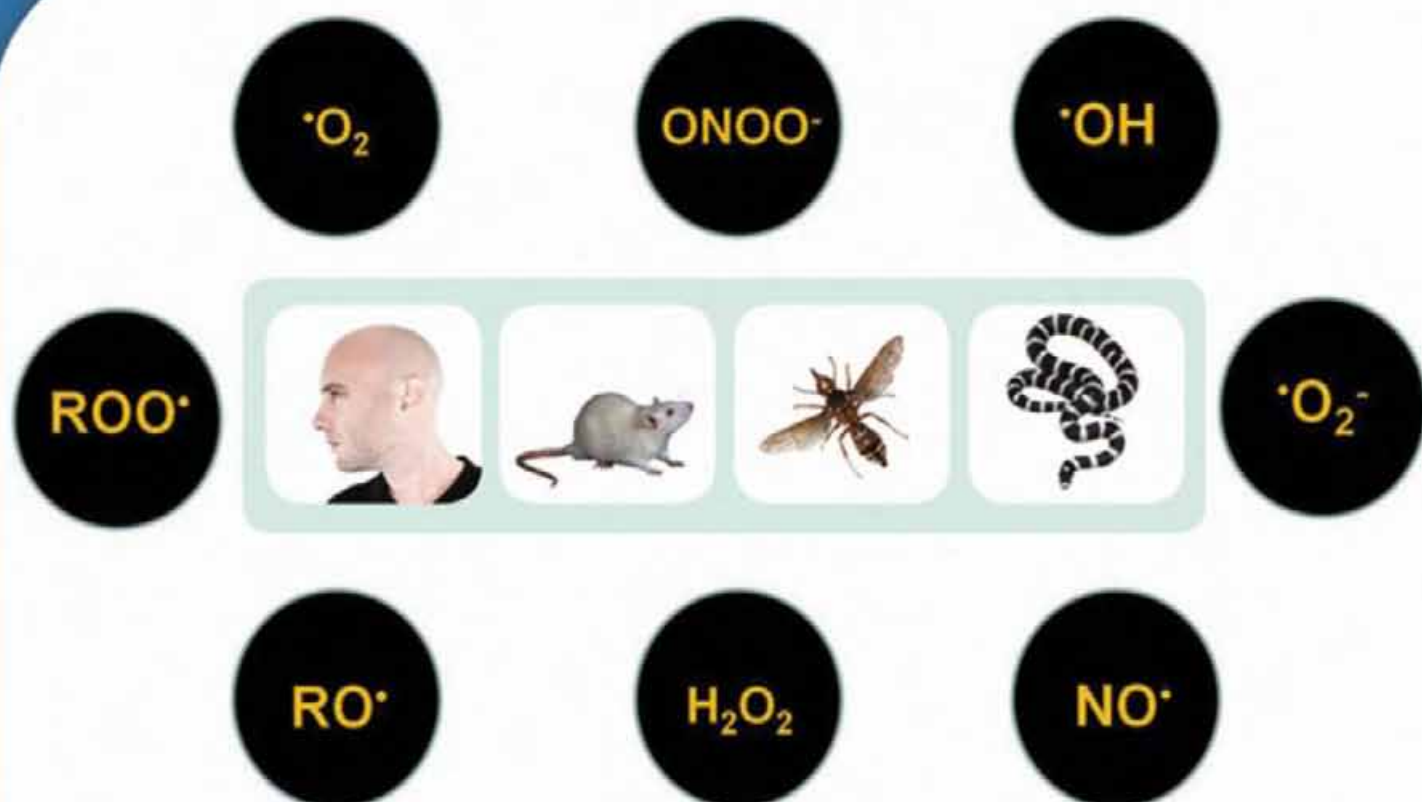


Oxidative Stress in Vertebrates and Invertebrates

MOLECULAR ASPECTS OF CELL SIGNALING

Edited by

Tahira Farooqui and Akhlaq A. Farooqui



OXIDATIVE STRESS IN VERTEBRATES AND INVERTEBRATES

Molecular Aspects of Cell Signaling

Edited by

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FOREWORD

Oxidative stress is a cytotoxic process that occurs in cells when antioxidant mechanisms are overwhelmed by reactive oxygen species (ROS). This imbalance not only causes damage to important biomolecules (lipids, proteins, and nucleic acids) in cells, but also has an impact on functional activities in both vertebrates and invertebrates. This new volume entitled "Oxidative Stress in Vertebrates and Invertebrates: Molecular Aspects of Cell Signaling" brings together important information from expert researchers in the oxidative stress-mediated cell signaling area in both vertebrate and invertebrate systems. Accumulation of high levels of ROS and significant reduction in cellular redox systems are common processes associated with acute and chronic visceral and neurodegenerative diseases, including hypertension, preeclampsia, arteriosclerosis, acute renal failure, diabetes, and Alzheimer and Parkinson diseases. This well-organized book presents up-to-date and comprehensive information on oxidative stress-related signaling events in vertebrates and invertebrates. The text is clear, concise, and easily accessible. Subject matter is divided into a vertebrate section (17 chapters) and an invertebrate section (10 chapters). The editors are known for their work on oxidative stress and neurodegeneration. They have done a commendable job in putting together this volume, and have contributed 5 chapters. These editors have taken great care in selecting the topics and describing progress that has been recently made in this field. The authors of this book also tried to ensure uniformity and mode of presentation in a simple and clear manner.

Topics addressed in the vertebrate section include the generation of ROS and their roles in cell survival and

suicide; ROS-induced signal transduction and human diseases; biochemical and pathological consequences and redox therapy; pathogenesis of neurotraumatic and neurodegenerative diseases; oxidative stress mediated by caloric restriction; the role of oxidative stress and neuroinflammation in Alzheimer disease and Parkinson disease; selenoproteins in cellular redox regulation and signaling; antioxidant therapy and its effectiveness in oxidative stress-mediated disorders; pharmacological and therapeutic properties of propolis; comparison of oxidative stress in aging between vertebrates and invertebrates; and finally, oxidative stress-mediated signaling pathways by environmental stressors. The invertebrate section includes oxidative stress-induced signaling in three important phyla, namely, arthropoda, annelida, and mollusca. Topics addressed in this section include effect of oxidative stress on insect endocrine control; the innate immune system in airway epithelial cells of *Drosophila*; age-related memory impairment in *C. elegans*; olfactory learning and memory in *Apis mellifera*; Keap1/Nrf2 signaling in *Drosophila*; circadian rhythm in *Drosophila*; molecular antioxidant protective processes in *Apis mellifera*; protective role of sestrins against chronic TOR; and oxidative challenge and redox sensing in mollusks.

The subject matter in this book develops logically and progresses from one topic to another with an extensive bibliography along with major primary references. These references will help readers in pursuing their areas of interest. In order to facilitate better understanding and easier reading, this book also contains a large number of figures and line diagrams of signal transduction pathways. This book fills the gap between basic science and

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TAHIRA FAROOQUI
AKHLAQ A. FAROOQUI

SURVIVAL STRATEGY AND DISEASE PATHOGENESIS ACCORDING TO THE Nrf2-SMALL Maf HETERODIMER

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5.1 INTRODUCTION

Organisms, including mammals, are constantly exposed to oxidative stress due to oxygen consumption during aerobic respiration and xenobiotic chemicals in their diet and/or the environment. Mammalian cells are equipped with sophisticated machinery for cell protection against oxidative and xenobiotic stress. NF-E2-related factor 2 (Nrf2) is a potent transcription activator that plays a central role in regulating the expression of genes encoding detoxifying enzymes and antioxidant proteins by binding to the antioxidant response element (ARE)/electrophile response element (EpRE). Nrf2 is a member of the Cap'n'Collar (CNC) transcription factor family that commonly contains a unique stretch of amino acids, designated the CNC domain, followed by a well-conserved basic region-leucine zipper (bZIP) motif. Under unstressed conditions, Nrf2 is ubiquitinated by kelch-like ECH-associated protein 1 (Keap1) and degraded by the proteasome in the cytoplasm. Upon exposure to oxidative or xenobiotic stress, Nrf2 is stabilized, translocates into the nucleus, heterodimerizes with small Maf, another bZIP protein, binds to the ARE/EpRE, and activates transcription. Recent studies have clarified the intricate molecular mechanisms of Nrf2 activation in response to stress and revealed the involvement of Nrf2 in many human diseases including neurodegeneration, airway disorders, cardiovascular disease, and cancer. This review covers the historical aspects of the discovery of Nrf2,

recent advances in molecular studies of Nrf2 function, updated reports on the involvement of Nrf2 in various pathological conditions, and perspectives of Nrf2 utilization for human welfare.

5.1.1 Identification of Nrf2

The antioxidant response element (ARE), also known as an electrophile response element (EpRE) [TGA(G/C)NNNGC], was first identified in analyses of induction mechanisms of the detoxifying enzyme genes in response to electrophilic chemicals [1–3]. ARE/EpREs are often found in the promoter regions of cytoprotective genes and are considered to be critical for the inducible expression of these genes. Although the importance of this *cis*-regulatory element was clearly shown, the *trans*-acting factor responsible for inducible transcription activation remained unknown.

During the search for the *trans*-acting factor, an interesting coincidence occurred in research on transcriptional regulation. Nuclear factor erythroid 2 (NF-E2) is a transcriptional activator important for erythroid-specific gene expression through interaction with NF-E2 binding sites [ATGA(G/C)TCAGCA] [4, 5]. Nrf2 was originally identified as a homolog of NF-E2 and was found to associate with the NF-E2 binding site as expected [6, 7] (Fig. 5.1). Similarities between the ARE/EpRE sequence and the NF-E2 binding site led to the discovery of Nrf2 as the ARE/EpRE binding factor [8] (Fig. 5.2A).

Nrf2 possesses a characteristic CNC domain followed by a well-conserved bZIP motif (Fig. 5.1). The CNC domain was defined based on the sequence homology of 43 amino acids to the *Drosophila* Cnc protein [9]. In addition to NF-E2 and Nrf2, four additional factors were isolated as members of the CNC family: Nrf1, Nrf3, Bach1, and Bach2 [10, 11, 12]. NF-E2, Nrf1, Nrf2, and Nrf3 are considered to be activators [4, 6, 7, 10, 11], while Bach1 and Bach2 possess characteristic Broad complex-Tramtrack-Bric-a-brac (BTB) domains at their N-terminal ends and are considered to be repressors [12–14] (Fig. 5.2B). Importantly, none of the members of the CNC family binds efficiently to DNA as monomer or homodimer, and each requires small Maf, another bZIP protein, as an obligate heterodimeric partner molecule [15] (Fig. 5.1 and Fig. 5.2B).

The CNC protein homolog in *Caenorhabditis elegans* is SKN-1, and it binds to DNA as a monomer because it does not possess the leucine zipper structure [16]. The SKN-1 recognition sequence is similar to half-sites of ARE/EpRE and NF-E2 binding site (Fig. 5.2A). One

side of the site is recognized by CNC proteins, and small Maf proteins recognize the other side of the site, which contains a GC dinucleotide. Nrf2 shares a heterodimeric partner molecule with other CNC proteins, and all of the CNC-small Maf heterodimers recognize a nearly identical DNA binding sequence. This implicates cross talk between Nrf2 and other members of the CNC and small Maf transcription factor families.

5.1.2 Susceptibility of Nrf2-Null Mice to Oxidative and Xenobiotic Stress

Analysis of Nrf2-null mice clearly demonstrated the substantial contribution of Nrf2 to the inducible regulation of cytoprotective genes [8]. Nrf2-null mice display an increased formation of DNA adducts in the lung following exposure to diesel exhaust [17], a more severe liver toxicity after administration of acetaminophen [18], an increased susceptibility to cigarette smoke-induced emphysema [19], and an aggravated bleomycin-induced pulmonary fibrosis

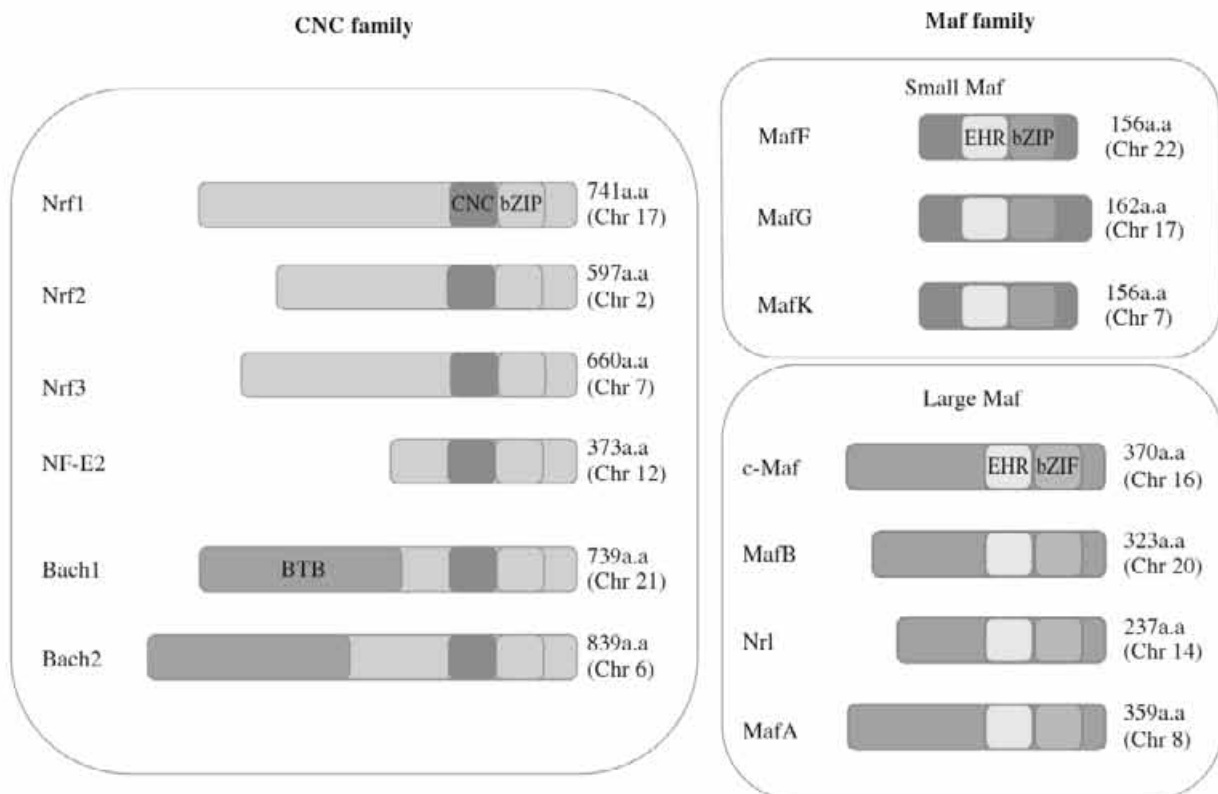


Fig. 5.1 The structures of CNC and Maf family proteins. The CNC family contains four transcriptional activators, Nrf1, Nrf2, Nrf3, and NF-E2, and two transcriptional repressors, Bach1 and Bach2. The Maf family consists of three small Maf transcription factors and four large Maf transcription factors. The bZIP motifs are a common structural feature of CNC and Maf family proteins. The CNC and EHR domains are unique to CNC and Maf family proteins, respectively. Bach1 and Bach2 possess BTB domains in their N-terminal regions. The amino acid number and chromosomal location of the human gene encoding each protein are shown.

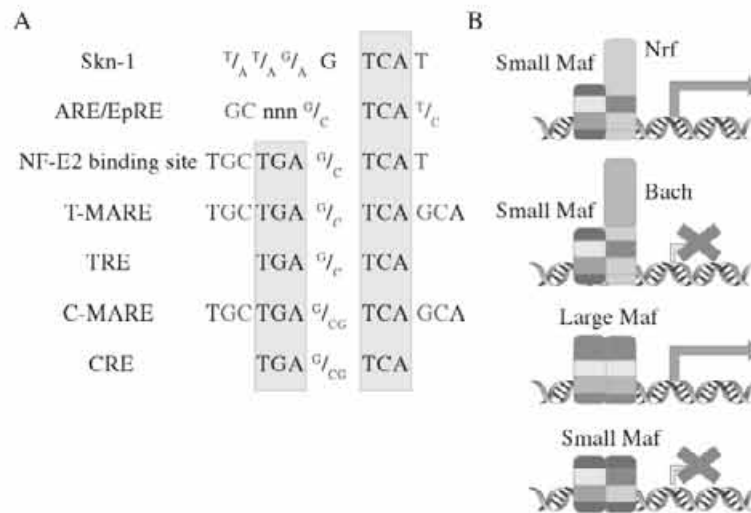


Fig. 5.2 DNA recognition sequences and the transcriptional activities of Maf-containing dimers. (A) Various MARE-related sequences are shown. The dinucleotide “GC” (marked in red) is essential for recognition by Maf proteins. The nucleotide “T” or “T/C” (marked in blue) enhances the binding of CNC proteins. Trinucleotides (boxed in gray) with the central G and GC sequence consist of the TRE and CRE, respectively, which make up the core region of the MARE. (B) Nrf1, Nrf2, Nrf3, and NF-E2 activate transcription by forming heterodimers with small Maf, while Bach1 and Bach2 repress transcription. A large Maf homodimer, possessing a *trans*-activation domain, activates transcription, while a small Maf homodimer, lacking a *trans*-activation domain, represses transcription. All of these Maf-containing dimers bind to T-MARE with high affinity. (See color insert.)

[20]. Nrf2 confers resistance to xenobiotic stress through activation of the genes encoding detoxifying enzymes, including glutathione *S*-transferases (GST), UDP glucuronate transferases (UGT), and NAD(P)H:quinone oxidoreductase 1 (NQO1) [8, 21–23]. The phenotypes observed in *Nrf2*-null mice after exposure to exogenous insults are summarized in Table 5.1.

Nrf2 also plays an important role in response to oxidative stress by activating antioxidant proteins and enzymes for glutathione synthesis [21, 24]. Cellular capacities for elimination of reactive oxygen species (ROS) are limited in the absence of Nrf2 [25]. Nrf2 regulates intracellular ROS levels not only in pathological conditions but also during the physiological cellular differentiation process of megakaryocytic maturation [26]. With decreased capacities to eliminate ROS of endogenous origins, *Nrf2*-null mice tend to spontaneously develop various inflammatory disorders, including glomerulonephritis, immune-mediated hemolytic anemia, and multiorgan autoimmune inflammation [27–29], all of which appear to be due to a chronic increase in ROS. The phenotypes observed in *Nrf2*-null mice that develop spontaneously or in response to endogenous insults are summarized in Table 5.2.

The *Nrf2* gene is ubiquitously expressed in various cell lineages and at both the embryonic and adult stages. The expression level is relatively higher in the lung, kidney, and liver in adults and in the intestine, lung, and choroid plexus in embryos [30]. Chicken Nrf2 is highly expressed in the kidney and intestine [7].

The expression level of the *Nrf2* gene is regulated by a positive feedback mechanism dependent on an ARE/EpRE in the promoter region of the *Nrf2* gene [31]. The *Nrf2* gene is also transcriptionally induced by the aryl hydrocarbon receptor [32].

Analysis of a single nucleotide polymorphism (SNP) in the promoter region of the *Nrf2* gene demonstrated the significance of the transcriptional regulation of the *Nrf2* gene in the determination of Nrf2 activity. A SNP in the promoter region of the mouse *Nrf2* gene is linked to the reduced expression of the *Nrf2* gene and susceptibility to hyperoxic lung injury in the C57BL/6J mouse strain [33]. Indeed, exposure to hyperoxia causes more severe lung damage in *Nrf2*-null mice than in wild-type mice [34]. SNPs found in the promoter region of the human *NRF2* gene [35] are linked to a higher risk of acute lung injury [36]. These data demonstrate the importance of transcriptional regulation of the *Nrf2* gene in stress response.

5.2 THE KEAP1-NRF2 SYSTEM IN RESPONSE TO ELECTROPHILES

Electrophiles have substantial impacts on biomolecules including proteins and nucleic acids that are rich in electron-dense parts. An atom with stronger electronegativity, such as oxygen, attracts pi electrons, and consequently an electron-deficient part is generated, which

TABLE 5.1 Phenotypes observed in *Nrf2*-null mice after exposure to exogenous insults

Organ	Reagent	Phenotype(s)	Inflammation-related genes affected in <i>Nrf2</i> -null mice	Reference
Skin	DMBA/TPA	Increased incidence of skin tumors and tumor numbers		119
	Diesel exhaust	Severe epithelial hyperplasia and increased levels of 8-OHdG in the lung		17
	Benzo[a]pyrene	Increased somatic mutations of the <i>Gpt</i> gene in lungs		121
Gastric	DSS	Susceptibility to DSS-induced colitis	IL-1 β , IL-6, iNOS, COX-2, TNF- α	156
	AOM/DSS	Susceptibility to AOM/DSS-induced colitis	TNF- α , IL-1 β , IL-12p40	157
	Benzo[a]pyrene	Increased tumor numbers in the stomach		65, 158, 159
Liver	AOM/DSS	Increased incidence of colonic tumors	COX-2, 5-LOX	122
	IQ	Increased incidence of liver tumors and tumor numbers		160
	D3T, CDDO-Im	Reduced expression of detoxifying genes and antioxidant genes		148, 161
Bladder	Acetaminophen	Acute hepatotoxicity		18
	BBN	Increased incidence of bladder tumors		23
Brain	LPS	Increased microglial infiltration	iNOS, IL-6, TNF- α	16
Lung	LPS, sepsis induced by cecal ligation and puncture	Exacerbated lung inflammation	TNF- α , enhanced activation of NF- κ B	163
	Carrageenin	Exacerbated lung inflammation, increased neutrophil infiltration		133
	Ovalbumin sensitization and challenge	Exacerbated allergen-driven asthmatic inflammation, hyperresponsiveness to cholinergic challenge	IL-4, IL-13, enhanced activation of NF- κ B	164
	Cigarette smoke	Susceptibility to cigarette smoke-induced emphysema	Activation of caspase-3, decreased SLPI	19, 134
	Elastase	Susceptibility to elastase-induced lung inflammation and emphysema	Decreased SLPI	135

DMBA/TPA, 7,12-Dimethylbenz(a)anthracene/12-O-tetradecanoylphorbol-13-acetate;

DSS, dextran sulfate sodium; AOM, azoxymethane; IQ, 2-amino-3-methylimidazo

[4,5-*f*]quinoline; D3T, 3*H*-1,2-dithiole-3-thione; CDDO-Im, 1-[2-cyano-3-,12-dioxooleana-1,9(11)-dien-28-oyl]imidazole; BBN, *N*-nitrosobutyl(4-hydroxybutyl)amine; LPS, lipopolysaccharide; SLPI, secretory leukoprotease inhibitor.

TABLE 5.2 Phenotypes observed in *Nrf2*-null mice that develop spontaneously or in response to endogenous insults

Organ	Phenotype(s)	Reference
Skin	Prolonged inflammation	165
Brain	Astrogliosis and vacuolar leukoencephalopathy after 1 year of age	166
	Exacerbated leukocyte infiltration, increased infarct size, and severe behavioral deficits after the intracerebral hemorrhage	167, 168
Immune system	Autoimmune inflammation and lymphoproliferation in multiple organs	29
	Lupus-like autoimmune nephritis	27
Hematopoietic	Immune-mediated hemolytic anemia	28
Craniofacial	De-colorization of incisors	169

attacks electron-dense parts of nucleic acids and proteins. The protein adduct leads to acute toxicity due to cellular dysfunction, and the DNA adduct formation leads to carcinogenesis. The Keap1-Nrf2 system is a

mechanism of cytoprotection from such electrophilic insults. The extremely sensitive nature of several thiol residues of Keap1 to electrophiles enables the system to respond to the stimuli in the first place.

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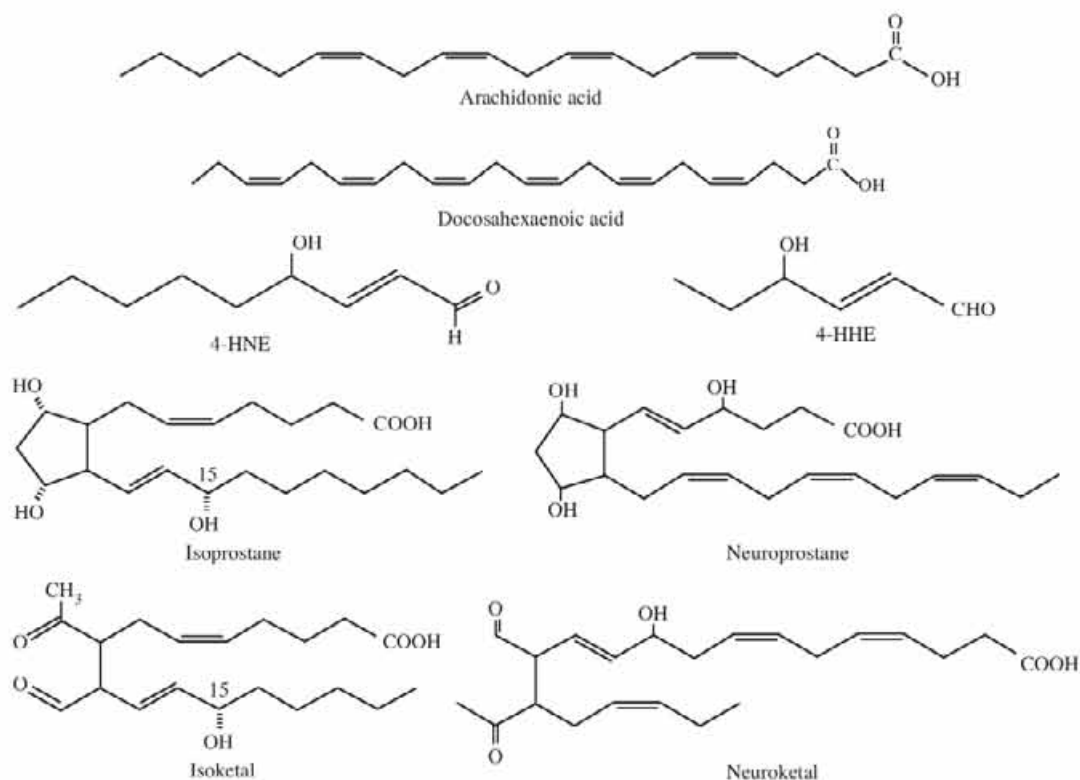


Fig. 7.5 Chemical structures of nonenzymic lipid mediators derived from ARA and DHA.

peroxyl radical isomers of ARA, which undergo endocyclization to form PGG_2 -like compounds. These compounds are reduced to PGF_2 -like compounds. F_2 -isoprostane (F_2 -IsoP) is subsequently released in free form by the action of PLA_2 [56–58]. F_2 -IsoP modulates the p38 MAPK pathway during monocyte adhesion [59]. Isoprostane-mediated monocyte adhesion does not depend on VCAM-1 but involves protein kinases, such as protein kinase A and mitogen-activated protein kinase kinase 1. Thus F_2 -IsoP not only affects vascular and bronchial smooth muscle function but also modulates cellular proliferation [57]. In addition to the above metabolites, nonenzymic oxidation of ARA produces isofurans and isoketals. Similarly, nonenzymic lipid mediators of DHA oxidation include neuroprostanes, neurofurans, and neuroketals. All these mediators are reliable indices of oxidative stress in vivo [6, 30, 52].

Ceramide and sphingomyelin are major components of lipid rafts in neural membranes. In neurodegenerative diseases, ceramide and ROS modulate intracellular ion channels, cell proliferation, and apoptotic cell death [52]. Ceramide triggers the generation of ROS and increases oxidative stress in many mammalian cells and animal models of neurodegenerative diseases [30, 38] (Fig. 7.3). Moreover, inhibition of ROS-generating enzymes or treatment of antioxidants impairs sphingomyelinase

activation and generation of ceramide. Ceramide-enriched raft platforms are important redox signaling platforms that amplify activation of ROS-generating enzymes (e.g., NADPH oxidase family enzymes) and sphingomyelinases [52].

The brain is the richest source of cholesterol in the body. Most brain cholesterol is present in myelin sheets and in cellular membranes. The presence of cholesterol in neural membranes is necessary for optimal fluidity, neural plasticity, and synaptic transmission. The structure of cholesterol makes it susceptible to a variety of radical attacks because of the 5,6-double bond and the concomitant vinylic methylene group at C-7 in the B ring. In addition, at C-17, cholesterol has an isooctyl side chain, which undergoes enzymic oxidation largely by cytochrome *P*450-dependent oxygenases, but this site of oxidation is typically not a target for ROS relevant to cellular biochemistry [60, 61]. ROS-mediated oxidation of cholesterol results in formation of three major products, cholesta-4,6-dien-3-ol, cholesta-4,6-dien-3-one, and cholesta-3,5-dien-7-one. In brain cytochrome *P*450-dependent oxygenases transform cholesterol into 24-hydroxycholesterol, 25-hydroxycholesterol, and 27-hydroxycholesterol. Cholesterol is also oxidized to cholesterol oxides and converted into cholesterol ester via acyl-CoA:cholesterol acyltransferase [62, 63].

glutathione peroxidase, catalase and small molecules such as vitamin C and E. Oxidative stress refers to the pathological states in which increased ROS production exceeds the antioxidant capacity of brain tissue to neutralize ROS. Although low levels of ROS are needed for normal neural cell function, high ROS levels damage neuronal plasma membrane and membranes of subcellular organelles directly (e.g., through peroxidation). ROS react with metals, nitrogen, or carbon to form intermediates that react with proteins (e.g., through nitration and nitrosylation). Oxidative stress also damages DNA or RNA, including mtDNA. Neuroinflammation is a neuroprotective process, in which the brain responds to infections, diseases, and injuries. Microglial cells play a major role in inducing and maintaining the intensity and duration of neuroinflammation through the release of glutamate, ROS, proinflammatory cytokines, and NO. Inhibition of microglial activation leads to the amelioration of neurodegeneration. It is becoming increasingly evident that increase in intensity and duration of interactions among excitotoxicity, oxidative stress and neuroinflammation, alterations in calcium homeostasis, mitochondrial and proteasomal dysfunction, and ROS-mediated protein aggregation play a crucial role in the development and progression of many neurodegenerative diseases.

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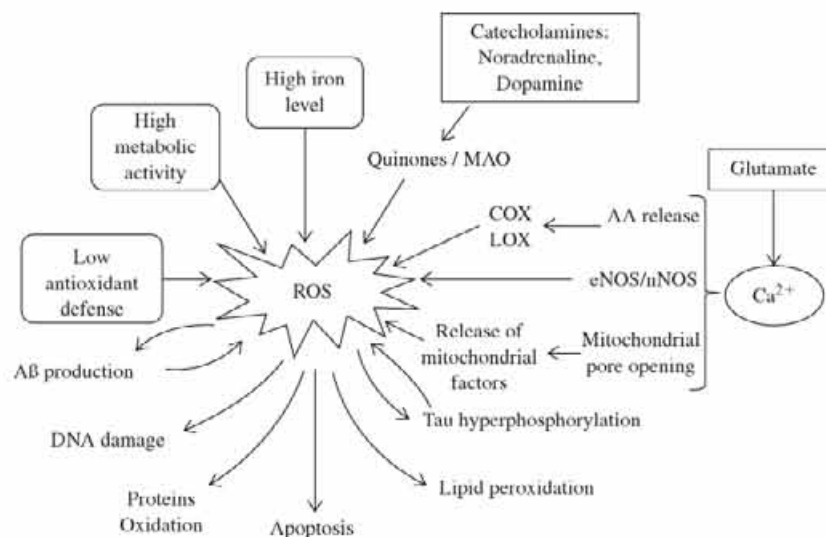


Fig. 8.1 Factors contributing to the accumulation of reactive oxygen species (ROS) in brain tissue and their implications in Alzheimer disease. See text for details. MAO, monoamine oxidase; COX, cyclooxygenase; LOX, lipoxygenase; AA, arachidonic acid; eNOS/nNOS, endothelial/neuronal nitric oxide synthase; A β , amyloid- β peptide.

biosynthetic pathways in nerve cells may therefore be interesting in development of neuroprotective strategies.

8.2 EVIDENCE FOR A PATHOLOGICAL ROLE FOR OXIDATIVE STRESS IN AD

AD is a neurodegenerative brain disease and the most common form of dementia among the elderly. It is characterized by clinical symptoms of severe and progressive loss of memory, language skills, as well as spatial and temporal orientation. The pathology of AD is characterized by extracellular senile plaques, composed of aggregated amyloid- β peptide, and intracellular neurofibrillary tangles, consisting of aggregates of abnormally hyperphosphorylated Tau protein, and is accompanied by mitochondrial dysfunction, but the mechanisms underlying AD-related dysfunction and neurodegeneration are poorly understood.

Identification of factors that contribute to the pathology of AD comes from epidemiological as well as genetic studies: AD can be classified into two different forms, rare familial forms (FAD), in which the disease onset is at an age younger than 60 years, and the vast majority of sporadic AD cases, in which onset occurs at an age over 60. Both forms of AD show the same clinical symptoms and neuropathology. Genetic studies in FAD patients have identified mutations in the genes encoding for the amyloid precursor protein (APP) and for the presenilins PS1 and PS2 that cause an autosomal inherited form of AD with 100% penetrance. These FAD mutations consistently lead to increased production of A β from

its precursor protein APP, which prompted Hardy and Higgins [5] to suggest a direct and pathological role for A β accumulation in the development of AD. In the sporadic form of the disease, several risk factors have been found that increase the risk to develop the disease but—unlike FAD mutations—do not necessarily lead to development of AD. Aging is by far the most important risk factor for AD, but the apolipoprotein E4 allele and female sex also predispose to the development of AD.

Immunohistochemical studies of postmortem AD brains have established that neurons undergo apoptotic cell death. Since ROS can elicit apoptotic signaling, the hypothesis that oxidative stress is involved in the pathogenic steps that lead to the development of AD was proposed in the 1990s by several groups [6–9]. There is a large body of evidence in support of this hypothesis: Oxidative stress has been repeatedly shown to be associated with A β toxicity and with risk factors for sporadic AD—mostly aging and the apolipoprotein E4 genotype.

8.2.1 Evidence for a Role for Oxidative Stress in Sporadic AD

Oxidative stress has been associated with the risk factors for sporadic AD, most prominently with aging [10], suggesting that an age-associated rise in accumulation of ROS can render the brain more vulnerable to the development of AD. Consequently, increased markers of oxidative stress have been found in AD patients: Several studies have reported elevated levels of lipid peroxidation products, oxidatively modified proteins, and oxidized DNA and RNA bases in brains and cerebrospinal

fluid from AD patients compared to age-matched nondemented control subjects [11–13]. Furthermore, tissue samples from AD brains display a higher susceptibility to *in vitro* oxidation [14], suggesting an impairment of antioxidant defense in AD patients. Reports on antioxidant parameters in AD brains have, however, been contradictory so far. Several antioxidant enzymes have been studied in AD brains with inconsistent results, but the majority of reports found elevations in antioxidant enzymes, suggesting an upregulation of antioxidant defense in response to increased ROS levels [15]. Interestingly, upregulation of antioxidant defence was more pronounced in female patients, and levels of 4-hydroxynonenal (HNE), a neurotoxic aldehyde derived from lipid peroxidation reactions, were elevated in female compared to male patients. These findings suggest that brains from female AD patients are under higher oxidative pressure [15], consistent with epidemiological findings that AD is more frequent in postmenopausal women compared to age-matched men. This observation can possibly be linked to the lack of sexual hormones, especially estrogens, that can modulate cognitive function and nonreproductive behaviours in humans and other mammalian species [16]. Potential sources of ROS in AD brains include ROS derived from impaired mitochondrial function [17, 18] and secondary ROS formation due to inflammatory reactions. Furthermore, increased monoamine oxidase B activity and increased levels of potentially pro-oxidative heavy metals like iron have been identified in AD brains [19, 20] and in patients with mild cognitive impairment (MCI), the “clinical precursor of AD,” suggesting that oxidative stress is an early event of the disease [21].

Apart from aging, the apolipoprotein E4 allele is the second most important risk factor for the development of AD. Apolipoprotein E seems to play a role in brain lipid metabolism and neuronal and glial development. It can exist in three different alleles, E2, E3, and E4, which differ in only two amino acids: The E2 isoform contains two and the E3 isoform one cysteine residue, while the E4 isoform contains none. Carriers of the apolipoprotein E4 are at increased risk to develop sporadic AD, especially when they are homozygous carriers. The apolipoprotein E4 allele has been associated with increased oxidative damage in AD brains, with the greatest impact in homozygotic carriers [22] and an increased susceptibility to cell death in lymphocytes from carriers bearing at least one E4 allele [23]. *In vitro* studies have evidenced that apolipoprotein E4 is less efficient in binding HNE, a cytotoxic lipid peroxidation product. These findings suggest that the Apo E4 isoform increases susceptibility to oxidative damage, thereby possibly predisposing to the development of AD.

8.2.2 Oxidative Stress and Toxicity of Mutant APP, Presenilins, and Tau

Since the proposal of the amyloid hypothesis of AD, toxic mechanisms caused by mutant APP and presenilins related to an increased production of A β have been extensively studied. Cells exposed to A β undergo apoptotic cell death, and the toxicity of A β has been shown to be related to the production of ROS [24, 25]. Furthermore, toxicity of A β depends on its aggregation state, which can be influenced by oxidation. Thus oxidative stress can cause formation of toxic A β species, which in turn can further exacerbate accumulation of ROS in a vicious cycle (Fig. 8.1). This could also explain why the prevalence of AD increases with advancing age—due to rising oxidative stress levels with aging favoring A β toxicity.

Toxicity of A β is also evident in cell cultures overexpressing APP/A β . PC12 cells transfected with mutant APP Swedish showed higher sensitivity to ROS-induced cell death and increased mitochondrial impairment after challenge with hydrogen peroxide [26]. Similar observations were obtained in human neuroblastoma cells (SH-SY5Y) overexpressing human wild-type APP (wtAPP) [27]. The study demonstrated that chronic exposure to A β protein resulted in activity changes of complexes III and IV of the oxidative phosphorylation system (OXPHOS) in mitochondria coupled with a drop of ATP levels and an increase of ROS production, which may finally instigate loss of synapses and neuronal cell death in AD. Furthermore, treatments of untransfected SH-SY5Y cells with A β or human amylin aggregates induced an increase of ROS production and had a negative impact on mitochondrial respiration by their action on OXPHOS system [25].

Toxicity of A β has also been evidenced in animal models of the disease. Mice transgenic for mutant APP have high levels of A β in their brains and show an age-dependent formation of A β plaques similar to the plaques found in AD patients. Increased markers of oxidative stress have been detected in brains of transgenic mice bearing mutant APP, accompanied by markers for mitochondrial damage [28]. Furthermore, mutant APP transgenic mice show reduced levels of the antioxidant enzyme copper/zinc superoxide dismutase [29]. In agreement, increased markers of oxidative stress and reduced antioxidant defense by catalase as well as a trend toward reduced activity of SOD were found in brains from FAD patients [30]. The results provide an important link of studies on toxicity of mutant APP in cell culture and animal models mimicking the pathogenesis of the disease in FAD patients, all of them bearing mutations finally causing an increased generation of A β .

Mutations in the presenilins PS1 and PS2 account for the majority of FAD cases and have similarly been linked with oxidative stress. Oxidative toxicity of mutant

presenilins can be either (i) due to increased formation of toxic A β , especially the A β ₁₋₄₂ isoform, or (ii) due to direct toxic effects of mutant presenilins. Several mutations in the presenilins have been found that consistently lead to increased production of the long A β ₁₋₄₂ from its precursor protein APP [31], resulting in increased A β levels and toxicity via the above-mentioned mechanisms. Expression of mutant presenilins in cell culture and transgenic mice sensitizes cells to apoptotic stimuli by increasing ROS production and mitochondrial damage [32, 33]. Furthermore, brains from PS1 mutant transgenic mice display reduced activities of antioxidant enzymes [34], and lymphocytes from these mice display increased sensitivity to apoptosis accompanied by high intracellular ROS and calcium levels [35]. Interestingly, increased ROS accumulation, disturbed calcium homeostasis, and diminished levels of antioxidants have also been identified in peripheral cells from FAD patients bearing APP or PS mutations as well as in cells from sporadic AD patients [36]. These results suggest that the oxidative toxicity observed in transgenic animal models of the disease can indeed play an important role to the pathogenesis of sporadic as well as familial AD in humans.

The second main hallmark lesion of AD is intracellular neurofibrillary tangles (NFTs) built up of hyperphosphorylated Tau. This protein may block the transport of mitochondria, leading to energy deprivation and oxidative stress at the synapse as well as to neurodegeneration [37]. Functional analysis showed mitochondrial dysfunction in transgenic mice (pR5 mice) expressing P301L mutation of Tau, with a reduced complex I activity and, with age, impaired mitochondrial respiration and ATP synthesis. Mitochondrial dysfunction was associated with higher levels of ROS in aged pR5 mice. Increased Tau pathology as in aged homozygous pR5 mice revealed modified lipid peroxidation levels and upregulation of antioxidant enzymes in response to oxidative stress. These findings demonstrated for the first time that not only the A β but also the Tau pathology acts on the enzyme metabolism of the brain and the oxidative conditions in AD. However, more recently, the successful development of double, and even triple, transgenic mouse models has facilitated the investigation of pathogenic mechanisms in AD and assisted in an understanding of the interplay of A β and Tau on bioenergetics processes *in vivo* [37]. These findings support the idea that A β and Tau act synergistically in amplifying mitochondrial respiratory deficits, mainly of complex I and IV activities [18].

8.2.3 Is Oxidative Stress an Early Event in the Pathogenesis of AD?

From the above evidence it can be concluded that oxidative stress is a feature of sporadic as well as familial forms

of AD. However, it remains to be elucidated whether oxidative stress is a primary factor in the pathogenesis of the disease or only a secondary contributing mechanism. The fact that oxidative damage and mitochondrial dysfunction can be detected at early stages in animal models [28]—even before the presence of A β plaques [38]—and that oxidative stress parameters have been detected at highest levels in early stages of the disease in AD patients [39] suggest that oxidative stress is a primary event in the course of the disease. This is supported by studies that reported a reduced risk of AD in users of antioxidant vitamin supplements [40]. Although further clinical trials are needed, antioxidant therapeutic approaches seem to be most effective at very early stages of AD and are even better utilized to modulate disease risk.

8.3 NEUROSTEROIDS

Steroid hormones are now well-defined molecules that are mainly produced by endocrine glands, such as adrenal gland, gonads, and placenta. They are involved in the control of a lot of physiological processes, from reproductive behavior to stress responsiveness. With their ability to cross cellular membranes, and thus the blood-brain barrier, steroid hormones have also an important role in the development, maturation, and differentiation of the central and peripheral nervous systems.

Three decades ago, Baulieu and co-workers were the first to show a steroid production within the nervous system itself. They discovered that some steroids, such as pregnenolone (PREG) and dehydroepiandrosterone (DHEA), were more concentrated in the brain than in the plasma [41]. In addition, they could show that the level of these steroids remained elevated in the brain even after adrenalectomy and castration. These molecules are now called “neurosteroids” and are defined as neuroactive steroids that are synthesized within the nervous system, independently of peripheral endocrine glands. Enzymatic activities of proteins involved in steroidogenesis have been shown in many regions of the central and peripheral nervous systems, in neurons as well as in glial cells [42]. Pharmacological and behavioral studies showed that neurosteroids were implicated in several physiological mechanisms, for example, cognition, anxiety, depression, neuroprotection, and even nociception [43]. Thus the conservation of the ability to produce neurosteroids during vertebrates’ evolution suggests that this category of molecules is important for living beings.

8.3.1 Biosynthesis of Neurosteroids

Neurosteroids derive from cholesterol and other blood-borne steroidal precursors. The first step of neurosteroidogenesis is the transfer of molecules of cholesterol from

the outer to the inner mitochondrial membrane. Free cholesterol accumulates outside of mitochondria and binds to the steroidogenic acute regulatory protein (StAR), a hormone-induced mitochondria-targeted protein that initiates cholesterol transfer into mitochondria. Then, molecules are transported inside mitochondria by a protein complex including translocator protein (TSPO), a cholesterol-binding mitochondrial protein also known under the name of peripheral-type benzodiazepine receptor (PTBR), which permits cholesterol transfer into mitochondria and subsequent steroid formation [44]. This translocation from the outer membrane to the inner membrane of mitochondria is the rate-limiting step in the production of neurosteroids. In fact, the ability of cholesterol to enter into mitochondria to be available to cytochrome *P*450 cholesterol side chain cleavage enzyme (*P*450_{scc}), located in the inner side of the mitochondrial membrane and responsible for the conversion of cholesterol to PREG, will determine the efficiency of steroidogenesis.

PREG, precursor of all steroid hormones, is then transported to the endoplasmic reticulum, where it is metabolized to form neuroactive steroids (Fig. 8.2). The next enzymatic step in neurosteroidogenesis is the conversion of PREG into DHEA by the cytochrome *P*450_{c17} enzyme (*P*450_{c17}), also called 17 α -hydroxylase/17,20lyase. This enzyme catalyzes the 17 α -hydroxylation of PREG in a two-step reaction that gives first 17-hydroxyPREG (17OH-PREG) and then the final product, DHEA. Each step requires the molecules NADPH and O₂.

PREG can also be catalyzed by another enzyme called 3 β -hydroxysteroid dehydrogenase (3 β -HSD) into progesterone (PROG). In general, 3 β -HSD uses NAD⁺ as a cofactor to oxidize hydroxysteroids, such as PREG, 17OH-PREG, and DHEA, into their respective ketosteroids, PROG, 17OH-PROG, and androstenedione. Then, neurosteroidogenesis follows two main pathways with PROG as precursor: the androgen/estrogen pathway and the corticoid pathway.

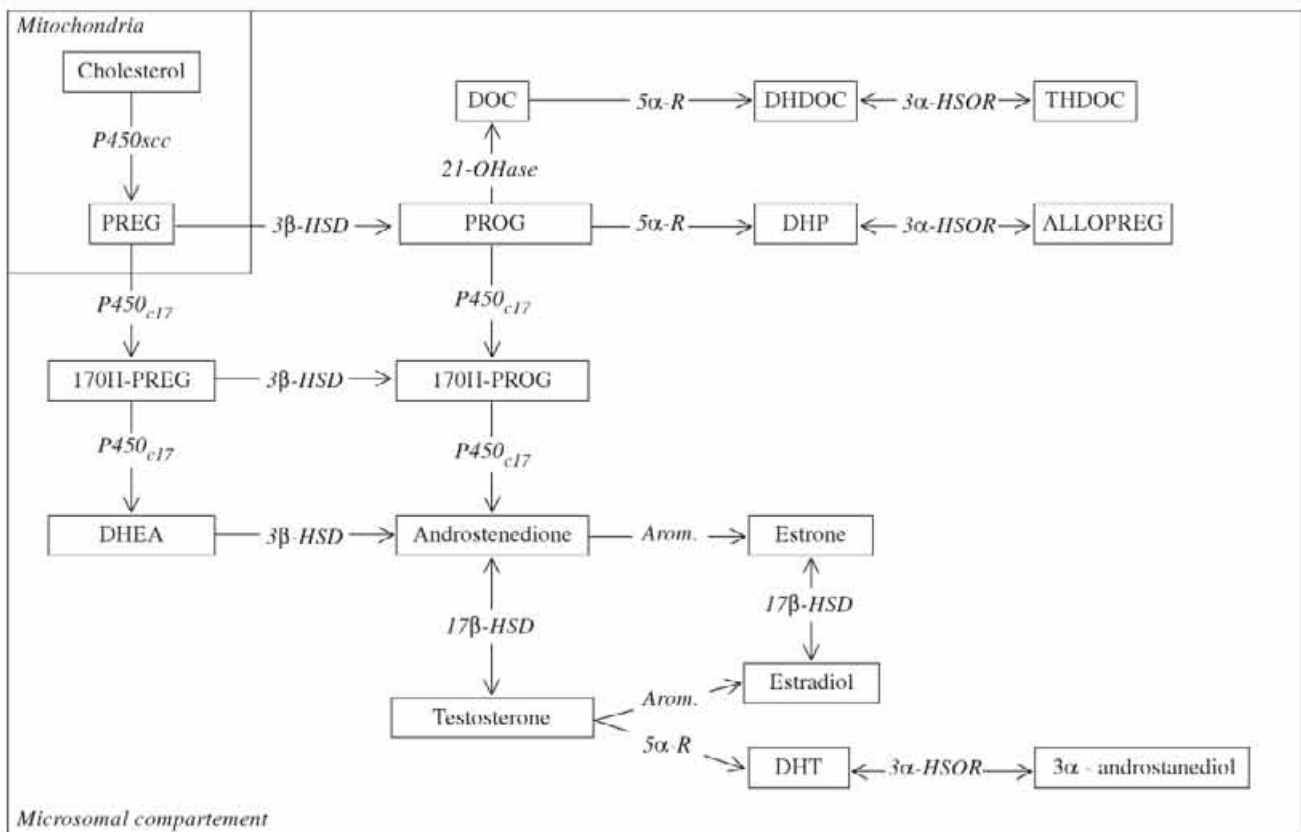


Fig. 8.2 Main biochemical pathways for neurosteroid biosynthesis and metabolism in the vertebrate brain. 17OH-PREG, 17-hydroxypregnenolone; 17OH-PROG, 17-hydroxyprogesterone; DHEA, dehydroepiandrosterone; DOC, deoxycorticosterone; DHDOC, dihydroxydeoxycorticosterone; THDOC, tetrahydroxydeoxycorticosterone; DHP, dihydropregnenolone; ALLOPREG, allopregnenolone; DHT, dihydrotestosterone; *P*450_{scc}, cytochrome *P*450 cholesterol side chain cleavage; *P*450_{c17}, cytochrome *P*450_{c17}; 3 β -HSD, 3 β -hydroxysteroid dehydrogenase; 5 α -R, 5 α -reductase; Arom., aromatase; 21-OHase, 21-hydroxylase; 3 α -HSOR, 3 α -hydroxysteroid oxyoreductase; 17 β -HSD, 17 β -hydroxysteroid dehydrogenase.

on the level of oxidative stress within cells estrogens can have a protective effect or, on the contrary, show a negative action on cell survival. On the other hand, oxidative stress itself can have an effect on neurosteroid production within nerve cells.

8.4.1 Regulation of Neurosteroidogenesis by Oxidative Stress and A β Peptide

It is established that steroids can be synthesized by nonglandular tissue within the nervous system. But the regulation of their biosynthesis is still poorly understood. Recent findings showed that several glial cells, in particular oligodendrocytes, upregulated their production of

DHEA under oxidative stress conditions induced by treatment with A β peptide or Fe²⁺ [54]. Modulation of neurosteroid production was also observed in neuroblastoma (SH-SY5Y) cells overexpressing the key AD proteins, APP/A β or Tau (Fig. 8.3) [52]. Indeed, overexpression of human wild-type Tau (hTau 40) protein induced an increase in production of progesterone, 3 α -androstenediol, and 17-hydroxyprogesterone, in contrast to overexpression of the abnormally hyperphosphorylated Tau bearing the P301L mutation and leading to a decrease in the production of these neurosteroids. In parallel, a decrease of progesterone and 17-hydroxyprogesterone production was observed in cells expressing human wild-type APP (wtAPP), whereas

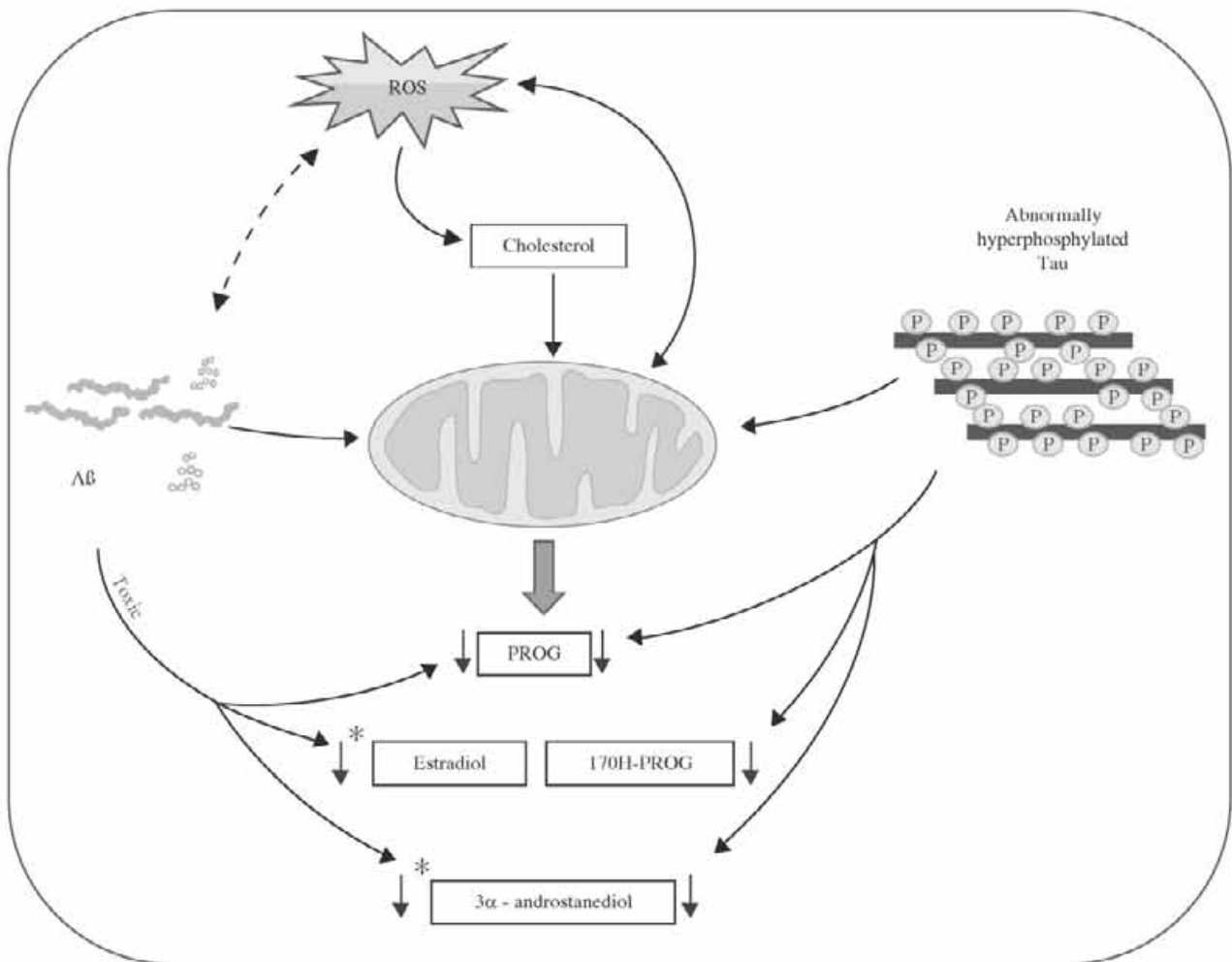


Fig. 8.3 Effect of toxic concentrations of A β peptides and abnormally hyperphosphorylated Tau protein on neurosteroid biosynthesis. A β induced a drop of the level of progesterone (PROG), estradiol, and 3 α -androstenediol by acting on reactive oxygen species (ROS) formation and mitochondrial function and/or directly on steroidogenesis. The presence of abnormally hyperphosphorylated Tau protein had the same effect by inducing a decrease of progesterone, 17-hydroxyprogesterone (17OH-PROG), and 3 α -androstenediol. On the other hand, it has been shown that nontoxic concentrations of A β induced an increase in estradiol and 3 α -androstenediol levels (this pathway is marked by *). (See color insert.)

3 α -androstenediol and estradiol level were increased. The latter finding was additionally confirmed with in vitro treatment experiments [4]. APPwt SH-SY5Y cells secrete A β levels within the nanomolar concentration range. Consistently, treatment of native SH-SY5Y cells with "nontoxic," that is, non-cell death-inducing, A β_{1-42} concentrations in vitro revealed an increase in estradiol production, whereas toxic A β_{1-42} concentrations within the micromolar range strongly reduced estradiol levels revealing the exact opposite effect. Of note, oxidative stress was able to modify neogenesis of neurosteroids in a similar pattern [55]. In fact, treatment with H₂O₂ for 24 h or 48 h induced a decrease of estradiol synthesis that was correlated to a downregulation of the aromatase, the enzyme responsible for estradiol formation from testosterone. Furthermore, an increase of cell death was observed in the presence of letrozole, an inhibitor of aromatase. This suggests that endogenous estradiol formation is very important for human neuroblastoma cells and plays a critical role in cell survival. Interestingly, when cells were pretreated with estradiol, it was possible to rescue neuroblastoma cells from H₂O₂ as well as from letrozole-evoked death. In agreement, similar results were also found in stress condition experiments using heavy metals, such as cobalt and mercury, and once again estradiol was able to reverse their deleterious effect by reducing oxidative stress and β -amyloid secretion [56].

8.4.2 Estrogens and Neuroprotection

Neuroprotective effects of estrogens against a variety of brain injuries have been described for many years. Treatment with 17 β -estradiol was able to protect the brain against excitotoxicity, A β peptide-induced toxicity, free radical generators, and ischemia in animal studies [53], but the basis of these effects is still poorly understood. It was recognized from former studies that estrogen depletion in postmenopausal women represents a significant risk factor for the development of AD and that an estrogen replacement therapy may decrease this risk and even delay disease progression [57, 58].

However, results from the "Woman's Health Initiative Memory Study" (WHIMS) including 4532 postmenopausal woman aged over 68 years indicated a twofold increase in dementia after 4.2 years of treatment (p.o. treatment with premaxin plus medroxyprogesterone). In addition, it indicated potential risks for breast cancer, pulmonary embolism, and stroke [59, 60]. Besides warrantable criticism with regard to the synthetic hormones used in the WHIMS trial, the outcome results were unexpected and disappointing. One can ask the question, "How could it be that so many scientific studies before the WHIMS trial were wrong?" Thus the currently prevailing view points about the "critical window

hypothesis" [16] are asking about the critical period in which one might expect a neuroprotective effect to occur. The results of the WHIMS study also initiated a discussion about a two-edged effect of estradiol. Thus estradiol can possibly also exhibit a "prooxidant effect" in the presence of ongoing oxidative stress [53]. Thereby, estradiol can be hydroxylated to give catecholestrogens that can enter a redox cycle generating superoxide radical. In an oxidative environment, this redox cycling can lead to a continuous formation of ROS that amplifies even more oxidative stress and increases neuronal loss.

On the contrary, animal studies, especially in rodents and transgenic mice models for AD, seem to confirm positive effects of estrogen treatment on the pathophysiology of the disease. It has been shown that treatment with estrogen in mice expressing mutations in human APP (Swedish and Indiana) had an impact on APP processing, decreasing levels of A β and so its aggregation into plaques [61]. In triple transgenic AD mice, depletion of sex steroid hormones induced by ovariectomy in adult females significantly increased A β accumulation and had a negative impact on cognitive performance [62]. Treatment of these ovariectomized mice with estrogens was able to prevent these effects. Of note, when PROG was administered in combination with estrogens, the beneficial effects on A β accumulation were blocked but not effects on cognitive performance. Furthermore, PROG reduced Tau hyperphosphorylation when administered alone. This suggests that estrogen and PROG can interact to regulate APP processing but can also act independently on different AD pathways.

At the cellular level, estrogen was able to activate antioxidant defense systems by reducing ROS production, limiting mitochondrial protein and DNA damage, and improving the activity of the electron chain transport during oxidative phosphorylation [53]. Thus estrogen can have direct antioxidant effects by increasing reduced glutathione (GSH) levels and decreasing oxidative DNA damage in mitochondria of ovariectomized female rats [63]. This is correlated with an upregulation of the expression of two enzymes: manganese superoxide dismutase (Mn-SOD) and glutathione peroxidase, both of them implicated in the antioxidant defense system. Of note, estrogen can modulate the redox state of cells by intervening in several signaling pathways, such as MAPK, G protein-regulated signaling, NF- κ B, c-fos, CREB, phosphatidylinositol-3-kinase, PKC, and Ca²⁺ influx [3, 64]. On the basis of this complex mode of action, estrogen seems to be able to decrease oxidative stress markers, including lipid peroxidation, protein oxidation, and DNA damage.

Recently, it has been proposed that estrogens exert their beneficial effects by acting directly on mitochondria

via estrogen receptor β (ER β) [65]. In fact, incubation of isolated mitochondria from rat brain with estradiol leads to a decrease of H_2O_2 production by this organelle coupled with an increase of the mitochondrial membrane potential. Moreover, estradiol seems to prevent the release of cytochrome c by mitochondria (a mechanism known to induce apoptosis of cells by activating the caspase cascade in the cytoplasm), which increases the efficiency of the respiratory chain. In addition, estrogens are able to bind to nuclear receptors, such as estrogen receptor α and β (ER α/β), and to act as transcription factors. Thus estrogens enhanced the expression of the antiapoptotic proteins, Bcl-2 and Bcl-xL, preventing the initialization of the cell death program by mitochondria [3]. They were also able to increase the expression of F1 subunits of ATP synthase and glucose transporter subunits and regulate enzymes involved in the tricarboxylic acid (TCA) cycle, which has the effect of improving glucose utilization by cells.

As described recently, estrogens can have an effect on the transcription of mitochondrial genes, especially on the electron transport chain components [66]. Treatment of ovariectomized female rats with estradiol induced an increase of mitochondrial respiratory function translated into an enhancement of O_2 consumption and coupled to an increased expression and activity of cytochrome c oxidase (electron transport chain complex IV).

Finally, another means for estrogens to avoid negative effects of oxidative stress is to regulate calcium homeostasis by inducing mitochondrial sequestration of cytosolic calcium [53]. In fact, an imbalance of calcium handling can lead to an increase of ROS production by activating the enzyme nitric oxide synthase, which can sensitize neural cells to oxidative damage. It has been shown that estradiol treatment of primary hippocampal neurons was able to potentiate glutamatergic response via NMDA receptor, which resulted in an increased influx of calcium in cells. This effect was coupled with an induction of mitochondrial sequestration of cytosolic calcium and an increase of the mitochondrial calcium load tolerability, to avoid calcium-induced excitotoxicity and to promote cell survival.

8.5 CONCLUSION

In summary, it is now clear that oxidative stress is an important actor involved in AD pathophysiology and intervenes already at an early disease stage. Furthermore, good evidence is provided that neurosteroids, such as estrogens, are able to limit oxidative damage by reducing lipid peroxidation, protein oxidation, Ca^{2+} overload in cytosol, and DNA damage in mitochondria as well as in the nucleus. These effects are mediated by

several mechanisms, from transcription of genes coding for antioxidant enzymes to the regulation of antiapoptotic pathways, by way of improvement of mitochondrial respiratory chain efficiency and glucose metabolism. Thus, with their abilities to counter excess oxidative stress, estrogens seem to be able to prevent AD-related toxic mechanisms, such as A β peptide aggregation, Tau hyperphosphorylation, and neuronal loss. Better human studies taking into account the critical window hypothesis are essential before drawing a final conclusion on efficacy of neurosteroids in prevention of AD.

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OXIDATIVE STRESS IN ADULT NEUROGENESIS AND IN THE PATHOGENESIS OF ALZHEIMER DISEASE

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9.1 INTRODUCTION

Alzheimer disease (AD) is the most common form of dementia among the elderly. It is a neurodegenerative disease that affects more than 26 million patients worldwide and for which there is still no cure [1, 2]. AD is characterized by memory and cognitive deficits, amyloid deposits, neurofibrillary tangles, neurodegeneration, aneuploidy, and genome damage [3]. Genetic mutations and genetic, acquired, and environmental risk factors, particularly neuroinflammation and oxidative stress, are the main causes of AD [4]. New research in adult neurogenesis and neural stem cells (NSCs) suggests that newly generated neuronal cells of the adult brain, particularly of the hippocampus, may be involved in AD and may be affected by reactive oxygen species (ROS) and oxidative stress [5, 6].

ROS are free radical reactive substances formed by the incomplete reduction of oxygen. They include superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$) and peroxynitrite anions ($ONOO^-$). Free radicals, such as ROS, are produced physiologically by cells and are involved in cell signaling and metabolism [7]. They are also damaging and toxic to the cells. They oxidize cytoplasmic and membrane proteins, lipids, and nucleic acids. Oxidation of proteins and nucleic acids and peroxidation of lipids compromise cellular functions. Cells are protected against the toxicity of ROS by enzymes, such as superoxide dismutase, catalase, and glutathione. These enzymes act as natural antioxidants.

Under physiological conditions, the balance between the generation and degradation of ROS by cells and organisms is highly regulated. Oxidative stress occurs when the level of free radicals exceeds the antioxidant capacity of the cells [8]. It results from an elevation of production of free radicals, from a decrease in the scavenging of free radicals, or from a decrease in the mechanisms used to repair oxidized macromolecules. Oxidative stress and the excessive production of ROS are deleterious conditions leading to cellular dysfunction and cell death, via apoptosis or necrosis [9]. Oxidative stress plays a key role in the development of numerous pathologies, particularly neurodegenerative diseases such as AD [9]. In this chapter we review and discuss the involvement and contribution of oxidative stress and ROS to adult neurogenesis and to the pathogenesis of AD.

9.2 ALZHEIMER DISEASE

AD is a neurodegenerative disease characterized by memory and cognitive deficits and anosmia [10–12]. The disease is initially associated with the loss of nerve cells in areas of the brain that are vital to memory and cognition, such as the entorhinal cortex, hippocampus, and neocortex. As the disease progresses, other regions of the brain are affected, leading to severe incapacity and death, [12]. AD is characterized in the brain by the presence of amyloid plaques and neurofibrillary tangles, the histopathological hallmarks of the disease, and

aneuploidy [13]. AD is the most common form of dementia among the elderly. The disease affects 30% of individuals over the age of 80 [14]. Age is the principal risk factor for AD. The incidence of the disease doubles every 5 years after age 65 [14]. AD affects more than 35 million patients worldwide, a number expected to quadruple by 2050 as the population ages [15]. Late-onset AD (LOAD) refers to cases of AD diagnosed after the age of 65. Early-onset AD (EOAD) refers to cases of AD diagnosed before age 65. LOAD accounts for the vast majority, over 93%, of all cases of AD.

9.2.1 Inherited and Sporadic Forms of Alzheimer Disease

The inherited form of AD, also known as familial Alzheimer disease (FAD), is caused by mutations in so-called familial Alzheimer genes, such as the gene of β -amyloid precursor protein (APP), the presenilin-1 (*PSEN-1*) gene, and the presenilin-2 (*PSEN-2*) gene [16]. It is a rare form of the disease, affecting about 200 families in the world. The sporadic form of AD is caused by a combination of genetic, acquired, and environmental risk factors [17]. These include the presence of the ApoE varepsilon 4 allele (*ApoE4*), the presence of variants in at least two different clusters of intronic sequences in the neuronal sortilin-related receptor (*SORL1*) gene, hypertension, diabetes, neuroinflammation, and oxidative stress [18–21]. The sporadic form of AD is the most common form of the disease. It accounts for most cases of LOAD, whereas the inherited form of AD accounts for most cases of EOAD.

9.2.2 Amyloid Plaques and Neurofibrillary Tangles

Amyloid plaques and neurofibrillary tangles are deposits of proteins distributed throughout the brain of patients with AD, particularly in the regions of degeneration such as the entorhinal cortex, hippocampus, and temporal, frontal, and inferior parietal lobes [22]. Amyloid plaques are primarily composed of extracellular aggregates of protein β -amyloid or amyloid fibrils and of the serine protease inhibitor α 1-antichymotrypsin [22]. Protein β -amyloid is a 42-amino acid peptide that is derived from the posttranscriptional maturation of APP [23]. The abnormal processing of APP results in the aggregation of protein amyloid and the formation of amyloid plaques. Neurofibrillary tangles are intracellular aggregates of hyperphosphorylated Tau protein. Tau protein is a microtubule-associated phosphoprotein [24]. The hyperphosphorylation of Tau protein by kinases results in the aggregation of Tau protein and the breakdown of microtubules that are involved in cell structure, intracellular transport, and cell division [25]. Amyloid plaques

and neurofibrillary tangles are two of the probable causes of the pathogenesis of AD; amyloid plaques and neurofibrillary tangles that would cause cell death in the brain [26]. In support of this contention, rat embryonic cortical neurons cultured with toxic concentrations of protein β -amyloid reenter the cell cycle and die by apoptosis in vitro [27].

9.2.3 Aneuploidy and Expression of Proteins of the Cell Cycle

Aneuploidy is a landmark of AD pathology. Preparations of lymphocytes from patients with AD, EOAD and LOAD, reveal an increase of aneuploidy in cells, particularly for chromosomes 13, 18, and 21 [28, 29]. Since the cells that are the most likely to develop aneuploidy are dividing cells, the nondisjunction of chromosomes during cell division in stem cells or somatic cells that retain their ability to divide is at the origin of aneuploidy in lymphocytes of patients with AD [30]. A substantial number of neurons, 4% to 10%, in regions of degeneration, such as the hippocampus, of the brain of AD patients express proteins of the cell cycle and are aneuploid. Among the proteins of the cell cycle expressed by nerve cells in regions of degeneration are the proliferating nuclear antigen, Ki-67, cyclin D, cyclin-dependent kinase 4, and cyclin B1 [31, 32]. Nerve cells are postmitotic. The forced expression of oncogenes in postmitotic nerve cells causes cell death rather than cell proliferation. Hence, nerve cells in regions of degeneration that express proteins of the cell cycle or are aneuploid would originate from abortive cell cycle reentry, leading to apoptosis, or cycle reentry and gene duplication, without cell division, leading to aneuploidy [33, 34].

The deregulation and/or reexpression of proteins controlling the cell cycle and aneuploidy in nerve cells would underlie the neurodegenerative process and pathogenesis of AD. In aneuploid cells, the genetic imbalance results in the overexpression of genes by the cells. This has tremendous consequences for the development of AD, as genes involved in the pathology of AD would further contribute to the pathogenesis of the disease, as a result of their overexpression. The genes of ApoE, APP, PSEN-1, PSEN-2 and TAU are located on chromosomes 19, 21, 1, 14, and 17, respectively [35–38]. Aneuploidy for chromosomes 19, 21, 1, 14, and/or 17 would result in the overexpression of ApoE and in an increased risk for individuals who have *ApoE4* in their genetic make-up of developing the sporadic form of AD, would result in the overexpression of APP and promote the formation of amyloid plaques, would promote the formation of amyloid plaques in patients carrying FAD mutations on PSEN genes and contribute to the pathogenesis of EOAD, and/or would result in the overexpression of Tau

TABLE 9.1 Processes Associated with Neurogenesis and Oxidative Stress in Alzheimer Disease

DNA damage	Mutational events, modified base 8-hydroxydeoxyguanosine, strand breaks, and large deletions
Cell cycle	Activity in controlling the cell cycle, cell cycle reentry of nerve cells, aneuploidy, particularly for chromosome 17 carrying the <i>TAU</i> gene
Protein oxidation	Enzymatic and mitogenic pathways, such as EGF and VEGF pathways, the stress-activated protein kinases JNK and p38, JAK/STAT, protein kinase C pathways, and histone deacetylase

Oxidative stress is a risk factor for developing Alzheimer disease (AD). Proteins, lipids, and nucleic acids elicit high rate of oxidation in patients with AD. The nuclear and mitochondrial DNA in degenerated regions of the brain of patients with AD elicit lesions. Reactive oxygen species (ROS) and oxidative stress promote cell cycle reentry of nerve cells and aneuploidy, particularly for chromosome 17 carrying the *TAU* gene. Aneuploidy for chromosome 17 in newly generated cells of the adult brain would promote the expression of Tau proteins in the hippocampus and neurodegeneration. ROS and oxidative stress contribute to the pathogenesis of AD by promoting cell death and neurodegeneration.

protein and promote the formation of neurofibrillary tangles. Cells of AD patients elicit an elevation of aneuploidy, particularly for chromosomes 13, 18, and 21 [28, 29, 39]. Hence, aneuploid cells in AD patients are likely to contribute to the pathogenesis of the disease with high probability as a result of the overexpression of genes involved in AD. It is proposed that the genetic imbalance in aneuploid nerve cells signifies that they are fated to die [33]. Their relatively high percentage at any one time in regions of degeneration in AD brains suggests that they will undergo a slow death process, unlike apoptosis. These cells may live in this state for months, possibly up to 1 year [33]. Hence, the deregulation and/or reexpression of proteins controlling the cell cycle in nerve cells would underlie the neurodegenerative process in AD.

9.2.4 DNA Damage

The nuclear and mitochondrial DNA in degenerated regions of the brain of patients with AD elicit lesions and mutational events, such as DNA oxidation, as evidenced by the presence of the modified base 8-hydroxydeoxyguanosine, strand breaks, and large deletions (Table 9.1) [40]. Proteins, lipids, and nucleic acids elicit a high rate of oxidation in patients with AD, particularly in regions containing amyloid plaques and neurofibrillary tangles in the brain [41]. AD is a neurodegenerative disease caused by an excessive rate of damage in the genome [42].

9.3 ADULT NEUROGENESIS AND ENHANCED NEUROGENESIS IN ALZHEIMER DISEASE

9.3.1 Adult Neurogenesis and Neural Stem Cells in Mammals

Neurogenesis occurs primarily in two regions of the adult mammalian brain, the dentate gyrus (DG) of the hippocampus and the anterior region of the subventricular zone (SVZ), in various species including in humans [43–46]. Newly generated neuronal cells in the

subgranular zone of the DG migrate to the granule cell layer, where they differentiate into granulelike cells and extend axonal projections to the CA3 region of the Ammon's horn [47, 48]. Newly generated neuronal cells in the SVZ migrate through the rostral migratory stream to the olfactory bulb, where they differentiate into granule and periglomerular interneurons [49, 50]. Newly generated neuronal cells in the DG establish synaptic contacts and functional connections with neighboring and target cells [48, 51–53]. They establish mossy fiber-like synapses with target cells of the CA3 region of the Ammon's horn [53]. The number of neuronal cells generated in the adult mammalian brain is relatively low. In the DG, it is estimated that 9000 neuronal cells, corresponding to about 0.1% of the granule cell population, are generated per day in young adult mice [54]. In higher primates, such as humans and monkeys, the number of neuronal cells generated per day is lower than in rodents. In adult macaque monkey, an estimated 0.004% of the granule cell population is generated per day in the hippocampus [55]. Newly generated neuronal cells in the adult brain survive for an extended period of time, at least 2 years in the human hippocampus [43]. Newly generated neuronal cells of the adult brain may replace nerve cells born during development. They may be involved in the physiology, pathology, and plasticity of the nervous system, particularly of the hippocampus and olfactory bulb.

Newly generated neuronal cells in the adult brain would originate from a pool of residual NSCs; the self-renewing multipotent cells that generate, through a transient amplifying population of cells, the main cell types of the nervous system, nerve cells, astrocytes, and oligodendrocytes [56, 57]. In support of this contention, self-renewing multipotent NSCs have been isolated and characterized *in vitro* from various regions of the adult brain of mammals, including from the hippocampus and SVZ [58–60]. Neural progenitor and stem cells express molecular markers, such as the intermediate filament nestin, the transcription factors sox-2, oct-3/4, and the RNA binding protein Musashi 1 [61–64]. Hence,

neurogenesis occurs in the adult brain and NSCs reside in the adult central nervous system. This reveals that the adult brain has the potential for self-repair and may be amenable to repair. The stimulation of endogenous neural progenitor or stem cells of the adult brain and the transplantation of adult-derived neural progenitor and stem cells are proposed to repair and restore the degenerated or injured nerve pathways. To this end, the isolation and characterization of population of neural progenitor and stem cells from human biopsies and postmortem tissues provide a source of tissue for cellular therapy for the treatment of a broad range of neurological diseases and injuries, including neurodegenerative diseases such as AD and Parkinson disease, cerebral strokes, and traumatic and spinal cord injuries [65, 66].

Neurogenesis occurs constitutively in the adult brain and is modulated by a broad range of environmental stimuli, physiological and pathological conditions and processes, trophic factors/cytokines, neurotransmitters, and drugs. This includes environmental enrichment, learning and memory tasks, physical activity, AD, and epilepsy [67, 68]. The contribution of adult neurogenesis and its modulation to the physiology and pathology of the nervous system remains to be determined.

9.3.2 Neurogenesis in Patients with Alzheimer Disease and in Animal Models of Alzheimer Disease

The expression of markers of immature neuronal cells, such as doublecortin and polysialylated nerve cell adhesion molecule, is increased in hippocampal regions, particularly the DG, of the brain of patients with clinical diagnosis of AD [69]. Neurogenesis is enhanced in the DG of adult transgenic mice that express the Swedish and Indiana APP mutations, a mutant form of human APP [70]. It is reduced in the DG of PDAPP adult transgenic mice, a model of AD with age-dependent accumulation of protein β -amyloid, in the DG and SVZ of adult transgenic mice deficient for PSEN-1 and/or APP and in the DG of adult transgenic mice overexpressing variants of APP or PSEN-1 [71–75]. These results show that neurogenesis is enhanced in the hippocampus of the brain of patients with AD, but they report conflicting data and discrepancies in animal models of AD.

The apparent discrepancies in the modulation of adult neurogenesis in humans and in animal models of AD may originate from the validity of animal models as representative of AD and from limitations and pitfalls in the conducted studies. AD is a neurodegenerative disease characterized by widespread neurodegeneration and multiple and complex processes, including the deposit of amyloid, the formation of neurofibrillary tangles, and aneuploidy. Hence, transgenic mice models of AD represent models to study the genes involved in

the disease rather than the disease itself. Transgenic mice may be limited in their validity to study adult phenotype, and particularly adult neurogenesis, as the mutated genes may alter the animal's development and this may affect the adult phenotype. Aggregation of protein β -amyloid during development in APP transgenic mice may alter the development of the nervous system and therefore may have adverse consequences on adult neurogenesis [76]. The apparent discrepancies in the modulation of adult neurogenesis in human and animal models of AD as well as discrepancies in studying adult neurogenesis in human and animal models in general, may originate from the methods used for studying and quantifying cell proliferation and neurogenesis. Bromodeoxyuridine (BrdU) labeling has been the most used method to study adult neurogenesis *in vivo*. BrdU is a thymidine analog used for birthdating and monitoring cell proliferation, as BrdU integrates into the DNA of dividing cells [77]. In this paradigm, BrdU is generally administered peripherally, intraperitoneally in rodents, and the fate of the BrdU-labeled cells is followed by immunohistochemistry [78]. Multiple immunohistochemistry staining with markers of neuronal lineages combined with confocal microscopy and stereology allows qualitative and quantitative study of adult neurogenesis [79–81]. BrdU labeling as a method for studying adult neurogenesis, is not without limitations and pitfalls, particularly for the study of adult neurogenesis in AD. BrdU is not a marker for cell proliferation and neurogenesis, but a marker of DNA synthesis. As a thymidine analog, BrdU integrates into DNA of dividing cells during the S-phase of the cell cycle. Hence, studying cell proliferation and neurogenesis with the BrdU-labeling paradigm requires discriminating cell proliferation and neurogenesis of other events involving DNA synthesis, particularly abortive cell cycle reentry leading to apoptosis and cell cycle reentry and gene duplication without cell division, leading to aneuploidy [82, 83]. Since aneuploidy in the region of neurodegeneration is a landmark of AD pathology, careful analysis must be carried out when studying adult neurogenesis in animal models of AD with the BrdU-labeling paradigm. In addition, the permeability of the blood-brain barrier is affected in AD [84]. As consequence, an increase in BrdU labeling in the brain could originate from an increase in BrdU uptake rather than an increase in cell proliferation and neurogenesis. There are other considerations that may affect the analysis of BrdU-labeling studies. Among these considerations, there is no consensus on the term "neurogenesis." Some studies only present proliferation data, whereas others present only neuronal differentiation or survival data, and in most studies, and in particular in human postmortem studies, only one time point along the pathology is analyzed. Therefore, careful

analysis and discussion of the studies must be carried out when using BrdU labeling to study adult neurogenesis, particularly in AD. Some of these limitations also apply when studying adult neurogenesis by immunohistology against markers of the cell cycle. In particular, immunohistochemistry for proteins of the cell cycle does not allow discrimination among cells reentering the cell cycle as a prelude to apoptosis, cells undergoing DNA duplication without cell division, as part of their pathological fate, and the genesis of neuronal cells [85].

Hence, these studies of adult neurogenesis in the hippocampus of the brain of patients with AD and in animal models of AD remain to be confirmed and validated. Indeed, neurogenesis might be differentially regulated along the pathogenesis. Enhanced neurogenesis in the DG of AD brain would contribute to a regenerative attempt, to compensate for the neuronal loss. It would result from damage or stimulation induction of neurogenesis and may be a consequence, rather than a cause, of the disease [86]. In the SVZ, studies show a reduction in the number of neural progenitor cells in patients with AD, as revealed by immunohistology for the markers of neural progenitor and stem cells, nestin and Musashi1 [87]. The reduction in the number of neural progenitor cells in the SVZ of AD brain may underlie the compromised olfaction associated with the disease.

9.4 OXIDATIVE STRESS: A RISK FACTOR FOR DEVELOPING ALZHEIMER DISEASE

Lipids, proteins, and nucleic acids elicit high rates of oxidation, particularly in regions of degeneration in the AD brain [41]. Oxidative stress is a risk factor for developing AD [9].

9.4.1 ROS and Oxidative Stress Promote Cell Death and Neurodegeneration

Protein β -amyloid promotes the generation of ROS in the brain of AD patients [88]. ROS, particularly generated by protein β -amyloid, and oxidative stress in the brain of patients with AD would contribute to the pathogenesis of the disease by promoting cell death and neurodegeneration. Abnormal mitotic signaling, such as abortive cell cycle reentry leading to apoptosis and cycle reentry and gene duplication, without cell division, leading to aneuploidy, underlies the process of neurodegeneration and contributes to the pathogenesis of AD [33, 34]. It is proposed that oxidative stress and abnormal mitotic signaling would both be necessary to propagate the disease. A "two-hit hypothesis" has been proposed to conciliate the activity of oxidative stress and abnormal mitotic signaling, such as abortive cell cycle

reentry and gene duplication without cell division, as causative factors of AD: Oxidative stress and abnormal mitotic signaling can act independently as initiators; however, both processes are necessary to propagate the pathogenesis of AD [89].

Abortive cell cycle reentry, cell cycle reentry and gene duplication without cell division, and DNA damage are underlying processes in the development of AD. ROS and oxidative stress promote cell cycle reentry of nerve cells and DNA damage [90]. The contribution of ROS and oxidative stress to the process of cell cycle reentry of nerve cells would support the "two-hit hypothesis" conciliating the activity of oxidative stress and abnormal mitotic signaling in the brain of AD patients. The mechanism by which ROS and oxidative stress increase the risk of developing AD, particularly through its activity on abnormal mitotic signaling, remains mostly unknown. ROS and oxidative stress would contribute to the pathogenesis of AD through their activity in controlling the cell cycle (Table 9.1). It would increase the risk of developing AD, directly or indirectly, through their oxidative activity on DNA and on various enzymatic and mitogenic pathways, such as the EGF and VEGF pathways, the stress-activated protein kinases JNK and p38, JAK/STAT, protein kinase C pathways, and histone deacetylase and through mutational events including strand breaks and large deletions (Table 9.1) [91]. Studies show that whether ROS-exposed cells undergo proliferation, growth arrest, or apoptosis depends in part on where the cell resides in the cell cycle when insulted [92]. This has implications for the mechanisms by which oxidative stress may affect neuronal cells in the adult brain, whether and how it affects mature neurons or newly generated neuronal cells in regions of neurodegeneration, such as the hippocampus. ROS and oxidative stress would also contribute to DNA damage, if the oxidation of DNA surpasses the DNA repair capacity of the cell, leading to the accumulation of mutations and the loss of genome stability, a landmark of AD pathology.

9.4.2 Oxidative Stress Promotes the Generation of Aneuploid Newly Generated Neuronal Cells in the Adult Brain

Stem cells and somatic cells that retain the ability to divide are the most likely to develop aneuploidy. Since newly generated neuronal cells of the adult brain originate from stem cells, they are most likely to develop aneuploidy. The nondisjunction of chromosomes in the process of cell division of adult neurogenesis would lead to newly generated neuronal cells that are aneuploids or to a population of aneuploid neural progenitor cells that would not proceed with their developmental program in the neurogenic regions, primarily the DG of the

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OXIDATIVE STRESS AND PARKINSON DISEASE

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10.1 INTRODUCTION

Parkinson disease (PD) is the most common neurodegenerative movement disorder, currently affecting more than 4 million predominantly elderly individuals worldwide [1]. Originally described as “the shaking palsy” in 1817 by the British physician James Parkinson, the disease is attended by a constellation of motoric deficits including bradykinesia (slowness in movements), postural instability, rigidity, and tremor that ultimately result in near total immobility. Although pathological changes are distributed in the PD brain [2], the principal neuropathology that underlies the characteristic motor phenotype of PD patients is unequivocally the loss of midbrain dopaminergic neurons in the substantia nigra pars compacta (SNpc), which results in a severe depletion of striatal dopamine (DA) and thereby an impaired nigrostriatal system that otherwise allows an individual to execute proper, coordinated movements. This specific pattern of neurodegeneration in PD is often accompanied by the presence of eosinophilic intracytoplasmic inclusions known as Lewy bodies (LBs) in surviving neurons in the SN as well as in other affected brain areas [2]. Accordingly, pharmacological replacement of brain DA via L-DOPA administration represents an effective symptomatic recourse for the PD patient (especially during the initial stages of the disease) and remains a clinical gold standard treatment for PD. However, neither L-DOPA nor any currently available therapies

can slow or stop the insidious degenerative process in the PD brain. Furthermore, the major drawbacks with current therapies are the inevitable loss of effectiveness and increasing drug-induced side effects as the disease progresses. Invariably, the debilitating nature and morbidity of the disease present significant social, emotional, and economic problems. As the world population rapidly ages, these problems undoubtedly will also increase.

Despite nearly two centuries of research, the etiology of PD remains elusive. However, a broad range of studies conducted over the past few decades, including epidemiological, genetic and postmortem analysis, as well as in vitro and in vivo modeling, have contributed significantly to our understanding of the pathogenesis of the disease. In particular, the recent identification and functional characterization of several genes, including *α-synuclein*, *parkin*, *DJ-1*, *PINK1*, and *LRRK2*, whose mutations are causative of rare familial forms of PD have provided tremendous insights into the molecular pathways underlying dopaminergic neurodegeneration [3, 4]. Collectively, these studies implicate aberrant mitochondrial and protein homeostasis as key contributors to the development of PD, with oxidative stress likely acting as an important nexus between the two pathogenic events.

Notably, the brain is often thought to be particularly susceptible to oxidation-induced damage because of its high metabolic rate and its relatively reduced capacity to replenish its postmitotic neuronal populations compared with other organs. Moreover, the brain contains high

levels of phospholipids and polyunsaturated free fatty acids, both of which are prone to modifications by oxidants. For SN dopaminergic neurons, the vulnerability toward oxidative stress is further enhanced by the abundance of redox-active iron in this region of the brain, as well as by the presence of DA, whose oxidation products are potentially cytotoxic [5, 6]. Although attractive, the hypothesis that oxidative stress plays a central role in PD pathogenesis continues to be keenly debated, particularly in view of recent findings in the clinic that antioxidant strategies have failed to produce convincing protection in PD patients. Furthermore, a major question that remains unresolved to this point is whether oxidative stress represents a cause or a consequence of neurodegeneration. Here, we review and discuss both historical evidence and current thoughts about the relationship between the oxidative stress and PD, which include our own views on the topic. For the benefit of the reader, we have also included a section on DA chemistry and oxidation that summarizes the oxidative pathways for catecholamines in the genesis of cytotoxic quinones.

10.2 REACTIVE OXYGEN SPECIES AND OXIDATIVE STRESS

It is perhaps paradoxical to note that the production of reactive oxygen species (ROS), often regarded as the cellular “bad guys,” is an inevitable consequence of aerobic (oxygen dependent) respiration—a process needed to keep all cells in our body, including postmitotic neurons, alive. During aerobic respiration, high-energy products (NADH and FADH₂) generated in the mitochondria via the Krebs cycle donate electrons to a series of electron carriers (complexes I–IV) on the electron transport chain (ETC) to produce a proton gradient across the inner mitochondrial membrane that is utilized by ATP synthase to drive oxidative phosphorylation of ADP to ATP (Fig. 10.1). Although molecular oxygen is typically reduced by electrons from Krebs cycle intermediates flowing through the ETC to produce H₂O, partial reduction of molecular oxygen to superoxide anion (O₂^{•−}) occurs when electrons leak from the ETC (particularly at complex I). This free radical can be converted to the highly reactive hydroxyl radical (OH[•]) via an iron-catalyzed reaction known as the Fenton reaction, or to peroxynitrite (ONOO[−]) upon reaction with nitric oxide (NO). Both hydroxyl radical and peroxynitrite are potent oxidants that can cause marked cellular damage by reacting with proteins, lipids, and nucleic acids. Further, these reactive species may also target the ETC, which results in a feedforward cycle of increasing oxidative stress and injury. It is noteworthy to

mention that NO, which is generated by nitric oxide synthase (NOS), can itself contribute to oxidative damage through *S*-nitrosylation, a reaction whereby the cysteine residues of protein are modified to nitrosothiols, often resulting in altered protein function [7]. (The role of *S*-nitrosylation and nitrosative stress in PD has been covered extensively in several excellent recent reviews [8] and is only discussed briefly in this chapter.)

To protect against potential ROS-induced damage, aerobically respiring cells have developed over the course of evolution an effective antioxidant defense mechanism that consists of a plethora of antioxidant enzymes [such as superoxide dismutase (SOD), glutathione peroxidase, and catalase] as well as nonprotein antioxidants (such as glutathione, α -tocopherol, and ascorbic acid) to keep the levels of cellular free radicals in check. For example, ROS by-products produced by mitochondrial respiration are rapidly detoxified by SOD2 (which converts superoxide to hydrogen peroxide) so that their basal levels are low and nontoxic. As glutathione (GSH) is a major antioxidant in cells, the ratio between the reduced and oxidized form of glutathione (i.e., GSH/GSSG), is often taken as an indicator of cellular redox status [9]. When a state of imbalance between the production of ROS and their clearance by the antioxidant defense system occurs, oxidative stress ensues. However, it is important to highlight that although the overproduction of ROS leading to oxidative stress is usually bad, ROS may be beneficial to cells in some instances. For example, ROS generated by phagocytes during inflammation helps to kill invading pathogens [10]. Furthermore, by virtue of the reversibility of oxidation and reduction, ROS are also used as secondary messengers in redox-based intracellular signaling in response to internal and external cues. This includes neuronal signaling processes that influence synaptic neurotransmission, synaptic plasticity, and long-term potentiation [11, 12]. Likewise, the role of NO as a signaling molecule in the brain is well characterized [13]. Thus ROS [or reactive nitrogen species (RNS)] may act as signaling or stress molecules depending on their cellular levels.

10.3 OXIDATIVE STRESS AND PD

There is certainly ample support from postmortem studies to suggest that the redox state in the PD brain is in disequilibrium. For example, several groups have reported that markers for lipid peroxidation (including 4-hydroxynonenal and malondialdehyde), protein carbonyl modifications, and even DNA and RNA oxidation are markedly elevated in the SN of postmortem PD brains [14–17], and that these ROS-induced events are

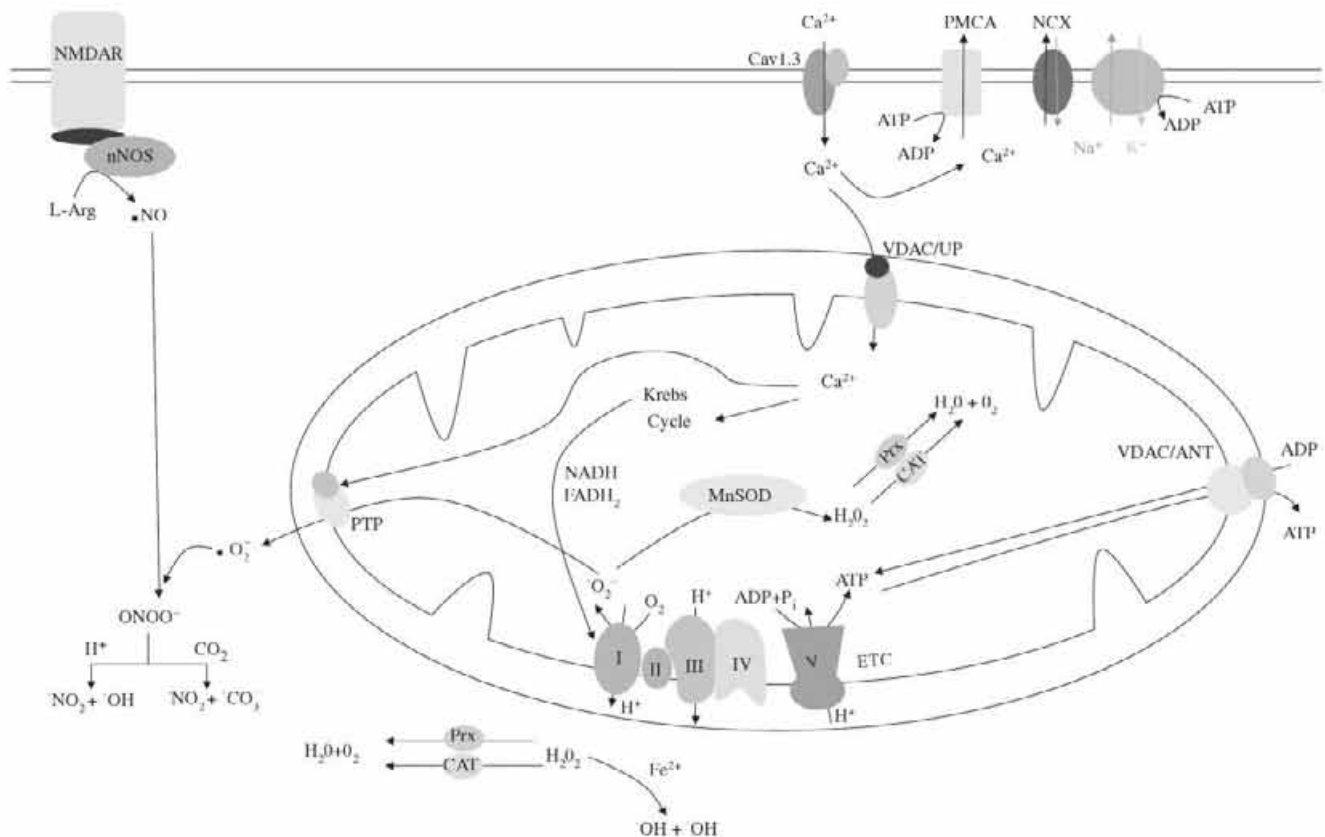


Fig. 10.1 Genesis of reactive oxygen and nitrogen species. NADH and FADH₂ produced via the Krebs cycle donate electrons to a series of electron carriers on the electron transport chain (ETC) to produce a proton gradient needed to drive the oxidative phosphorylation of ADP to ATP by ATP synthase. Leakage of electrons from the ETC (especially from complex I) occurs, leading to production of superoxide ($\text{O}_2^{\bullet-}$), which is typically detoxified into hydrogen peroxide (H_2O_2) by superoxide dismutases (SOD). H_2O_2 is then converted into H_2O and oxygen by catalase (CAT) and peroxiredoxin (Prx). H_2O_2 can also be converted to highly reactive hydroxyl radical ($\text{OH}^{\bullet}$) via the iron-catalyzed Fenton reaction. Activation of nitric oxide synthase (nNOS) as a result of *N*-methyl-D-aspartate receptor (NMDAR) over-stimulation leads to the production of nitric oxide (NO) that can react with $\text{O}_2^{\bullet-}$ to form the reactive peroxynitrite (ONOO^-). ONOO^- can react with H^+ or CO_2 in the cytosol to generate nitrogen dioxide (NO_2) and highly reactive $\text{OH}^{\bullet}$ or $\text{CO}_3^{\bullet-}$. Dendritic influx of Ca^{2+} , which apparently occurs via Cav1.3 Ca^{2+} channels in SN dopaminergic neurons during pace-making, can be taken up by the mitochondria via voltage-dependent anion channels (VDAC) and Ca^{2+} uniporter (UP). Ca^{2+} entering the mitochondrial matrix can stimulate enzymes of the Krebs cycle and thereby oxidative phosphorylation and concomitantly $\text{O}_2^{\bullet-}$ production. The Ca^{2+} that entered through Cav1.3 channels is transported back across the plasma membrane at the expense of cellular energy through either the Ca^{2+} -ATPase (PMCA) or through a $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX) that relies upon the Na^+ gradient maintained by the Na^+/K^+ -ATPase.

accompanied by a dramatic depletion of reduced glutathione (presumably leading to a considerably weakened antioxidant defense system) [18]. Interestingly, the magnitude of GSH depletion appears to correlate well with disease progression, suggesting that GSH depletion may be taken as an (early) indicator of nigral degeneration. Changes in iron disposition also occur in the PD brain [19]. Notably, total iron is increased while ferritin is decreased in the diseased state, a condition that is expected to promote the availability of free iron capable of catalyzing the formation of ROS via the Fenton reaction. Correlating with the rise in iron content is an elevated expression of the

divalent metal transporter 1 (DMT1) in the SN dopaminergic neurons of PD patients compared to age-matched control subjects [20]. DMT1 is a major transport protein responsible for the uptake of iron and other divalent metal ions into cells, and its expression has also been reported to increase with age [21]. Finally, these studies also revealed a significant reduction in the activity of mitochondrial complex I as well as ubiquinone (coenzyme Q_{10}) in the SN of PD brains [22–24].

The relevance of the above findings derived from postmortem diseased tissues has been supported by several toxin-induced models of PD. Indeed, parkinsonian

neurotoxins such as MPTP, paraquat, and rotenone that selectively destroy nigral dopaminergic neurons in experimental models are often inhibitors of mitochondrial complex I function, the impairment of which would enhance superoxide production and thereby the formation of highly reactive free radicals that can initiate neuronal death [25]. Importantly, in these models, there is apparently a temporal and causal relationship between oxidative damage and degeneration in nigral dopaminergic neurons [26, 27]. Interestingly, MPTP-induced dopaminergic neurodegeneration and associated motor deficits in treated animals can be mitigated by iron chelation either genetically via transgenic expression of ferritin or pharmacologically via administration of clioquinol [28]. Consistent with this, newborn mice administered iron display progressive midbrain neurodegeneration that is paralleled by an increase in markers of oxidative damage as they age [10]. Together, these studies suggest that iron accumulation is a key accelerator of oxidative stress that is relevant to dopaminergic cell loss.

Recent results generated from genetic models also support a role for oxidative stress in PD pathogenesis [25]. For example, several studies have linked oxidative stress to the aggregation of α -synuclein, a presynaptic protein that is also a major component of LBs. Consistent with this, LBs are enriched with nitrated α -synuclein [29], and the protein is similarly modified in MPTP-treated mice [30]. Importantly, whereas α -synuclein overexpression in mice leads to nigral mitochondrial abnormalities [31], its genetic ablation reduces ROS production in these animals and renders them markedly resistant to degeneration when challenged by MPTP and other mitochondrial toxins [32, 33]. These results suggest that the PD-associated protein may induce oxidative stress by promoting mitochondrial dysfunction. Related to this, several studies have also demonstrated the ability of iron-mediated oxidative stress to promote α -synuclein aggregation [34–36]. Separately, we and others have shown that parkin, a ubiquitin ligase associated with recessive parkinsonism that normally functions as a potent neuroprotectant, is also susceptible to oxidative modifications [37, 38]. After its modification by a wide spectrum of oxidative stress-inducing agents such as MPTP, paraquat, and iron, the protein exhibits altered solubility properties and thereby impaired protective function [37, 38]. It is now clear that parkin function is also linked to mitochondrial homeostasis, as is the case with another recessive parkinsonism-associated protein, PINK1, suggesting that the loss of parkin or PINK1 function can precipitate oxidative stress [39]. This notion is certainly well supported by numerous studies performed in different experimental models, including mouse models [40, 41]. Interestingly, parkin also helps to regulate oxidation level in DA-containing cells by

limiting the expression of monoamine oxidases (MAO)-mitochondrial enzymes responsible for the oxidative deamination of DA (see next section), apparently by promoting the degradation of estrogen-related receptors (ERR), orphan nuclear receptors that play critical roles in the transcription regulation of many nuclear-encoded mitochondrial proteins [42, 43]. Perhaps the most direct genetic evidence supporting the role of oxidative stress in PD pathogenesis is the finding that mutations in the redox-sensitive protein DJ-1 cause an early-onset form of PD. DJ-1 is thought to operate as an antioxidant protein [44, 45], although a more recent study has classified DJ-1 as an atypical peroxiredoxin-like peroxidase that functions to scavenge mitochondrial H_2O_2 through oxidation of its cysteine at position 106 (a residue previously demonstrated by others via mutagenesis and structural analyses to be modified by oxidative stress) [45]. Notably, a pool of DJ-1 is known to be localized to the mitochondria [45, 46], and increased levels of H_2O_2 in mitochondria can be isolated from DJ-1 knockout mice [47]. Accordingly, the absence of DJ-1 may predispose dopaminergic neurons to oxidative stress-induced degeneration. Indeed, DJ-1-deficient animals are hypersensitive to pharmacological inducers of oxidative stress [48–53]. Taken together, the evidence from genetic studies supporting a relationship between oxidative stress and PD is rather compelling. However, a caveat is that none of these PD-associated genes exhibits a selective pattern of expression that could explain the vulnerability of SN dopaminergic neurons toward oxidation-mediated injury.

Why SN dopaminergic neurons are predisposed to degeneration in PD remains unclear, although the preferential accumulation of iron in this region of the brain may contribute to their vulnerability to endogenous and/or exogenous stress. Another obvious factor is DA itself. The sections below discuss the cytotoxic potential of DA reactions and address whether SN dopaminergic neurons are uniquely susceptible to degeneration by virtue of the neurotransmitter they carry.

10.3.1 DA Chemistry and Oxidation

There are two distinct pathways by which reactive metabolites can be generated from DA in the cell. The first pathway is driven by the MAO enzymes (located on the outer mitochondrial membrane) and aldehyde dehydrogenase where DA is converted to DOPAC (or dihydrophenylacetic acid) with the generation of H_2O_2 in the process. DOPAC can undergo further oxidation to form DOPAC quinones and ROS. Normally, DOPAC quinones will be conjugated to GSH. Those that fail to do so may undergo conversion to 5-S-cys-DOPAC, which can be further oxidized to other reactive species. The second

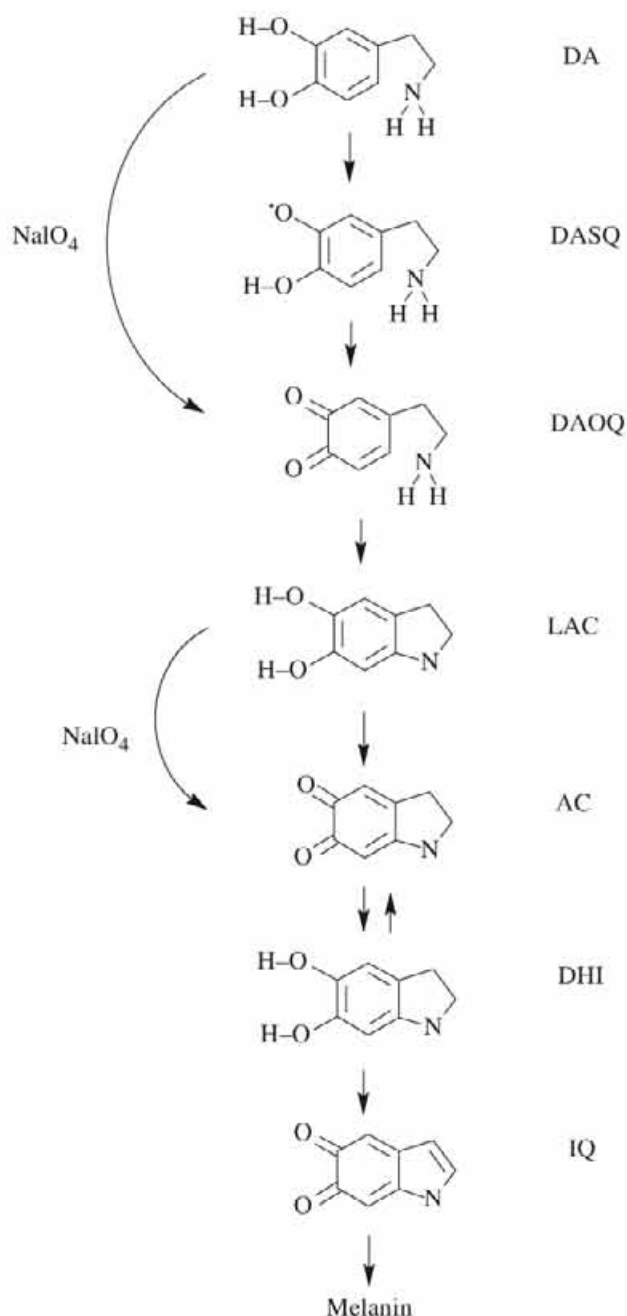


Fig. 10.2 Oxidative pathway for DA in the genesis of cytotoxic quinones. DA, dopamine; DASQ, dopamine semiquinone; DAOQ, dopamine orthoquinone; LAC, leukoaminochrome; AC, aminochrome; DHI, dihydroxyindole; IQ, indole quinone.

pathway, which is described in detail below, involves the oxidation of the catechol ring of DA to form ROS and the electron-deficient quinone (Fig. 10.2).

It has been long appreciated that the neuromelanin that accumulates in the lysosomes of catecholaminergic

neurons is the result of DA oxidation [54]. That this accumulation begins early in life and is progressive has been interpreted to mean that the process of oxidation of catecholamines to their quinone oxidation products is continuous throughout one's lifetime. Unlike the oxidation of L-3,4-dihydroxyphenylalanine (L-DOPA) by the tyrosinase of melanosomes in melanocytes, the oxidation of catecholamines in neurons is not tyrosinase dependent and is termed "autooxidation," a process initiated by ROS as well as by molecular oxygen with transition metal catalysis. (A recent report demonstrating the presence of tyrosinase in the brain [55] and thereby supporting a role for tyrosinase in the oxidation of catecholamines should be interpreted with caution, as the observation may be a result of contamination by leptomeningeal melanocytes.) The autooxidation of catecholamines is in addition to their oxidation by MAO. The process of autooxidation increases with increased pH and is countered by reducing agents, such as ascorbic acid. Thus the synaptic vesicle is an acidic and reducing environment where catecholamines are stable, whereas the higher pH of the cytosol enhances autooxidation [56].

In 1978 Graham described for the first time in complete form the autooxidative pathways for the catecholamines to the quinone species that polymerize to form neuromelanin [5]. As illustrated in Figure 10.2, the one-electron oxidation by O₂ can convert DA to its semiquinone radical (DASQ), which can be further oxidized to DA orthoquinone (DAOQ), reactions that generate ROS in addition. The electron-withdrawing carbonyls of DAOQ render the quinone ring electron-deficient and thus a potent electrophile that can react with external nucleophiles, such as the sulfhydryl functions of proteins and reduced glutathione. The amino function of DAOQ can then attack the quinone ring at the 6-position, an irreversible reaction that reduces the carbonyls to hydroxyl functions and yields leukoaminochrome (LAC). Once formed, LAC is then also vulnerable to autooxidation to yield the cyclized quinone aminochrome (AC). AC can then undergo tautomerization (an intramolecular rearrangement that is neither oxidation nor reduction) to 5,6-dihydroxyindole (DHI), and DHI in turn can undergo autooxidation to indole quinone (IQ) (Fig. 10.2). In parallel to the formation of eumelanin in melanosomes, it is likely that multiple species in this reaction sequence participate in the polymerization reactions that result in neuromelanin.

As one would expect, other catecholamines are also vulnerable to autooxidation to their corresponding quinone species. However, DA is much more vulnerable to autooxidation than either norepinephrine (NE) or epinephrine (E) [5]. Furthermore, the orthoquinone

products of NE and E cyclize, respectively, to leuko-noradrenochrome and leukoadrenochrome much faster than the cyclization of DAOQ to LAC. The net result of these two observations is that while DA, NE, and E can all contribute to the neuromelanin polymer as the result of autooxidation, DA stands out with its greater potential for cytotoxicity, through both generation of ROS and its oxidation to DAOQ, with its greater availability for reaction with external nucleophiles. It should be appreciated that DAOQ, and especially the orthoquinones derived from NE and E, are transient species that cannot be observed during autooxidation, as the oxidations to the orthoquinones occur more slowly than the subsequent cyclization and oxidation steps. The only methods by which the orthoquinones can be observed are electrochemical oxidation [57] and through oxidation with periodate, as first described by Graham and Jeffs [58]. Indeed, the latter paper and Graham (1978) [5] established that catechols, like 1,2-glycols, form cyclic iodate intermediates that are then hydrolyzed to the orthoquinone. The use of periodate allows specific, rapid oxidation of catecholamines that is not confounded by additional products seen with other chemical oxidants [5, 59]. It is noteworthy to mention that orthoquinones have $\lambda_{\max}$ at 390 nm [5], not at 450 nm as recently reported [60], as this is the $\lambda_{\max}$ of AC at high pH through the dissociation of a proton (the $\lambda_{\max}$ of AC at neutral pH is 480 nm).

10.3.2 DA Oxidation and Neurotoxicity

Under normal conditions, DA is sequestered safely within synaptic vesicle. However, vesicular DA storage can be disrupted by various cellular stressors, resulting in increased cytosolic DA that can become toxic. The cytotoxicity of DA thought to play a role in neurodegeneration in PD is a product of ROS and reactive quinones generated during oxidation. Being electron deficient, DA quinones readily seek out cellular nucleophiles such as the cysteine residues on proteins. Quinone reactivity can thus deplete cellular stores of reduced glutathione. Indeed, elevated levels of 5-S-cys-DA and 5-S-cys-DOPAC are observed in the SN of PD brains [61]. Quinone reactivity can also result in the adduction and cross-linking of proteins, thereby altering their normal functions. For example, the modification of the PD-linked ubiquitin ligase parkin by the quinones derived from DA oxidation (DAQ) inactivates its enzymatic activity and promotes its aggregation [62]. Consistent with this, an increase in catecholamine-modified parkin was observed in the SNpc of postmortem PD brains [62]. Furthermore, parkin appears to be uniquely susceptible to DA-induced modifications compared to several related E3 members such as HHARI, Cbl, and

CHIP [38, 62, 63]. Given that functional parkin plays an important neuroprotective role in the brain, its modification by DAQ provides a mechanism for its dysfunction that is relevant to the pathogenesis of sporadic PD. Notably, a recent proteomic study performed by the Hastings laboratory demonstrated that DJ-1 can also be modified by DAQ [64], although it is not known whether DJ-1 function is compromised as a result. In some cases, DA conjugates can be directly toxic. For example, modification of α -synuclein by DAQ stabilizes the PD-associated protein in its protofibrillar form that can permeabilize synaptic DA-containing vesicles [65]. The resulting vesicular DA leakage, in turn, would augment the pathogenic process. Moreover, DAQ-synuclein adducts can seed the formation of oligomeric complexes that not only inhibit the normal degradation of the protein by chaperone-mediated autophagy (CMA) but also block the degradation of other CMA substrates in the process [66]. The net result is the accumulation of α -synuclein (and other proteins) and an impaired CMA function. Interestingly, α -synuclein can also interact with DA transporters and facilitate their clustering at the cell surface, the consequence of which is an acceleration of DA uptake leading to increased ROS and sensitivity toward DA-induced apoptosis [67]. Finally, several groups have shown that DA oxidation products can promote mitochondrial dysfunction through the impairment of the activity of ETC, as well as inducing mitochondrial permeability transition pore (mPTP) opening leading to organelle swelling [68–71]. Irreversible opening of the mPTP is known to promote the release of proapoptotic factors into the cytosol.

Supporting a toxic role for DA, mice administered with exogenous DA via intrastriatal injection exhibit degeneration of SN dopaminergic neurons that begins in the terminal fields and progresses to eventual loss of cell bodies, the extent of which appears to correlate with the levels of quinone-modified proteins and can be reduced by antioxidant treatment [72, 73]. Furthermore, mice engineered to express a mere 5% of normal VMAT2, a transporter that normally sequesters cytosolic DA into vesicles, display progressive nigrostriatal dopamine dysfunction that ultimately results in neurodegeneration [74]. Alongside this, elevated cysteinyl adducts to L-DOPA and DOPAC are seen early and are followed by increased striatal protein carbonyl, 3-nitrotyrosine formation, and the accumulation of α -synuclein [74]. More recently, an elegant study in mice performed by Chen and colleagues demonstrated that striatal neurons engineered to take up extracellular DA exhibit degeneration within weeks, a phenotype that is accompanied by substantial oxidative protein modifications and decrease in glutathione level [56]. That forced uptake of DA by a neighboring cell (that

is normally GABAergic in nature) can result in its loss is a testimony to the cytotoxic role of DA.

10.3.3 Is DA Really the Culprit?

In view of the toxic potential of oxidative reactions involving DA, and the experimental evidence supporting the cytotoxic effects of DA oxidation products *in vivo*, it is curious that a considerable regional variability exists in the vulnerability of DA neurons toward degeneration in PD. Notably, the mesencephalon contains two major dopaminergic neuronal populations, namely, the A9 neurons of the SNpc that project to the striatum along the nigrostriatal pathway and the A10 neurons of the ventral tegmental area (VTA) that project to the limbic and cortical areas along the mesolimbic and mesocortical pathways [12]. Despite their anatomical proximity and biochemical and electrophysiological similarities, the A9 neurons are the ones that are selectively lost in PD. Furthermore, this subset of dopaminergic neurons are also lost at a significantly faster rate compared to other neuronal types under conditions of normal aging in the apparent absence of environmental toxin exposure [75]. Although the exact reason for this selectivity remains unclear, Chung and colleagues have recently demonstrated that the expression of antioxidant genes is intrinsically higher in A9 neurons than in A10 neurons [76], suggesting that A9 neurons may be constantly experiencing a sustained heightened level of oxidative stress. Given this, one would assume that the ability of A9 neurons to cope with sudden or chronic increase in ROS production may be more limited than that of their A10 counterparts. Supporting this, treatment of the two neuronal populations with ROS inducers such as MPTP, 6-OHDA, and paraquat (all of which are parkinsonian neurotoxins) results in the death of A9 but not A10 neurons [26, 77, 78]. Conversely, transgenic mice with increased activity of SOD1 or glutathione peroxidase, key ROS scavenging enzymes, are resistant to MPTP-induced dopaminergic neurodegeneration [57, 79].

Why the A9 neurons are in an apparent state of heightened oxidative stress is, however unclear, although a recent study by Mosharov and colleagues demonstrated that cytosolic DA concentrations in these neurons are significantly (2- to 3-fold) higher than in the more resistant VTA neurons [80]. Not surprisingly, cytosolic DA-enriched A9 neurons are found to be more sensitive than A10 neurons to the toxic effects of an acute L-DOPA challenge [80]. The difference in DA levels between these two groups of neurons may be attributed to differences in synthesis (as there is no significant difference with regard to precursor uptake, storage, or degradation of DA), which appears to be

related to the use of distinct calcium (Ca^{2+}) channels by these neurons. Unlike the VTA neurons, A9 dopaminergic neurons use L-type Ca^{2+} channels to help maintain autonomous pacemaking [81]. By virtue of the activity and thereby opening of this type of channel in the absence of synaptic inputs, the A9 neurons experience a significantly larger magnitude and spatial extent of Ca^{2+} influx [82]. Importantly, antagonizing L-type Ca^{2+} channel diminishes the differences in cytosolic DA levels between A9 and A10 neurons and concomitantly reduces the toxic effects of acute L-DOPA treatment in the former group of neurons [80], suggesting a complex interplay between calcium and DA in the degeneration of A9 neurons in PD.

The reliance of SN dopaminergic neurons on voltage-dependent L-type Ca^{2+} channels obviously comes with a price, as intracellular Ca^{2+} concentration is under very tight homeostatic control by the actions of ATP-dependent pumps whose operations are metabolically expensive. A sustained entry of Ca^{2+} in these neurons would presumably work the mitochondria machinery harder and concomitantly raise the level of ROS that would predispose them to oxidative stress-induced degeneration. Supporting this, a recent elegant study conducted by Guzman et al. [83] in transgenic mice showed that the basal oxidation of a mitochondrially localized redox-sensitive form of GFP (mito-roGFP) is indeed significantly higher in SN dopaminergic neurons relative to their VTA counterparts. Furthermore, the level of mito-roGFP oxidation in SN (but not VTA) dopaminergic neurons can simply be lowered by the administration of L-type Ca^{2+} channel antagonists in these transgenic mice. Importantly, the ablation of DJ-1 expression results in the amplification of basal oxidant stress in SN dopaminergic neurons. Collectively, the findings by Guzman et al. emphasize the intimate relationship between L-type Ca^{2+} channels and mitochondrial oxidant stress. This "L-type Ca^{2+} hypothesis" [82] is certainly an attractive proposition, as neurons in the locus ceruleus (LC) that are also lost in the PD brain are similarly autonomous pacemakers dependent on the activity of L-type Ca^{2+} channels [84]. Furthermore, the majority of A10 neurons contain Ca^{2+} -binding proteins such as calbindin D-28K that are capable of buffering intracellular Ca^{2+} to prevent it from rising to damaging levels within the cell [85]. Comparatively, the A9 neurons express calbindin D-28K at significantly lower levels [76]. However, it is noteworthy that PD pathology according to the neuropathological staging proposed by Braak is not confined to the SN or LC but progressively extends from the caudal to the rostral brain regions, involving not just the dopaminergic system but also noradrenergic, cholinergic, and serotonergic systems that may or may not rely on L-type Ca^{2+} channels [86]. Thus, as attractive as the "L-type Ca^{2+} hypothesis" may seem, it does not

adequately explain all the predilection sites in the PD brain, although it remains an extremely attractive factor underlying the vulnerability of SN dopaminergic neurons to degeneration.

10.3.4 Extracellular Oxidative Stress

It is important to recognize that in addition to intrinsic factors, extracellular oxidative stress mediated by activated glial cells may also accelerate the degeneration of SN dopaminergic neurons in disease conditions. In their normal state, glial cells participate in the maintenance of neuronal homeostasis in several ways, including providing trophic support to neurons and helping to clear neurotransmitter released into synapses [87, 88]. Upon their activation during neuroinflammation, reactive astrocytes and microglia release various inflammatory cytokines and complement factors to repair or dispose of damage cells and thereby reestablish the regional micro-environment. Associated with this is the production of ROS and RNS [89]. Although beneficial, excess or chronic neuroinflammation can obviously exert unintended deleterious effects on neighboring dopaminergic neurons and contribute to disease progression [90]. The activation of glial cells is well documented in affected regions of the PD brain, as well as in genetic and toxin-induced models of PD [91]. Glial cell activation is usually accompanied by the upregulation of iNOS, which is either absent or expressed at very low level in the normal brain. Notably, iNOS null mice are less susceptible to MPTP-induced neurotoxicity compared to their wild-type counterparts and, accordingly, display reduced staining for markers of oxidative stress [80]. Similarly, inactivation of the inflammatory enzyme NADPH-oxidase, which catalyzes the production of superoxide from oxygen and NADPH, results in the attenuation of MPTP-induced neurotoxicity in mice [92]. The expression of both iNOS and NADPH-oxidase are elevated in the PD brain [92, 93]. Activated microglia is also known to upregulate the expression of cyclooxygenase-2 (Cox-2) and thereby the synthesis of prostaglandins that could, in turn, kill neurons directly through the activation of caspase-3 or indirectly via the excessive release of glutamate by astrocytes [94]. Interestingly, SN dopaminergic neurons express Cox-2, suggesting that dysregulated Cox-2 levels may lead to cell-autonomous suicide [95]. Inhibition of Cox-2 apparently mitigates the formation of the deleterious DA-quinone and reduced toxicity in a MPTP mouse model [95]. Notwithstanding the obvious damage to neighboring cells that chronic neuroinflammation could bring, the glial response is often taken to be a secondary cause of the neurodegenerative process. Furthermore, neuroinflammation is not limited to PD but a consistent feature

of neurodegenerative diseases in general. Nonetheless, the role of non-cell-autonomous neuronal death is regaining its prominence in the field as more and more researchers are revisiting the cross talk between neurons and glial cells in neurodegeneration.

10.4 THERAPEUTIC IMPLICATIONS

Given the apparently compelling role of oxidative stress in PD pathogenesis, strategies aimed at reducing oxidative stress should in theory mitigate disease progression and provide clinical benefits. Indeed, the success of such strategies will provide the ultimate proof of concept that oxidative stress is a key factor underlying neuronal death in PD. The first controlled clinical trial to evaluate the potential of antioxidants as neuroprotective agents was the Deprenyl and Tocopherol Antioxidative Therapy of Parkinsonism (DATATOP) study [96]. Deprenyl (selegiline) is an irreversible MAO-B inhibitor that is expected to reduce the formation of DOPAC and hydrogen peroxide from oxidative deamination of DA. In so doing, deprenyl would decrease the formation of oxyradicals from hydrogen peroxide generated by DA metabolism. α -tocopherol is vitamin E, an established antioxidant. However, this study together with several other subsequent studies utilizing similar antioxidant strategies failed to produce convincing protection against PD. If oxidative stress is indeed the primary cause of PD pathogenesis, why have these trials apparently failed? There could be several explanations for the generally disappointing outcomes of antioxidant clinical trials in PD, including the study design involved, the availability of administered compounds in the brain, and the heterogeneity of PD patients. Furthermore, as much as we like to believe that oxidative stress is a central player in PD pathogenesis, it is obviously not the only player but one important factor among several others all interwoven in a tapestry of events underlying the degeneration process. To expect a singular therapy to work effectively for a complex disease like PD may just be too tall an order. Perhaps a combination of therapies targeted at major problem centers in the pathogenic cascade may be the way to go, although getting administrative approval for such cocktail drug trials would invariably pose a challenge. A "druggable" target related to the oxidative stress cascade is obviously the L-type Ca^{2+} channel, which can be antagonized by dihydropyridines (DHPs). DHPs have been approved for human use for the treatment of hypertension. Interestingly, a large Danish study conducted recently demonstrated that reducing Ca^{2+} influx in the brain via the administration of DHPs decreases the risk for PD [97]. Given the interplay between DA and calcium in

dopaminergic neurodegeneration, and the availability of FDA-approved Ca^{2+} channel antagonists, it may be worthwhile to explore the clinical benefits of combining antioxidants with Ca^{2+} channel blockers for the PD patient.

10.5 CONCLUSION

It is apparent from the discussion above that oxidative stress has a definite role in PD pathogenesis. Indeed, virtually all the epidemiological PD risk factors identified to date are directly or indirectly linked to oxidative stress. Similarly, the majority of the genetic causative factors of the disease are associated in one way or another with oxidative stress. Even normal aging, recognized as an unequivocal risk factor for PD, is accompanied by increased indices of oxidative stress. Coupling these phenomena to the knowledge that intrinsic pro-oxidant factors like iron, DA, and L-type Ca^{2+} channel all can confer neuronal vulnerability to degeneration, it is hard not to be persuaded by the oxidative stress hypothesis in PD pathogenesis. However, a nagging question that constantly surfaces is whether oxidative stress represents a cause or a consequence of PD. The "chicken and egg" argument here is that oxidative stress can be as much a promoter as it is a result of neuronal death. This is a longstanding debate that is difficult to resolve.

As discussed above, several studies have highlighted an unequal distribution of pro- and antioxidant factors in different regions of the brain [98]. Notably, the basal oxidant level in vulnerable neurons is typically higher than in neighboring neurons that are unaffected, as in the case between A9 and A10 dopaminergic neurons, even in seemingly healthy brain. The most logical assumption to make here is that neurons with high intrinsic oxidant levels are predisposed to degeneration but not necessarily already exhibiting signs of degeneration or undergoing degeneration. The corollary is that oxidative stress is unlikely to be a mere passive consequence of neuronal death, notwithstanding that dying neurons can further elevate pro-oxidative events. A related question that many often ask is whether oxidative stress represents a primary or secondary event in PD. Perhaps a more pertinent question to ask here is whether we should be taking a reductionist approach in pinpointing causative factors of PD. Many would agree that the PD pathogenesis involves an intricate network of interacting pathways rather than a linear sequence of events. For example, oxidative stress can cause mitochondrial dysfunction and vice versa. Likewise, oxidative stress can damage the proteasome, and, conversely, proteasome dysfunction can also lead to oxidative stress [99]. In this circle of events, oxidative

stress can therefore be a primary cause as much as it can represent a secondary cause of the disease. The important thing to recognize is that while oxidative stress is a persuasive key player in PD pathogenesis, it is likely to be one among several other key players.

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OXIDATIVE STRESS IN CARDIOVASCULAR DISEASES

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11.1 INTRODUCTION

Cardiovascular diseases comprise several complex conditions, such as inflammatory, cerebrovascular, ischemic, hypertensive, and rheumatic diseases. Collectively, cardiovascular diseases contribute to 30% of all causes of mortality around the world, whereas ischemic heart diseases alone represent ~13% (Fig. 11.1). Besides the invaluable loss of lives, cardiovascular diseases also contribute to economic burden [1].

As for all complex diseases, there is not a single cause for cardiovascular diseases. However, several factors have been described as risk factors for the development of cardiovascular diseases [2]. Among these are high-fat diet, smoking, alcohol consumption, age, sex, and race. Interestingly, despite the broad range of manifestation, atherosclerotic plaque formation could be considered the common starting point for all cardiovascular diseases. It has a chronic inflammatory component, but its effects may manifest acutely, as stroke or acute myocardial infarction.

Reactive oxygen species (ROS), reactive nitrogen species (RNS), and oxidative stress are the basis of many hypotheses about the development of cardiovascular diseases. Both ROS and RNS have important physiological functions as chemical weapons in immune cells, vascular tonus regulators, and signaling molecules [3]. Conversely, ROS and RNS can have roles as bad agents during the onset and progression of cardiovascular diseases. Why does this happen? Mainly because risk factors are supposed to cause imbalance, either favoring ROS/RNS

production or impairing antioxidant mechanisms. Thus it is easy to understand why smoking, which can decrease antioxidant defenses and generate reactive aldehydes, represents a risk factor for cardiovascular diseases [4].

The discovery of the imbalance between ROS/RNS generation and its consumption in cardiovascular diseases led to the false belief that the Holy Grail was found and that antioxidant therapy could be life saving. The truth is that researchers have not found a common ground and the evidence is still controversial, as we shall see further in this chapter.

11.2 FREE RADICALS—ORIGINS AND FATES

Free radicals are molecules or atoms that are able to have a free existence despite having one unpaired electron, which confers a high reactivity. Among the most-studied free radicals are superoxide ($O_2^{\bullet-}$), hydroxyl ($HO^{\bullet}$), peroxy ($RO_2^{\bullet}$), and nitric oxide ($NO^{\bullet}$). Reactive oxygen species (ROS) are a wide class of reactive molecules that includes free radical and other nonradical molecules that are also highly reactive. Also considered ROS are molecules such as hydrogen peroxide (H_2O_2), hypochlorous acid ($HOCl$), ozone (O_3), and peroxynitrite ($ONOO^-$) [3].

Superoxide is the most abundant free radical in cells. Despite this, it is not highly reactive. Even so, it can act as a signaling molecule or as a substrate for H_2O_2 and peroxynitrite generation. Perhaps the main source of superoxide is the electron transport chain in mitochondria, since it has been estimated that 1–3% of total oxygen is leaked as

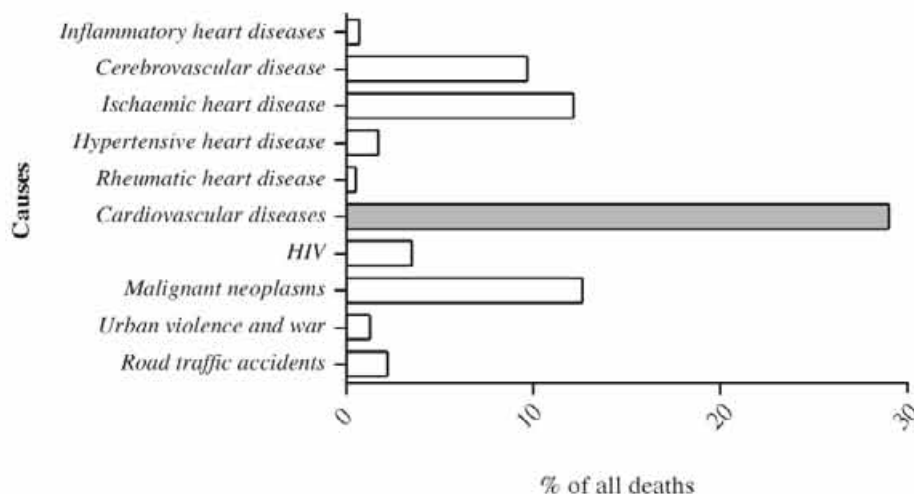


Fig. 11.1 Estimated deaths by cause worldwide. Data from World Health Organization for 2004.

superoxide in physiological processes. NAD(P)H oxidase oxidizes the reducing agent NAD(P)H and transfers the electron to the molecular oxygen, yielding superoxide. It is mainly employed as a chemical weapon by neutrophils and macrophages, but it is highly important in endothelial and myocardial dysfunction [5]. Xanthine oxidoreductase is also an important source of superoxide that oxidizes xanthine or hypoxanthine in uric acid and transfers the electron to NAD^+ rather than to O_2 . In some cases, such as after myocardial infarction, this enzyme suffers spatial modifications that make it an oxidase that transfers the electron to oxygen rather than to NAD^+ (see Fig. 11.2).

Superoxide dismutase is the enzymatic defense against superoxide produced in cells and exists in three isoforms: a manganese-dependent mitochondrial isoform (Mn-SOD), a copper- and zinc-dependent cytosolic isoform (Cu,Zn-SOD), and an extracellular tetrameric

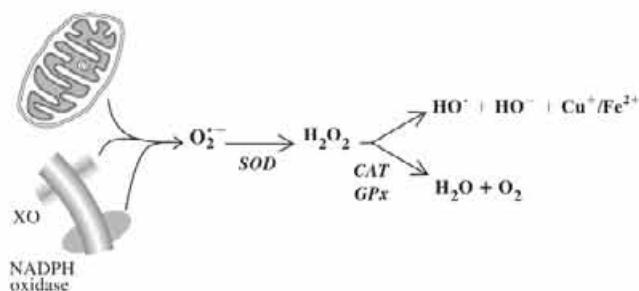


Fig. 11.2 The three main sources of superoxide radical in cells are mitochondria, xanthine oxidase (XO), and NAD(P)H oxidase. After superoxide production, a chain of coupled enzymatic reactions leads to the consumption of reactive oxygen species to avoid oxidative stress. In the case of hydrogen peroxide accumulation, the reaction with transitional metals generates the most harmful radical, hydroxyl.

Cu,Zn-dependent isoform (EC-SOD). These enzymes act on superoxide, and the product is hydrogen peroxide, according to reaction (1) in Figure 11.3.

Although hydrogen peroxide is not a free radical, it is important as a signaling molecule, since it is highly diffusible. Moreover, the reaction of hydrogen peroxide with transitional metals (e.g. Cu^+ , Fe^{2+}) yields the most reactive and harmful radical in biological systems, hydroxyl. To avoid hydroxyl generation, the antioxidant enzymes such as catalase and glutathione peroxidase (GPx) are able to use hydrogen peroxide to generate water and molecular oxygen, according to reactions (2) and (3) in Figure 11.3, respectively. This chain of events is depicted in Figure 11.2.

NO is a diffusible gas that has important physiological roles. The source of NO can be any of the three isoforms of nitric oxide synthase NOS: neuronal (nNOS), endothelial (eNOS), or inducible (iNOS). NOS converts the amino acid L-arginine into NO and another amino acid, L-citrulline. Certain cofactors are needed, such as FAD, FMN, and tetrahydrobiopterin (BH_4). NOS can also produce superoxide in the presence

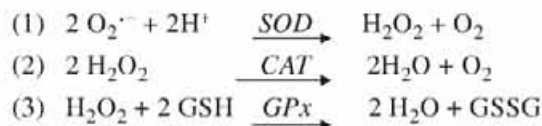


Fig. 11.3 The main enzymatic antioxidant system is shown. Equation (1) represents the action of superoxide dismutase (SOD) on superoxide radical. Equation (2) represents the action of enzyme catalase (CAT) on SOD product hydrogen peroxide. Equation (3) represents the action of glutathione peroxidase (GPx) on the same hydrogen peroxide. The difference is the necessity of reduced glutathione (GSH) as cofactor.

of low levels of BH_4 , which may contribute to atherosclerosis generation, as we shall see. NO exerts its signaling effects binding to iron-heme groups in guanylate cyclase. The signaling effects are best comprehended as antihypertensive, since NO can stimulate cGMP synthesis in smooth muscle of blood vessels and thus induce relaxation and lower blood pressure. Besides its physiological roles, NO can react with superoxide to give peroxynitrite. This nonradical ROS is able to oxidize reduced cysteine, lipids, and DNA. The most-studied damage in proteins is the modification of tyrosine to 3-nitrotyrosine.

To summarize, in case of any disturbance between free radical production and its consumption by the antioxidant system, harmful species can arise. These species are prone to react with any molecule, such as DNA, protein, and lipids, which may lead to errors in transcriptional activity, signaling, and membrane permeability. When this damage overcomes the capability of the organism for repairing it, oxidative stress takes place.

11.3 CARDIOVASCULAR DISEASES

Although cardiovascular diseases might vary in their symptoms, endothelial dysfunction, hypertension, and atherosclerosis appear to be at the core of all cardiovascular complications. The formation of atheromatous plaques can increase blood pressure and vice versa. Narrowing of blood vessels may lead to ischemic events that increase free radical production that damages cells, contributing to endothelial and cardiac dysfunction. From development to progression, redox imbalance and subsequent oxidative stress play a crucial role in all these diseases.

11.3.1 Atherosclerosis

Atherosclerosis is characterized by thickening of the arterial wall due to macrophage infiltration, smooth muscle migration, and cholesterol deposition. Symptoms can occur late in the course of atherosclerotic plaque formation and are mainly due to the narrowing of vascular lumen or to the rupture of plaque, which leads to the obstruction of a small capillary. Regardless of the type of manifestation, the final event is the decrease of blood supply to the organ. Initiation and progression of atherosclerosis involve complex mechanisms, and many aspects are still not understood.

Again, the participation of free radical in atherosclerotic plaque formation seems to be a unifying theory, since all known risk factors contribute to ROS/RNS generation. For example, F_2 -isoprostanes, a well-accepted marker of

lipid oxidation, nitrated proteins, and lipid hydroperoxides, are all associated with increased risk for developing atherothrombotic diseases. Moreover, several stimuli that in some way participate in atherosclerotic plaque formation also trigger NAD(P)H oxidase-mediated superoxide production [6]. For instance, fluid shear stress, the frictional force generated by blood flow over the vascular endothelium, is a major factor in atherogenesis. Branched and curved arteries are considered prone to lesion, whereas straight arteries are less prone. Interestingly, cultured endothelial cells subjected to shear stress *in vitro* produce twice as much superoxide and monocyte binding than cells subjected to laminar stress. Also, this effect does not occur in cells deficient in p47 protein, a component of NAD(P)H oxidase complex [7].

NO can be synthesized constitutively by endothelial isoform of NOS (eNOS) or, after an induction, by the inducible isoform (iNOS). The former participates in vascular tonus regulation, antagonism of platelet aggregation, and leukocyte binding, whereas the latter is involved in the proinflammatory process [8]. The inducible isoform is detected in human atheroma as soon as monocyte infiltration and foam cell differentiation starts [9]. Also, it was recently shown that iNOS colocalizes with other proatherosclerotic factors (ACE and AT_1) whereas eNOS vanishes in endothelial cells from autopsy [10].

Despite being considered essential as antiatherosclerotic, eNOS deletion in mice is not enough to induce plaque formation because nNOS compensatory activity can keep NO production in a normal range [11]. It is noteworthy that eNOS can undergo a process called uncoupling in which the reduction of molecular oxygen is no longer coupled with L-arginine oxidation. Thus eNOS generates superoxide rather than NO [12]. Among several factors that could lead to eNOS uncoupling, BH_4 deficiency and presence of asymmetrical dimethyl arginine (ADMA) appear to be the main factors. BH_4 acts by improving L-arginine binding and donating the second electron to oxygen reduction. Thus, in the absence of BH_4 , eNOS still partially reduces oxygen without oxidizing L-arginine, generating superoxide [12]. ADMA may act as an eNOS inhibitor by competitive inhibition of L-arginine oxidation or may uncouple eNOS activity. In fact, it has been found that coronary artery disease patients carrying the low-activity form of the rate-limiting enzyme in BH_4 synthesis (GTP cyclohydrolase I) produce more superoxide and oxidized low-density lipoprotein (oxLDL) and demonstrate diminished response to acetylcholine induced vasorelaxation [13]. Plasma levels of ADMA were inversely related to acetylcholine-induced vasorelaxation in saphenous veins collected from patients undergoing coronary bypass surgery, whereas it was directly related to superoxide production by both saphenous vein and internal mammary artery [14].

Oxidative stress can have two main consequences: First, after oxidizing catalytically active or structural biomolecules, it can lead to a loss or gain of function and thus precipitate some dysfunction. Second, oxidatively modified molecules become a new class of molecules, and some of them can bind to membrane receptors and trigger signals. A good example is the increased level of oxidation of fibrinogen seen in patients with coronary artery disease. Formation of NO-derived species can lead to 3-nitrotyrosine formation in fibrinogen. The consequence is an accelerated and aberrant fibrin polymerization that presents resistance to lysis [15–17].

In addition to oxidative modification, a process called glycation also occurs in diabetes and is believed to contribute to the generation of atherosclerotic plaque. Briefly, glycation consists in a series of processes that involve a nonenzymatic reaction between a reducing sugar (e.g., glucose) and amino groups of proteins to form a reversible Schiff base. This reaction is prone to some arrangements, and free radicals are part of this complex process. After that, a new and heterogeneous class of molecules called collectively advanced glycation end-products (AGEs) arises [18]. Among the epitopes generated are *N*^ε-(carboxymethyl)lysine (CML), pentosidine, and GA-pyridine. CML has been extensively studied and has been associated with a variety of inflammatory processes. Immunohistochemical studies have already found CML and GA-pyridine in human atherosclerotic lesions both intracellularly in foam cells and in the extracellular environment [19, 20].

Whether AGE formation is a consequence of an inflammatory process or it has any active role in atherosclerotic plaque generation seems not to be under debate nowadays. AGEs constitute a class of signaling molecules that exert their effects mainly through receptor for AGE (RAGE). RAGE is a transmembrane receptor belonging to the immunoglobulin superfamily that acts as a multiligand receptor [21]. Among the RAGE ligands are AGEs, S100/calgranulin proteins, high mobility box group 1 protein (HMGB1), and oxLDL [22]. One of the best characterized consequences of RAGE activation is the stimulation of free radical-generating pathways, which in turn will lead to the generation of additional AGEs and oxLDL. Moreover, RAGE stimulation triggers intracellular signaling that culminates with NF- κ B translocation to the nucleus, which is largely described as a transcriptional factor associated with proinflammatory response [23]. In line with this, diabetic mice lacking RAGE showed less incidence of atherosclerotic plaque formation in aorta than mice expressing RAGE [24]. The central role of RAGE in atherosclerosis development suggests that this receptor should be seen as a therapeutic target in that condition. In fact, this suggestion has already been tested in animal models with good results

[25], but unfortunately there are no current clinical trials testing this approach.

Environmental factors are well-established risk factors for atherosclerosis development, and many of them are linked to oxidative stress generation inside the body or are themselves reactive species. Cigarette smoking is one of the most important and modifiable risk factors associated with the generation and progression of cardiovascular diseases [26]. Cigarette smoke contains high concentrations of free radicals, such as superoxide, NO, carbon-centered radicals, and other toxic compounds, such as acrolein and formaldehyde. Even passive smokers have increased risks for cardiovascular diseases that are mediated by increased oxidative stress [27]. Guinea pigs subjected to cigarette smoking for 28 days develop atherosclerosis, which could be avoided with dietary vitamin C supplementation [28]. This rationale indicates that free radicals are important in the onset and progression of atherosclerotic plaque.

However, antioxidant supplementation in humans (smokers and nonsmokers) has yielded contradictory results. Vitamin E supplementation (800 IU) seems to be protective in a cohort of atherosclerotic patients, decreasing the risk of nonfatal myocardial infarction by half [29]. Despite this, a study with a large cohort followed up for 6 years demonstrated that vitamin E alone has no effect on atherosclerosis progression [30]. Even so, when associated with vitamin C, the antioxidant therapy was able to diminish the intima-media thickening in the common carotid artery of smoker subjects [30].

Diet is another source of toxic compounds that could contribute to atherosclerosis development. AGEs can be generated during the cooking process, and after ingestion they can reach the circulatory system and participate in some mechanisms. Diabetic patients with a poor AGE diet presented decreased levels of circulatory AGE, VCAM, and C-reactive protein compared with a regular diet [31]. The relation between dietary AGEs and atherosclerosis is better understood when looking at preclinical models. For instance, mice subjected to femoral artery injury and fed with low-AGE diet presented less neointima area and larger luminal area than the high-AGE diet group. These data reinforce the role of AGEs in neointima proliferation after vascular lesion [32].

11.3.2 Heart Infarction and Heart Failure

The heart is a highly oxidative organ, and fatty acid oxidation is responsible for 60–80% of its energy requirement [33]. Because of this oxidative metabolism, 25–35% of total cardiomyocyte content is occupied by mitochondria [34]. Even under physiological states (normoxia), 1–3% of oxygen is leaked as free radicals by

mitochondria, and this value can be increased several-fold during or after infarction.

Acute myocardial infarction is defined as an interruption in the blood flow in myocardium. The most common cause is the occlusion of coronary artery following the rupture of an atherosclerotic plaque, and the obvious consequence is the death of starved cells. During the infarction, oxidative stress is caused both by inflammatory cells that infiltrate and by cardiomyocytes after the onset of necrosis. There is no doubt that the ischemic process must be stopped and that the reestablishment of blood flow is necessary to avoid acute heart failure. This can be done through pharmacological thrombolytic therapy or surgically, and these interventions help to decrease the mortality rates after acute myocardial infarction [35]. It turns out that this procedure is considered a “double-edged sword” since it is as harmful as it is necessary and results in “ischemia-reperfusion injury” (IRI). The clinical manifestation of IRI is seen as myocardial stunning, arrhythmia, myocyte death, and endothelial/microvascular dysfunction [36]. Several intricate steps occur after IRI, and free radicals act centrally in this process. Chronic treatment with β -blockers, aspirin, angiotensin-converting enzyme (ACE) inhibitors, and statins have improved the survival and the quality of life in infarcted patients. However, myocardial infarction remains a prevalent event with devastating consequences since it cause loss of cardiomyocytes, which are terminally differentiated cells. With few myocytes remaining it is hard to sustain adequate contractile function and heart failure develops.

11.3.3 The Ischemic Phase

During the ischemic process low levels of oxygen and nutrients arrive in the heart. The only way to keep the levels of ATP for contractile function is by increasing the glycolysis rate, which will lead to an overload of lactate and acidosis. As the glycogen vanishes, there will be a fall in ATP levels and an increase in its degradation products (hypoxanthine). These changes in energetic balance are the main cause of mitochondrial permeability transition (MPT) pore opening [37]. The MPT pore is a voltage- and Ca^{2+} -dependent high-conductance channel, whose opening leads to the increased permeability of inner mitochondrial membrane with consequent dissipation of the electrochemical proton gradient that drives mitochondrial functions. Also, MPT pore opening promotes the release of free radicals and cytochrome *c*, a proapoptotic factor that, once in cytosol, triggers caspase-3 and the apoptotic process [38]. The increase in permeability caused by MPT pore opening causes an influx of water into the mitochondrion, which makes it swell. The final step is the rupture of this organelle.

When the insult is severe, there will be neither time nor ATP for the cell to enter the apoptotic process, and necrosis will occur. In fact, it was recently confirmed by atomic force microscopy that mitochondrial swelling happens in infarcted rat hearts [37].

11.3.4 The Reperfusion Phase

Prolonged periods of ischemia are related to necrosis, whereas reperfusion is related to the start of apoptosis. The reestablishment of glucose and oxygen restores the ATP levels necessary for apoptosis but also restores the levels of molecular oxygen needed to generate free radicals. Then, as soon as the reestablishment of blood flow takes place, the IRI starts. The ischemic process that precedes reperfusion represents a priming phase in oxidative stress. The ischemic process itself is not free radical generating, since it causes the lack of the main substrate, oxygen. However, as a priming phase, it causes changes in xanthine oxidase (as discussed above), in antioxidant enzymes and in several mitochondrial proteins, which predisposes the cell to an oxidative burst as soon as oxygen enters the cell [39, 40].

All of the mechanisms involved in IRI are not well known, but the overproduction of ROS, mainly by mitochondria and xanthine oxidase, has been related to IRI. During the ischemic process ATP levels fall and the ADP formed is also further oxidized to AMP, adenosine, hypoxanthine, and xanthine. The enzyme responsible for the last two steps is xanthine oxidoreductase that is present in the organism as the reductase isoform. Xanthine reductase oxidizes xanthine and transfers the electrons to NAD^+ instead of to O_2 . During an insult process, such as heart infarction, the ion gradient disruption leads to Ca^{2+} accumulation and activation of Ca^{2+} -stimulated proteinases. In the reoxygenation process, oxidation of $-\text{SH}$ groups or limited proteolysis of xanthine reductase converts it to an oxidizing isoform, and now the oxidation of xanthine also leads to the monovalent reduction of molecular oxygen, generating superoxide anion [3].

In fact, since 2003 a clear relationship has been recognized between high serum uric acid and mortality in patients with chronic heart failure [41]. Probably uric acid in serum represents the increased xanthine oxidase activity and, eventually, increased oxidative stress in those patients. In light of these data, some studies have attempted to link the use of allopurinol with decreased mortality in heart failure patients. For instance, allopurinol administration in patients enrolled for revascularization caused less cardiac damage, measured by creatine phosphokinase and troponin I release. Moreover, patients receiving allopurinol showed 13% less incidence of death, infarction, or tachycardia in a 30-day follow-up [42].

Perhaps a major role in IRI is developed by mitochondria. Besides being a source of free radicals in the reperfusion phase, it is also a depository of proapoptotic molecules. Thus any dysfunction would lead to oxidative damage and cell death. Isolated hearts subjected to IRI have provided information about free radical generation in a more confident way, since data from isolated mitochondria may present some bias regarding the isolation process and in vivo data are hard to obtain. In this way, it was evidenced by electron paramagnetic resonance (EPR) that ubisemiquinone, alkyl-peroxy radical, and nitrogen-centered radical were generated after the reperfusion [43]. The mitochondrial source of ubisemiquinone and alkyl-peroxy radical could be confirmed in hearts perfused with sodium amobarbital, an inhibitor of complex I of the mitochondrial electron transport chain. The major consequence was a decreased membrane lipid oxidation and high pressure developed by hearts at the end of reperfusion [43].

An early target of mitochondrial free radicals is cardiolipin, a phospholipid located inside the inner mitochondrial membrane that is responsible for attaching cytochrome *c*. The oxidation of this phospholipid is related to a decreased electron transport activity, increased free radical generation, and cytochrome *c* release with eventual caspase-9 activation [44]. Genetic approaches and pharmacological studies confirm that cardiomyocyte apoptosis plays a crucial role in the pathogenesis of many cardiac syndromes and pathologies. For instance, the inhibition of cardiac myocyte apoptosis reduces infarct size up to 50–70% and decreases cardiac dysfunction after IRI [45, 46].

The impairment of mitochondrial complex I, III, and IV activity due to ROS-induced cardiolipin damage may increase the electron leak from the electron transport chain, generating more superoxide anion radical and perpetuating a cycle of oxygen radical-induced damage, which ultimately leads to a decrease in oxidative phosphorylation and to heart failure on reperfusion. Perhaps a good strategy should be to reperfuse with low pO_2 in order to try to attenuate free radical generation, as demonstrated in isolated rat hearts. This strategy improved mitochondrial electron transport, decreased H_2O_2 production, and improved cardiolipin oxidation compared with normoxic reperfused hearts [47].

Antioxidant enzyme modulation (e.g., SOD, catalase, GPx) seems not to contribute largely to the oxidative damage in IRI. Some studies showed that Mn-SOD decreases after IRI [48], whereas other studies showed no modulation in Mn-SOD, EC-SOD, and Cu,Zn-SOD content [49, 50]. Moreover, IRI also promoted a decrease in catalase and GPx in an in vivo model of transient ischemia [51]. Taken together, these data suggest that the increase in free radical production without a consistent compensation

of the antioxidant machinery contributes to oxidative damage in IRI. Artificially increasing antioxidant enzymes by transgenic overexpression or supplementing with exogenous enzymes/vitamins/antioxidants may contribute to limit the damaged area. These data suggest that antioxidant supplementation during reperfusion could avoid IRI and render better results over the long term.

11.3.5 Cardiac Remodeling

Cardiac remodeling occurs in response to a variety of stimuli, such as hypertension or myocardial infarction. It represents functional and structural changes, and crucial aspects are involved in remodeling: ventricular hypertrophy, development of interstitial fibrosis, loss of contractility, and chamber dilation. As the signs worsen, heart failure takes place. These changes are associated with an extensive inflammatory process and are, to some extent, necessary to replace the damaged myocardium. As remodeling progresses, it becomes harmful by compromising normal cardiac function [52].

11.3.6 Hypertrophy

ROS production is present in a variety of animal models of cardiac hypertrophy (response to α -adrenergic agonists, angiotensin II stimulation, and cyclic stretch) [53]. Free radicals have an important part in coordinating the cardiac remodeling, and NAD(P)H oxidase seems to be an important source. A study with mice lacking subunit gp91^{phox} (Nox2) of NAD(P)H oxidase showed that there was no hypertrophic response to angiotensin II in those mice [5]. In contrast, mice lacking p91^{phox} were not protected against hypertrophy by aortic banding (a model of response to pressure overload), which seemed to be compensated by an increase in expression of another related subunit of NAD(P)H oxidase, Nox4. The role of free radicals in hypertrophy was further confirmed by treating these mice with the antioxidant *N*-acetylcysteine, which could prevent the hypertrophic response [54]. Superoxide anion appears to be central in the free radical-guided hypertrophy, since NAD(P)H oxidase modulation can avoid hypertrophic response. The participation of other ROS in cardiac hypertrophy can not be neglected, since *N*-acetylcysteine is a nonspecific antioxidant.

11.3.7 Interstitial Fibrosis

The deposition of an excess of extracellular matrix components between cardiomyocytes contributes to decreased cardiac elasticity and electrical conductance (see Section 11.3.10 Arrhythmia). The proliferation of myofibroblasts is another key component of interstitial fibrosis.

NAD(P)H oxidase also participates in collagen I and III deposition in both perivascular space and left

ventricular (LV) tissue. Rats rendered hypertensive by aldosterone treatment showed lower levels of collagen I and III when treated with apocynin, an NAD(P)H oxidase inhibitor [55]. Superoxide from other sources, such as xanthine oxidase, could also participate in this process. Infarcted mice develop extensive cardiac remodeling, but allopurinol, a xanthine oxidase inhibitor, could reverse collagen deposition in myocardium and preserve echocardiographic parameters, such as ejection fraction and chamber diameter [56].

11.3.8 Contractility

Multiple mechanisms contribute to contractile dysfunction, including abnormalities of cardiomyocyte excitation-contraction, mitochondrial dysfunction and energetic deficit, loss of myocytes from the ventricular walls, and alterations in the extracellular matrix. Interestingly, NAD(P)H oxidase has a role in contractility of cardiomyocytes independent of other effects that also interfere in contractility (chamber dilation and fibrosis). Cardiomyocytes isolated from mice lacking gp91^{phox} and wild-type mice subjected to aortic banding clearly demonstrated that NAD(P)H oxidase absence improved contractility independent of other effects on the tissue [57]. Contractility has been shown to be preserved when another source of superoxide is blocked. Allopurinol, an inhibitor of xanthine oxidase, was able to preserve cardiac contractility in hearts from dogs with dilated cardiomyopathy [58]. The downstream effects of superoxide may involve the generation of other ROS and regulation of sarcolemmal ion channels, sarcoplasmic reticulum calcium release channel (the ryanodine receptor), sarcoplasmic reticulum calcium pump (SERCA 2a), and the contractile proteins themselves [53, 59].

11.3.9 Chamber Dilation

Matrix metalloproteinases (MMPs), a family of enzymes that catabolize collagen, elastin, and gelatin, and their tissue inhibitors (TIMPs) are central during cardiac remodeling. Several MMPs become activated in heart failure [60]. In animal models, it has been demonstrated that deletion of MMPs is accompanied by reduced LV [61] dilation and inflammation after myocardial infarction. Conversely, deletion of TIMPs is accompanied by increased LV dilation after myocardial infarction (MI) [62]. Free radical participates in activation of MMPs. Mice subjected to a model of pressure overload by aortic constriction present increased levels of superoxide and MMP-2 activation. Mice lacking iNOS did not present MMP-2 activation or suffer all these adverse remodeling effects on the heart. Such effects could also be avoided by the selective iNOS inhibitor administered

in wild-type mice subjected to aortic constriction [63]. In the same line, infarcted hearts from mice that were treated with allopurinol presented attenuated chamber dilation compared with those who were treated with saline [56]. Moreover, other studies have demonstrated that heart-specific deletion of Mn-SOD promoted a progressive dilated cardiomyopathy [64]. Collectively, these data suggest the importance of free radicals in the progression of chamber dilation.

11.3.10 Arrhythmia

Arrhythmia is defined as any change in the electrical activity in the heart. It is classified according to the alteration produced. Tachycardia consists of accelerated heartbeat, while bradycardia is characterized by a slower pump rate. Although events of arrhythmia can result in cardiac arrest and sudden death, some arrhythmias occur as sparse events and are not considered as threats. The most important causes of arrhythmia are coronary artery disease and atrial fibrillation (AF), the latter being closely associated with postsurgery inflammation.

11.3.11 Atrial Fibrillation

AF is characterized by rapid electrical activity of the atria that leads to abnormal ventricular activity [65]. One general cause of AF is heart failure (HF), although intrinsic mechanisms vary and are not well understood. Besides HF, diabetes mellitus and cardiac surgery increase the risk of AF [66].

Animal models have shown that increased atrial pacing is associated with increased superoxide production. In addition, most of this radical is produced by the enzyme NAD(P)H oxidase [67]. In humans, NAD(P)H oxidase activity is a strong predictor of postoperative AF [68]. The process of atrial fibrosis itself is closely related to the activation of the AT₁ receptor by angiotensin II and the subsequent increase in NAD(P)H oxidase activity. Inhibition of AT₁ is known to prevent superoxide production [69].

Along with free radical production, inflammation is considered as a risk factor and also a component of AF. Postoperative high levels of interleukin 6 (IL-6) are predictors of AF onset [70]. Moreover, serum levels of hydroperoxides are associated with atrial function and predict AF in patients after radiofrequency catheter ablation [71].

Antioxidants have been proven as efficient in treatment and prevention of AF and arrhythmias, although their role is no more than that of coadjuvants. The pool of glutathione (GSH) is a major physiological defense against oxidative stress in the myocardium. Indeed, its precursor *N*-acetylcysteine was shown to decrease the

incidence of postischemia arrhythmias [72, 73]. The use of drugs such as thiazolidinediones might act by reducing the activity of xanthine oxidase or by increasing NO production. This NO is capable of reacting with the overproduced superoxide radical, due to the increased activity of NAD(P)H oxidase. Ascorbic acid also seems to be an important antioxidant, as its levels were found decreased in a dog model of atrial tachypacing [74], and its administration to patients with persistent AF reduced the recurrence of the condition after cardioversion [75]. NO precursors such as L-arginine and sodium nitroprusside have strong potential as therapeutics, since NO acts as an antioxidant and anti-inflammatory [76, 77].

Although their origins might rely on other cardiovascular complications such as heart failure and atherosclerosis, AF and arrhythmias are strong life-threatening conditions with specific mechanisms of development that require specific interventions.

11.4 EXERCISE AND PROTECTION AGAINST FREE RADICAL-MEDIATED CARDIOVASCULAR DISEASES

As stated above, environmental factors contribute greatly to increased risk of cardiovascular diseases. Among these are smoking and dietary habits (see above). Another important habit that contributes to cardiovascular diseases is lack of physical activity. Physical activity can either confer resistance to cardiovascular events, such as myocardial infarction, or help during the recovery process.

11.4.1 Exercise as a Protector

There is a large amount of epidemiological evidence showing that physical activity habit has a protective effect for several cardiovascular complications, such as atherosclerosis development and hypertension [78]. But does exercise also work for improving life after the myocardial infarction episode? The answer seems to be positive, since men who had physical activity after an episode of myocardial infarction were more prone to survive [79, 80]. Unfortunately, direct evidence of protective effect of physical activity is hard to obtain, thus we can rely on animal models to understand the mechanism involved. From these models we can learn that exercise preconditioning exerts its effects by decreasing the size of infarct [81], a parameter that is closely linked with the mortality rate in humans [82]. But how does exercise decrease mortality of cardiomyocytes after an infarction? The following effects are involved: first, morphological changes concerning myocyte hypertrophy and increased coronary vascularization; second, changes in metabolic efficiency; and third, increase in

the myocardium antioxidant potential after a transient release of free radicals.

ROS such as hydrogen peroxide and superoxide were already described as important signaling molecules in a variety of processes, including myocyte hypertrophy and death. Isolated cardiac myocytes subjected to mechanical stretch present increased levels of superoxide and develop hypertrophy [83]. These events are mediated by mitogen kinases, such as ERK1/2, which is implicated in cell growth and can be stimulated by free radicals in cardiac myocytes. When antioxidants are given to these cells, not only superoxide is abolished, but also ERK1/2 activation and cell growth [83]. The participation of ROS in myocyte hypertrophy becomes clearer with the demonstration that hydrogen peroxide delivery to cultured adult cardiomyocytes has a biphasic response, being proliferative in low doses and apoptotic/necrotic at higher doses [84]. Lower doses of H_2O_2 (10–30 μM) induce activation of ERK1/2 and cell growth, whereas higher doses (>100 μM) induce activation of ERK1/2, JNK, and p38 MAPK, and JNK activation is related to the deleterious effects of H_2O_2 treatment [84].

From studies with animals we can learn that high-intensity exercises can increase MnSOD activity in both young and old rat hearts [85, 86], indicating that the heart retains the ability to induce antioxidant defense despite age. Interestingly, the cytoplasmic isoform of superoxide dismutase (Cu,ZnSOD) is, in general, not induced by physical exercise [87]. The chemical product of SOD action is hydrogen peroxide, and it serves as substrate for catalase and glutathione peroxidase [3]. Thus these hydrogen peroxide-consuming enzymes should be kept in balance with the SOD increment to avoid an excess of peroxide and a possible start of oxidative stress. However, it seems that the exercised heart proceeds otherwise, since there is no compensatory increase in catalase or glutathione peroxidase [87]. Thus, taken collectively, these data indicate that the increase in H_2O_2 in cardiac myocytes during the exercise can lead to myocyte growth and thus to cardioprotection against future events.

Besides this, free radicals are centrally involved in angiogenesis. Vascular endothelial growth factor (VEGF) is a family of key mediators of angiogenesis after wound or exercise that exert their effects through VEGF receptors (VEGFR1, VEGFR2, and VEGFR3) [88]. A well-conducted study in animals showed that the delivery of low doses of H_2O_2 to the wound can increase VEGF mRNA and CD31⁺ cells and accelerate wound healing [89]. Moreover, either NAD(P)H oxidase deficiency or overexpression of catalase abolished VEGF synthesis and impaired wound healing [89]. These data are consistent with the impaired wound healing seen in chronic granulomatous disease patients, who also present dysfunctional NAD(P)H oxidase [90]. In the same way,

antioxidant therapy is considered antiangiogenic, and some studies have employed it as adjuvant therapy to cancer treatment. Several compounds, such as glutathione, selenium, and resveratrol have been shown to impair angiogenesis [91–93].

But how does hydrogen peroxide induce VEGF synthesis? The pathway is not completely elucidated yet, but some studies employing different cell types have shown that the MAPK pathway, mainly involving MEK, ERK1/2, and p44/42 MAPK, seems to be important [88]. Moreover, VEGF signaling is further stimulated because hydrogen peroxide also triggers NF- κ B translocation to the nucleus and stimulates the transcription of VEGF receptors [88].

Finally, physical activity increases the antioxidant potential in the heart in order to counterbalance the permanent increase in free radical generated as a consequence of heart work. Thus, in the case of a myocardial infarction, the heart is more prone to buffer the oxidative burst during the reperfusion process. However, this view is not fully elucidated, as we shall see below. First, there are some studies with chronically exercised animals that do not show any improvement in the heart antioxidant profile [94]. SOD, for instance, appears to be upregulated only after high-intensity exercise [87]. Also, exercise induced MnSOD activity in both young and old rats [86]. Moreover, the systems responsible for consuming hydrogen peroxide do not respond after exercise in most studies [87]. Glutathione increase after exercise is also under debate, and its role in heart adaption is not fully elucidated. In fact, because hydrogen peroxide acts as signaling molecule in heart adaption, we should not to expect an increase in the systems responsible for consuming it.

11.4.2 Exercise as a Therapy

After the onset of myocardial infarction a lot of attention is devoted to decreasing the remodeling process that takes place and leads to heart failure. The remodeling process involves cardiomyocyte death, fibroblast proliferation, and extracellular matrix deposition [52], all processes coordinated in some way by free radicals. Besides the classical therapies, exercise training may attenuate cardiac remodeling and may even reverse this process [95]. Unfortunately, few studies have focused on the effect of exercise on free radical after myocardial infarction. In one study, 17 patients were enrolled in a training program for 3 weeks. At the end, the patients presented better hemodynamic performance and lesser hydrogen peroxide production after the physical test [96]. Similar results were found in another study that evaluated antioxidant enzymes in skeletal muscle biopsies from chronic heart failure patients who entered into a physical activity

program [97]. Despite these studies, which clearly showed that physical activity performed after a myocardial insult is efficient in restoring free radical/antioxidant balance, there has been no evaluation of the effect of exercise on survival rate in a long-term study.

11.5 ANTIOXIDANT THERAPIES FOR CARDIOVASCULAR DISEASES

Atherosclerosis is one of the leading causes of morbidity and mortality in Western countries. Its occurrence is in great part explained by the way of life in a technologically advanced world. Lack of exercise activities and high-fat diet are major risk factors for atherosclerosis. Indeed, evidence suggests that diets low in saturated fat and rich in vegetables contribute against the development and progression of cardiovascular diseases [26]. It is clear that antioxidants present in the diet play an important role by acting against the intrinsic mechanisms of atherosclerosis and other cardiovascular diseases, but clinical trials and follow-up studies are controversial [98].

Studies with animals often support antioxidant therapies to prevent or attenuate atherosclerosis. A low-vitamin C and E diet accelerated atherosclerosis in ApoE-deficient mice [99]. In the same model of atherosclerosis, *N*-acetylcysteine, a precursor of glutathione, reduced plaque formation and superoxide production [100]. Although it may seem fairly obvious that antioxidants can prevent cardiovascular diseases, tests in humans raise many questions about the specificity of each antioxidant and of each disease. Furthermore, some clinical studies with vitamins A and E report an increase in mortality and incidence of cardiovascular disease [101–103].

On the other hand, some antioxidants have been demonstrated to be effective. After infarction, administration of L-arginine has been proved to ameliorate the response to the ischemic event by increasing the levels of NO, which promotes vasodilation and can also neutralize reactive species. Besides NO production, L-arginine also increases the activity of SOD and the levels of thiols, and both are believed to act synergistically with the vasodilation [104]. Potent free radical scavengers like edaravone have also been tested in humans and proven to improve LV ejection fraction and reduce rehospitalization after infarction [105]. However, it is clear that no antioxidant will be found to prevent all cardiovascular diseases or restore cardiovascular function after any deleterious event.

Some success has been achieved using xanthine oxidase inhibitors, such as allopurinol and oxypurinol. Patients with ST segment elevation myocardial infarction who underwent reperfusion showed better recovery markers when treated with allopurinol than those treated with placebo. Allopurinol was able to completely recover

ST elevation in 76% of the patients, against only 58% in placebo-treated subjects. Moreover, markers of myocyte damage, such as cardiac troponin I and CK-MB, were also lower in patients treated with allopurinol [42]. Also, congestive heart failure patients with ejection fraction <40% receiving oxypurinol over a month demonstrated 7% of improvement in ejection fraction compared with the placebo group [106]. Thus xanthine oxidase indeed seems to be important in the course of heart failure, and superoxide may be a therapeutic target in the future.

In this sense, antioxidants are important agents in prophylaxis and treatment, regulating the intra- and extracellular redox status. Fine-tuning of the antioxidant enzymes and production of glutathione is associated with lower incidence of cardiovascular diseases and increase of life span. However, while the specific mechanisms lying behind cardiovascular diseases are still to be elucidated, the use of antioxidants in medicine might remain underrated.

11.6 CONCLUSION

Oxidation of biomolecules accelerates cell senescence and is closely associated with neurodegenerative and cardiovascular diseases. As the organism gets older, antioxidant defenses and repair mechanisms become weaker and cannot counteract the production of reactive species and accumulation of oxidative damage. Although this scenario seems cruel, fine regulation of the oxidative status of cells and tissues is a key point in the maintenance of homeostasis. Regarding the cardiovascular system, oxidative stress shows up as a major culprit, participating in endothelial dysfunction, development and progression of atherosclerosis, and cardiac dysfunction. In any case, reducing the production of reactive species or increasing the levels of antioxidants has proven to be important for prevention and treatment. Nevertheless, clinical trials are inconclusive and sometimes controversial. From this perspective, better knowledge of the mechanisms regulating oxidative stress generation, as well as new therapeutic interventions, capable of acting more specifically in each disease, can be foreseen.

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OXIDATIVE STRESS AND AGING: A COMPARISON BETWEEN VERTEBRATES AND INVERTEBRATES

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12.1 INTRODUCTION

Aging and death, the two ubiquitous phenomena, have always been an enigma for mankind. Except for certain primitive organisms such as bacteria that propagate through simple division, all organisms including humans must age and subsequently face death, yet their life span can vary. Among major model organisms, the worm *Caenorhabditis elegans* lives for 2 weeks, the fly *Drosophila melanogaster* lives for 2 months, the mouse *Mus musculus* lives for 2 years, and humans live for approximately 80 years [1].

Old age in most species is associated with impaired adaptive and homeostatic mechanisms leading to susceptibility to environmental/internal stresses with increasing rates of disease and death [2]. Since ancient times humans have pondered over the mysterious question: How do we age? How do different organism/species show different life spans, and what are the factors that determine longevity? Hippocrates (460–377 B.C.) defined aging as an irreversible and actual event dictated by the gradual loss of heat. Darwin established the scientific background to understand the process of aging; he explained aging as the loss of excitability over time.

The term “aging” refers to the biological process of growing older in a deleterious sense, which some authors call “senescence” [3, 4]. It is a unique feature of the life cycle of all organisms. In scientific terms, aging may be defined as an inevitable process of accumulation of molecular, cellular, and organ damage, leading to loss

of function and increased vulnerability to disease and death [5].

12.2 THEORIES OF AGING

Aging is a multifactorial process caused by damage by a variety of cellular components, followed by their accumulation. It is characterized by progressive deterioration of physiological functions and metabolic processes [6, 7]. Within an organism, manifestation of aging occurs at genetic, molecular, cellular, organ, and system levels [8]. Long-term studies on numerous animal models (in vivo and in vitro) have generated a number of theories that attempt to elucidate the cause/mechanism(s) of aging, since it is doubtful that a single theory can explain all the mechanism of aging. Presently more than 300 theories of aging are in existence [9].

The evolutionary theory of aging states that aging occurs because the force of natural selection declines with age in populations, making it possible for hazardous late-acting genes to exist [10, 11]. The life history principle describes aging as an emergent phenomenon that takes place primarily in the protected environment and that allows survival beyond the natural life span that would occur in the wild. The natural or essential life span (ELS) of a species is the time required to fulfill the Darwinian purpose of life, that is, successful reproduction. The period of extended survival beyond the ELS is defined as the period of aging [12]. The mutation accumulation theory argues that detrimental, late-acting mutations

may accumulate in the population and ultimately lead to pathology and aging [13]. According to the energy consumption hypothesis, animals are born with a limited amount of potential energy or physiological capacity, the faster they use it, the faster they will die [14]. Later this hypothesis became the rate of living theory: The faster the metabolic rate, the faster the biochemical activity, the faster an organism will age. The protein error theory also tried to explain the mechanism of aging. According to this theory an induction and increase in protein errors can accelerate aging in human cells and bacteria [15–17]. Similarly, the accuracy of protein synthesis can slow aging and increase the life span in fungi [18, 19].

The cellular senescence/telomere theory explains that cells have a limited proliferative potential. After a finite number of divisions, cells enter into a state of senescence, and this process of cell senescence limits the number of cell divisions [20]. The number of divisions a cell can undergo in culture is known as the Hayflick limit and has been postulated to determine the maximum life span of an organism [21, 22]. Furthermore, it was elaborated that this specific type of cellular senescence results from the loss of a small amount of DNA at the end of the chromosome, resulting in ever shorter telomeres. This limit in the replicative capacity results in terminally arrested cells with altered cellular physiology that might contribute to aging and cancer through secondary effects on neighboring cells in tissue [23, 24].

The role of genes in regulation of longevity has also been put forward; this theory postulates that aging results from changes in gene expression [25–27]. The free radical theory of aging, developed by Denham Harman in 1956, is one of the most influential theories in describing the aging mechanism. According to this theory free radicals, specifically hydroxyl and hydroperoxyl, are formed endogenously from normal oxygen-utilizing metabolic processes as by-products that play a essential role in aging and age-related processes. Mitochondria play a central role in generation of free radicals through impaired function of the electron transport chain, and these free radicals elicit damaging properties. Aging results in accumulation of free radical damages as a function of time [28–31]. Support for the free radical theory of aging has increased progressively over the years, and growing numbers of studies implicate free radical reactions in aging and the pathogenesis of specific diseases such as diabetes, cancer, and heart diseases [32–34].

12.3 FREE RADICAL/OXIDATIVE STRESS THEORY OF AGING

Among several theories that attempt to explain the aging mechanism, the free radical/oxidative stress theory of

aging offers the best mechanistic elucidation of the aging process and other age-related events [28, 35].

Interest in the free radical theory was at first very limited because of persistent doubt about the existence of oxygen free radicals in biological systems despite the reports by Gerschman et al. [36] and the detection of radicals by Commoner and co-workers [37]. The discovery of superoxide dismutase (SOD) by McCord and Fridovich [38] and the demonstration of the existence of H_2O_2 in vivo by Chance [39] gave credibility to and raised the profile of the hypothesis. In 1972, Harman modified his free radical theory, ascribing a central role to mitochondria [29] because mitochondria generate a disproportionately large amount of free radicals/reactive oxygen species (ROS) in cells [39]. Later on, correlative evidence supporting the free radical theory of aging was published. Later on, several reports substantiated Harman's hypothesis that free radical oxidative damage increases during aging [40–42].

In the last few decades, Harman's hypothesis has been refined; it is now accepted that not only free radicals but also other forms of activated oxygen such as peroxides and aldehydes (which are not technically free radicals) play a role in oxidative damage in cells. A collective term, "reactive oxygen species (ROS)," has been introduced to define these oxidants including free radicals. This realization led to a modification of the free radical theory, that is, the oxidative stress theory of aging [43].

Apart from respiratory chain in mitochondria, there are other endogenous sources of ROS including immune reactions, enzymes such as xanthine oxidase and nitric oxide synthase, and transition metal-mediated oxidation [44]. A diverse range of exogenous sources of ROS are reported, which incorporate ionizing and nonionizing radiations, pollutants, natural toxic gases such as ozone, drugs, and toxins including oxidizing disinfectants [34, 45]. ROS production and accumulation is a common denominator in many diseases and environmental insults and can lead to severe cellular damage resulting physiological dysfunction and cell death in virtually all aerobes. When oxidative stress occurs, cells function to counteract the oxidant effects and to restore redox balance by resetting critical homeostatic parameters. Such cellular activity leads to activation or silencing of genes encoding defensive enzymes, transcription factors, and structural proteins [34, 46]. According to the free radical theory of aging, oxidative stress increases with increasing age; this condition leads to accumulation of oxidation products of lipids, nucleic acids, proteins, sugars, and sterols ultimately causing cellular dysfunction and making the body prone to external deleterious agents (Fig. 12.1). In agreement with the free radical/oxidative stress theory of aging, it was investigated that mtDNA deletions are induced by oxidative stress and dramatically accumulate with age in organisms ranging from worms to humans [40, 47].

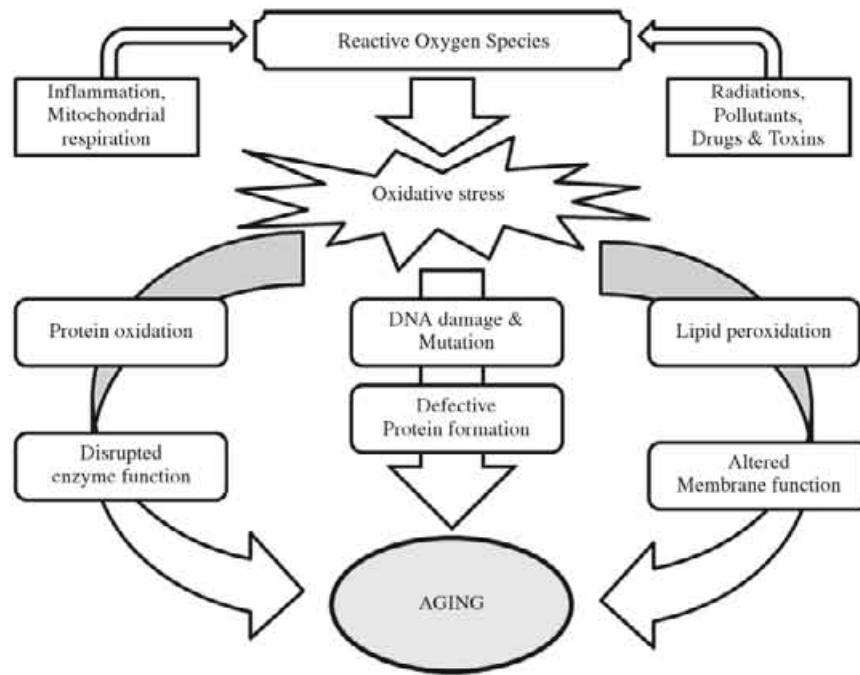


Fig. 12.1 Reactive oxygen species generated by endogenous as well as exogenous sources cause oxidative damage and accumulation of proteins, lipids and DNAs, when defensive mechanisms of body become weak. These disturbances cause organelle damage, changes in gene expression followed by altered cellular responses which ultimately results into aging.

12.4 AGING IN INVERTEBRATES: ROLE OF OXIDATIVE STRESS

The role of oxidative stress in aging is clearly seen in *Podospira anserina*, a fungus that belongs to the ascomycete family of fungi. The growth of hyphae in *P. anserina* is not indeterminate; however, its growth arrests on reaching a certain length and hyphae eventually wither and die. The period to onset of the condition of senescence is referred to as the life span of *P. anserina*. Tudzynski and Esser in 1979 stated the central involvement of mitochondria in determining the timing of senescence [48]. Recently, Dufour et al. [49] proved the involvement of ROS in life span limitation of *P. anserina* and demonstrated that elimination of the mitochondrial electron transport chain extended the hyphal life span of *Podospira* by at least threefold. It has been proposed that oxidative damage to mitochondrial DNA by the mitochondrial ROS triggers senescence [40, 50, 51].

Saccharomyces cerevisiae, the common yeast, is the other invertebrate whose life span is directly determined by oxidative stress/ROS. *S. cerevisiae* also belongs to the ascomycete family. This single-cell budding yeast is a good organism for studying the aging process. For multiplication *S. cerevisiae* uses asymmetric cell division, which results in a small daughter cell; the process is known as budding. Interestingly, a yeast cell (mother

cell) can only bud a finite number of times, after which it loses its capacity to multiply and becomes sterile. This budding period of the yeast cell is called the clonal life span [40].

Studies have revealed oxidative damage accumulation during clonal life span. Accumulation of oxidatively damaged proteins has been reported in yeast mother cells during this period [52]. The attack by ROS against proteins modifies amino acid residues generating carbonyl moieties, which has been identified as an early marker for protein oxidation and is used as a measure of protein damage [53]. In yeast cells, protein carbonyls have been shown to accumulate during chronological aging, in a manner dependent on the rate of mitochondrial ROS production [54]. Besides overexpression of the methionine oxidation repair enzyme, MsrB and MnSOD have been unambiguously shown to increase chronological life span of yeast cells [40, 55]. Collectively, these data suggest that ROS limit life span during aging in *S. cerevisiae*; however, the role of oxidative stress in determining the clonal and chronological life span in yeast cells is still not fully understood.

The nematode *Caenorhabditis elegans* is one of the most frequently used invertebrate models for studying the aging process because of its many interesting characteristic features ideally required to study mechanism(s) of aging. Large numbers of offspring, short generation

time, and the ability to be stored frozen have attracted the attention of biogerontologists. Unlike the unicellular yeast, it allows studies of different cell types and organs, such as the nervous or digestive systems; it is also more closely related to mammals [5]. During the past few years, a large number of long-lived mutants of *C. elegans* have been identified that are now frequently used as models in aging research. The commonly used mutants of *C. elegans* are *daf-2* and *age-1* [56, 57]. The *daf-2* gene encodes a protein with structural similarity to the mammalian insulin and the insulin-like growth factor receptors, while the *age-1* gene encodes a catalytic subunit of phosphatidylinositol-2-OH kinase, which is involved in a conserved signal transduction pathway downstream of the insulin-like receptor [56]. Studies on *daf-2* reveal that these mutants show resistance to oxidative stress, elevated antioxidant enzymes, and expression of some antioxidant genes; all these strongly support the oxidative stress hypothesis of aging [58, 59]. Several recent studies report that there is indeed slower age-related accumulation of protein carbonyl groups in long-lived *C. elegans* strains compared to wild type [60, 61] and faster accumulation in a short-lived strain [61].

In support of the free radical theory in the aging process, Lithgow and co-workers showed that catalytic antioxidants like catalase (CAT) and SOD markedly extend the life span of *C. elegans* [62]. Although some reports do contradict the oxidative stress theory in *C. elegans* aging, there is enough evidence for oxidative stress as a life span determinant in *C. elegans* [40].

The free radical theory of aging has also been extensively studied in the fruit fly (*Drosophila melanogaster*). The fly is important for establishing evolutionary conservation of mechanisms and for studying events in different tissues that are more numerous and differentiated than in *C. elegans*. Early experiments have indicated that there is a quasi-linear, inverse relationship between life span and oxygen tension in *Drosophila*, and it was seen that increase in oxygen tension above 21% shortened the life span [63, 64]. In 2004, Landis et al. [65] in their microarray study compared gene expression patterns in old and young flies by treating young flies with 100% oxygen. The results were interesting: Young flies treated with 100% oxygen exhibited many gene expression changes resembling those in old flies. This indicated that oxidative injury plays a prominent role in normal fly aging and suggests that the life span shortening under mild hyperoxia (40% O₂) may be true accelerated aging [40].

Genetic manipulation of the SOD activity in *Drosophila* emphasizes the direct association between oxidative stress and life span. Increasing the activity of different forms of SOD by two- to fourfold suggests that elevated antioxidant defense is necessary for

extended longevity in *Drosophila* [66]. In 1995, Sohal et al. reported that concomitant overexpression of SOD and CAT increased both average and maximum life span of *D. melanogaster* [67]. Parkes et al. also targeted the overexpression of CuZnSOD in *Drosophila* and reported that overexpression of CuZnSOD in motor neurons resulted in an increase in life span as well as an increase in resistance to paraquat and γ -irradiation [68]. Phillips and co-workers in 2000 reported that MnSOD overexpression increased life span in *Drosophila* [69].

Some recent transgenic studies provide more evidence for the oxidative stress theory of aging in *Drosophila sp.* Overexpression of the protein oxidative damage repair enzyme peptide-S-methionine sulfoxide reductase (MsrA) was found to increase average life span in several independent insertion lines [70]. Many contradictory reports have been published that argue the role of oxidative stress in the aging process of *Drosophila sp.* Nevertheless, there is a fair amount of evidence to show the involvement of ROS as a limiting factor in determining the life span of *D. melanogaster* [40].

In addition to the above-discussed invertebrate models there are many other invertebrate species such as helminths in which the direct influence of oxygen radicals in longevity determination has been reported [40].

12.5 AGING IN VERTEBRATES: ROLE OF OXIDATIVE STRESS

Vertebrates are an evolved group of animals that include mammals and humans. Invertebrate model organisms have been used for the discovery of genes and mechanisms involved in extension of life span, but the mouse is the most practical mammal for establishing whether homologous genes and processes can extend healthy life span [5]. In vertebrate models caloric restriction has been shown to increase life span. Caloric restriction also protects against age-related decline in function and disease in rodents and monkeys, and in humans it reduces risk factors for diabetes, cardiovascular disease, and cancer [5]. The most accepted explanation behind caloric restriction life extension is based on the free radical theory of aging: The rate of mitochondrial free radical production is directly proportional to the oxidative damage. A decreased rate of operation of mitochondrial electron transport chain leads to a lower oxidative burden and a higher life span [71].

Studies on transgenic mice have been carried out to understand the relation between free radical generation, oxidative stress, and longevity. Takagi and co-workers in 1999 reported an increase in life span of mice after approximately threefold overexpression of human thioredoxin 1 (*hTrx1*) in different tissues [72]. An increased

resistance to cerebral ischemia and to UV-induced oxidative stress was also demonstrated in *hTrx1* mice. Mitsui et al. in 2002 documented that *hTrx1* mice lived about 35% longer than littermate control mice. In addition, a 22% increase in maximal life span was also observed [73]. In continuation, transgenic mice overexpressing metallothionein have been shown to have an increased mean life span of about 15% relative to control mice. Metallothionein is a low-molecular-weight protein rich in cysteine residues; it exhibits heavy metal detoxification and free radical scavenging abilities [74].

Transgenic mice that overexpress human catalase in peroxisomes, nucleus, or mitochondria have also been tested [76]. The mitochondrial overexpression of catalase (MCAT) in mice caused significant delay in the development of age-related cataracts. This was an interesting finding in support of the free radical theory of aging since the development of age-related cataracts has been shown to be inversely related to life span in humans, and its incidence is reduced in mice that exhibit extended longevity [40, 75]. It was seen that Ames dwarf mice have an increased life span compared to their wild-type littermates [76]. Further investigations revealed that the dwarf mice have increased levels of CAT and SOD [77, 78]. The Ames dwarf mice also showed reduced levels of DNA and protein oxidation in liver [78]. Embryonic fibroblasts from these mice are resistant to UV radiation, heat stress, paraquat, hydrogen peroxide, and cadmium [56, 79].

In addition, interspecies comparisons of the life span in vertebrates show a very clear role of oxidative stress in determining longevity. In 1999, Kapahi et al. performed comparative experiments to measure the resistance of fibroblasts and lymphocytes from hamster, rat, marmoset, rabbit, sheep, pig, cow, and human to various oxidative stresses such as paraquat, sodium arsenite, hydrogen peroxide, and *tert*-butyl hydro-peroxide (tBHP). Interestingly, they observed that cells from species with a longer life span had increased resistance to oxidative stress and proposed that cellular defenses that help cells to survive acute stresses are associated with longevity [80]. These observations are consistent with the free radical/oxidative stress theory of aging.

In humans there is an array of experimental evidence in agreement with the free radical/oxidative stress theory of aging [16, 34]. Over the past 10 years, major developments have been made in the assay of oxidative damage to lipids, DNA, and protein to measure specific types of oxidative damage in specific types of cells/tissues during aging. Several reports document the age-dependent increase in lipid peroxidation, protein oxidation, and nucleic acid damage in humans, and there exists a significant negative correlation with antioxidant capacity of plasma [34, 81–83]. Overexpression of

antioxidative enzymes/defense mechanisms during aging in humans has also been reported in recent studies that support the oxidative stress theory of aging [83–85].

12.6 CONCLUSION

The phenotype and the rate of progression of aging are highly variable in different species and in organisms within a species. Although there is no doubt that aging is a multifactorial process that is governed by more than one factor, it is becoming increasingly clear that oxidative stress and the resultant damage to biomolecules play a vital role in several age-related pathologies. Despite some contradictory reports, there is sufficient experimental evidence to conclude that oxidative stress, if not alone, is one of the main limiting factors for aging and life span in invertebrates and vertebrates under normal atmospheric conditions.

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OXIDATIVE STRESS-MEDIATED SIGNALING PATHWAYS BY ENVIRONMENTAL STRESSORS

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13.1 INTRODUCTION

Oxidative stress in the form of excess reactive oxygen species (ROS) or reactive nitrogen species (RNS) can affect cells deleteriously or beneficially. Such stress might be generated by intracellular or extracellular sources. Furthermore, oxidative stress can cause various biological effects. Environmental stress is a key contributor to human disease. A number of substances such as metals, particulate materials, smoke, pesticides, and physical agents are environmental stressors [1] that contribute to many diseases. Concerns related to environmental stressor-related diseases such as cancer, chronic lung disease, diabetes mellitus, neurodegenerative diseases, and reproductive disorders have been raised recently. Research efforts elucidating the modes by which environmental stressors influence the development and progression of diseases or exploring preventive approaches are expected to engender further improvements in our knowledge. Understanding environmental stressor-induced influences at the molecular level will also provide a wealth of information related to the exploration of biomarkers for environmental stressor-related diseases [2–4].

The mechanisms of redox adaptation in living bodies and cells might involve multiple influences on an active redox-sensitive signaling pathway, such as ROS metabolism and antioxidant defenses, p53 pathway signaling, nitric oxide (NO) signaling pathway, hypoxia signaling, transforming growth factor (TGF)- β -bone morphogenetic protein (BMP) signaling, tumor necrosis factor (TNF)

ligand-receptor signaling, and mitochondrial function (Table 13.1). For example, transcription factors such as nuclear factor- κ B (NF- κ B), nuclear factor erythroid 2-related factor 2 (Nrf2), c-Jun, and hypoxia-inducible factor-1 (HIF-1) engender increased expression of antioxidant molecules such as superoxide dismutase (SOD), catalase, thioredoxin, and the GSH antioxidant system. Metal ions such as arsenic (III/V) or copper (II) directly influence expression levels of those transcription factors and induce various oxidative stress events including thiol molecule perturbation, generation of oxidative DNA adducts, and induction of oxidative molecular biomarkers [5–8]. Nonmetal chemicals such as retinoic acids and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) are also known to influence the expression of oxidative stress-related genes and proteins during carcinogenesis and during embryonic development [9–12]. In relation to cancer, a growing tumor might also produce intracellular and extracellular oxidative stress, which can modify its malignant features. Endogenous sources of tumor ROS or RNS include impaired intracellular genomes or proteomes, metabolism pathways, and xenobiotic metabolism. Consequently, the study of transcriptional regulation of gene expression in the research field of oxidative stress has been useful for identifying new transregulatory factors or new biomarkers induced by exposure to environmental stressors.

Microarray technology has been used in environmental toxicology and biology studies and has led to the establishment of gene expression signatures profiling

TABLE 13.1 Summary of oxidative stress-mediated signaling pathways

Categorical pathways	Canonical Pathway (orthology)
Reactive Oxygen Species (ROS) Metabolism and Antioxidant Defenses	Glutathione Peroxidases (GPx) Peroxiredoxins (TPx) Superoxide Dismutases (SOD) Genes Involved in Superoxide Metabolism Genes Involved in ROS Metabolism Other Peroxidases and Antioxidant-Related Genes
p53 Signaling (including DNA damage)	Apoptosis-Related Genes Cell Cycle Arrest and Checkpoint Regulation of the Cell Cycle Regulation of Cell Proliferation, Cell Growth, and Differentiation Damaged DNA Binding Mismatch, Base-Excision and Double-Strand Break Repair
Nitric Oxide (NO) Signaling Pathway	Genes with NO Synthase and Regulators of NO Biosynthesis Genes regulated by NO and NO Signaling Pathway Genes Involved in Superoxide Release Antiapoptosis Genes Genes with Antioxidant and Superoxide Dismutase Activity Genes with Glutathione Peroxidase, Oxidoreductase, or Peroxidase Activity Transcription Regulators
Hypoxia Signaling	Response to Hypoxia and Signal Transduction, Oxidative Stress Genes Related to Stress and Immune Response Hemoglobin Complex-Associated Genes Peroxidase, Oxidoreductase-Related Genes Transcription Factors and Regulators and Protein Binding Antiapoptosis Induction of Apoptosis and Caspase Activity Protein Biosynthesis, Phosphorylation, and Metabolism Cytoskeleton and Other Extracellular Molecules Cell Cycle, Cell Proliferation, and Growth Factors Carbohydrate, Lipid, One-Carbon Compound Metabolism RNA Metabolism Cardiac Excitation-Contraction (E-C) Coupling
TGF- β -BMP Signaling	TGF- β superfamily, bmp (bone morphogenetic protein) family members, gdf (growth differentiation factor), activin, and activin receptors Smad family members, TGF- β /activin-responsive genes, bmp-responsive genes, molecules regulating signaling of the TGF- β superfamily, adhesion molecules, extracellular matrix structural constituents, other extracellular molecules, transcription factors and regulators
Tumor Necrosis Factor (TNF) Ligand-Receptor Signaling	Caspase activation, caspase inhibition, anti-apoptosis genes, induction of apoptosis, other apoptosis-related genes, jnk signaling pathway, nf- κ b signaling pathway, tnfr1 and tnfr2 signaling pathway, inflammatory response, transcription regulators
Mitochondria	Mitochondrial processing, mitochondrial transportation Fatty acid biosynthesis

the toxicity of environmental stressors [13, 14]. Statistical methods used for DNA microarray studies are mostly multivariate approaches. Although basic methods treat genes as traits, which is consistent with the rules of experimental design, several approaches have been developed using expression ratio data sets. Such approaches regard the genes as cases and the array plates as variables. Most well-known methods based on singular value decomposition have used principal component analysis [15, 16]. In alternative approaches, our previous reports have described that a Bayesian

network technique, which is a probabilistic graphical model that represents a set of variable identities, is applicable to investigation of the gene expression interaction networks and the detection of differences arising in them from exposure to different doses of chemicals [17, 18]. Bayesian network techniques can provide predictive information related to the relations between agents and gene expression signatures in the life science fields [19–21].

This chapter addresses various environmental stressor-induced toxicities in experimental animals such as

rats and humans to elucidate the molecular mechanisms underlying toxicity-induced oxidative stress.

13.2 OXIDATIVE STRESS-MEDIATED SIGNALING PATHWAYS

Cells respond and adapt to environmental signals such as stressors [22–24] through multiple mechanisms that involve communication pathways and signal transduction processes. The impact of oxidative stress on various diseases and aging has been reviewed comprehensively. In particular, free radical-induced oxidative stress plays an important role in cancer development, metabolism-related diseases like diabetes and hypertension, and neurodegenerative disorders [4, 25–36]. Our survey of microarray databases and many other published references has revealed the categorical pathways induced by oxidative stress presented in Table 13.2.

ROS metabolism and antioxidant defenses center upon ROS, which are necessary for biological functions and which regulate many signal transduction pathways by directly reacting with and modifying the structure of proteins, transcription factors, and genes to modulate their functions. Actually, ROS induce expression levels of genes associated with signaling cell growth and differentiation, regulating the activity of enzymes (such as ribonucleotide reductase and peroxidase). Control of ROS levels is achieved by balancing ROS generation with their elimination through ROS-scavenging systems such as superoxide dismutases (SOD1, SOD2, and SOD3), glutathione peroxidase, peroxiredoxins, glutaredoxin, and thioredoxin catalase. The ROS can modulate the activities and expression of many transcription factors and signaling proteins that are involved in stress response and cell survival through multiple mechanisms. Therefore, this category includes glutathione peroxidases (GPx), peroxiredoxins (TPx), superoxide dismutases (SOD), genes involved in superoxide metabolism such as arachidonate 12-lipoxygenase (ALOX12), and copper chaperone for superoxide dismutase (CCS). In fact, p53 signaling plays a central role in coordinating the cellular responses to a broad range of cellular stress factors; p53 functions as a node for organizing whether the cell responds to various types and levels of stress with apoptosis, cell cycle arrest, senescence, DNA repair, cell metabolism, or autophagy. Moreover, p53 controls transactivation of target genes, which is an essential feature of stress response pathways [37–39]. In other words, p53 activation leads to a complicated network of responses to the various stress signals encountered by cells [40–44]. The mitochondrial respiratory chain produces NO, which can generate other RNS when cells are under hypoxic conditions. Although excess ROS and

RNS can engender oxidative and nitrosative stress, moderate to low levels of both function in cellular signaling pathways. Especially important are the roles of these mitochondrion-generated free radicals in hypoxic signaling pathways, which have important implications for cancer, inflammation, and various other diseases [25, 45]. Hypoxic signaling events include vasodilation, modulation of mitochondrial respiration, and cytoprotection following ischemic insult. These phenomena are attributed to the reduction of nitrite anions to NO if local oxygen levels in tissues decrease [46], which activates the expression of genes through oxygen-sensitive transcription factors including HIF and NF- κ B. Hypoxia-dependent gene expression can have important physiological or pathophysiological consequences for an organism, depending upon the cause of the hypoxic insult [47]. These NO signaling and hypoxia signaling pathways are linked to the p53 pathway [48], because recent studies have shown that HIF2 α inhibition promotes p53-mediated responses by disrupting cellular redox homeostasis, thereby permitting ROS accumulation and DNA damage [49]. Reportedly, hypoxia activates the tumor suppressor protein p53 by upregulating Sema3E expression [50].

TGF- β -BMP signaling is involved in developmental morphogenesis and cancer morphogenesis. Morphogens such as those of the TGF- β family inhibit and stimulate basic cell proliferation, respectively, at high and low concentrations. A signaling gradient of declining TGF- β concentration regulates the inhibition and stimulation of cell proliferation [51]. ROS can activate TGF- β either directly or indirectly via the activation of proteases. In addition, TGF- β itself induces ROS production as part of its signal-transduction pathway. Pulmonary tissues are vulnerable to the toxic effects of inhaled air. The oxidant pathways are especially relevant in the lung, where TGF- β is known to have a role in tissue repair and connective tissue turnover. In pulmonary fibrosis and renal endothelial cells, TGF- β activation is considered a hallmark of disease progression [52, 53]. In ovarian cancer, overexpression of FOXG1 contributes to TGF- β resistance through inhibition of p21WAF1/CIP1 expression, which is repressed by p53 [54]. Recent studies have revealed some additional novel functions of the p53 pathway. These include the downregulation of two central cell-growth pathways, the IGF/AKT-1 and mTOR pathways, and the upregulation of the activities of the endosomal compartment [55–57]. The mTOR pathway including the IGF-1/AKT pathway plays critical roles in regulation of cell proliferation, survival, and energy metabolism to shut down cell growth and division to avoid the introduction of infidelity into the process of cell growth and division [58, 59]. In response to stress, IGF-BP3, PTEN, TSC2, AMPK beta 1, and Sestrin1/2 are transcribed by p53, play a critical

TABLE 13.2 Oxidative stress-mediated signaling pathways and their related genes

Categorical Pathways	Gene Name (Symbol)
Canonical Pathway (ontology)	
Reactive Oxygen Species (ROS) Metabolism and Antioxidant Defenses	
Glutathione Peroxidases (GPx)	GPX1, GPX2, GPX3, GPX4, GPX5, GPX6, GPX7, GSTZ1
Peroxioredoxins (TPx)	PRDX1, PRDX2, PRDX3, PRDX4, PRDX5, PRDX6
Other Peroxidases	CAT, CSDE1, CYGB, DUOX1, DUOX2, EPX, GPRI56, LPO, MGST3, MPO, PIP3-E, PTGS1, PTGS2, PXDN, PXDNL, TPO, TTN
Other Antioxidants	ALB, APOE, GSR, MT3, SELS, SRXN1, TXNDC2, TXNRD1, TXNRD2
Superoxide Dismutases (SOD)	SOD1, SOD2, SOD3
Other Genes Involved in Superoxide Metabolism	ALOX12, CCS, CYBA, DUOX1, DUOX2, GTF2L, MT3, NCF1, NCF2, NOS2A, NOX5, PREX1, PRG3
Genes Involved in ROS Metabolism	AOX1, BNIP3, EPHX2, MPV17, SFTPD
Oxidative Stress-Responsive Genes	ANGPTL7, ATOX1, CAT, CCL5, CSDE1, DGKK, DHCR24, DUSP1, EPX, FOXM1, GLRX2, GPRI56, GSS, KRT1, LPO, MBL2, MPO, MSRA, MTL5, NME5, NUDT1, OXR1, OXSR1, PDLIM1, PIP3-E, PNKP, PRDX2, PRDX5, PRDX6, PRNP, RNF7, SCARA3, SELS, SEPP1, SGK2, SIRT2, SRXN1, STK25, TPO, TTN
p53 Signaling Pathway	
Induction of Apoptosis	BAX, BID, CDKN1A, CRADD, E124, FADD, FASLG (TNFSF6), FOXO3, PCBP4, PRKCA, TNFRSF10B, TP53, TP73, TP73L
Antiapoptosis	BCL2, BCL2A1, BIRC5, CASP2, HDAC1, IGF1R, MCL1, NFKB1, REL, TNF, TNFRSF10
Other Apoptosis Genes	APAF1, BRCA1, CASP9, E2F1, GADD45A, GML, LRDD, P53AIP1, SIAH1, SIRT1, TP53BP2, TRAF2
Cell Cycle Arrest	CDKN1A, CDKN2A, CHEK1, CHEK2, GADD45A, GML, MYC, PCAF, PCBP4, RPRM, SESN1, SESN2
Cell Cycle Checkpoint	ATR, BRCA1, CCNE2, CCNG2, CDKN2A, RB1, TP53
Negative Regulation of the Cell Cycle	BAX, BRCA1, CDKN2A, MSH2, NF1, PTEN, RB1, TP53, TP73, TP73L, TSC1, WT1
Regulation of the Cell Cycle	BRCA2, CDC2, CDC25A, CDK4, E2F1, E2F3, HK2, IGF1R, KRAS, PPMID, PRKCA, STAT1, TADA3L, TP53BP2
Other Cell Cycle Genes	BIRC5, CCNH, CCNB2, ESRI, MLH1, PCNA, PRC1
Negative Regulation of Cell Proliferation	BAL1, BCL2, BTG2, CDKN1A, CDKN2A, CHEK1, GML, IFNB1, IL6, MDM2, MDM4, NF1, PCAF, PPM1D, SESN1
Positive Regulation of Cell Proliferation	IGF1R, IL6
Cell Proliferation	BRCA1, CDC25A, CDC25C, CDK4, E2F1, MYC, PCNA, PRKCA
Cell Growth and Differentiation	ESR1, MCL1, MYOD1
Other Genes Related to Cell Growth, Proliferation, and Differentiation	EGRI, FOXO3A, JUN, KRAS, PTTG1
DNA Repair Genes	ATM, ATR, BRCA1, BTG2, CCNH, DNMT1, GADD45A, MSH2, PCNA, PTTG1, TP53, XRCC5
Human Nitric Oxide Signaling Pathway	
Genes with Nitric Oxide Synthase or Oxidoreductase activity	NOS1, NOS2A, NOS3, NQO1
Positive Regulators of Nitric Oxide Biosynthesis	HSP90AB1 (HSPCB), INS
Negative Regulators of Nitric Oxide Biosynthesis	DNCL1, GLA, IL10

Other Genes Involved in NO Biosynthesis	AKT1, ARG2, DDAH2, DNCL1, EGFR, GCHI, GCHFR
Genes Induced by NO	CDKN1A, IL8, JUN, VEGFA
Genes Suppressed by NO	CCNA1, MYB, TROAP
Genes Involved in NO Signaling Pathway	CAMK1, DLG4, GRIN2D, NOS1, PPP3CA, PRKAR1B, PRKCA
Genes Involved in Superoxide Release	ALOX12, DUOX1, DUOX2, NOX5, PRG3
Genes with Oxidoreductase Activity	ALOX12, CYBA, DUOX1, DUOX2, NOS2A, NOX5, SOD1, SOD2, SOD3
Genes with Peroxidase Activity	DUOX1, DUOX2
Genes with Superoxide Dismutase Activity	SOD2
Other Genes Involved in Superoxide Metabolism	CCS, NCF1, NCF2, PREX1
Antiapoptosis Genes	MPO, MTL5, NME5, PRDX2, RNF7
Genes with Antioxidant Activity	APOE, MT3, SELS, SOD1, SOD3, SRXN1 (C20orf139)
Genes with Glutathione Peroxidase Activity	GPX1, GPX2, GPX3, GPX4, GPX5, GPX6, LOC493869
Genes with Oxidoreductase Activity	CAT, EPX, GPX1, GPX2, GPX3, GPX4, GPX5, GPX6, LPO, MPO, MSRA, PRDX2, PRDX6, SOD1, SOD2, SRXN1 (C20orf139), TPO, TXNRD2
Genes with Peroxidase Activity	CYGB, EPX, GPX156, LPO, MPO, PRDX2, PRDX5, PRDX6, TPO, TTN, UNR
Transcription Regulators	FOXO1, GLRX2, SCRT2, SIRT2, SOD2, UNR
Other Genes Involved in Oxidative Stress	ATOX1, DUSP1, GSS, KRT1, MBL2, NUDT1, OXR1, PNKP, PRNP, SCARA3, SEPP1, SGK2
DNA Damage Signaling	
Apoptosis	ABLI, BRCA1, CIDEA, GADD45A, GADD45G, GML, IHPK3, PCBP4, AIFM1 (PDCD8), PPP1R15A, RAD21, TP53, TP73
Cell Cycle Arrest	CHEK1, CHEK2, DDIT3 (CHOP), GADD45A, GML, GTSE1, HUS1, MAP2K6, MAPK12, PCBP4, PPP1R15A, RAD17, RAD9A, SESN1, ZAK
Cell Cycle Checkpoint	ATR, BRCA1, FANCG, NBN (NBS1), RAD1, RBBP8, SMC1A (SMC1L1), TP53
Damaged DNA Binding	ANKRD17, BRCA1, DDB1, DMC1, ERCC1, FANCG, FEN1, MPG, MSH2, MSH3, N4BP2, NBN (NBS1), OGG1, PMS2L3 (PMS2L9), PNKP, RAD1, RAD18, RAD51, RAD51L1, REV1 (REV1L), SEMA4A, XPA, XPC, XRCC1, XRCC2, XRCC3
Base-Excision Repair	APEX1, MBD4, MPG, MUTYH, NTHL1, OGG1, UNG
Double-Strand Break Repair	CIB1, FEN1, XRCC6 (G22P1), XRCC6BP1 (KUB3), MRE11A, NBN (NBS1), PRKDC, RAD21, RAD50
Mismatch Repair	ABLI, ANKRD17, EXO1, MLH1, MLH3, MSH2, MSH3, MUTYH, N4BP2, PMS1, PMS2, PMS2L3 (PMS2L9), TP73, TREX1
Other Genes Related to DNA Repair	APEX2, ATM, ATRX, BTG2, CCNH, CDK7, CRY1, ERCC2 (XPD), GTF2H1, GTF2H2, IGHMBP2, LIG1, MNAT1, PCNA, RPA1, SUMO1
Mitochondria	
Membrane Polarization & Potential	BAK1, BCL2, BCL2L1, BNIP3, SOD1, TP53, UCP1, UCP2, UCP3
Mitochondrial Transport	AIP, BAK1, BCL2, BCL2L1, BNIP3, CPT1B, CPT2, DNAJC19, FXC1 (TIMM10B), GRPEL1, HSP90AA1, HSPD1, IMMP2L, MFN2, MIPEP, MTX2, STARD3, TP53, TSPO, UCP1, UCP2, UCP3
Small Molecule Transport	SLC25A1, SLC25A10, SLC25A12, SLC25A13, SLC25A14, SLC25A15, SLC25A16, SLC25A17, SLC25A19, SLC25A2, SLC25A20, SLC25A21, SLC25A22, SLC25A23, SLC25A24, SLC25A25, SLC25A27, SLC25A3, SLC25A30, SLC25A31, SLC25A37, SLC25A4, SLC25A5
Targeting Proteins to Mitochondria	AIP, DNAJC19, FXC1 (TIMM10B), GRPEL1, HSPD1, IMMP2L, MFN2, MIPEP, TSPO
Mitochondrial Protein Import	AIP, COX10, COX18, DNAJC19, FXC1 (TIMM10B), GRPEL1, HSPD1, MIPEP, SH3GLB1

(Continued)

TABLE 13.2 Continued

Categorical Pathways Canonical Pathway (ontology)	Gene Name (Symbol)
Outer Membrane Translocation	TOMM20, TOMM22, TOMM34, TOMM40, TOMM40L, TOMM70A
Inner Membrane Translocation	FXC1 (TIMM10B), IMMP1L, IMMP2L, OPA1, TAZ, TIMM10, TIMM17A, TIMM17B, TIMM22, TIMM23, TIMM44, TIMM50, TIMM8A, TIMM8B, TIMM9
Mitochondrial Fission & Fusion	COX10, COX18, FIS1, MFN1, MFN2, OPA1
Mitochondrial Localization	DNM1L, LRPPRC, MFN2, MSTO1, NEFL, OPA1, RHOT1, RHOT2, UXT
Apoptotic Genes	AIFM2, BAK1, BBC3, BCL2, BCL2L1, BID, BNIP3, CDKN2A, DNML, PMAIP1, SFN, SH3GLB1, SOD2, TP53
Hypoxia Signaling	ANGPTL4, ARNT2, CREBBP, EP300, HIF1A, MT3, PRKAA1
Response to Hypoxia	CAT, CYGB, GPX1, PIP3-E
Response to Oxidative Stress	GPI, IL1A, IL6, IL6ST, NOS2A, NOTCH1, PTX3, RARA
Immune Response	ADM, EPO, HYOU1, VEGFA
Other Genes Related to Stress Response	CYGB, EPO, HBB, HMOX1, NOS2A, PIP3-E
Hemoglobin Complex-Associated Genes	CAT, CYGB, GPX1, PIP3-E
Peroxidase	HIF1AN, HMOX1, MT3, NOS2A, PLOD3, TH
Other Oxidoreductase-Related Genes	CREBBP, DRI, ENO1, EP300, EPAS1, HTATIP, RARA
Transcription Cofactors	ARNT2, BHLHB2, CREBBP, ENO1, EP300, EPAS1, HIF1A, HIF3A, KHSRP, MYBL2, PPARA, RARA
Transcription Factors	HIF1AN, NOTCH1
Other Transcription Factors and Regulators	BAX, ANGPTL4, BIRC5, IL1A, MYBL2, PEA15, PRKAA1, VEGFA
Apoptosis	BIRC5, CASP1
Caspase Activity	BAX, DAPK3, NUDT2
Induction of Apoptosis	EP300
Other Apoptosis Genes	ADM, ARNT2, CASP1, CDC42, CREBBP, EP300, EPAS1, EPO, GNAI1, HIF1A, HIF3A, HMOX1, IGFBP1, IL1A, IL6, IL6ST, IQGAP1, KIT, LEP, PLAU, RARA, VEGFA
Signal Transduction	EEF1A1, PDIA2 (PDIP), PRKAA1, RPL28, RPL32, RPS2, RPS7
Protein Biosynthesis	ARNT2, HIF1A, RARA, SAE1
Protein Heterodimerization	ARNT2, RARA, VEGFA
Protein Homodimerization	DAPK3, KIT, PRKAA1
Protein Amino Acid Phosphorylation	CASP1, CREBBP, ENO1, EP300, IQGAP1, NOS2A, PEA15, PPP2CB, RARA
Protein Binding	ARD1A, CDC42, GNAI1, HYOU1, MAN2B1, PLOD3, PSMB3, SUMO2, TUBA4A (TUBA1)
Other Genes Related to Protein Metabolism	BIRC5, CSTB
Protease Inhibitors	AGTPBP1, CASP1, ECE1, PLAU, PSMB3
Protease Molecules	ADM, ANGPTL4, CHGA, COL1A1, EPO, IGF2, IGFBP1, IL1A, IL6, LEP, NPY, PTX3, VEGFA
Other Extracellular Molecules	DCTN2, IGF2, IL1A, IL6, MT3, NPY, RARA, VEGFA
Cytoskeleton	GPI, IGF2, IGFBP1, IL1A, IL6, KIT, VEGFA
Cell Cycle	ENO1
Cell Proliferation	
Growth Factors	
Other Genes Related to Cell Growth	

Carbohydrate Metabolism	GPI, HK2, LCT, MAN2B1, PEAI5, PRKAA1, SLC2A1, SLC2A4
Lipid Metabolism	AGPAT2, ANGPTL4, PPARA, PRKAA1
One-Carbon Compound Metabolism	CA1
Superoxide Metabolism	MT3, NOS2A
RNA Metabolism	PRPF40A (FBNP3), KHSRP, RARA, RPL28, RPS2, SNRP70
Other Genes Related to Metabolism	ADM, AGPAT2, MOCS3, NUDT2, TH, TST, UCP2
Cardiac Excitation-Contraction (E-C) Coupling	ARNT2, CHGA, DAPK3, GNAI1, IQGAP1, KIT, NOS2A, NOTCH1, NPY, PRKAA1, SPTBN1
TGF- β BMP Signaling	TGFB1, TGFB2, TGFB3
TGF- β	BMP1, BMP2, BMP3, BMP4, BMP5, BMP6, BMP7
BMP	AMH, GDF2 (BMP9), GDF3 (Vgr-2), GDF5 (CDMP-1), GDF6, GDF7, IGFI, IGFBP3, IL6,
GDF	INH A (inhibin a), INHBA (inhibin BA), LEFTY1, LTBPI, LTBP2, LTBP4, NODAL, PDGFB
Activin	INH A (inhibin a), INHBA (inhibin BA), INHBB (inhibin BB), LEFTY1, NODAL
Receptors	ACVR1 (ALK2), ACVR2A, ACVR1L (ALK1), AMHR2, BMPRI A (ALK3), BMPRI B (ALK6), BMPR2, ITGB5 (integrin B5), ITGB7 (integrin B7), LTBPI, NR0B1, STAT1, TGFB11, TGFB1, (ALK5) TGFB2, TGFB3, TGFB4
SMAD	SMAD1 (MADH1), SMAD2 (MADH2), SMAD3 (MADH3), SMAD4 (MADH4), SMAD5 (MADH5)
TGF- β /Activin-Responsive	CDC25A, CDKN1A (p21WAF1 / p21CIP1), CDKN2B (p15LNK2B), COL1A1, COL1A2, COL3A1, FOS, GSC (goosecoid), IGFI, IGFBP3, IL6, ITGB5 (integrin B5), ITGB7 (integrin B7), JUN, JUNB, MYC, PDGFB, SERPINE 1 (PAI-1), TGFB11, TSC22D1 (TGFB114), TGFB1, TGFI
BMP-Responsive	BGLAP (osteocalcin), DLX2, ID1, ID2, JUNB, SOX4, STAT1
Molecules Regulating Signaling of the TGF- β	BAMBI, BMPER, CDKN2B (p15LNK2B), CER1 (cerberus), CHRD (chordin), CST3, ENG (Evi-1), EVI1, FKBP1B, FST (follistatin), HIPK2, NBL1 (DAN), NOG, PLAU (uPA), RUNX1 (AML1), SMURF1
Superfamily	BGLAP (osteocalcin), ENG (Evi-1), ITGB5 (integrin B5), ITGB7 (integrin B7), TGFB11, TGFB1
Adhesion Molecules	BGLAP (osteocalcin), COL1A1, COL1A2, COL3A1, LTBPI, LTBP2, LTBP4, TGFB1
Extracellular Matrix Structural Constituents	AMH, BMP1, BMP2, FST (follistatin), GDF2 (BMP9), GDF3 (Vgr-2), IGFI, IGFBP3, IL6, INHA (inhibin a), INHBA (inhibin BA), INHBB (inhibin BB), PDGFB, PLAU (uPA), SERP1NE1
Other Extracellular Molecules	DLX2, EVI1, FOS, GSC (goosecoid), HIPK2, ID1, JUN, JUNB, MYC, NR0B1, RUNX1 (AML1), SMAD1 (MADH1), SMAD2 (MADH2), SMAD3 (MADH3), SMAD4 (MADH4), SMAD5 (MADH5), SOX4, STAT1, TGFB11, TSC22D1 (TGFB114), TGFI
Transcription Factors and Regulators	
Tumor Necrosis Factor (TNF) Ligand and Receptor	
Induction of Apoptosis	CASP2, CASP3, CASP8, CRADD, FADD, FAS, FASLG, LTA, TNFSF8, TNFSF10, TNFSF10A, TNFSF10B, TNFSF14, TNFSF19, TNFSF25, CD27 (TNFSF7), TNFSF9, TRADD
Caspase Activation	TNFSF10A, TNFSF10B, TNFSF15
Caspase Inhibition	CD27 (TNFSF7), TNFSF14
Anti-apoptosis Genes	CD40LG, FAS, TNF, TNFSF10D, TNFSF6B, CD27 (TNFSF7), TNFSF18
Other Apoptosis-Related Genes	CD40, CD70 (TNFSF7), TNFSF9, LTBR, NGFR, TNFSF10C, TNFSF11B, TNFSF12A, TNFSF14, TNFSF1A, TNFSF1B, TNFSF21, DFFA, PAK1, TRAF2
Inflammatory Response	CD40LG, TNF, TNFSF1A
NF- κ B Signaling Pathway	CASP8, FADD, TNF, TNFSF1A, FASLG, TNF, TNFSF10, TNFSF14, TNFSF15, CD40, EDA2R, LTBR, TNFSF10A, TNFSF10B, CD27 (TNFSF7), TRADD

(Continued)

TABLE 13.2 Continued

Categorical Pathways	Gene Name (Symbol)
Canonical Pathway (ontology)	
JNK Signaling Pathway	EDA2R, TNFRSF19, CD27 (TNFRSF7), MAP2K4, MAPK8, PAK1
Other TNF Superfamily Members and ligands	LTB, PGLYRP1, TNFSF11, TNFSF12, TNFSF13, TNFSF13B, TNFSF4, TNFSF5IP1, TNFSF11A, TNFSF13B, TNFSF13C, TNFSF17, TNFSF19L, TNFSF4, TNFSF8
Transcription Regulators	JUN, PARP1, RBL, TNF, TNFRSF1A, TNFSF25, CD27 (TNFRSF7), TNFSF9
TNFR1 Signaling Pathway	ARHGD1B, CAD, HRB, LMNA, LMNB1, LMNB2, MADD, MAP3K1, MAP3K7, PAK2, PRKDC, SPTAN1
FAS signaling pathway	IKBKG, LTA, TRAF3, TNFRSF14, TNFRSF1A, TNFRSF1B
Induction of Apoptosis	NFKB1, TNFAIP3
Anti-apoptosis Genes	NFKB1A, TNFRSF1B, TRAF1, TRAF2
Other Apoptosis Genes	NFKB1
Inflammatory Response	CHUK, IKBKB, IKBKG, NFKBIA, TNFAIP3
NF- κ B Signaling Pathway	IKKBK, IKBKG, NFKB1, NFKBIA
Transcription Regulators	DUSP1, HRB, IKBKAP, MAP3K1, MAP3K14, TANK
TNFR2 Signaling Pathway	

role as negative regulators, and lead to the reduction in the activities of these two pathways. Furthermore, p53 transcriptionally regulates TSAP6, Chmp4C, Caveolin-1, and DRAM, which are critical genes in the endosomal compartment, increases exosome secretion and the rate of endosomal removal of growth factor receptors from cell surface, and enhances autophagy [60–63]. It is thought that these p53-mediated activities slow down cell growth and division, conserve and recycle cellular resources, communicate with adjacent cells and dendritic cells of the immune system, and inform other tissues of the stress signals [55, 64, 65].

TNF ligand-receptor signaling occurs because TNF, as a multifunctional cytokine, can induce cell death through receptor-mediated caspase activation and mitochondrial dysfunction by a trigger of oxidative stress induced in cardiovascular disease, neuronal disease, and cancer [66]. Opposing these cell death-promoting signals, binding of TNF receptors can also trigger survival signal activation. A critical balance among various intracellular signaling pathways determines the predominant *in vivo* bioactivity of TNF, as best exemplified by the differential responses of various organs.

A major source of ROS in cells is the mitochondria. Electron leakage from the mitochondrial respiratory chain can react with molecular oxygen, resulting in the formation of the superoxide anion radical, which can subsequently be converted to other ROS. In phagocytes and some cancer cells, ROS can be produced through a reaction that is catalyzed by NADPH oxidase complexes. When attackers from the outside, such as environmental stressors, damage mitochondria, electron leakage is also induced; this dysfunction induces severe problems in tissues [67–70]. Mitochondrial dysfunction causes the onset of some diseases [71–74]. Recent evidence has shown that mitochondrial dysfunction is related closely to insulin resistance and metabolic syndrome. The underlying mechanism of mitochondrial dysfunction is very complex, including genetic factors from both the nucleus and mitochondrial genome, with numerous environmental factors also impacting [75].

Exposure to air pollution, including particles, metals, and other organic compounds as environmental stressors, is associated with pulmonary diseases and cancer. The mechanisms of induced health effects are believed to involve oxidative stress. Oxidative stress mediated by airborne particles and/or fibers might arise from direct generation of ROS from the surfaces of particles and fibers, soluble compounds such as transition metals or organic compounds, and activation of inflammatory cells capable of generating ROS and RNS. Generation of ROS/RNS can cause covalent modifications to DNA directly, or they can initiate the formation of genotoxic lipid hydroperoxides. The resulting oxidative DNA damage can engender

changed gene expression such as upregulation of tumor promoters and downregulation of tumor suppressor genes; the DNA damage might therefore be implicated in cancer development. This chapter describes the important role of free radicals in particle- and fiber-induced cellular damage, the interaction of ROS with target molecules, especially with DNA, and the modulation of specific genes and transcription factor caused by oxidative stress. Consequently, various environmental stressors cause cellular damage through oxidative stress induction and many signaling pathways. However, what environmental stressor is dominant in which signaling pathway is not always clear. Therefore, identifying gene expression signatures extracted from microarray data can clarify how environmental stressors may damage cells and engender diseases.

13.2.1 Case Studies in Tissues or Organs from Rats Exposed to Environmental Stressors

Many animal models have been studied to elucidate mechanisms of action of oxidants or antioxidants. Research on oxidative stress and its defense has expanded dramatically because of its potential benefit in disease prevention and health promotion. In particular, rats exposed to stress-induced chemicals have been extensively studied in biological systems such as cell cultures, animal models, and clinical trials [76–79]. Therefore, 33 independent studies in rats are focused on in this chapter because these studies used microarrays for which gene expression data are publicly available from the Gene Expression Omnibus (GEO; <http://www.ncbi.nlm.nih.gov/gds>). Those microarray data with the same platform GPL341 (Affymetrix) sets in rats were downloaded for this study. All data sets were normalized across all arrays by Z-score transformation methods after combination with respect to probe IDs. The normalized values were filtered with oxidative-related genes listed in this work (Table 13.2), and then the top 10 genes from the upregulated and downregulated genes were chosen to analyze gene expression signatures (Table 13.3). The selected genes were classified by using principal component analysis to create gene expression signatures of oxidative stress and were divided into six groups. Most selected genes could be assigned to gene ontology (GO) categories: DNA repair, oxygen and reactive oxygen species metabolism, and response to stress, but cyclins and cyclin dependent kinase contained in “Apoptosis related genes, Cell Cycle Arrest and Checkpoint, Regulation of the Cell Cycle, Regulation of Cell Proliferation, Cell Growth and Differentiation” of “p53 signaling” and “TGF-beta signaling” were not observed. Experimental conditions selected from GPL341 data sets in this work were almost all of short-period exposure using *in vivo* and *in vitro* culture systems of rats. It is noteworthy that

TABLE 13.3 Environmental stressors induce different gene expression signatures

Environmental Stressors (target organ or tissues)	Up-gene	Down-gene	GEO ID
Cluster 1			
Methylprednisolone (kidney)	Apoe, Gpx2, Ngb, Nos2, Prdx6, Tmod1, Tnpl, Tpo	Brcal, Cry2, Fen1, Hus1, Ptgs2, Ptgl1, Rad50, Srxn1, Xrcc6	GDS964
Methylprednisolone (liver)	Aass, Atrx, Nefl, Nqo1, Sed1, Slc41a3, Srd5a2, Tmod1, Tnpl	Chck1, Cry2, Lig1, Mgmt, Pold1, Pold3, Rad50, Rad52, Smc3, Xrcc6	GDS972
Streptozotocin (penile cavernosal)	Apc, Cat, Duox2, Gpx2, Gpx6, Gsr, Lpo, Slc38a1, Smc3, Tpo	Atrx, Gpx7, Nos2, Park7, Ptgs2, Sed1, Slc38a4, Slc41a3, Srxn1, Zmynd17	GDS1393
Trimethyltin (hippocampus)	Apex1, Dnm2, Fance, Gpx7, Lpo, Mgmt, Park7, Prnp, Txnip, Ucp3	Apc, Apoe, Hbz, Mpp4, Ptgs2, Smc3, Srd5a2, Tnpl, Tpo	GDS2555
Octreotide (gastric ECL)	Brcal, Brcal, Dnm2, Duox2, Msh2, Nox4, Tmod1, Tpo, Xirp1	Apex1, Atrx, Cry2, Gpx6, Nos2, Slc38a1, Slc38a4, Slk, Tmod1, Tpo	GDS2558
Cluster 2			
Fibronectin (ventricular myocytes)	Apoe, Atrx, Chaf1a, Ngb, Rad51c, Smc3, Srxn1, Tpo, Zmynd17	Actb, Atrx, Gsr, Mutyh, Ngb, Prdx6, Rad52, Smc3, Tpo, Txnd1	GDS696
Protein restriction (visceral adipose tissue)	Aass, Apc, Gpx6, Gsk1, Ngb, Prnp, Rad51c, Sed1, Tmod1, Tnpl	Brcal, Chaf1a, Lpo, Mutyh, Nos2, Ptgl1, Slc38a1, Slc38a4, Tpo, Ung	GDS880
Heregulin (ureteric buds)	Dhcr24, Hus1, Ldha, Mif, Park7, Rad1, Rad50, Sed1, Tdg, Ung	Actb, Atrx, Nos2, Nox4, Nqo1, Ptgs1, Rad23a, Srxn1, Txnd1	GDS1518
Kainic acid (hippocampi)	Apoe, Brcal, Cry2, Hus1, Nos2, Ptgs2, Ptgl1, Rad50, Rad51c, Srd5a2, Tmod1	Chaf1a, Hbz, Lpo, Mb, Pold3, Tnpl, Tpo, Ucp3, Ung, Zmynd17	GDS1626
Ethanol (pancreas)	Apoe, Atrx, Hbz, Ogg1, Ptgs2, Sed1, Srxn1, Tmod1, Txnd2, Zmynd17	Cry2, Hus1, Mb, Msh2, Nox4, Nthl1, Prdx6, Rad52, Slk, Srd5a2	GDS2107
Sulfur dioxide (lung)	Aass, Brcal, Cry2, Hus1, Nos2, Ptgs2, Ptgl1, Rad50, Tpo, Zmynd17	Apex1, Brcal, Gpx6, Nos2, Nox4, Rad23a, Rad51c, Srd5a2, Tnpl, Tpo	GDS2372
Hypoxia (adrenal gland)	Chaf1a, Duox2, Ldha, Ngb, Pold3, Rad23a, Slc41a3, Tpo, Txnd2	Aass, Apc, Apoe, Atrx, Cry2, Lpo, Nox4, Rad52, Srd5a2, Tnpl	GDS2457
Methylprednisolone (skeletal muscles)	Aass, Atrx, Hbz, Ngb, Rad1, Sed1, Slc38a5, Tmod1, Tpo, Xirp1	Als2, Atrx, Brcal, Cat, Gsr, Nefl, Nox4, Nqo1, Slc41a3, Trpc2	GDS2688
Cluster 3			
Forskolin (pheochromocytoma cell)	Aass, Apex1, Brcal, Chck1, Duox2, Gpx2, Hbz, Nxn, Ptgs1, Ptgl1	Atrx, Cat, Cygb, Ehd2, Gpx3, Gpx4, Gpx7, Sed1, Sod3, Vim	GDS1363
N-methyl-N-nitrosourea (mammary tumors)	Cat, Ehd2, Gadd45a, Gsk1, Mgmt, Prdx3, Prdx6, Sed1, Srxn1, Ube2a	Dpagt1, Gab1, Gpx3, Lpo, Mpg, Nxn, Prdx4, Prnp, Rad52, Txnip	GDS1452
Retinoic X receptor ligand LG100268 (mammary gland)	Brcal, Dnm2, Gpx6, Hbz, Mpp4, Nefl, Nos2, Slc38a1, Tpo	Aass, Atrx, Chaf1a, Gsr, Idh1, Nox4, Prdx1, Rad23a, Xrcc1, Zmynd17	GDS1922
Angiotensin-1 (aortic rings)	Apex1, Dnm2, Mgmt, Ngb, Pold3, Rad50, Slc38a1, Srd5a2, Srxn1, Ucp3	Atrx, Brcal, Chaf1a, Gpx6, Mb, Nox4, Rad23a, Slk, Tpo, Zmynd17	GDS2037

Isoflurane (basolateral amygdalae)	Brc2, Gpx2, Ifi172, Mif, Nos2, Ptg1, Rad1, Rad51c, Tpo, Ung	Atr, Atr, Gsr, Nox4, Pold3, Prmp, Pigs2, Sed1, Smc3, Xrc6	GDS2073
Fe deficiency (jejunum)	Aass, Gadd45a, Gsr, Nqo1, Srxn1, Tdg, Tmod1, Txnd1, Xrc1,	Gpx7, Hba-a2, Lpo, Mgmt, Nthl1, Pms2, Rad52, Smc3, Xpc, Xrc6	GDS2093
Pregnenolone/6alpha-carbonitrile (liver)	Dnm2, Gpx6, Lpo, Nqo1, Prdx5, Pigs2, Sed1, Srxn1, Tpo, Txnd1	Aass, Als2, Apoe, Hbz, Nos2, Rad51c, Sld38a5, Srd5a2, Tpo	GDS2194
Particulate matter (TPM)/of cigarette smoke (lung)	Aass, Apc, Brcal, Brc2, Cry2, Gpx2, Hus1, Sld38a4, Tpo, Txnd1	Chaf1a, Mb, Mutyh, Nos2, Pold3, Pigs2, Rad50, Tmod1, Tnpl, Tpo	GDS2616
Genistein (mammary epithelial cells)	Atr, Brc2, Hba-a2, Ngb, Rad23a, Rad52, Smc3, Tpo, Ung, Zmynd17	Apex1, Brcal, Gpx6, Lpo, Ptg1, Sld38a4, Srd5a2, Tnpl, Tpo	GDS2639
Aging (hippocampi)	Atr, Ehd2, Gadd45a, Gt2h1, Mgmt, Nefl, Nthl1, Pigs2, Ptg1, Srxn1	Erc6, Mlh1, Pms2, Rad50, Rad52, Sld38a1, Trpe2, Txnip, Wrnpl, Xpc	GDS2774
Depolarization. (midbrain)	Apc, Apoe, Atr, Brcal, Pold3, Pigs2, Rad23a Sld38a4, Smc3, Zmynd17	Apex1, Atr, Chaf1a, Gpx2, Hba-a2, Nos2, Ptg1, Srxn1, Tmod1, Tnpl	GDS2901
Aristolochic acid (kidney)	Apoe, Atr, Cry2, Ngb, Ppp1r15b, Sed1, Srxn1, Tpo	Apoe, Atr, Fen1, Gadd45a, Gpx6, Ifi172, Pold3, Rad52, Txnip, Zmynd17	GSM1038
Cluster 4			
Pyridine activator (ventricular myocytes)	Aass, Chaf1a, Dhcr24, Nthl1, Pmx1, Pold3, Rad52, Sed1, Sld38a1, Xirp1	Apex1, Brc2, Cry2, Gpx6, Hus1, Lpo, Mutyh, Pold1, Rad51c, Tpo	GDS902
Reinnervation (tibialis anterior muscles)	Apex1, Atr, Chk1, Gpx6, Mgmt, Nefl, Nox4, Pold3, Smc3, Tnpl	Atr, Brcal, Chaf1a, Lpo, Nthl1, Rad50, Sld41a3, Txnd2, Ung, Zmynd17	GDS2243
Hyperinsulinemia (kidney)	Apoe, Chaf1a, Gpx6, Hbaa2, Lpo, Ngb, Pigs2, Sed1, Sld, Srd5a2	Apc, Atr, Duox2, Hbz, Mb, Nefl, Sld38a4, Tmod1, Tnpl, Txnip	GDS2361
Cluster 5			
Sulfur mustard bis-(2-chloroethyl) sulfide (lung)	Apoe, Gadd45a, Gpx2, Hba-a2, Mif, Prdx5, Pigs2, Sed1, Smc3, Srxn1	Apc, Atr, Dnm2, Duox2, Gab1, Gpx6, Mutyh, Nox4, Srd5a2, Tpo	GDS1027
Amoxicillin (intestine)	Apc, Apoe, Atr, Lpo, Mutyh, Sld38a4, Tnpl, Tpo	Apex1, Chaf1a, Cry2, Gpx2, Ngb, Nox4, Sed1, Tpo, Trpe2, Zmynd17	GDS1273
Ischemia (heart)	Apc, Apoe, Gpx7, Nos2, Nox4, Nxn, Prdx4, Rad52, Sed1, Smc3	Atr, Brcal, Chaf1a, Hus1, Lpo, Pold1, Prdx5, Rad51c, Sld38a4, Xirp1	GDS1959
Cluster 6			
Carbon tetrachloride (liver)	Chaf1a, Ehd2, Gpx2, Hba-a2, Nefl, Prmp, Pigs2, Sld38a4, Vim, Zmynd17	Apoe, Dpagt1, Gab1, Hus1, Nos2, Nxn, Pigs1, Sld, Trpe2, Txnip	GDS1354
Dexamethasone (marrow-derived stromal cells)	Apoe, Ehd2, Gpx6, Mgmt, Mpp4, Srd5a2, Tmod1, Tpo	Apex1, Apoe, Chaf1a, Dnm2, Nos2, Rad50, Rad51c, Sld, Sme1a, Smc3	GDS2231

microarrays capture only transient responses to oxidative stimuli. However, we can predict the underlying mechanism of environmental stressors through oxidative signatures for gene expression. For example, methylprednisolone [80, 81], streptozotocin [82], trimethyltin [83], and octreotide [84] upregulate GPXs, NOS, and NOX, suggesting that environmental stressors in cluster 1 can activate the NO signaling that leads inflammation or other cellular damage. Thioredoxin interacting protein, Txnip, was identified as a unique gene in this category. In cluster 2 (GDS696 [85], GDS880 [86], GDS1518 [87], GDS1626 [88], GDS2107 [89], GDS2372 [90], GDS2457 [91], GDS2688 [92]), Rad23, Rad50, and Rad51c, which are DNA repair and recombination proteins, and the other DNA replication proteins DNA-directed DNA polymerase delta (Pold1) and Pold3 were classified. This classification suggests that environmental stressors in cluster 2 such as fibronectin, protein restriction, heregulin, kainic acid, hypoxia, and ethanol harmed mitochondria or damaged DNA more than the stressors in cluster 1. In cluster 3 (GDS1363 [93], GDS1452 [94], GDS1922 [95], GDS2037 [96], GDS2073 [97], GDS2093 [98], GDS 2194 [99], GDS2616 [100], GDS2639 [101], GDS2774 [102], GDS2901 [103], GDM1038 [104]), Gadd45a, Nth11, Mgmt, Mpp4, Chk1, Cry2, and Txnrd1 were observed as upregulated genes. Since these genes interact with DNA repair and are p53 signaling activated, it is possible that environmental stressors in cluster 3 cause DNA damage and remodeling. In cluster 4 (GDS902 [105], GDS2243 [106], GDS2361 [107]), DNA replication proteins Pinx1 and Slk were detected as unique genes. In particular, STE20-like kinase (Slk) appears to influence cell survival and proliferation. In fact, Slk has been suggested to have a central growth-suppressive role for Mst orthologs, with intriguing possible links to other established tumor suppressors through work in model organisms. A part of the genes in cluster 5 (GDS1027 [108], GDS1273 [109], GDS1959 [110]) were overlapped in clusters 1 and 3. In cluster 6 (GDS1354 [111], GDS2231 [112]), a part of the genes were overlapped in clusters 2 and 4. However, Vim was detected as a unique gene in GDS1354, which is an experiment in cirrhotic rats [111], since upregulation of this gene was also observed in renal cell carcinoma [113], cerebral tumors [114], and germ cell and trophoblastic neoplasms [115].

13.2.2 Prediction of Biological Influences from Gene Expression Signatures in Rats Exposed to Environmental Stressors

These clusters were characterized by several biological functions. Data of gene expression signatures in Table 13.3 were analyzed through the use of Ingenuity

Pathways Analysis (Ingenuity® Systems, www.ingenuity.com). The Functional Analysis identified the biological functions that were most significant to the data set. Molecules from the data set that met the expression value associated with biological functions and/or diseases in Ingenuity's Knowledge Base were considered for the analysis. Right-tailed Fisher's exact test was used to calculate a *P*-value determining the probability that each biological function and/or disease assigned to that data set is due to chance alone. In Table 13.4, the highest probability predictive function in cluster 1 showed "DNA Replication, Recombination, and Repair"; that in cluster 2 showed "Small Molecule Biochemistry"; that in cluster 3 also showed "Small Molecule Biochemistry"; that in cluster 4 showed "DNA Replication, Recombination, and Repair"; that in cluster 5 showed "Cancer"; and that in cluster 6 showed "Lipid Metabolism." In "Small Molecule Biochemistry," genes related with "degradation or catabolism of hydrogen peroxide" like CAT, GPX3, and GPX4 and peroxidation of lipid were affected in clusters 2, 3 and 6. In "Gene expression," genes related with "binding of p53 consensus binding site" like APEX1, BRCA1, and PTTG1 were affected. For instance, the top-rated network activated by retinoic X receptor ligand LG100268 is shown in Figure 13.1. This network consists of 33 molecules meet 14 molecules, which belong to biological functions of DNA replication, recombination and repair, cancer, and cell cycle.

13.2.3 Oxidative Stress-Mediated p53 Pathways in Human Tissues

Among many oxidative responsive pathways, p53 signaling has been studied extensively and has been thought to play a main role in the orchestration of oxidative events in cells. It coordinates the cellular responses to a broad range of cellular stress factors. In fact, p53 functions as a node for organizing whether the cell responds to various types and levels of stress with apoptosis, cell cycle arrest, senescence, DNA repair, cell metabolism, or autophagy, as described above in this chapter [37–39]. To control and fine-tune responses to various stress signals encountered by cells, as a transcription factor that both activates and represses a broad range of target genes, p53 demands an exquisitely complicated regulatory network (Fig. 13.2). The classical model for activation of p53 specifically examines three simple and rate-limiting steps: p53 stabilization induced by ataxia telangiectasia mutated (ATM)/ataxia telangiectasia and Rad3 related (ATR)-mediated phosphorylation, sequence-specific DNA binding, and target gene activation through interaction with the general transcriptional machinery [29]. Recent studies with animal models describe that mouse double minute

TABLE 13.4 Predicted biological functions by gene expression signatures shown in Table 13.3

Cluster	Predictive Biological Functions	<i>P</i> Value
1	DNA Replication, Recombination, and Repair	1.02E-07
	Hematological Disease	1.33E-06
	Cardiovascular System Development and Function	3.62E-06
	Lipid Metabolism	3.62E-06
	Organ Morphology	3.62E-06
2	Small Molecule Biochemistry	2.34E-07
	Cell Cycle	3.80E-06
	DNA Replication, Recombination, and Repair	3.80E-06
	Cell-to-Cell Signaling and Interaction	3.22E-05
	Cell Death	6.18E-05
3	Small Molecule Biochemistry	2.41E-07
	Gene Expression	7.30E-07
	Cellular Compromise	1.93E-06
	Cell Cycle	4.02E-06
	DNA Replication, Recombination, and Repair	4.57E-06
4	DNA Replication, Recombination, and Repair	7.14E-11
	Cell Cycle	1.18E-05
	Cell Death	2.52E-05
	Respiratory System Development and Function	2.52E-05
	Reproductive System Development and Function	1.10E-04
5	Cancer	8.09E-09
	Gastrointestinal Disease	8.09E-09
	Cell Death	1.20E-07
	Dermatological Diseases and Conditions	5.01E-07
	Organismal Functions	3.57E-06
6	Lipid Metabolism	1.45E-09
	Small Molecule Biochemistry	1.45E-09
	Cell-to-Cell Signaling and Interaction	1.26E-08
	Nervous System Development and Function	1.26E-08
	Cell Death	3.49E-08

(Mdm) 2 and MdmX might determine whether a cell responds to p53 activation with growth arrest or apoptosis, but the molecular mechanism of these differential effects remains unknown. In fact, Mdm2 and MdmX can both be recruited to p53 promoter regions. Via a multitude of mechanisms, they can repress transcription of p53 target genes [116–118]. p53 protein binds sequence-specific regions of DNA of the target gene to process sensing and removal of oxidative damage to nuclear DNA and genetic instability. Furthermore, p53 acts as a transcription factor to regulate the expression of many prooxidant and antioxidant genes. A newly refined model for p53 activation includes three key steps: (1) p53 stabilization, (2) antirepression, and (3) promoter-specific activation. Among the three steps, most environmental stressors contribute mainly to p53 stabilization and promoter-specific activation. Several reports describe that low-weight molecules engender induction of stress-induced genes such as NAD(P)H dehydrogenase, quinone (NQO)1, and NQO2, which stabilize and transiently activate p53 and downstream

genes leading to protection against adverse effects of stressors [119–121].

Therefore, to understand how stress-induced genes are downstream within the p53 pathway, we analyzed gene expression of p53 signaling pathways in array data sets GDS2780 [122] and GSE7967 [123] that had been obtained from the GEO database. In the GDS2780 study, six heavy metals and three organic compounds to which liver carcinoma HepG2 cells were exposed responded dramatically to gene expression of CHK1, CHK2, Cyclin B, Cdc2 p21, p53R2, Cop1-1, and Gadd45 [1]. Interestingly, expression levels of p53R2 and Gadd45 responded differently to the heavy metals: p53R2 is likely to associate with mitochondrial DNA and play a critical role in embryogenesis and neurogenesis [124–128]; in contrast, Gadd45 plays a vital role as a cellular stress sensor in the modulation of cell signal transduction in response to stress. Increasing Gadd45 can stabilize p53 activation, leading to cell cycle arrest or progression to apoptosis [129–131]. Consequently, exposure of cultured human cells to heavy metals

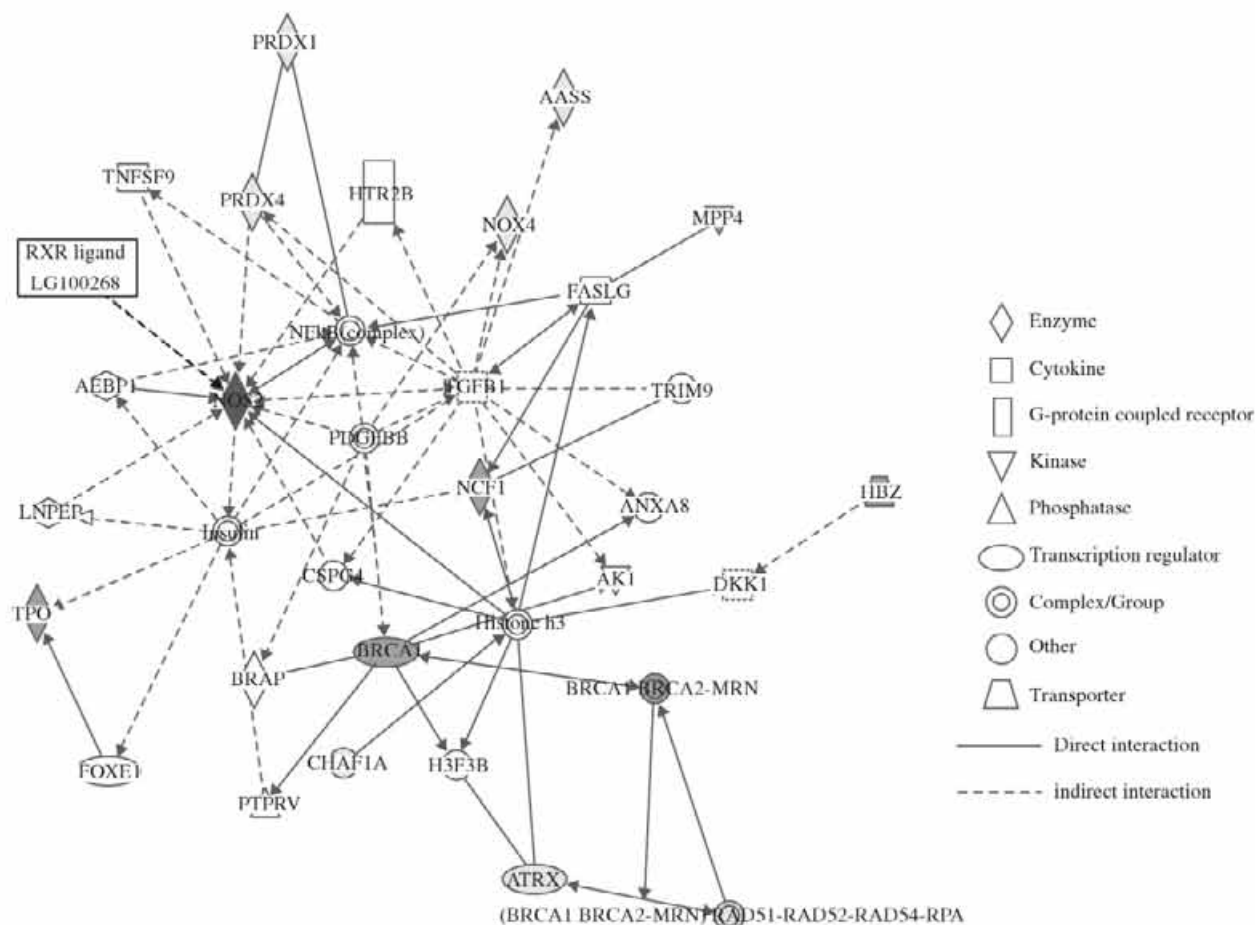


Fig. 13.1 The network generated by retinoic X receptor ligand LG100268 in cluster 3 shown in Tables 13.3 and 13.4: the top-rated network as an example.

dramatically altered the gene expression of oxidative stress-responsive genes. However, in human tissues of the GSE7967 study, the p53 signaling pathway differed from that of heavy metals in the GDS2780 study. Overall, the gene expression signals were weaker than those examined in the GDS2780 study. The GSE7967 study examined cord blood collected at birth from infants whose mothers were exposed or unexposed to arsenic (0.1–68.63 mg/g), showing activation of inflammation and NF- κ B signaling in infants born to mothers exposed to arsenic at high concentration. Therefore, after downloading the data sets, we selected four subjects according to blood concentrations of 0.1, 1.76, 9.66, and 68.63 mg/g; then gene expression of the arsenic exposure-induced responses were visualized in the p53 signaling pathway map. The highest concentration subject showed Gadd45, p53-inducible ribonucleotide reductase small subunit 2 (p53R2), spermatogenic leucine zipper 1 (TSP1), cyclinB, Cdc2, Fas, Noxa, and ATR that were higher than those of the subject with the

low concentration. However, p53 was opposite: high in the low-exposure subject and low in the high-exposure subject, suggesting that the downregulation of p53 facilitates apoptosis and promotes cell proliferation.

Previous works described in our study showed that GSS (glutathione synthetase) and PRDX2 (peroxiredoxin 2) regulated TRADD (TNFRSF1A-associated via death domain), NUDT1 (nucleoside diphosphate linked moiety X-type motif 1), SOD1 (superoxide dismutase 1, soluble), and INSIG1 (Insulin induced gene 1) in the low-exposure group (mean blood concentration 0.142 μ g/g) and that NUDT1 regulated TRADD, TXNRD2 (thioredoxin reductase 2), and PRDX2 in the high-exposure group (21.41 μ g/g), using the theoretical algorithm for identifying optimal gene expression networks (TAO-Gen), which is a Bayesian network algorithm used to describe gene interaction networks [18, 132–134] (Fig. 13.3). In fact, NUDT1 is a DNA repair and recombination protein. The H_2O_2 treatment significantly increased this gene and other oxidative

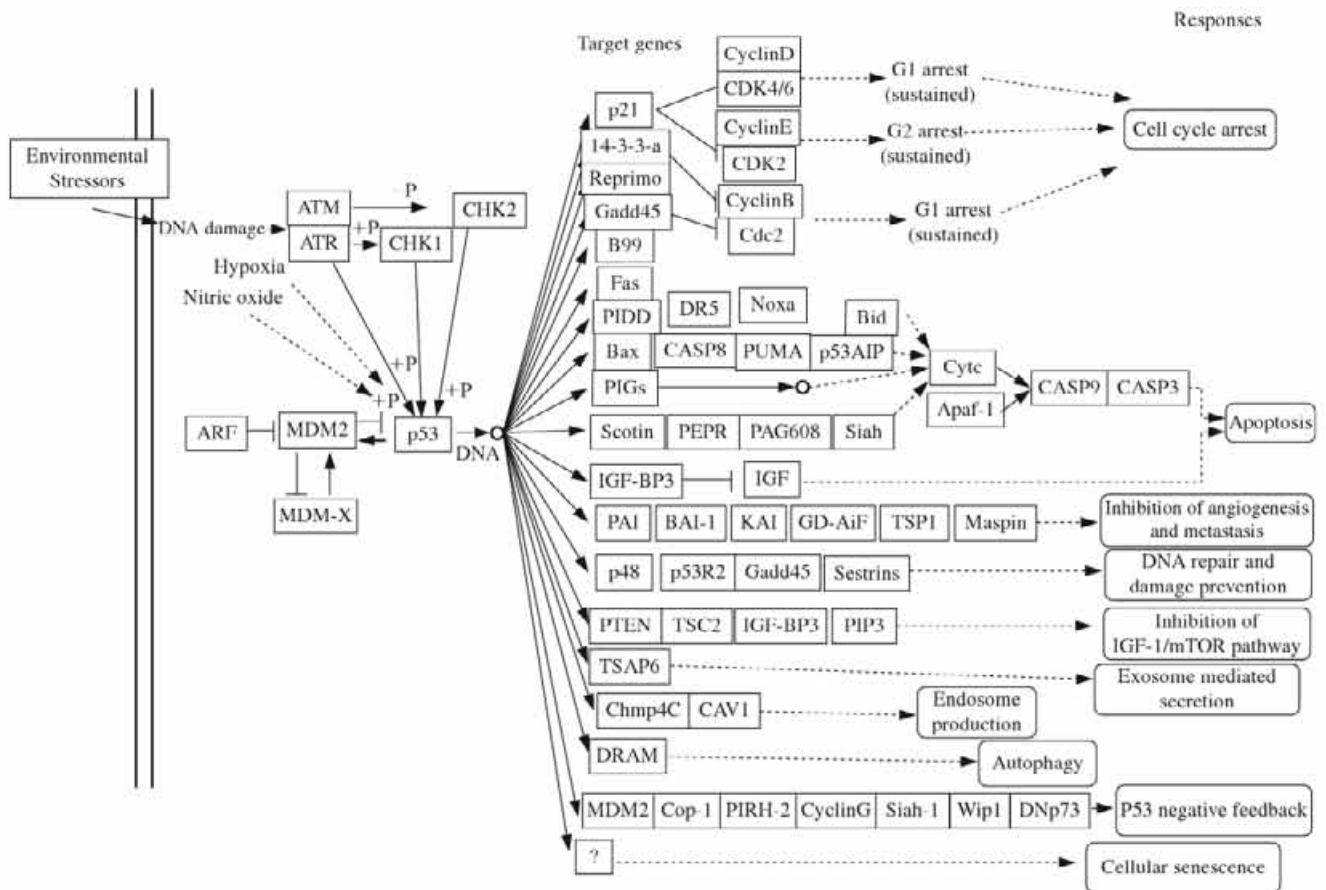


Fig. 13.2 Environmental stressor-mediated p53 signaling pathways. Maps of the p53 signaling pathway partly consulted the KEGG pathway.

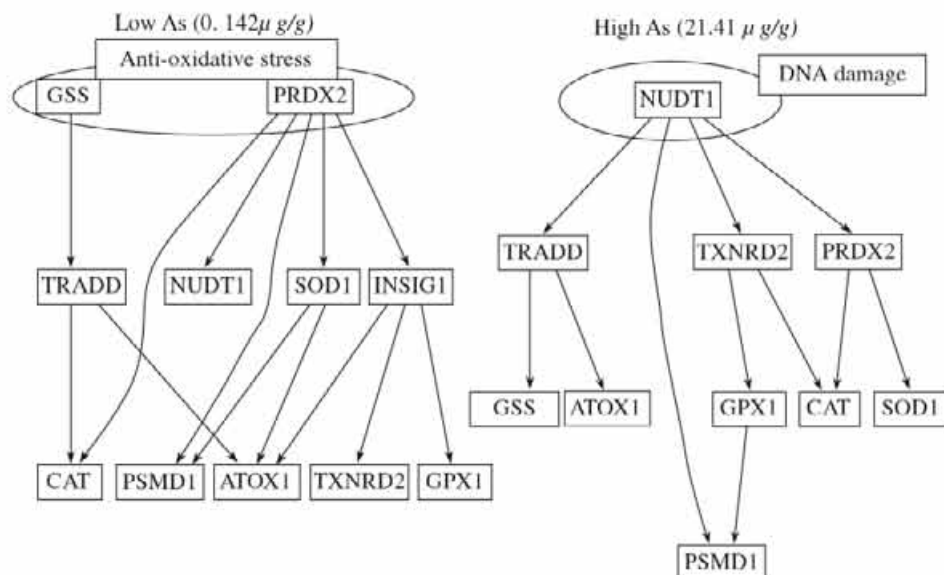


Fig. 13.3 Networks for 11 oxidative stress-related genes selected from the GSE7967 study. TAO-Gen algorithms can predict different mechanisms in low and high exposure to arsenic.

stress genes involved in cell cycle arrest [135]. Results of our analyses suggest that anti-oxidative stress-related genes play key roles in protection against cellular damage in the low-exposure group, but a DNA damage-related gene was dominant in the high-exposure group, in which cell damage would progress. Data sets used in this chapter are from fundamental exposure to environmental stressors in normal tissues and cell lines. Therefore, this discrepancy indicates that gene expression signatures in human clinical tissues or epidemiological studies apparently reflect more inflammation than those of experimental materials, which show acute toxicity in animals after short exposure to oxidants in cell cultures.

13.3 CONCLUSION

In this chapter, we have reviewed gene expression signatures of oxidative stress-mediated signaling pathways by environmental stressors and proposed categorical pathways and canonical pathways of oxidative stress in rat and human systems. Analyses of gene expression signatures in environment-related disease such as neuronal disorders, cancer, and diabetes is an important approach in etiology and risk assessment for human health to elucidate the underlying mechanisms of induced health effects. This will take many more genetic and reverse genetic analyses, combined with functional analysis studies. Furthermore, we have shown that oxidative stress has been associated with many signaling pathways and different environmental stressors impacting different molecules, but they are all connected to the same goals like apoptosis or cell cycle. From a therapeutic point of view, researchers must consider that the best biomarker and/or therapy for oxidative stress-related disease may rely on a combination of several different agents, each specifically targeting one aspect of the oxidative stress machinery.

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SELENOPROTEINS IN CELLULAR REDOX REGULATION AND SIGNALING

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14.1 INTRODUCTION

In 1817 the Swedish chemist Jöns Jacob Berzelius discovered selenium (Se), a chemical element with the atomic number 34 and an atomic weight of 78.96 g/mol. It is a trace nonmetal of the chalcogen group that includes oxygen and sulfur and is present in the Earth's crust and in ocean water at abundances ranging from 0.05 to 2.0 ppm. Sulfur has physical and chemical properties similar to those of Se; however, their noninterchangeable nature in biological systems highlights the substantial differences between these two elements. Se exists naturally as structural analogs of sulfurous molecules, including inorganic selenides (Se^{2-}), selenites (SeO_3^{2-}), and selenates (SeO_4^{2-}), and the organic amino acids selenocysteine and selenomethionine. Although Se is a dietary antioxidant and is required in nanomolar concentrations by organisms in all three domains of life, it is simultaneously toxic, having a median lethal dose in humans of 1.5–6.0 mg/kg body weight with the inorganic salts being more acutely toxic than organic forms [1]. In vertebrates, Se is obtained almost exclusively by ingestion of plants and animals, and the amount of Se in dietary sources depends on the concentration of environmental Se. Currently, the recommended daily allowance of dietary Se for healthy adults is 55 µg/day and the upper tolerable intake level is 400 µg/day [2].

Low-molecular-weight selenocompounds are bioactive, but most cellular biological functions of Se are attributed to genetically encoded selenoproteins. Selenoproteins are

defined by incorporation of the 21st amino acid selenocysteine (Sec), which is an analog of cysteine with the sulfur-containing side chain replaced by a Se-containing side chain. Although Se and sulfur have similar chemical and physical properties, key differences generate divergent character between Sec and cysteine, with Sec being a much more efficient catalyst. The cause of the higher catalytic capacity of Sec is uncertain, but a few ideas have been proposed. For example, Sec has been described as having stronger nucleophilic and electrophilic character than cysteine, potentially aiding in catalysis. Additionally, the selenol of Sec has a lower acid dissociation constant than the thiol of cysteine (pK_a of 5.2 vs. 8.3), rendering the selenol a superior leaving group because of its higher acidity. This also means that at physiological pH, Sec tends toward ionization whereas cysteine tends toward protonation, and at low pH Sec retains much higher reactivity than cysteine. The biological rationale for Sec utilization is enigmatic, but rapid recycling of oxidized Sec to the reduced form as well as resisting irreversible oxidation to selenonic acid could potentially explain the biological pressure to use Sec over cysteine in proteins during evolution [3].

Sec is synthesized from serine after aminoacylation to a Sec-specific tRNA (tRNA^{Sec}) that recognizes the UGA “stop” codon, which typically signals to terminate mRNA translation. Recognition of the UGA codon as a Sec insertion site, as opposed to a termination site, requires a Sec insertion sequence (SECIS) element in selenoprotein mRNA molecules, which is a stem-loop structure that is

typically downstream of the UGA codon [4]. Although selenoproteins are present in eukarya, archaea, and bacteria, the mechanism of Sec incorporation differs between domains in several respects. For example, the bacterial SECIS element is located immediately following the Sec UGA codon, whereas the eukaryotic and archaeal SECIS elements are typically located much farther downstream in the 3' untranslated region (UTR). Additionally, the archaeal genus *Methanococcus* has a 5' UTR SECIS element, while eukaryotes have two distinct forms of 3' UTR SECIS elements [5, 6]. An elongation factor specific to Sec-tRNA^{Sec} has been identified as selB in bacteria, whereas the coordinated function of a SECIS binding protein (SBP2) and the eukaryotic Sec-tRNA^{Sec} elongation factor (EFSec) allows the cotranslational incorporation of Sec instead of termination [7]. Biosynthesis of Sec in eukarya and archaea is accomplished by the sequential actions of *O*-phosphoseryl-tRNA kinase (PSTK) and *O*-phosphoserine-tRNA:Sec-tRNA^{Sec} synthase (SEPSECS, SLA), which convert seryl-tRNA^{Sec} to selenocysteinyl-tRNA^{Sec} with a phosphoseryl-tRNA^{Sec} intermediate. Thus Sec formation in archaea and eukarya is a two-step process, whereas bacterial selenocysteine synthase (selA) can synthesize Sec directly from seryl-tRNA^{Sec}. The Se donor utilized by both SEPSECS and selA is selenophosphate, which is generated from selenide (H₂Se) by selenophosphate synthetase (SPS) enzymes. Selenophosphate is first transferred to *O*-phosphoseryl-tRNA^{Sec} by SEPSECS while displacing the phosphoseryl moiety, and subsequent hydrolysis of the phosphate group yields Sec charged to its cognate tRNA [8]. Bacteria have one SPS enzyme termed selD, while two (SPS1, SPS2) have been identified in eukarya, of which SPS2 is itself a selenoprotein. Selenocysteine lyase (SCLY), a putative Se recycling enzyme, is able to catalyze the hydrolysis of Sec into selenide and alanine and potentially delivers Se to SPS enzymes for phosphorylation and subsequent reinsertion into nascent selenoproteins [9]. Pyridoxal phosphate is a prosthetic group derived from vitamin B6 and is required by enzymes involved in metabolism of amino acids, glucose, lipids, and neurotransmitters. The enzymatic activity of both SCLY and SEPSECS is pyridoxal phosphate dependent, implying that Sec synthesis shares regulatory elements with standard metabolic pathways [8, 10, 11].

Several steps in selenoprotein synthesis are regulated by Se availability (dietary or environmental depending on the organism), as well as by the level of oxidative stress. For example, in addition to being a limiting substrate for SPS2, Se levels affect different SECIS elements differentially, potentially regulating selenoprotein synthesis efficiency at the level of translation [7]. Oxidation of the cysteine-rich, redox-sensitive domain of SBP2 masks the nuclear-export signal (NES), causing importation into the nucleus. Subsequently, it is either

sequestered there during high oxidative burden or else reduced by nuclear-specific isoforms of selenoproteins, unmasking the NES for binding and exportation by the nuclear export receptor CRM-1 [12]. As discussed below, individual selenoprotein expression also responds differentially to Se availability and oxidative stress, providing another level of regulatory control for selenoprotein synthesis. Thus there is a complex, nonlinear interaction between Se status and oxidative burden that coordinates the synthesis of the numerous selenoproteins. Despite a low environmental availability of Se and the various elaborate mechanisms for Sec incorporation, selenoproteins are widespread in organisms, with certain plants and fungi being the only major exceptions that lack selenoproteins and an essential biological function for Se [13].

14.2 OXIDATIVE STRESS AND SELENOPROTEINS

Oxidative stress is a broadly used term, but it generally signifies an imbalance in prooxidants and antioxidants in cells and their environment, causing disruption of redox control and macromolecular damage. Here we consider free radicals and nonradical oxidants separately as prooxidants, and distinguish macromolecular damage as a mechanistically different outcome from disrupted redox control. Macromolecular damage to DNA, proteins, and lipids is caused by redox reactions with free radicals, which contain an unpaired electron and are scarce in biological systems because of enzymes and chemicals that rapidly scavenge them and typically generate nonradical oxidants [14]. For example the superoxide radical (O₂^{•−}) is rapidly converted by superoxide dismutase to the nonradical oxidant hydrogen peroxide (H₂O₂). Superoxide can also react with nitric oxide (NO), another free radical, to produce the nonradical oxidant peroxynitrite (ONO₂[−]). Although free radical-mediated macromolecular damage is a common end point observed in aging and disease, the rest of this chapter focuses on the role of selenoproteins in redox systems control and signaling.

Because of such rapid conversion in organisms, free radicals are quantitatively miniscule compared to nonradical two-electron oxidants such as hydrogen peroxide, fatty acid hydroperoxides, disulfides, peroxynitrite, aldehydes, and quinones. In a prototypical redox control system, these nonradical oxidants reversibly react with protein thiols to elicit a redox signal that functions in physiological regulation. Details on the mechanisms of reversible thiol oxidation as a biological control system are beyond the scope of this chapter, but they generally include reaction with an active site cysteine,

modification of a distal allosteric cysteine, and alteration of macromolecular interactions [15]. If the relevant cysteine is in an active site such modifications may serve as a simple binary on-off "switch" to activate or inactivate the protein. Another scenario in which the cysteine residue is not in the active site allows for allosteric regulation, which can throttle protein function along a continuous gradient, akin to a "dimmer." Finally, oxidation of cysteine thiols is known to control intra- and intermolecular protein interactions by formation of disulfides. Among other things, disulfide cross-linking is well established in maintaining proper protein structure, regulating the viscosity of mucus, and connecting actin filaments and tethering proteins to the cytoskeleton [16, 17].

Selenoproteins are closely linked with the cellular thiol-disulfide couples, particularly the glutathione (GSH) and thioredoxin (TXN) couples. GSH is a tripeptide made of glycine, cysteine, and glutamate and is the most abundant thiol in cells, present at millimolar concentrations. Oxidation of the cysteine thiol links two molecules of GSH to form glutathione disulfide (GSSG). This reaction can be spontaneous in the presence of electrophiles or, alternatively, can be catalyzed by a number of enzymes that utilize GSH as an electron donor, including glutathione peroxidase (GPX), glutaredoxin, and glutathione *S*-transferase enzymes. Reduction of GSSG, producing two molecules of GSH, is performed by the homodimeric flavoenzyme glutathione reductase.

All of the characterized selenoproteins that function as enzymes are oxidoreductases that catalyze thiol-disulfide oxidation-reduction (redox) reactions and contain Sec in the active site. In the early 1970s the first Se-dependent enzyme discovered was glutathione peroxidase 1 (GPX1), which catalyzes the reduction of hydrogen peroxide (H_2O_2) by oxidation of two molecules of GSH to GSSG [18]. Cytosolic and mitochondrial forms of the GPX1 enzyme are transcribed from the same gene containing one Sec-encoding TGA. The enzyme functions as a homotetramer utilizing four Se atoms per active enzyme [19]. GPX1 is the most abundant glutathione peroxidase and the most abundant selenoprotein in rats, representing a significant fraction of the total circulating Se pool [20].

Four homologous GPX selenoproteins have subsequently been identified in humans. GPX2, also known as gastrointestinal GPX, is a cytosolic enzyme that is specific to epithelial cells and is abundant in the gut [21]. The extracellular GPX3 has broad substrate specificity and is found in most extracellular compartments but is abundant in kidney and blood plasma [22]. GPX6 is closely related to GPX3 but is only expressed in the olfactory system and exists as a cysteine homolog in rodents [23]. GPX4 is structurally and functionally different from the other GPX enzymes because it is

active as a monomer rather than a tetramer and can directly reduce membrane lipid hydroperoxides and free fatty acid hydroperoxides. Alternative splicing and transcription initiation generates three distinct isoforms of GPX4 that localize to the cytosol, mitochondria, and nucleus. Additionally, GPX4 translation is regulated by the mRNA binding protein guanine-rich sequence-binding factor 1 [24]. Genetic deletion of GPX4 in mice causes embryonic lethality, and knockdown of GPX4 in cells leads to rapid lipoxygenase-mediated lipid peroxidation and subsequent apoptosis, suggesting that removal of lipid hydroperoxides by GPX4 is essential for cell viability [25, 26]. There are three additional GPX enzymes (GPX5, GPX7, and GPX8) that are not selenoproteins in humans.

TXN is a small protein of ~12 kDa and is present at concentrations several orders of magnitude below GSH. It contains an active site dithiol that is highly conserved in evolution and widely distributed among the TXN superfamily. Through oxidation of the dithiol to a disulfide, TXN can directly reduce cysteine sulfenic acids and control the state of dithiol-disulfide motifs in target proteins, and can also serve as an electron-donating cofactor for enzymes such as ribonucleotide reductase, peroxiredoxins, and methionine sulfoxide reductases [27]. In turn, reducing oxidized TXN is mediated exclusively by the thioredoxin reductase (TXNRD) family of selenoproteins [28]. At least four selenoproteins (GPX3, GPX4, SEPPI, SEPI1) can utilize TXN as a cofactor for enzymatic reduction, and it is possible that others do as well [29, 30]. There are also numerous thioredoxin-like proteins that may depend on TXN or act in parallel to provide additional substrate specificity beyond that provided by TXN. Several selenoproteins contain a TXN-like fold, which is a well-described secondary/tertiary structure pattern with a conserved Cys-X-X-Cys or Cys-X-X-Ser/Thr active-site motif characteristic of oxidoreductases [31], where X is any amino acid. While it is tempting to speculate that these selenoproteins operate similarly to TXN, namely, by controlling the redox state of cysteine residues and dithiol motifs, there is at present little evidence to support or deny this notion.

Three mammalian thioredoxin reductases are selenoenzymes encoded by individual genes. TXNRD1, TXNRD2, and TXNRD3 encode homodimeric flavoproteins that localize to the cytosol, mitochondria, and testes, respectively. They are members of the pyridine nucleotide-disulfide oxidoreductase family and contain two redox-sensitive sites in the N- and C-termini that interact in a head to tail dimer conformation of the active enzyme. These enzymes are capable of reducing a number of substrates, but they depend on NADPH for donating electrons, which are first transferred to the FAD group, then passed to the N-terminal dithiol of one subunit and

subsequently to the C-terminal selenenyl-sulfide of the other subunit. The highly conserved Sec-containing C-terminal motif is absolutely critical for catalytic function of TXNRD enzymes [27, 32]. The main substrate for TXNRDs is the small redox-sensitive protein TXN, which is integral to physiological processes such as cell communication, metabolism, proliferation, and apoptosis. In general, the reactive dithiol of TXN will become oxidized to a disulfide during reduction of an oxidized target protein. Regeneration of reduced TXN proteins requires TXNRD, and thus the TXN/TXNRD system is completely dependent on Se in mammals. The importance of this system is highlighted by the fact that knockout of either TXNRD1 or TXNRD2 is embryonic lethal in mice [33, 34]. It is worth noting that TXNRDs from mammals differ from the Se-independent enzymes of archaea, bacteria, yeast, and plants.

Reactions between target proteins and TXN can be spontaneous, but several enzymes can catalyze the reduction of target proteins using TXN as an electron-donating cofactor. The human genome codes for four methionine sulfoxide reductase (MSR) enzymes that reduce oxidized methionine residues in proteins utilizing TXN as a cofactor. There is now considerable evidence that, like cysteine, reversible methionine oxidation can regulate protein function [35]. For example, calcium/calmodulin-dependent protein kinase II and the phosphatase calcineurin, among many other proteins, are regulated by methionine redox status [36, 37]. A single MSRA and three MSRB enzymes stereospecifically reduce S- and R-sulfoxidated methionines, respectively. MSRB1 is a selenoprotein also known as Selenoprotein R and SEPX1, while MSRA as well as MSRB2 and MSRB3 are Se-independent enzymes. SEPX1 is a zinc-containing protein present in the cytosol and nucleus and exhibits the highest methionine-R-sulfoxide reductase activity because of the presence of Sec in its active site [38]. Interestingly, redox status of the cysteine-rich metallothionein/thionein couple dictates zinc loading in that reduced thionein binds zinc and oxidation of metallothionein releases it. Moreover, thionein can reduce nonselenoprotein MSRB3 in the presence of TXN, TXNRD, and NADPH more efficiently than without thionein [39]. Therefore regulation of specific kinases, phosphatases, and other proteins by methionine-R-sulfoxide reduction is mediated by two selenoproteins (MSRB1, TXNRD1) and NADPH.

14.3 SELENOPROTEINS AND REDOX SYSTEMS

The GSH and TXN couples are central to a global system of connected redox elements involving reversible

oxidation of proteins containing cysteine, methionine, and Sec residues. GPX and TXNRD selenoproteins, being critical effectors of peroxide and thioredoxin reduction, have a direct role in the redox systems biochemistry of organisms (Fig. 14.1A). Oxidative stress in the form of disrupted redox control arises when cysteine (or methionine) residues become abnormally oxidized or irreversibly modified, stripping the reversible redox reactivity that is required for physiological processes. Several covalent and noncovalent modifications to distal or active site cysteine residues modify protein structure and function. A redox-active cysteine residue can become reversibly oxidized to sulfenic acid, which can be reduced stepwise by two molecules of reduced GSH. Additionally, cysteinyl-S-glutathione conjugates are semistable in certain proteins, and S-glutathionylation can lead to altered protein function as observed with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and caspase 3 [40, 41]. If two neighboring reduced cysteine residues (dithiol) are reactive, the pair can be reversibly oxidized to a form a cysteine disulfide bridge, as with TXN. The gaseous molecules NO and hydrogen sulfide (H₂S) also react with cysteine residues to produce modified derivatives that are functionally relevant. For example, S-nitrosylation of Cys118 in Ras activates the protein by stimulating guanine nucleotide exchange, while sulfhydration, or the addition of a sulfhydryl group from hydrogen sulfide gas to a cysteine thiol, has recently been demonstrated to augment the activity of GAPDH [42, 43]. Proteins often contain multiple cysteine residues that can be reversibly modified (e.g., dithiol-disulfide, S-glutathionylation, S-nitrosylation, S-sulfhydration, etc.) to affect protein function and/or interactions.

From a systems perspective, cellular redox status is best understood in terms of redox potential. The redox potential (E_h), or electron motive force, for an oxidation/reduction couple depends on the inherent tendency of the molecule to accept/donate electrons relative to the standard hydrogen electrode and the concentrations of the acceptor and donor (oxidized and reduced species of the couple). In biological systems the redox potential of the major thiol/disulfide couples (GSH and TXN) is maintained at stable nonequilibrium conditions, or in other words, the mean E_h of the GSH and TXN couples in subcellular compartments is nonzero. These redox couples, along with the Cys/CySS (cysteine/cystine) couple and protein Cys residues involved in redox signaling, are in equilibrium neither with each other nor with the NADPH/NADP⁺ couple. In addition to nonequilibrated redox potentials between couples within a subcellular compartment, redox potential is also not equilibrated across subcellular and extracellular compartments. The mitochondria and nucleus tend to be highly reduced (redox potential farther from zero),

mechanisms are able to control reaction rates. Under aerobic conditions peroxide and oxygen are always present in cells; therefore coupling of electron transfer to peroxidase (e.g., GPX) or oxidase reactions can provide an additional energetic driving force to maintain function of low-current redox control circuits. Kinetic and spatial insulation of reactive thiol couples allows biosystems to be highly responsive and dynamic, and concurrently provides specificity because noncatalyzed oxidation-reduction and thiol-disulfide exchange reactions are slow in the cellular environment [44].

S-glutathionylated Sec and diselenide bridges have not been described *in vivo*, but transient and stable selenenyl-sulfide linkages are observed in selenoproteins as either active intermediates or structural features, respectively. For example, the reaction mechanisms for GPX and TXNRD enzymes involve a transient mixed selenenyl-sulfide bond, while rat selenoprotein P (SEPP1) has two such linkages that appear to be a structural feature [32, 46]. Transient nitrosylation and sulphydration of Sec is thermodynamically possible, but its existence is speculative at present. Free Sec is virtually absent from cells, making a selenocysteine/selenocystine couple unlikely, but selenoproteins are present and could be maintained in a nonequilibrium steady state favoring the reduced form. Kinetic (reaction rate and substrate specificity) and spatial (subcellular and tissue distribution) insulation of the selenoproteins has been described; however, whether selenoproteins represent kinetically *limiting* sites of redox control remains an important question. The standard reduction potential (E_0) of selenoproteins is unexplored, and determining whether the mean E_0 of selenoproteins, particularly the TXN-like selenoproteins, is maintained in a nonequilibrium steady state will help determine whether they are central redox control points. Given the known kinetic advantages of the Sec amino acid compared to cysteine in catalysis, as well as the pivotal positions of GPX and TXNRD selenoproteins in respect to redox circuitry (Fig. 14.1A), it seems possible that one or more selenoproteins could be identified as kinetically limiting redox control points that are rapidly disrupted during oxidative stress. Additionally, several selenoproteins have unknown function, but, given the unique reactivity of Sec, they are well suited to transduce oxidant signals by formation of disulfide bonds in downstream targets even in the reducing cellular environment.

14.4 SELENOPROTEINS IN VERTEBRATE SIGNALING

Specific selenoproteins function at the intersection of cellular and organism metabolism by modulating insulin

and thyroid hormone signaling. The iodothyronine deiodinases (DIO) function in activation and deactivation of thyroid hormone and were the second family of enzymes determined to be Sec-containing selenoproteins in the early 1990s [47]. Thyroid hormone metabolism at the level of both production in the thyroid and local hormone activity in the periphery is reliant on the DIO family of selenoenzymes. Most vertebrates have three DIO enzymes that can deiodinate thyroid hormones to control local availability. These integral membrane protein enzymes are thiol-requiring oxidoreductases that remove iodine atoms from the aromatic rings of thyroxine (T_4), triiodothyronine (T_3), and reverse triiodothyronine (rT_3) [48]. DIO1 is a plasma membrane protein found mainly in cells of the liver and kidney, is capable of deiodinating both the inner and outer rings, and produces most of the circulating T_3 . DIO2 is found in the endoplasmic reticulum of cells in several tissues including the thyroid, heart, skeletal muscle, fat, and the central nervous system and selectively removes the outer ring iodine, making it the primary tissue activator of thyroid hormone by converting T_4 to T_3 . DIO3 is also a plasma membrane protein; however, it is mainly found in fetal tissue and the placenta, selectively removes the inner ring iodine, and thus contributes to thyroid hormone inactivation.

A specific role for a selenoprotein in redox regulation of insulin signaling was established when it was found that overexpression of GPX1 causes hyperinsulinemia and insulin resistance in mice [49]. Moreover, genetic deletion of GPX1 promotes glucose tolerance and insulin sensitivity in mice on a high-fat diet by enhancement of insulin-induced PI3K/Akt signaling [50]. Dietary studies in humans have further suggested that supranutritional levels of Se are associated with type 2 diabetes, while animal studies confirm that both excessive dietary Se and GPX1 overexpression lead to hyperinsulinemia and insulin resistance [51–54]. Peroxide-induced oxidation of PTEN and *S*-glutathionylation of protein tyrosine phosphatase 1B are affected by GPX1 activity, which thereby modulates insulin receptor activation and insulin resistance [55].

Selenoprotein P (SEPP1) is a unique selenoprotein that contains multiple Sec residues and is also implicated in insulin resistance. Diabetic patients display an increase in hepatic SEPP1 mRNA and serum SEPP1 protein, and purified SEPP1 administered to mice is able to induce insulin resistance and glucose intolerance. Furthermore, knockdown or knockout of SEPP1 in mice improves glucose tolerance and insulin sensitivity, and SEPP1 knockout mice are protected against glucose intolerance and insulin resistance even when on an obesity-inducing diet [56]. Since SEPP1 expression can dictate expression of other selenoproteins including GPX1, its effect on insulin resistance may be direct or indirect. Primate and rodent

SEPP1 contains up to 10 Sec residues, while as many as 17 Sec residues are present in zebrafish. SEPP1 is the most abundant selenoprotein in blood and accounts for as much as 65% of plasma Se in rats [57]. Aside from the multiple Sec residues and high Se content, SEPP1 is also distinct in that it is one of only two extracellular selenoproteins (the other being GPX3 mentioned above), and it appears to be a vertebrate adaptation as it has not been found in the genome of *Caenorhabditis elegans* or *Drosophila melanogaster*. SEPP1 is abundantly produced by the liver and secreted into blood; however, local production and secretion in nearly all tissue systems has been described [58]. Bodily transport of Se to extrahepatic tissues, particularly the brain, testes, and kidneys, appears to be facilitated by receptor-mediated uptake of SEPP1 by the low-density lipoprotein receptor family members ApoER2 (LRP8) and Megalin (LRP2) [59]. The abundant Sec residues of SEPP1 are divided into two regions, with the bulk being located in the C-terminal domain that is required for the Se transport function. SEPP1 also contains an N-terminus Cys-X-X-Sec motif and catalyzes the reduction of lipid hydroperoxides in vitro, utilizing TXN as a cofactor [30, 60]. In addition to a Se transport function and peroxidase activity, SEPP1 exhibits pH-dependent heparin binding and heavy metal binding that likely also function in redox-dependent processes.

The examples of GPX1, SEPP1, and the DIO enzymes modulating insulin and thyroid hormone signaling highlight the fact that vertebrate metabolism is tightly integrated with selenoprotein function. This connection between Se and metabolism in multicellular organisms suggests that cell-autonomous regulation of redox systems and signaling may similarly depend on one or more selenoproteins. The regulatory control of cellular redox signaling by Selenoprotein W is discussed next as an example, bearing in mind that several selenoproteins with uncertain functions could similarly have a role in regulating target protein oxidation state.

Selenoprotein W (SEPW1) was purified in the early 1990s but putatively identified much earlier because of its absence in Se-deficient lambs suffering a myopathy called white muscle disease [61]. Mammalian SEPW1 is a highly conserved cytosolic protein of just 87 amino acids, and SEPW1 orthologs are among the most widely distributed selenoproteins in all species including prokaryotes [6, 62]. The expression level of SEPW1 in vertebrates is very sensitive to dietary Se intake as well as the expression level of SEPP1 [63–65]. Abundant SEPW1 expression is observed in muscle, and SEPW1 transcription during myocyte differentiation is maintained by binding of the myogenic transcription factor MyoD to the SEPW1 promoter [66]. A putative metal-response element in the promoter of the SEPW1 gene was probed in vitro with the use of a luciferase reporter fusion construct, and

luciferase specific activity was found to be stimulated by copper and zinc but not cadmium [67]. Although a bona fide enzymatic activity has not been attributed to SEPW1, the presence of a Cys-X-X-Sec motif in a thioredoxin-like fold may indicate thioredoxin-like redox activity [31].

Recently, SEPW1 was shown to pull down and coimmunoprecipitate with the β and γ isoforms of 14-3-3 protein. This interaction was further confirmed by NMR spectroscopy and extended to identify three loops of SEPW1 that interact with 14-3-3 proteins [68]. 14-3-3 β and γ proteins are scaffolding proteins derived from the YWHAB and YWHAG genes, respectively, and bind a diverse assortment of proteins including kinases, phosphatases, and receptors. In this way 14-3-3 proteins coordinate molecular interactions and participate in cell cycle regulation, metabolism, apoptosis, protein trafficking, and gene transcription [69]. A computational study of SEPW1/14-3-3 interaction suggests that a conserved cysteine of 14-3-3 β and γ (Cys191 and Cys195, respectively) can be reversibly oxidized, with SEPW1 acting as a reducing agent [70]. The oxidized cysteine sulfenic acid of 14-3-3 can putatively react with Sec of SEPW1, producing a mixed complex. Subsequently, the formation of an intramolecular selenenyl-sulfide within SEPW1 would result in 14-3-3 being fully reduced. Oxidized SEPW1 can then migrate away and likely be reduced to its parent state by GSH, which is supported by evidence that a SEPW1 cysteine residue conserved from rodents to primates can be S-glutathionylated [71, 72]. Redox regulation of 14-3-3 proteins by SEPW1 could serve several cellular functions, but an intriguing possibility is presented by the in vitro finding that SEPW1 expression is regulated by the cell cycle and knockdown of SEPW1 induces cell cycle arrest [73]. Therefore SEPW1, through redox regulation of 14-3-3 proteins, may coordinate Se availability and oxidative burden with cellular proliferation, differentiation, and death. This redox-regulated functionality may serve as the basis for the association of SEPW1 with myopathies in livestock and multiple myeloma in humans [74].

The example of SEPW1 is meant to highlight the fact that selenoproteins with unknown roles can impact the cellular response to environmental changes, particularly in relation to growth and stress. In the following section a brief summary of what is known of the remaining selenoproteins is presented, with the biological functions described possibly owing to the activity of the selenoproteins in undefined redox circuits.

14.5 THE SELENOPROTEIN FAMILY

The human genome codes for 25 selenoproteins, most of which have been identified recently by bioinformatics

approaches looking for SECIS elements downstream of in-frame UGA codons [23]. The various members display wide subcellular and tissue distribution, and several are known to have multiple transcript variants and protein isoforms. Selenophosphate-synthetase 2 (SPS2) is a eukaryotic selenoprotein that is required for the synthesis of all selenoproteins including itself. Selenophosphate is generated by SPS2 in the presence of selenide and ATP [75]. A related protein called SPS1 contains a cysteine residue in place of Sec, but its involvement in Sec and selenoprotein biosynthesis is uncertain [76]. It has been suggested that SPS2 assimilates selenite, whereas SPS1 recycles Sec in a Se-salvage pathway [77]. Intriguingly SPS2, but not SPS1, is required for selenoprotein synthesis in NIH3T3 mouse fibroblasts [78]. However, an *in vivo* requirement of SPS2 and specificity of SPS1 and SPS2 with different Se substrates have not been reported.

Oxidative stress can trigger dysfunction of organelles including the mitochondria, nucleus, and endoplasmic reticulum. A subset of selenoproteins is observed in mitochondrion to combat against electron leak during oxidative respiration and phosphorylation. Mitochondrion-specific isoforms of GPX1, GPX4, and TXNRD2 regulate peroxide metabolism and oxidative tone within this organelle. The selenoproteins GPX4, TXNRD1, MSRB1, and SELH have been shown to exhibit varying degrees of nuclear localization (Fig. 14.1B). Apart from SELH, the other three selenoproteins are presumably involved in reduction of lipid peroxides, oxidized TXN, and sulfoxidized methionine residues within the nuclear envelope.

SELH is the only DNA-binding selenoprotein described and has a role in regulation of gene expression. Similar to SEPW1, Selenoprotein H (SELH) is a small selenoprotein that is highly expressed during development and is sensitive to dietary Se intake [79, 80]. Like several selenoproteins, it contains a Cys-X-X-Sec sequence within a thioredoxin-like fold, but unlike any other selenoprotein described to date, it is a DNA-binding protein of the AT-hook family. SELH is primarily located in the nucleus and is implicated in redox-sensitive transcription of genes whose products are involved in *de novo* glutathione synthesis and phase II detoxification [81]. Multiple metal-response elements are present in the SELH gene [82], and one group has confirmed *in vivo* that SELH mRNA and protein are upregulated under conditions of elevated copper in mouse liver [83]. Although it is a nuclear protein, mitochondrial biogenesis and function are also linked with SELH. Overexpression of SELH in a transformed neuronal cell line attenuates the UVB-induced increase of p53 protein and caspase-mediated apoptosis [84]. Additionally, SELH overexpression increases mitochondrial size, cytochrome c content, and expression of

mitochondrial biogenesis proteins while boosting respiration [85]. Collectively these findings suggest that SELH is a Se- and metal-regulated selenoprotein that is able to transduce oxidant signals by modulating gene expression in conjunction with other redox-sensitive transcription factors. Further investigation is warranted to determine whether SELH modifies cysteine *S*-glutathionylation or disulfide formation in target proteins such as p53 to regulate gene expression.

The endoplasmic reticulum (ER) regulates the synthesis, folding, and transport of proteins and additionally constitutes the main intracellular store for calcium ions, which are integral in cell signaling. Seven selenoproteins are enriched in the ER (Fig. 14.1B), and some are postulated to have a role in protein folding and ER calcium handling, since oxidative mechanisms within the ER are known to regulate these processes [86]. The ER is a relatively oxidizing environment compared to other intracellular organelles and contains oxidase enzymes to facilitate the formation of disulfide bonds in proteins destined for export. Simultaneously, GSH and TXN system components are transported into the ER, providing both oxidation and reduction mechanisms for dynamic redox regulation associated with protein processing and secretion. Redox state affects calcium homeostasis by modulating ER calcium channels and chaperones, and oxidative stress and ER stress are intimately related in signaling for apoptosis [87]. Similar to the ER, the secretory and endosomal/lysosomal pathways are also more oxidized than other subcellular compartments [88, 89]. Ligand binding to various receptors stimulates endocytosis of redox-active endosomes whose luminal redox activity directs spatiotemporally regulated signaling and prevents nonspecific redox reactions [90, 91]. Therefore redox-mediated processes are vital for secretory and endocytic function, and the ability of selenoproteins to transmit oxidative signals from reactive intermediates to disulfide bonds of target proteins may help to explain the enrichment of selenoproteins in the ER.

Selenoprotein T (SELT) is an ER- and Golgi-localized selenoprotein that is ubiquitously expressed from development through adulthood and shares some sequence similarity with SEPW1 and SELH including the thioredoxin-like fold containing a Cys-X-X-Sec motif [31]. Deficiency of SELT in murine fibroblasts causes an upregulation of SEPW1, in addition to altering cell adhesion and redox regulation [92]. A biological role for SELT in neuroendocrine secretion and calcium mobilization *in vitro* has also been presented. SELT was identified as a target gene of the neuropeptide pituitary adenylate cyclase-activating polypeptide (PACAP), and manipulation of SELT expression altered PACAP-induced intracellular Ca^{2+} changes and growth hormone secretion [93].

Two of the more abundant and ubiquitous ER selenoproteins, Selenoprotein M (SELM) and 15-kDa selenoprotein (SEP15), are both 15-kDa proteins that share 31% sequence homology and have Cys-X-X-Sec and Cys-X-Sec redox motifs, respectively. SELM and SEP15 have thioredoxin-like tertiary structure and homology to protein disulfide isomerases that suggest oxidoreductase activity, but direct evidence in support of this notion is lacking [94]. SEP15 associates with UDP-glucose:glycoprotein glucosyltransferase (UGTR), a protein involved in protein conformation quality control, and has been suggested to facilitate proper protein folding [95]. A role for SELM in protein folding has been proposed, and recent evidence suggests that SELM may also be involved in regulating the flux of calcium ions. Overexpression of SELM in a neuronal cell line *in vitro* reduced peroxide-induced calcium influx, whereas knockdown of SELM increased the baseline intracellular calcium concentration [96].

Selenoprotein N (SEPN1) is a large (70 kDa) single-spanning transmembrane protein localized to the ER membrane with two known isoforms generated by alternative splicing of exon 3 [97]. Several congenital muscular dystrophy syndromes such as multimimicore disease, rigid spine muscular dystrophy, and desmin-related myopathy with Mallory body-like inclusions are directly associated with mutations in the SEPN1 gene and have been classified as SEPN1-related myopathies [98]. Interestingly, mutations in SEPN1 leading to congenital fiber type disproportion are associated with insulin resistance [99]. To date, SEPN1 is the only selenoprotein gene in which mutations are directly and causatively linked to human disease. An *in vivo* study using zebrafish determined that SEPN1 associates with ER/SR ryanodine receptors, and that this interaction is necessary for the calcium-induced release of calcium from intracellular stores [100]. Ryanodine receptor channels are homotetramers with several redox-regulated cysteine residues, and SEPN1 contains a Cys-Sec-Gly-Ser motif, suggesting that SEPN1 regulates ryanodine receptor-mediated calcium flux in muscle by redox-dependent signaling. Finally, SEPN1 is integral to the generation and/or maintenance of skeletal muscle satellite cells, which are an adult stem cell population involved in muscle growth and regeneration [101].

Selenoprotein K (SELK) and Selenoprotein S (SELS) are also predominantly ER-localized single-spanning transmembrane proteins; however, they are much smaller than SEPN1 and also show some localization to the plasma membrane [23, 102, 103]. Both are widely expressed in a variety of tissues and have been implicated in the cellular response to ER stress. Specifically, ER stress agents regulated the expression of SELK in HepG2 hepatoma cells, and knockdown of SELK exacerbated

cell death when challenged with ER stress [104]. Genetic deletion of SELK in mice decreases receptor-mediated calcium flux in immune cells, impairs calcium-dependent immune cell function, and increases West Nile virus-induced lethality [105]. An interesting link between metabolism and inflammation is presented in the case of SELS, which was originally identified as a glucose-regulated protein in a rodent model of diabetes [106]. The relationship between SELS and type 2 diabetes was confirmed in humans, and there is evidence that SELS can be secreted from liver and identified in blood sera, where it associates with LDL [107, 108]. SELS is now known to also be regulated by inflammatory cytokines [109], and reciprocally, reduced expression of SELS, due to polymorphisms in the gene promoter, influences the levels of IL-1, TNF- α , and IL-6 [110]. SELS participates in removal of misfolded proteins from the ER lumen [103, 111] and was demonstrated to prevent ER stress and have antiapoptotic function in macrophages and astrocytes [112, 113].

Three selenoproteins remain largely unexplored, with very little published data currently available. The sequences of Selenoprotein I (SELI), Selenoprotein O (SELO), and Selenoprotein V (SELV) were identified in the human genome several years ago; however, almost no information is available on the cellular localizations or physiological functions of these selenoproteins. SELI mRNA is known to be expressed in several tissues, and it is a putative transmembrane protein hypothesized to function in phospholipid biosynthesis based on the presence of a CDP-alcohol phosphatidyltransferase motif that is conserved in phospholipid synthases [23, 114]. SELO is predicted to be a 669-amino acid selenoprotein containing a Cys-X-X-Sec motif, but experimental data demonstrating a redox function are unavailable [23]. SELV appears to be a testes-restricted protein with a predicted thioredoxin-like fold housing a Cys-X-X-Sec motif and also has some sequence homology with SEPW1, SELH, and SELT [23].

14.6 CONCLUSION AND FUTURE PERSPECTIVES

The implications of selenoproteins functioning as part of the circuits mediated by central couples for redox signaling are widespread. These circuits control networks that integrate and coordinate cellular processes through a series of parallel redox elements, and selenoproteins are positioned in key places to drastically affect circuit function. The GPX enzymes rapidly consume peroxide to limit the distance of locally generated oxidant signals, and in doing so impact the redox potential of the GSH/GSSG couple. TXNRD selenoproteins reduce TXN to

maintain the nonequilibrium steady-state redox potential of the couple and thus are the basis of a functionally diverse array of signaling processes mediated by TXN. Reducing sulfoxidized methionines is one such process that is partially realized by another selenoprotein, MSR1. DIO enzymes and SEPP1 modulate vertebrate metabolism, while SEP1 and SELH modulate cellular metabolism. At least four ER-localized selenoproteins (SELT, SELM, SEP1, SELK) are involved in regulating intracellular calcium flux and consequent cellular signaling, and another two (SEP15, SELS) participate in protein folding. SPS2 is a selenoprotein that regulates synthesis of all selenoproteins including itself, while SELI, SELO, and SELV still have unknown functions. The interaction of selenoproteins with thiol-disulfide pools in humans highlights their importance in redox regulation and signaling, particularly under conditions of oxidative stress. Oxidative stress is associated with diverse disease processes, and redox-sensing selenoproteins may provide a mechanistic link between sources of oxidative stress and the fundamental cell signaling that contributes to disease pathology. Specific selenoproteins could optimize target protein function by modifying reversible reactions (i.e., oxidation, nitrosylation, acylation, sulfhydration or metal binding) that affect macromolecular structure, activity, interactions, and trafficking. Elucidation of specific targets and circuits for selenoprotein activity will create new strategies for targeting relevant redox systems that go awry during disease pathology. Almost two hundred years since Berzelius named Se after the Greek moon goddess Selene, this unique trace element is poised to illuminate a world of redox systems biology.

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ANTIOXIDANT THERAPY AND ITS EFFECTIVENESS IN OXIDATIVE STRESS-MEDIATED DISORDERS

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15.1 INTRODUCTION

Antioxidants are chemical compounds that give an electron to free radical molecules and convert them into a harmless configuration. This prevents damage to chain reaction and can involve lipids, proteins, enzymes, carbohydrates, DNA, cell, and nuclear membranes up to the death of the cell. When the body's scavenging ability is not able to deal with free radical species, that is, reactive oxygen species (ROS) and reactive nitrogen species (RNS), they cause oxidative damage in all the body's tissues, leading to disease. The effect of oxidative stress at the cellular level is illustrated in Figure 15.1. This oxidation-induced damage may be prevented by exogenous or endogenous antioxidants. ROS seem to be an important factor involved in endothelial dysfunction, diabetes, atherosclerosis, and ischemia, while RNS have been associated with arthritis, diabetes, degenerative neuronal diseases, cancer, and atherosclerosis. Under physiological conditions, the overproduction of ROS and RNS and their neutralization are prevented by the activity of the endogenous antioxidative defense system (AOS). It includes enzymes like superoxide dismutase, catalase, glutathione peroxidase, and other antioxidant regenerating enzymes such as glutathione reductase, dehydroascorbate reductase, and glucose-6 phosphate dehydrogenase that maintains reduced NADPH, hydrophilic scavengers like urate ascorbate glutathione,

flavonoids, and lipophilic scavengers, like tocopherols, carotenoids, and ubiquinone. Antioxidants are generally supplied in the diet and include polyphenols, lipoic and ascorbic acid, carotenoids, resveratrol, epigallocatechin-3-O-gallate, lycopene, quercetin, genistein, ellagic acid, ubiquinone, and indole-3 carbinole. The properties of these compounds are involved in the physiological redox balance as they can prevent damage to the tissues due to the oxidation typical of all the biological systems and characterized by the production of highly reactive free radicals [1]. There is growing evidence that ROS play a key role in several pathological conditions and in the aging process. No final conclusion about possible therapy protocols based on the administration of antioxidant compounds has been reached yet, but it can be hypothesized that, in the near future, biochemical investigations, able to detect an oxidative imbalance, may become routine tests necessary to restore the antioxidant natural protective barrier, avoiding irreversible damage. An example of the power of antioxidant supplementation to prevent excessive oxidative stress is given by a study conducted in 400 healthy subjects to assess the effect of physical activity combined with antioxidant treatment (330 ml/day of Funciona™). This study shows that, after 10 months, the oxidative stress caused by exercise was prevented by the antioxidant treatment [2]. There is also a lack of agreement concerning the parameters of oxidative stress or antioxidant state in specific

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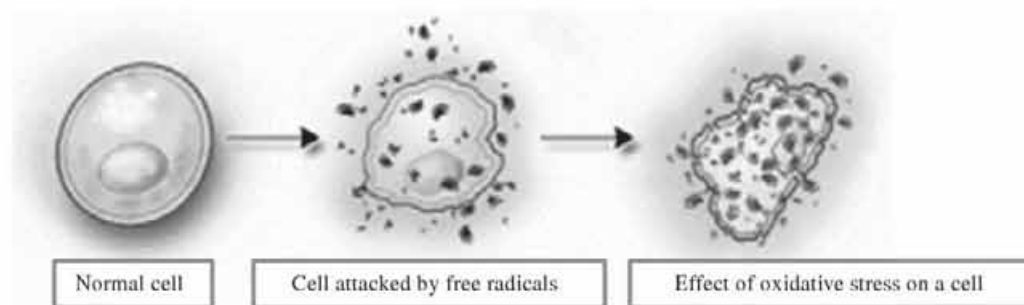


Fig. 15.1 Effect of oxidative stress at cellular level.

patients or diseases, and researchers are trying to find new techniques to monitor oxidative stress in humans. Among other techniques are high-performance liquid chromatography (HPLC) and immunochemical determinations, which have become the common standard. The number of centers using Points of Care is growing among the scientific community because of their low invasiveness, reduced costs, practicality, and ease of use. In particular, Free Oxygen Radicals Testing (FORT) and Free Oxygen Radicals Defence (FORD) have proven their reliability and ease of use [3]. Among the main techniques for free detection is electron spin resonance (ESR), which can provide valuable information on free radicals in solution chemistry, while to examine free radicals in biological materials, spin trapping is the most appropriate technique, which may allow investigators to detect radicals that are normally present at very low concentrations or whose lifetime is too short to be detected by direct ESR analysis. Specific products of DNA oxidation have been proposed as potential general markers of this process. For example Bruce Ames's laboratory, at the Berkeley campus of the University of California (USA), has proposed that thymine glycol and 8-hydroxydeoxyguanosine might be appropriate markers of DNA base oxidation. Analysis of 8-hydroxydeoxyguanosine in extracted DNA is a straightforward method of studying DNA base oxidation in biological tissues, and this substance is also excreted in urine and hence might provide a noninvasive means of assessing whole-body DNA base oxidation. GSH is the major intracellular reductant, and analysis of GSSG:GSH can provide a valid index of cellular oxidative stress. Assays relying on the ability of bleomycin to bind Fe, facilitating the oxidation of DNA (the bleomycin-Fe assay), and of phenanthroline to undertake the same role with Cu (the phenanthroline-Cu assay) have also been developed. Data indicate that small amounts of this "free" or "catalytic" Fe or Cu may be found in biological fluids such as cerebrospinal fluid (CSF) and that these levels increase in some disease states (e.g., Cu or Fe overload).

They provide strong support, as increased free radical production may occur in these situations [4]. An evaluation of 67 randomized trials including participants who were either healthy (primary prevention trials) or affected by different diseases (secondary prevention trials; gastrointestinal, cardiovascular, neurological, ocular, dermatological, rheumatoid, renal, endocrinological, or unspecified) analyzed the effect of several antioxidant supplements, such as beta-carotene, vitamin A, vitamin C, vitamin E, and selenium versus placebo or no intervention, concluding that only vitamin A, beta-carotene, and vitamin E seem to increase mortality [5]. α -Lipoic acid (ALA), also named 1,2-dithiolane-3-pentanoic acid or thioctic acid, is synthesized in humans by the liver, heart, and kidney and has also been widely studied for its antioxidative properties. It is water- and fat soluble and is widely distributed in both cellular membranes and cytosol. A number of experimental and clinical studies point to the usefulness of ALA as a therapeutic agent for several medical conditions, namely, diabetes, atherosclerosis, insulin resistance, neuropathy, neurodegenerative diseases, and ischemia-reperfusion injury [6]. ALA exerts an antioxidant effect in biological systems through direct ROS quenching and via transition metal chelation [7]. Carpal tunnel syndrome (CTS) is the most common peripheral mononeuropathy. One study [8] has compared the efficacy of ALA (600 mg/day) and γ -linolenic acid (GLA; 360 mg/day) to that of a multivitamin B preparation (Vit B6 150 mg, Vit B1 100 mg, Vit B12 500 μ g daily) administered for 90 days in 112 subjects with moderately severe CTS. The following results were observed:

1. A significant reduction in both symptom scores and functional impairment in the ALA/GLA group, while the multivitamin group experienced a slight improvement in symptoms and a deterioration in functional scores.
2. Electromyography showed a statistically significant improvement in the ALA/GLA group, but not in the multivitamin group.

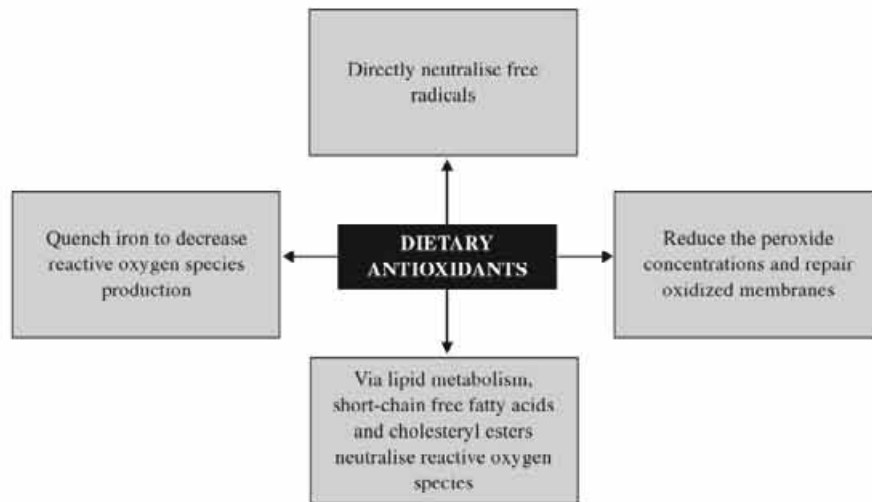


Fig. 15.2 Dietary antioxidants' main functions.

(Dietary Approaches to Stop Hypertension) diet share many characteristics with the Okinawan diet. Features such as the low levels of saturated fat, high antioxidant intake, and low glycemic load in these diets are likely to contribute to a decreased risk of cardiovascular disease, some cancers, and other chronic diseases through multiple mechanisms, including reduced oxidative stress. A comparison of the nutrient profiles of the three dietary patterns shows that the traditional Okinawan diet is the lowest in fat intake, particularly in terms of saturated fat, and the highest in carbohydrate intake, in keeping with the very high intake of antioxidant-rich yet calorie-poor orange-yellow root vegetables, such as sweet potatoes, and green leafy vegetables. The high longevity that characterizes this population is thought to be related to a healthy lifestyle, particularly to the traditional diet that is low in calories but rich in nutrients, especially with regard to phytonutrients in the form of antioxidants and flavonoids [55]. The main function of dietary antioxidants are summarized in Figure 15.2, while the main antioxidants, their mechanisms of action, and their main dietary sources are summarized in Table 15.1.

15.3 CARDIOVASCULAR DISEASE, RISK, AND ISCHEMIA-REPERFUSION INJURY

Coronary heart disease and stroke have been associated to several risk factors, namely, tobacco use, alcohol use (having 1–2 alcohol drinks a day may lead to a 30% reduction in heart disease, but above this level alcohol consumption will damage the heart muscle), high blood pressure (hypertension), obesity and related comorbidities (metabolic syndrome, diabetes, and so on), physical inactivity (increases the risk of heart disease and stroke

by 50%), and unhealthy diet (excessive consumption of saturated fat increases the risk of heart disease and stroke; it is estimated to cause about 31% of coronary heart disease and 11% of stroke worldwide). Generally it has been pointed out that abnormal blood lipid levels (hyperlipemia), in the form of high total cholesterol, high levels of triglycerides, and high levels of low-density lipoprotein or low levels of high-density lipoprotein (HDL) cholesterol, increase the risk of heart disease and stroke. Moreover, the contraceptive pill and hormone replacement therapy (HRT) may increase the risk of heart disease [56]. Nowadays, oxidative species seem to play a key role in these pathological conditions. Relevant evidence has supported the theory that free radical-mediated oxidative processes and specific related products play a key role in atherogenesis [3].

Aging has also been related to an increased risk of stroke, in fact the latter doubles every decade after the age of 55. Family history of cardiovascular disease is also a risk indicator. A man is at greater risk of heart disease than a premenopausal woman. Ethnic origin plays a role, too. People with African or Asian ancestry are at higher risk of developing cardiovascular disease than other racial groups [56]. Several studies have linked a high consumption of plant polyphenols with a decreased risk of cardiovascular disease and hypertension [57–61]. Vitamin C (500 mg twice a day) and vitamin E (400 IU twice a day), administered for 1 year, reduce the progression of cardiac transplant-associated arteriosclerosis (TxAA) in patients with normal or abnormal endothelial function, with a particular benefit in patients with endothelial dysfunction [62]. Antioxidant vitamin intake [vitamins A, C, and E, 1683 mg (± 1245), 371 mg (± 375), and 97 mg (± 165) respectively] does not seem to be significantly related to coronary artery calcification, implying that

TABLE 15.1 Common antioxidants, their action, and main dietary sources

Antioxidant	Action	Foods containing antioxidant
Vitamin A (beta-carotene)	Protection against lipid oxidation	Variety of dark orange, red, yellow, and green vegetables and fruits such as broccoli, kale, spinach, sweet potatoes, carrots, red and yellow peppers, apricots, cantaloupe, and mangos
Vitamin C (ascorbic acid)	Inhibition of reactive oxygen species. Stimulation of the vitamin E antioxidant power and selenium. Protection against damages caused by LDL-ox	Citrus fruits and their juices, berries, dark green vegetables (spinach, asparagus, green peppers, brussels sprouts, broccoli, watercress), red and yellow peppers, tomatoes and tomato juice, pineapple, cantaloupe, mangos, papaya, and guava
Vitamin E (α -tocopherol)	Protection of membrane polyunsaturated fatty acids LDL peroxidation	Vegetable oils such as olive, soybean, corn, cottonseed and safflower, nuts and nut butters, seeds, whole grains, wheat, wheat germ, brown rice, oatmeal, soybeans, sweet potatoes, legumes (beans, lentils, split peas), and dark leafy green vegetables
Cu, Zn, Mn, Se	Cofactors of antioxidant enzymes SOD-Cu/Zn, SOD-Mn and glutathione peroxidase	Brazil nuts, brewer's yeast, oatmeal, brown rice, chicken, eggs, dairy products, garlic, molasses, onions, salmon, seafood, tuna, wheat germ, whole grains, and most vegetable.
Other carotenoids (lycopene)	Protection against lipid oxidation, LDL, proteins, and DNA; elimination and inactivation of free radicals	Tomato, ketchup, hot pepper, fishes, watermelon, grapefruit
Resveratrol, catechin, quercetin, phenolic acid (phytochemicals)	Protection against lipid and DNA oxidation	Grapes, berries, peanuts, pine nuts

there is no effect on the development of early coronary atherosclerosis. Vitamin E, according to this study, is positively associated with an increased risk of calcified atherosclerosis [63]. Intravenous infusion of Edaravone, a powerful neuroprotective free radical scavenger, has been investigated in 141 patients with cardioembolic stroke, and it was concluded that it may be only effective in patients with mild cardioembolic stroke [64]. Supplementation with antioxidant vitamins and B-group vitamins, separately or together [96 acute ischemic stroke patients randomized to receive either daily oral 727 mg vitamin E and 500 mg vitamin C ($n = 24$), B-group vitamins (5 mg folic acid, 5 mg vitamin B2, 50 mg vitamin B6, and 0.4 mg vitamin B12; $n = 24$), both vitamins together ($n = 24$), or no supplementation ($n = 24$) for 14 days], enhance antioxidant capacity, mitigate oxidative damage, and may have an antiinflammatory effect immediately after an acute ischemic stroke [65]. Administration of 15 g *N*-acetylcysteine (NAC) infused over 24 h, in combination with streptokinase, significantly diminishes oxidative stress and improves left ventricular (LV) function in patients with acute myocardial infarction (MI) [66]. In addition, serum carotenoids have been

associated, some interactively with smoking, with some benefits such as markers of inflammation, oxidative stress, and endothelial dysfunction [67]. It has also been proposed that antioxidant supplementation (vitamins E and C) may be able to counteract the progressive oxidative stress associated with Chagas disease, underlining that future perspectives for treatment of Chagas disease might include an antioxidant therapy in order to attenuate the consequences of oxidative insult related to this disease [68]. Administration of vitamin C (1 g) and vitamin E (400 IU) for 8 weeks in untreated essential hypertensive patients improves flow-mediated dilation (FMD), significantly reduces central pulse wave velocity (PWV), and decreases augmentation index (Aix). This treatment also increases plasma vitamin levels and antioxidant capacity and levels of oxidative stress. The decrease and changes in central PWV have been related to changes in levels of oxidative stress. Therefore this treatment has beneficial effects on endothelium-dependent vasodilation and arterial stiffness in these particular patients [69]. In a study involving 19 patients with heart failure (HF) after MI, administration of vitamin C has proven to enhance the contractile response to

dobutamine and improve myocardial efficiency [70]. A double-blind parallel study, consisting of five weeks of 100 mg/day or 200 mg/day gamma-T supplementation, has shown that gamma-T supplementation seems to have a permissive role in decreasing the risk of thrombotic events by improving lipid profile and reducing platelet activity [71]. Interestingly, it has been reported that edaravone can salvage the boundary zone of the infarct and is a useful cytoprotective antiedema agent, as shown by magnetic resonance imaging in six patients with extensive hemispheric ischemic stroke [72]. Three months of lipoic acid supplementation seems to relieve exercise pain according to a randomized, double-blind controlled study in 28 participants with peripheral arterial disease (PAD) [73]. Vitamin E (400 U/day) supplementation appears to reduce cardiovascular events in individuals with diabetes mellitus (DM) and Haptoglobin (Hp) 2-2 genotype, a subgroup that comprises 2–3% of the general population [74]. A randomized placebo-controlled study in 26 healthy male subjects and eight male patients with PAD has shown that ischemia-reperfusion (IR)-induced vascular injury can be prevented by administration of vitamin C [75]. A randomized double-blind placebo-controlled clinical trial conducted in 110 men with grade 1 essential hypertension (EH) (35–60 years of age without obesity, dyslipidemia, and diabetes mellitus, nonsmokers, not undergoing vigorous physical exercise, without the use of any medication and/or high consumption of fruit and vegetables) who were randomly assigned to receive either vitamins C + E [vitamin C (1 g/day) plus vitamin E (400 IU/day)] or placebo for 8 weeks has shown that enhancement of antioxidant status by supplementation with vitamins C and E in patients with EH is associated with lower blood pressure [76]. The effects of AGI-1067 on coronary atherosclerosis has been investigated in a placebo-controlled, randomized trial. Atherosclerosis regression was observed, although it was not significantly different from placebo. The antiinflammatory effect of AGI-1067 was supported by reduced levels of myeloperoxidase [77]. An extensive review [78] has shown that dietary advice reduces total serum cholesterol and LDL cholesterol after 3–24 months. It also reduces blood pressure and 24-hour urinary sodium excretion after 3–36 months. Furthermore, dietary advice increases fruit and vegetable intake by 1.25 servings/day. Dietary fiber intake increases with advice by 5.99 g/day, while total dietary fat, as a percentage of total energy intake, falls by 4.49% with dietary advice. In addition, saturated fat intake falls by 2.36%. This study points out that dietary advice may be an effective tool to achieve modest beneficial changes in diet and cardiovascular risk factors over approximately 10 months. Melatonin has significant protective action against the cardiac damage and altered physiology that occur during IR injury

[79–81]. Furthermore, it has been demonstrated that melatonin, at pharmacological concentrations, strongly protects against IR myocardial damage [82]. This protective effect of melatonin may be explained through its action at the mitochondrial level.

15.4 ROS AND NEURODEGENERATIVE DISORDERS

Oxidative stress is involved in the pathogenesis of neurodegenerative diseases and, in particular, the central nervous system (CNS) can be targeted by free radicals because of its high metal content, leading to the formation of ROS and to the relatively low content of antioxidant defenses. The brain seems to be particularly targeted by ROS since it consumes approximately 20% of total body oxygen. It contains high levels of PUFA and may contain low proportions of endogenous antioxidants. Moreover, iron accumulates in brain-specific regions, and iron-binding proteins, such as ferritin, may be relatively deficient in them [83]. Specifically, PUFA are easily targeted by lipid peroxidation because of the ROS interaction with them. This can be explained by the fact that the increase in the number of the molecule double bonds is related to the ease of the hydrogen atom removal. It is not clear yet whether oxidative damage causes neurodegeneration or what its consequences are.

Unregulated metal metabolism may lead to oxidative stress that, in turn, leads to neuronal cell death, but metals play a key role in cellular metabolism and cell signaling. Mutations in mtDNA or metal overload in aged brain lead to oxidative stress and free radical-mediated pathological changes in neurons. Neuronal proteins and structural components are modified because of oxidative stress in different neurological disorders, leading to neuroinflammation and loss of cognitive function in Alzheimer disease (AD), Parkinson disease (PD), multiple sclerosis (MS), and amyotrophic lateral sclerosis (ALS) [84]. Increased oxidative stress has been linked to AD, MS, Batten disease, PD, and brain tumors [85]. In particular, AD is characterized by memory loss with age, a symptom that is linked to ROS-induced neuron damage. AD has been first reported by Alois Alzheimer, a German psychiatrist and neuropathologist, a hundred years ago, and it is the most common course of dementia in the elderly above 65 years of age. It is characterized by two distinct pathological features, namely, intracellular neurofibrillary tangles (NFTs) and extracellular amyloid plaques in the brain, which are both related to neuronal demise and consequential onset of dementia symptoms. NFTs contain abnormally hyperphosphorylated forms of the microtubule-binding

protein tau. Amyloid plaques are made up of insoluble aggregates of amyloid β ($A\beta$) peptides [86]. Clinical characteristics are memory dysfunction, loss of lexical access, spatial and temporal disorientation, and impairment of judgment. The neurotransmitter acetylcholine, necessary for cognition and memory, and oxidative stress may play an important role in AD development [87]. The complex nature and genesis of oxidative damage in AD can be partly due to mitochondrial and redox-active metal anomalies. AD is essentially an acceleration of the aging mechanism in affected brain regions that progressively become more damaged by free radicals. Neurodegeneration, occurring in specific brain areas, namely, substantia nigra and striatum, plus dopamine depletion is considered the key feature. In addition, increased oxidative stress, abnormal mitochondrial function, and excitotoxicity are among the most relevant initiators or mediators of neuronal damage. The disease is inherited and familial (FALS) in 10% of all ALS cases. About 20% of FALS cases are associated with mutations and lowered activity of CuZnSOD, which is known to catalyze the formation

of hydrogen peroxide through the dismutation of superoxide radical anions, playing a relevant role in regulating oxidative damage to cells. Oxidative damage to DNA, protein, and lipids has been observed [83]. The oxidation of arachidonic and docosahexanoic acids, which are the main constituents of the brain membrane phospholipids, leads to the production of malondialdehyde (MDA) and 4-hydroxynonenal, which increase in autopsied specimens from multiple brain regions and in CSF in AD subjects [88–90]. Advanced glycation end products have been found present in AD brains and closely associated with the senile plaques [91].

Vitamin E is a lipid-soluble, chain-breaking, natural antioxidant that is able to cross the blood-brain barrier and accumulate in the CNS, where it reduces markers of oxidative stress [92; see Fig. 15.3 for other properties and its interaction with vitamin C]. A study performed in more than 4000 elderly patients has shown that decreased circulating levels of vitamin E are consistently associated with decreasing memory levels, while the same association has not been observed for plasma levels of vitamins

VITAMIN C	VITAMIN E
Inhibitor of lipid peroxidation	Inhibits oxidative modifications of LDLs (prevents atherosclerosis)
Regenerates vitamin E in lipoproteins and membranes	Inhibits isoprostanes
Reduces α -tocopheroxyl radicals rapidly in membranes and reduces LDL	α -tocopherol is quantitatively the major form of vitamin E in humans
Decreases high levels of isoprostanes in animal models	Crosses the blood brain barrier and reduces markers of oxidative stress in the central nervous system
Reduces homocysteine concentration	Aids in the formation of red blood cells and in the use of Vitamin K

Exert a cooperative effect by residing and scavenging radicals at different positions in the lipophilic compartment

Fig. 15.3 Vitamin C and vitamin E main characteristics and functions [227–229].

A and C and β -carotene [93]. The Honolulu–Asia Aging Study has shown that supplementary intake of vitamins E and C are both associated with better cognitive performance [94]. The Chicago Health and Aging Project, which has analyzed almost 3000 subjects aged 65 to 102 years, has shown that supplementary or dietary intake of vitamin E, but not C, is inversely correlated with cognitive decline [95]. In addition, the Nurses' Health Study, which includes almost 15,000 women aged 70–79 years, has shown that long-term use of vitamin C and E supplements results in a better cognitive status [96]. A lower risk of developing AD [97] has been associated with dietary intake of vitamin E. Another study found that dietary and not supplement-derived vitamin E intake is associated with a lower risk of AD only in individuals who were not carriers of apolipoprotein E4 [98]. Another report showed no association between dietary or supplementary vitamin E intake and a decrease in the risk for AD [99]. The Cache County study also reported no benefit or significant reduction in the risk of AD from supplementation of vitamin E alone. However, the beneficial effect of a reduced risk of developing AD is seen in the combination of vitamin E and vitamin C [100]. A subgroup of the Honolulu–Asia Aging Study, which examined dietary intake of antioxidants and risk of late-life dementia, showed that vitamin C, vitamin E, β -carotene, and flavonoids are not associated with a reduced risk of dementia [101]. In 1997 the results of a double-blind, placebo-controlled 2-year randomized multicenter trial in 341 patients with moderate AD who received the selective monoamine oxidase inhibitor selegiline (10 mg/day), α -tocopherol (vitamin E, 2000 IU/day), both selegiline and α -tocopherol, or placebo were reported. This study shows that treatment with selegiline or α -tocopherol slows the progression of disease [102]. Recently, in a double-blind 3-year-follow-up study, the same high doses of vitamin E had no benefit in subjects with a clinical diagnosis of mild cognitive impairment (MCI). Consequently, the subjects receiving the antioxidants did not show any difference from placebo in preventing the progression of MCI to AD [103]. The same authors have also shown that in a subgroup of these patients the MRI-annualized percent changes of the volumes for some areas of the brain (hippocampus, entorhinal cortex) are less evident in the group receiving the vitamin E than in the placebo group [104].

Among other antioxidants, selenium (Se) seems to be involved in the maintenance of brain function and is particularly present in the brain, especially in the gray matter (area responsible for chemical synaptic communication), even with prolonged Se dietary deficiency [105]. Cognitive impairment, depression, anxiety, and hostility have been associated with low body levels of Se in humans [106]. Levels of Se are elevated in amygdala

[107] and microsomes in the temporal lobe of patients with AD [108]. Selenoproteins, such as GPX1, GPX4, SelP, thioredoxin reductases, selenoproteinW (SelW), and selenoprotein M (SelM), have been implicated in brain maintenance and may play a protective role in several neurodegenerative conditions [109]. Patients affected by AD have shown a significant age-dependent decrease in the Se-dependent glutathione peroxidase activity in plasma and red blood cells [110], while another study has shown an increase in thioredoxin reductase activity in patients affected by the same disease [111]. Interestingly, another study has shown that the levels of Se in plasma and CSF from patients affected by AD are comparable to normal individuals [112]. In the future it would be interesting to investigate the role of selenoproteins in neurological aging and their potential interaction with telomeres. In fact, the length of the telomeres in cells, derived from the hippocampus, has been proven to be longer in AD patients than in control individuals, while in buccal and white blood cells from the same AD patients the telomere lengths are significantly shorter than those of the control groups [113]. This shows that AD patients have evolved a unique mechanism for telomere maintenance. Tanaka and colleagues [114] have shown that age-dependent telomere shortening in human brain microvascular endothelial cells is negated by phosphorylated α -tocopherol, possibly through α -tocopherol's ability to decrease intracellular oxidative stress. Se, in the form of selenite, prevents telomere length shortening during cellular aging in the normal human L-02 hepatocyte [115].

PD is a chronic, progressive neurodegenerative disorder that affects at least 1% of people by the age of 70. This disease was first described by James Parkinson in his 1817 monograph "An Essay on the Shaking Palsy." After that, Charcot described the cardinal clinical features of PD, namely, rest tremor, rigidity, balance impairment, and slowness of movement [116]. The majority of the movement-related symptoms of PD are caused by a lack of dopamine due to the loss of dopamine-producing cells in the substantia nigra. As a consequence, communication between the substantia nigra and corpus striatum becomes impaired, and so does movement. Among others, flavonoids possess potential neuroprotective mechanisms. Their antiinflammatory properties added to their ability to act as scavengers for ROS to maintain the correct glutathione levels and to inhibit Ca^{2+} influx, which represents the end of the cell death cascade, make them important candidates for treatment of AD and PD [117, 118].

We have previously described melatonin's antioxidant properties. PD, together with other age-related neurodegenerative disorders, have been associated with a malfunction of mitochondrial complex I [119, 120],

strongly suggesting that melatonin, through the prevention of complex I deficiency, may improve mitochondrial physiology in brain aging and age-associated brain diseases. The effects of melatonin in the field of cognitive impairment or dementia have been summarized in a review that collected all randomized controlled trials involving the use of orally administered melatonin in any dosage compared with a control group. Unfortunately, the conclusion was that there is insufficient evidence to support the effectiveness of melatonin in managing the cognitive and noncognitive sequelae of dementia [121]. Experimental studies have suggested that mitochondrial decay is a major contributor to brain tissue alterations associated with aging [122], and a potential role of melatonin in mitigation of changes associated with brain aging has been observed [123, 124], but conclusive clinical data are not available yet. A randomized controlled trial involving 16 patients receiving 1 hour of morning light exposure Monday to Friday for 10 weeks and 5 mg melatonin (LM) compared to a placebo group receiving usual indoor light has been performed. The following results were reported: significant improvement in daytime somnolence consisting in a reduction in daytime sleep duration, an increase in daytime activity, an improvement in day-night sleep ratio, and a significant increase in rest-activity rhythm amplitude. Unfortunately, this study does not confirm that the improvements can be attributed to melatonin [125]. A large study has shown that the use of vitamins E and C, alone or in combination, did not reduce the risk of AD or overall dementia over 5.5 years of follow-up, and therefore their use is not recommended for prevention of dementia in older adults [126]. Tocopherol, CoQ₁₀, and glutathione have been used in the prevention or treatment of PD, and their effectiveness has been related to their potential to alter the course of two common theories of PD pathogenesis, namely, free radical generation and mitochondrial complex I deficiency. A collection of three large clinical studies involving the use of tocopherol, four trials of CoQ₁₀, and a study of glutathione for the prevention and treatment of PD has shown that antioxidant supplementation, in particular tocopherol, does not alter the course of PD. CoQ₁₀ and glutathione, according to the studies analyzed, have shown a statistically significant improvement in PD symptoms [127]. A randomized, double blind, placebo-controlled study evaluated the effect of melatonin on sleep and motor dysfunction in PD. This study involved 18 patients with motor dysfunction randomized to receive melatonin (3 mg) or placebo 1 hour before bedtime, for 4 weeks. Melatonin significantly improved the subjective quality of sleep, but polysomnography (PSG) abnormalities were not changed. Motor dysfunction was not improved by the use of melatonin [128].

15.5 COMPLEX REGIONAL PAIN SYNDROME

Complex regional pain syndrome (CRPS) was described for the first time more than a hundred years ago, and it is characterized by chronicity and relapses that can result in significant disability over time. Fractures and surgical insult are considered the main triggers for CRPS, but it can also develop after a seemingly benign trauma. We still do not fully understand how this condition works. It goes by many names, "reflex Sympathetic Dystrophy," "causalgia," "Sudeck atrophy," "algodystrophy," "neurodystrophy," and "posttraumatic dystrophy." The term CRPS was adopted in 1995 by the International Association for the Study of Pain (IASP) to standardize the taxonomy [129–131]. On the basis of signs and symptoms of CRPS, four subgroups have been created: (1) a unique set of signs and symptoms indicating abnormalities in pain processing, for example, allodynia and hyperalgesia; (2) a different set of clinical signs characterized by skin color and temperature changes, which are indicative of vasomotor dysfunction; (3) a third group characterized by edema and sudomotor dysfunction, for example, sweating changes; and (4) a fourth subgroup including motor and trophic signs and symptoms [132]. The progression of CRPS involves three stages: (1) acute, warm, or inflammatory; (2) dystrophic; and (3) atrophic. MRI findings of diffuse bone marrow edema on both sides of the joint, accompanied by periarticular soft tissue edema, with or without a joint effusion, are present in about half of all patients in CRPS stage 1 [133]. It has been hypothesized that ROS may contribute to CRPS. Experimental studies have shown that *N*-acetyl-L-cysteine and 4-hydroxy-2,2,6,6-tetramethylpiperidine reduce the signs of hyperalgesia and allodynia in animal models of CRPS [134]. In addition, topical treatment with 50% dimethyl sulfoxide cream can be effective in decreasing the hypoxia-related production of free oxygen radicals [135]. The use of vitamin C in CRPS has been widely investigated. A double-blind, prospective multicenter trial involving 416 patients with 427 wrist fractures randomly allocated to placebo or treatment with 200, 500, or 1500 mg of vitamin C daily for 50 days showed that vitamin C reduces the prevalence of CRPS after wrist fractures, and a daily dose of 500 mg for 50 days is recommended [136]. It has been reported that a 5-year-old girl with clinical and radiographic evidence of scurvy who developed features of CRPS 2 years after a left ankle fracture improved with the administration of vitamin C [137].

Zollinger and colleagues [138] performed 32 arthroplasties for first carpometacarpal arthritis in 27 patients, using a cementless total trapeziometacarpal joint prosthesis that may be complicated by CRPS. All their patients took vitamin C 500 mg/day starting 2 days

before surgery and continuing for 50 days. There were no cases of CRPS under vitamin C prophylaxis. Besse et al. [139] studied a group of patients having surgery on the foot or ankle with the exception of diabetic foot cases. Four hundred and twenty feet were included in this study: 185 in Group I (no Vitamin C treatment), and 235 in Group II (1 g/day of preventative oral vitamin C treatment). CRPS I occurred in 18 cases in Group I (9.6%) and 4 cases in Group II (1.7%) (57). The authors conclude that vitamin C is effective in preventing CRPS I of the foot and ankle since it was not an infrequent complication in their control group (9.6%). It has been hypothesized that both inflammatory and neural mechanisms may contribute to CRPS type I (CRPS-I). The levels of antioxidants in the serum and saliva of 31 patients with CRPS-I and in a control group of 21 healthy volunteers have been investigated, showing that serum lipid peroxidation products (MDA) and all antioxidative parameters analyzed were significantly elevated in CRPS-I patients. Median salivary peroxidase and superoxide dismutase (SOD) activity values, uric acid (UA) concentration, and total antioxidant status (TAS) values were higher in CRPS I patients by 150%, 280%, 60%, and 200%, respectively, as compared with control subjects. Oxidative changes were also found in the serum, namely, mean serum UA and MDA concentrations, and TAS value in the CRPS-I patients were higher by 16%, 25%, and 22%, respectively, than in the control group. Median salivary albumin concentration and median salivary LDH activities in the patients were 2.5 times and 3.1 times higher than in the control group. The accumulated data show that free radicals are involved in the pathophysiology of CRPS I, which is reflected both in serum and salivary analyses [140].

15.6 CANCER

The Hippocratic "corpus" consists of 70 books written by Hippocrates of Cos (460 BCE–370 BCE) and other physicians. It was the first text to use the words *karkinos* and *karkinoma* to describe a nonhealing swelling or ulceration and a malignant nonhealing tumor, respectively. Hippocrates also introduced the word *scirrhus* to describe hard tumors [141]. The word "tumor" derives from the Latin word *tumere* meaning "to swell." It is synonymous with the term "neoplasm" and can be defined as a lesion resulting from the autonomous or relatively autonomous abnormal growth of cells that persists after the initiating stimulus has been removed [142]. ROS formed in vivo are powerful oxidizing agents, capable of damaging DNA and other biomolecules. An increased formation of the molecules can promote the development of malignancy [143]. Histone deacetylase

inhibitors are important regulators of many oxidative stress pathways, including those involved in the cellular response to oxidative stress. Aberrant regulation of these pathways by histone deacetylases may play a critical role in cancer progression [144]. The relationship among lipid soluble antioxidant vitamins, lipid peroxidation, disease stage, and systemic inflammatory response has been studied in 14 healthy subjects, 20 patients with benign prostate hyperplasia (BPH), and patients with localized ($n = 40$) and metastatic ($n = 38$) prostate cancer. This study suggests that in patients with prostate cancer lower concentrations of carotenoids, in particular, lycopene, reflect a disease progression rather than a systemic inflammatory response [145].

An interesting study evaluated the association between the intake of antioxidants from foods and supplements and the risk of prostate cancer among men in the screening arm of the Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial. This study does not provide strong support for population-wide increase of high-dose antioxidant supplementation for the prevention of prostate cancer. However, vitamin E supplementation in male smokers and beta-carotene supplementation in men with low dietary beta-carotene intake are associated with a reduced risk of this disease [146]. The relationship between Se supplementation and prevalent and incident colorectal adenomas and colorectal cancer (CRC), detected during the Nutritional Prevention of Cancer trial follow-up, has been studied in 1312 recipients randomized to 200 mcg of selenized yeast [only 598 underwent endoscopic screening (flexible sigmoidoscopy or colonoscopy) for CRC sometime during the follow-up period]. This study suggests that Se supplementation is associated with a significantly reduced risk of prevalent adenomas, but only among subjects with a low baseline Se level or among current smokers [147]. A study performed in French adults (7876 women and 5141 men) who were randomized to take an oral daily capsule of antioxidants (120 mg vitamin C, 30 mg vitamin E, 6 mg beta-carotene, 100 µg Se, and 20 mg zinc) or a matching placebo has shown that antioxidant supplementation affects the incidence of skin cancer (SC) differentially in men and women. In fact, in women the incidence of SC was higher in the antioxidant group, while in men the incidence did not differ between the two treatment groups [148]. A study in 1732 Finnish male smokers who were diagnosed with incident prostate cancer has evidenced that higher pre-diagnostic serum concentrations of α -tocopherol, but not dietary vitamin E, are associated with a lower risk of developing prostate cancer, particularly advanced prostate cancer [149]. A systematic review, searching multiple electronic databases from their dates of inception until August 2005, has evidenced that beta-carotene

play a key role in this condition. The antioxidant depletion or deficiency in antioxidants may contribute to oxidative stress. Furthermore, airflow limitation seems to be related to dietary deficiency of antioxidants. The effects of antioxidant polyphenol-rich pomegranate juice (PJ) supplementation for 5 weeks on patients with stable COPD has been investigated, but no benefit has been reported [178]. The association between antioxidant nutrients and markers of oxidative stress with pulmonary function in patients with chronic airflow limitation has been also investigated, showing that serum beta-cryptoxanthin, lutein/zeaxanthin, and retinol and dietary beta-carotene, beta-cryptoxanthin, lutein/zeaxanthin, vitamin C, and lycopene were positively associated with FEV1% (0.05, all associations). In addition, serum vitamins beta-cryptoxanthin, lutein/zeaxanthin, lycopene, dietary beta-cryptoxanthin, beta-carotene, and vitamin C were positively associated with FVC%. Erythrocytic glutathione was negatively associated with FEV1%, while plasma thiobarbituric acid-reactive substances (TBARS) were negatively associated with FVC% [179]. It has been observed that erdosteine treatment in current smokers with mild COPD leads to a significant drop in blood ROS and IL-8 in bronchial secretions (observed after day 4 from the beginning of treatment), while 8-isoprostane drop was significant only after day 10. An e-NO decrease was also reported, but it was not significant [180].

15.11 DIABETES TYPE 1 AND TYPE 2

Diabetes is a blood glucose metabolism impairment subdivided into type 1, which is due to the inability of the body to produce insulin, and type 2, when the body produces the insulin needed but the cells are not able to respond to it, making it ineffectual. Type 2 diabetes has been associated with obesity, and it is a condition that can lead to severe illness and even death. In the peripheral nervous system, diabetes causes a progressive deterioration of primarily sensory nerves, and the damage also extends to motor nerves. Approximately 50% of diabetics experience some degree of neuropathy, which is ultimately the leading cause of lower-extremity amputation [181]. In addition, variations in blood sugar level, namely, hypoglycemia or hyperglycemia can lead to atherosclerosis, neuropathy, retinopathy, and nephropathy. The hyperglycemic state that characterizes the diabetic patient is responsible for the high oxidative stress that accompanies this condition. In particular, this seems to be due to superoxide anion production by the cell glucose metabolism, which, in turn, is able to damage oxidative blood balance, leading to protein glycation and O_2 and H_2O_2 release. It has been shown

that high α -tocopherol levels, among patients with renal disease and in those using vitamin supplements are associated with lower coronary artery disease (CAD) risk in type 1 diabetes [182]. A randomized and open study [183] has been performed on a total of 36 patients with type 2 diabetes and hypercholesterolemia. They were randomly assigned to a probucol group (500 mg/day, $n = 18$) or an atorvastatin group (10 mg/day, $n = 18$). Over 3 months, total and LDL-cholesterol decreased significantly in both groups. LDL-cholesterol was significantly lower in the atorvastatin group than in the probucol group. HDL-C decreased significantly in the probucol group and did not change in the atorvastatin group. Urinary 8-hydroxy-2'-deoxyguanosine (8-OHdG) decreased significantly in both groups after 3 months. Urinary 8-OHdG was significantly lower in the probucol group than in the atorvastatin group after the second and third months of administration.

A study involving 12 patients who were treated with oral ALA, 600 mg twice daily, over a period of 4 weeks has shown that short-term oral ALA treatment increases peripheral insulin sensitivity in patients with type 2 diabetes mellitus [184]. Cilostazol (100 mg) was administered to hypertensive type 2 diabetic patients twice daily. At 1 month follow-up the Cilostazol group (26 patients) showed significant decrease in hsCRP, erythrocyte sedimentation rate (38.7%), total leukocyte count, plasma MDA, HbA1c, and an increase in serum albumin and blood reduced glutathione from baseline. The cardiovascular risk assessment in terms of 10-year risk of cardiovascular heart disease (%), calculated using the United Kingdom Prospective Diabetes Study (UKPDS) guidelines, decreased by 6%. Therefore inflammatory and oxidative stress is high in hypertensive type 2 diabetic patients, and Cilostazol reduces those factors as well as coronary heart disease risk in diabetes mellitus [185].

A study involving 36 type 1 diabetic patients showed that neither normalization of glycemia nor vitamin C treatment alone was able to normalize endothelial dysfunction or oxidative stress; when insulin and vitamin C were combined, endothelial dysfunction and decreased oxidative stress normalized to normal levels. Telmisartan significantly improved basal endothelial function and decreased nitrotyrosine plasma level. In patients treated with telmisartan, a near-normalization of both flow-mediated vasodilation and oxidative stress was achieved when glycemia was normalized. Instead, adding vitamin C infusion did not show further effect on endothelial function or nitrotyrosine plasma levels [186]. A double-blind placebo-controlled clinical trial, conducted for 2 months and involving 35 patients with type 2 diabetes mellitus, has shown that lycopene, probably by increasing TAC and inhibiting MDA-LDL formation, may attenuate T cell-dependent adaptive (proatherogenic) immune

response. With enhancement of innate immunity and hence prevention of ox-LDL uptake by macrophage and foam cell formation, lycopene should be effective in prevention of long-term diabetic complications, notably cardiovascular disease [187]. One thousand two hundred and two people seen in dermatology clinics who did not have type 2 diabetes at baseline were studied in a randomized, double-blind, placebo-controlled trial to receive oral administration of Se (200 µg/d) or placebo. The average follow-up was 7.7 years. According to this study, Se supplementation does not seem to prevent type 2 diabetes, and it may increase the risk for the disease [188]. Vitamin C has a dose-dependent effect on the cellular contents of antioxidants and on vitamin E content of LDL in elderly patients with type 2 diabetes mellitus, but these changes are not sufficient to decrease the LDL susceptibility to peroxidation [189]. DEX (dextrothiamine: R-lipoic acid) therapy appears to reduce endothelial dysfunction in type 2 diabetes mellitus, especially in men with long history of type 2 diabetes mellitus and having poor glucose control (study involving 114 diabetic recipients). DEX is safe and well tolerated, and dyspepsia appears to be the most relevant side effect of DEX treatment [190]. Oxidative stress plays a key role in long-term β-cell dysfunction in type 2 diabetes. It has been shown that treatment with ALA improves functional outcomes, like insulin sensitivity in type 2 diabetic subjects [191]. Supplementation with vitamin E appears to lower plasma glucose in type 2 diabetic subjects [192], but 900 mg vitamin E/day lowers plasma glucose but may not improve pancreatic response to glucose. In humans no relationship between vitamin E intake and severity of retinopathy in type 2 diabetics has been observed [193, 194]. It has also been observed that there is no association between risk of retinopathy and intake of vitamins C and E from foods and supplements [195]. Plasma vitamin E-to-lipid ratio is lower in diabetic subjects than control subjects, and this effect is even more pronounced in diabetic subjects with neuropathy [196]. The same study has also shown that plasma vitamin E-to-lipid ratio is also inversely related to an assessed score of neuropathy, suggesting that diabetic subjects with neuropathy have higher levels of oxidative stress than those without this complication.

15.12 LIVER DISEASES

The liver is the largest organ in the body, weighing approximately 1.36 kg, and exerts several vital functions. It metabolizes substances present in blood, preparing them for excretion, synthesizes many essential proteins, produces bile, and regulates some key nutrients such as glucose, cholesterol, and amino acids. An increase in liver free radicals can lead to inflammation. Normally

the liver uses internally generated antioxidants to neutralize the toxin-derived free radicals, but when the liver antioxidants are low because of alcohol or chronic drug use damage from free radicals increases, resulting in inflammation and formation of scar tissue (fibrosis). Therefore it is important to maintain a constant supply of antioxidants and a healthy lifestyle (abstaining from all alcohol and avoiding environmental toxins) to reduce the strain on the liver. Alcohol lowers liver antioxidant levels, including vitamin E and S-adenosyl-L-methionine, making the liver vulnerable. In addition, alcohol lowers glutathione, an important internal antioxidant. Because heavy drinkers consume a substantial number of calories as alcohol, they consume less vitamin- and mineral-rich food, exacerbating alcohol-induced nutritional deficiencies that include low levels of vitamin C, riboflavin, zinc, pyridoxine (vitamin B6), and vitamins A. A randomized clinical trial has compared antioxidant and corticosteroid treatments, showing that corticosteroids, in the form of prednisolone 30 mg daily, are superior to a broad antioxidant cocktail in treatment of severe alcoholic hepatitis [197]. A combination of seven different antioxidants administered orally (glycyrrhiza capsules, 500 mg, bid; schizandra capsules, 500 mg, tid; ascorbate capsules, 2000 mg, tid; L-glutathione capsules, 150 mg, bid; silymarin capsules, 250 mg, tid; lipoic acid capsules, 150 mg, bid; d-α-tocopherol, 800 IU/day) in patients with chronic hepatitis C virus (HCV) infection has been associated with a significant decline in ALT levels in 52% of patients versus 20% of patients who received placebo. Histology activity index (HAI) score at the end of treatment was reduced in 48% of patients versus 26% of patients who received placebo. HCV-RNA levels decreased by 1 log or more in 28% of patients who received antioxidant therapy versus 12% of patients who received placebo (not significant) [198]. Antioxidant therapy, alone or in combination with corticosteroids, does not improve 6-month survival in severe alcoholic hepatitis patients [199]. An analysis of nine randomized clinical trials including 434 patients with alcoholic liver diseases and the supplementation of SAMe has shown no significant effects on all-cause mortality, liver-related mortality, liver transplantation, or complications [200].

15.13 PANCREATITIS

Acute pancreatitis (AP) is an acute inflammatory condition probably due to the activation of enzymes in the pancreatic acinar cells. Chronic pancreatitis (CP) is a progressive inflammatory disorder that is characterized by recurrent episodes of severe abdominal pain. There is evidence that the pathogenesis of both AP and CP can be associated with oxidative stress. In fact, it has been

better define optimal protocols, in terms of scheduling, timing, and synergy with the emerging drugs (especially biologicals), in order to reduce their side effects and enhance their ability to heal.

15.17 CONFLICT OF INTEREST STATEMENT

The authors certify that there is no conflict of interest with any financial organization regarding the material discussed in this chapter.

15.18 STATEMENT OF AUTHORSHIP

The authors hereby certify that all work contained in this review is original work of Tommaso Iannitti and Beniamino Palmieri. The authors claim full responsibility for the contents of the article.

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THE PROTECTIVE ROLE OF GRAPE SEED POLYPHENOLS AGAINST OXIDATIVE STRESS IN TREATING NEURODEGENERATIVE DISEASES

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16.1 INTRODUCTION

Alzheimer disease (AD) is the most common type of dementia in the United States. Victims of AD commonly display a loss of memory, inability to learn new things, loss of language function, deranged perception of space, inability to do calculations, depression, delusions, and other cognitive deficiencies. AD is ultimately fatal within 5–10 years of its onset. It is estimated that approximately 5 million people in the United States currently have AD [1], with an estimated cost to society of more than \$100 billion per year. Up to 14 million people in the United States are projected to be affected by AD by the middle of this century if effective therapies are not developed [1]. To date, there is no cure for AD; thus researchers are continually exploring novel avenues for the prevention and treatment of this condition. It is important to note that even delaying AD onset by a few years would lead to significant reductions in disease prevalence and, consequently, its burden on health care systems. The few agents that are approved by the FDA for the treatment of AD have only modest efficacy in modifying clinical symptoms, and none appears to affect disease progression or prevention [2]. Historically, the majority of candidate therapeutics have been directed toward cholinergic neurons, which are especially vulnerable in AD and cognitive alterations [2, 3]. More recently, agents are being developed to interfere with the β -amyloid ($A\beta$) protein precursor pathway, which is thought to be

responsible for $A\beta$ -mediated neuronal dysfunction and death [4, 5].

16.2 ALZHEIMER DISEASE NEUROPATHOLOGY FEATURES: IMPLICATIONS FOR THERAPEUTIC DEVELOPMENTS

While the classification and diagnosis of AD are based on the cognitive behavior of an individual, the roots of the disease lie in the neurological pathology of its victims [6]. The two defining neuropathology features of AD are abnormal aggregation and deposition of $A\beta$ peptides and tau protein in the brain as, respectively, extracellular neuritic plaque (NP) and intracellular neurofibrillary tangles (NFT) [7].

$A\beta$ species with different amino and carboxyl termini are generated from the ubiquitously expressed amyloid precursor protein (APP) through sequential proteolysis by β - and "proamyloidogenic" γ -secretases [8, 4, 5]. A 40-amino acid form of $A\beta$ ($A\beta$ 1–40) is the major secreted species. However, a 42-amino acid form of $A\beta$ ($A\beta$ 1–42), which contains two additional residues at its carboxyl terminus, is thought to initiate AD pathogenesis [9]. $A\beta$ 1–42 aggregates much more readily than $A\beta$ 1–40 in vitro and is also deposited earlier and more consistently in AD brain. In humans, AD-causing mutations elevate plasma $A\beta$ 1–42 levels by approximately 30–100% [10].

Even mutations showing small increases in A β 1-42 levels are associated with the onset of dementia in the fourth or fifth decade of life. In the Tg2576 transgenic mouse model of AD, the same mutations produce increases in A β 1-42 levels and markedly accelerate A β deposition [11]. In light of this evidence, a major effort from both academia and industry is presently focused on developing pharmacological strategies that would delay the initiation and/or slow the progression of AD-type A β neuropathology in the brain. Recent evidence from experimental AD mouse models indicates that accumulation of soluble high-molecular-weight oligomeric A β species in the brain, rather than deposition of NP per se, may be specifically related to spatial memory reference deficits. In particular, experimental evidence demonstrated that high-molecular-weight oligomeric A β species may disrupt hippocampal long-term potentiation and synaptic plasticity and induce synaptic deficits [12–15]. Consistent with the hypothesis that soluble high-molecular-weight oligomeric A β species play a key role in AD memory dysfunction, experimental therapeutic evidence demonstrated that neutralization of soluble high-molecular-weight A β oligomeric species from the brain causally improves spatial memory functions in a mouse model of AD [16].

Despite strong genetic data arguing that A β neuropathology is sufficient to cause AD [17], progressive cognitive decline and neuron and synapse loss in AD are best correlated with tau neuropathology [18]. In the AD brain, tau proteins, particularly hyperphosphorylated tau, are found aggregated into progressively larger polymeric species that are ultimately deposited as insoluble NFTs [19]. NFTs themselves are not necessarily the tau species inducing neurotoxicity, as evidence in experimental mouse models suggests that neuronal loss and memory impairment may occur before NFT formation in the brain [20, 21]. Instead, evidence indicates that accumulation of multimeric tau aggregates may play a more important role in AD memory dysfunction [21]. Consistent with this hypothesis, in transgenic mouse models levels of tau multimers are significantly correlated with memory index [21]. Moreover, neuronal loss and memory impairment in a mouse model of tauopathy can be mitigated by reducing tau expression without affecting the number of NFTs [20]. Aggregation of tau is a seed-nucleation process. Formations of hyperphosphorylated oligomers serve as nucleation sites that sequester additional hyperphosphorylated tau as well as normal nonphosphorylated tau [22]. Thus a predominant theory of tau-mediated neurodegeneration is based on a “toxic gain of function” model, in which abnormally phosphorylated tau promotes sequestration of both hyperphosphorylated and normal tau from microtubules, leading to microtubule instability and alteration

of microtubule-mediated processes, including abnormalities in axon transport among others [22].

These considerations strongly suggest that reducing the accumulation of soluble oligomeric A β peptides and tau species in the brain, as opposed to dissociating or preventing NP and/or NFT formation or deposition, may be a more productive approach to AD therapy. Conceptually, it might be possible to reduce brain accumulation of oligomeric tau species by reducing tau aggregation and/or by promoting tau clearance. Similarly, it might be possible to reduce oligomeric A β peptides in the brain by reducing A β generation, reducing A β aggregation, and/or promoting A β clearance. As discussed in more detail below, we recently demonstrated for the first time that dietary supplementation with red wines that is equivalent to moderate wine consumption in humans effectively attenuated the development of A β -mediated AD-type neuropathology and cognitive dysfunction in a mouse model of AD. Moreover, our evidence demonstrated the polyphenolics in the red wines as principle bioactive components that may modulate neuropathology A β phenotypes by reducing A β generation as well as A β aggregation.

16.3 POTENTIAL ROLES OF RED WINES AND WINE-DERIVED POLYPHENOLS IN ALZHEIMER DISEASE PREVENTION AND/OR THERAPY

While genetic factors are highly relevant in early-onset AD cases, their significance diminishes in late-onset sporadic AD cases, which is the most common form of AD [2]. Nongenetic factors, including modifiable lifestyle dietary regimens such as moderate consumption of alcoholic beverages, are receiving increasing attention in AD research, especially in light of the recent epidemiological studies indicating that moderate wine consumption may influence the relative risk for AD clinical dementia [23]. For example, while little is known about the beneficial role of red wine in AD dementia onset, recent studies suggested that the neuroprotective efficacy of red wine may be mediated, in part, by the antioxidant activities of polyphenols in the wine. Wine-derived polyphenols are known to have strong inhibitory effects on lipid peroxidation. Moreover, evidence indicates that several types of natural polyphenols may have neuroprotective effects both in vivo and in vitro, possibly through their abilities to scavenge reactive oxygen species, which may have profound implications for the overall protective effects of red wine in neurodegenerative disorders and stroke outcomes [24].

Presently, little is known about the potential role of red wine in AD. Recent prospective studies showed that moderate intake of red wine may decrease the relative

risk for AD [23]. We therefore explored whether red wines may beneficially modulate AD phenotypes in the Tg2576 AD mouse model.

16.4 THE Tg2576 AD MOUSE MODEL

Tg2576 mice are transgenic mice engineered to express a mutant form of the human APP harboring the Swedish [Lys670→Asn, Met671→Leu] mutation that is found in a subset of familial AD patients. Recapitulating select features of AD, the Tg2576 mouse model is characterized by progressive development of A β neuropathology and cognitive decline. Originally generated by Dr. Karen Hsiao [25], Tg2576 is the most commonly used animal model for studying the mechanisms underlying A β -mediated AD neuropathology and cognitive deterioration. This animal model is also commonly used for testing novel AD therapeutic strategies.

16.5 EXPLORING THE POTENTIAL BENEFITS OF MODERATE RED WINE CONSUMPTION IN Tg2576 MICE

In our initial studies, we treated Tg2576 mice with a dietary supplement of a red Cabernet Sauvignon wine to explore the impact of wine consumption on A β neuropathology and cognitive functions [26]. This was generated from *Vitis vinifera* by our collaborators at the University of Florida as previously described [27]. Our Cabernet Sauvignon contained ~12% alcohol, as determined by ebulliometry, and had a titratable acidity (as tartaric acid) of 6 g/l and a pH of 3.6 [26]. The wine was delivered to the mice by dilution into the drinking water to a final ethanol concentration of 6% [26]. In a parallel control study, Tg2576 mice were provided with drinking water containing 6% ethanol [26]. Wine (or ethanol control) treatment was initiated at 3 months of age before animals developed A β neuropathology or cognitive impairment, and continued to about 10.5 months of age when Tg2576 mice are typically characterized by moderate A β neuropathology and cognitive dysfunction.

In our Cabernet Sauvignon study, we observed that each mouse consumed ~4 ml of wine-adulterated water per day. We calculated that ~7% of the total daily energy consumption in Cabernet Sauvignon-treated Tg2576 mice was derived from wine. This is equivalent to wine-derived energy intake in the human following moderate wine consumption of one 5-oz glass of red wine for a woman (accounting for 6.2% of energy intake) and two 5-oz glasses of red wine for a man (accounting for 10% energy intake) [26]. We also used

an FDA-recommended criterion that incorporates body surface area for calculating equivalent drug doses across species [human equivalent dose in mg/kg = animal dose in mg/kg $\times$ (animal wt in kg/human wt in kg)] [27] as an independent means of estimating human wine consumption that is equivalent to what mice received in our study. We calculated that Cabernet Sauvignon-treated mice consumed ~8 g of alcohol/kg body wt/day, which is equivalent to a human daily intake of 39.5 g of alcohol per day, or daily consumption of 2.3 5-oz glasses (329.2 ml) of Cabernet Sauvignon. Thus, on the basis of two independent calculations, we determined that mice in our study consumed an amount of Cabernet Sauvignon considered moderate by the U.S. Departments of Agriculture and Health and Human Services [26].

16.6 MODERATE CONSUMPTION OF A RED CABERNET SAUVIGNON WINE ATTENUATES AD-TYPE NEUROPATHOLOGY AND COGNITIVE DETERIORATION IN Tg2576 MICE

At the end of the Cabernet Sauvignon treatment, we assessed cognitive functions in Tg2576 mice with the Barnes maze protocol. We found that moderate consumption of Cabernet Sauvignon attenuated the onset of cognitive deterioration in the Tg2576 transgenic AD mouse model (Fig. 16.1A). In parallel control studies we found that moderate Cabernet Sauvignon consumption had no detectable impact on cognitive behavioral performance in age-, sex-, and strain-matched wild-type (WT) mice [26]. We found that cognitive benefits of wine treatment were associated with significant reduction of AD amyloid neuropathology in the brains of Tg2576 mice (Fig. 16.1B). Moreover, we found that Cabernet Sauvignon treatment might modulate A β neuropathology, in part, by promoting brain activity of α -secretase that interferes with generation by cleaving the APP within the A β sequence [26].

In light of evidence that natural polyphenols may exert neuroprotective effects, we explored whether polyphenolic components in Cabernet Sauvignon may be responsible, in part, for the efficacy of this red wine to modulate A β -mediated neuropathology responses. We extracted total polyphenolic components from the Cabernet Sauvignon with acetonitrile-butanol (1:1, v/v), followed by vacuum centrifugation, to concentrate the extracted polyphenol compounds and remove volatile organic components, including ethanol from the wine as well as organic solvents used in the extraction [26]. HPLC analysis confirmed that the contents of polyphenol components in the extract were comparable to Cabernet Sauvignon (data not shown).

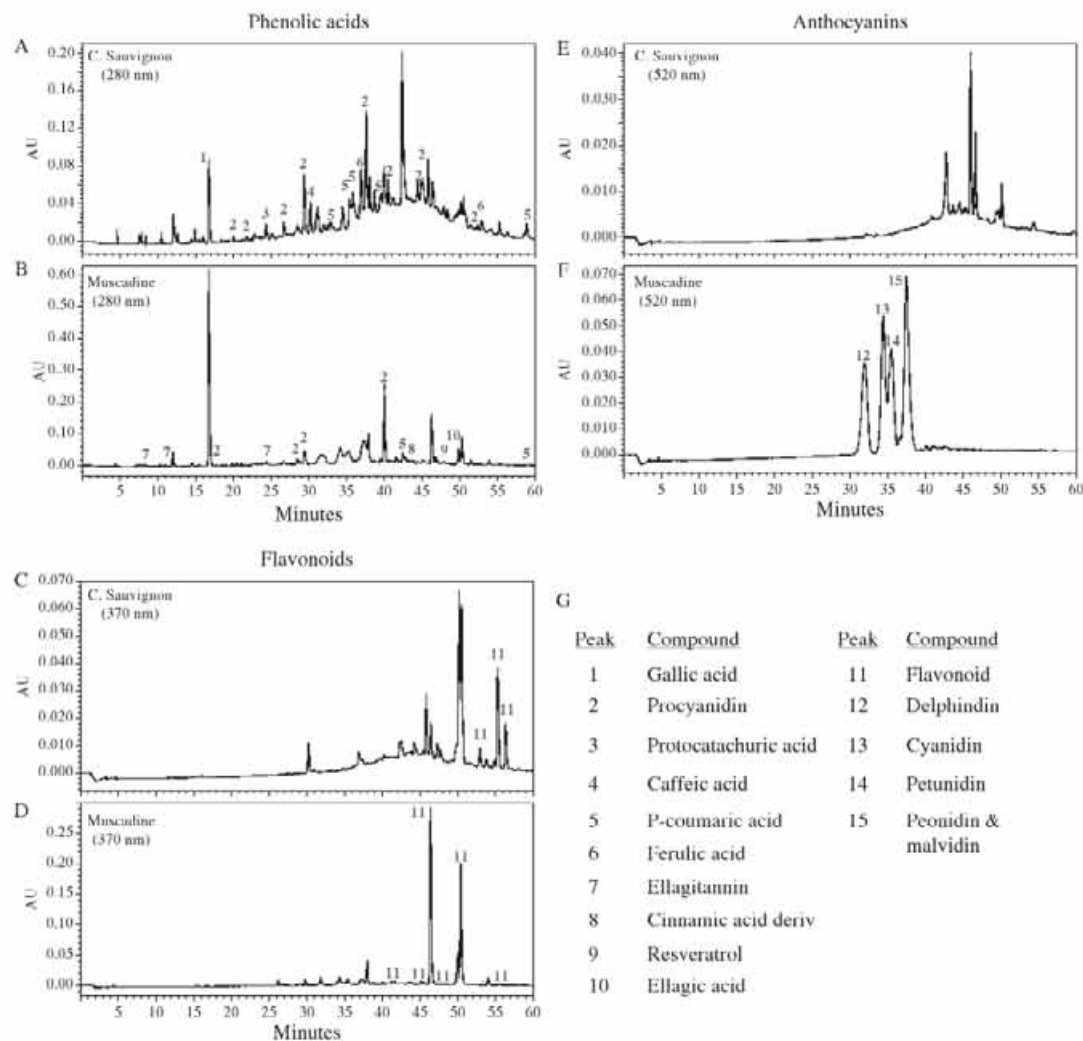


Fig. 16.3 Chemical analysis of Cabernet Sauvignon and muscadine wines. Constituent polyphenolic components in Cabernet Sauvignon (A,C,E) and muscadine (B,D,F) wines were analyzed by reverse phase HPLC using a C18 column. (A,B) Detection of phenolic acid compounds at 280 nm. (C,D) Detection of flavonoids at 370 nm. (E,F) Detection of anthocyanins at 520 nm. (G) Identification of polyphenols corresponding to peaks detected in panel (A-F) based on spectroscopic interpretations.

composition (as gallic acid and measured by the Folin–Coicalteau method) was 1.731 mg/l [27].

We analyzed chemical compositions of muscadine and Cabernet Sauvignon by reverse-phase chromatography using HPLC and an octadecyl silane column [29]. Different classes of polyphenolic compounds from the wines were detected at 280 nm (phenolic acids) (Fig. 16.3A,B), 370 nm (flavonoids) (Fig. 16.3C,D), and 520 nm (anthocyanins) (Fig. 16.3E,F), with select compounds identified based on spectroscopic interpretations from 200–600 nm (Fig. 16.3G) [29]. Our analysis demonstrated that the two wines are characterized by distinct component compositions of phenolic acid (Fig. 16.3A, B), flavonoid (Fig. 16.3C,D), and anthocyanin polyphenolic compounds (Fig. 16.3E,F).

16.8 MODERATE CONSUMPTION OF RED MUSCADINE WINE ATTENUATES AD-TYPE NEUROPATHOLOGY AND COGNITIVE DETERIORATION IN Tg2576 MICE

We treated Tg2576 mice with the red muscadine wine at a dosage equivalent to moderate wine consumption, using a procedure comparable to that we used with our Cabernet Sauvignon studies [29]. Similar to our observation with Cabernet Sauvignon, we found that moderate consumption of the muscadine wine significantly attenuated the development of spatial memory decline (Fig. 16.4A,B) and AD-type A β neuropathology (Fig. 16.4C) in the Tg2576 AD mouse model. However, in contrast to our previous observation with Cabernet

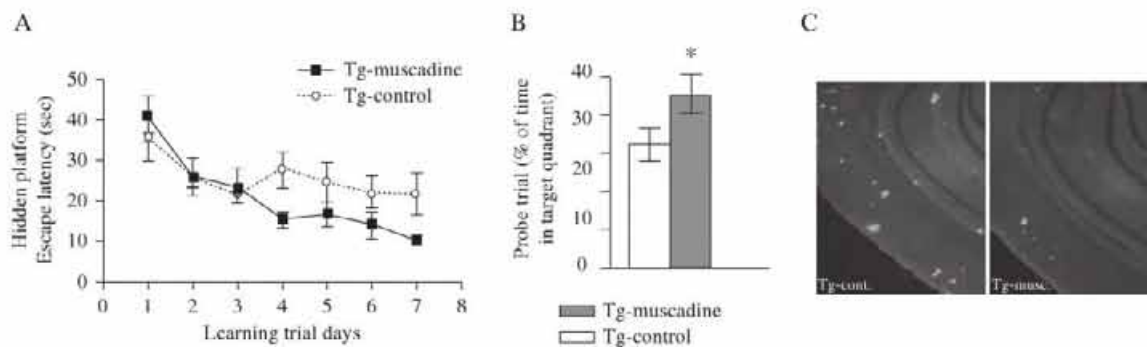


Fig. 16.4 Muscadine treatment improves spatial memory function and A β neuropathology in Tg2576 mice. (A,B) Assessments of spatial memory behavioral functions of 14 month old muscadine-treated (Tg-muscadine) and control, gender- and age-matched non-treated (Tg-control) Tg2576 mice using the Morris water maze protocol. (A) Learning trial hidden-platform acquisition curves. Tg-muscadine group performed significantly better than the control, non-treated group (Tg-control) [2-way ANOVA analysis of Tg-muscadine vs. Tg-control groups for muscadine treatment ($p < 0.05$, $F = 4.24$, $DFn = 1$, $DFd = 84$) and for training days ($p < 0.05$, $F = 6.43$, $DFn = 6$, $DFd = 84$)]. (B) Probe trial conducted 24 hours after completion of hidden-platform training. Muscadine-treated Tg2576 mice exhibited a significantly higher preference for the target platform compared to control, non-treated Tg2576 mice ($p < 0.05$, 2-tailed Student t test). In (A,B) Values represent group mean ($\pm$ SEM); $n = 7-9$ mice per group. (C) Assessments of A β neuropathology reflected by amyloid neuritic plaque density in cerebral cortex and in the hippocampal formation of brain specimens from muscadine-treated and control, non-treated Tg2576 mice. Representative micrograph of brain specimen stained for amyloid neuritic plaques in muscadine-treated (Tg-musc.) or in control, non-treated (Tg-cont.) Tg2576 mice. (See color insert.)

Sauvignon [26], we found that muscadine treatment did not modulate α -secretase activity or activities of other enzymes known to be involved in generation of A β peptides in the brain from APP [29]. Instead, we found that muscadine treatment reduced the accumulation of soluble high-molecular-weight oligomeric A β species in the brain, suggesting that muscadine treatment interferes with A β oligomerization (Fig. 16.5A). In parallel studies, we confirmed the efficacy of muscadine polyphenolics to interfere with the assembly of A β peptides into neurotoxic, high-molecular-weight oligomeric species (Fig. 16.5B). As illustrated by using a gel electrophoresis to assess steady-state A β aggregates, we demonstrated that polyphenolic components from the muscadine wine potently interfere with the formation of a synthetic A β peptide into high-molecular-weight aggregates (Fig. 16.5B). Collectively, our *in vivo* and *in vitro* evidence suggests that muscadine polyphenol may have benefited AD phenotypes in the Tg2576 mouse model of the disease by inhibiting the assembly of A β peptides into neurotoxic high-molecular-weight aggregates.

16.9 DIETARY GRAPE-DERIVED BIOACTIVE POLYPHENOLIC COMPONENTS: IMPLICATIONS IN AD THERAPY AND PREVENTION

The evidence from our studies with two independent red wines summarized above strongly supports the hypothesis that moderate red wine consumption might provide preventive and/or therapeutic value in AD. The

potential health benefits of wine consumption are generally ascribed to the polyphenol compounds that are present in high abundance, particularly in red wines [30, 31]. Since many of the wine-derived polyphenols are strong antioxidants, it is thought that red wine polyphenols may benefit AD (and other neurodegenerative disorders) by reducing the content of reactive oxygen species in the brain [32]. Aside from potential antioxidant activities, our accumulating preclinical evidence suggests that red wine polyphenols may also benefit AD by directly modulating A β -related mechanisms in the brain. Results from our studies demonstrated that polyphenolic components from the Cabernet Sauvignon wine may protect against the onset of AD-type A β neuropathology and cognitive deterioration by promoting α -secretase activity in the brain. In contrast, our studies suggest that polyphenolic compounds from a red muscadine wine may modulate AD phenotypes by interfering with aggregation of A β peptides into neurotoxic high-molecular-weight oligomeric A β species in the brain. Because the two red wines are characterized by distinct polyphenolic component compositions, our evidence suggests that selective bioactive polyphenolic compounds may be responsible for A β -lowering and anti-A β aggregation activities. More studies will be necessary to identify and characterize specific polyphenolic components from red wines capable of exerting A β -lowering and/or anti-A β aggregation activity in the brain. Collectively, our evidence suggests the possibility of developing a "combination" of dietary polyphenolic compounds for AD prevention and/or therapy by modulating multiple A β -related mechanisms (Fig. 16.6).

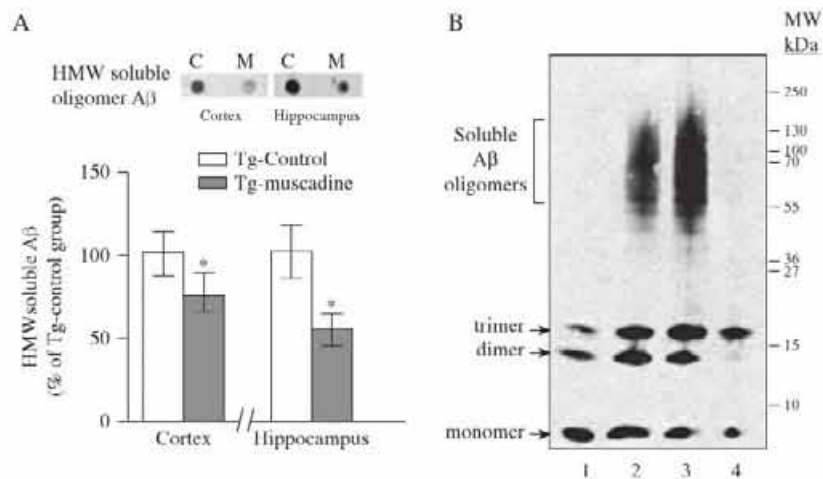


Fig. 16.5 Muscadine treatment reduces aggregation of A β peptides into neurotoxic high molecular weight aggregates. (A) Muscadine treatment significantly attenuated the accumulation soluble high molecular weight A β species in the brain of Tg2576 mice. The contents of soluble high molecular weight A β oligomeric species in the cerebral cortex and hippocampal formation of muscadine-treated or control, non-treated 14 month old Tg2576 mice were assessed by an immunological dot-blot (A, inset) Representative A11-immunoreactive dot-blot analysis of cortical and hippocampal formation brain specimens. Bar graphs represent means $\pm$ SEM., $n = 6-8$ per group; * $P < 0.05$ vs. non-treated control Tg2576 group (2-tailed Student's t test). (B) Muscadine wine interferes with aggregation of synthetic A β 1-42 peptides into high molecular weight oligomer A β species, *in vitro*. Synthetic A β 1-42 peptides were aggregated in the absence or in the presence of muscadine wine. A β species were then resolved by molecular size, transblotted onto a nitrocellulose membrane, followed by immunodetection of A β peptides using and the 6E10 antibody. Lane 1 represents non-aggregated A β 1-42 peptides; Lane 2, aggregated A β 1-42 peptides; Lane 3, A β 1-42 peptides aggregated in the presence of 1.2% ethanol (the same amount of ethanol presented in the aggregation assay in the presence of muscadine in Lane 4); Lane 4, A β 1-42 peptides aggregated in the presence of 1 μ l muscadine wine. In this assay, aggregation of A β without in the absence or presence of ethanol in lanes 2 and 3, respectively, lead to generation of high molecular weight A β species that migrated slowly in the assay. In contrast, addition of muscadine completely eliminate the generation of high molecular weight A β aggregates.

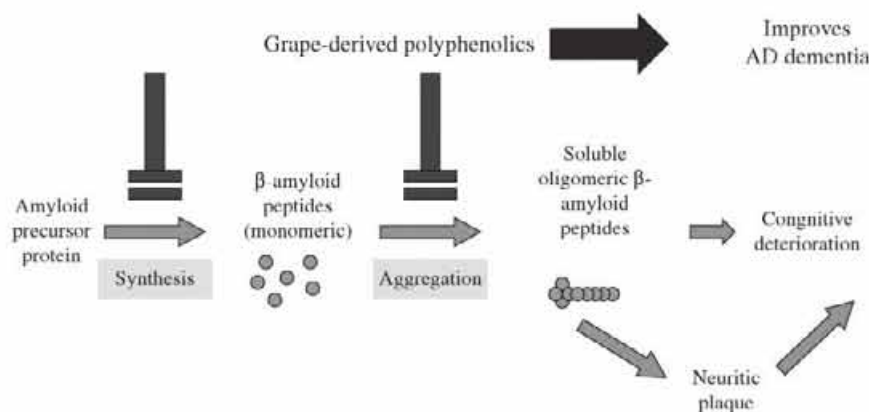


Fig. 16.6 Schematic of how grape-derived polyphenolics may benefit AD by modulating A β -mediated mechanisms. A β peptides are generated from amyloid precursor protein. Thereafter, monomeric A β peptides can be assembled into neurotoxic, soluble high-molecular-weight aggregates that may directly induce neuronal dysfunction that leads to cognitive deterioration. Further aggregation of soluble high-molecular-weight A β aggregates leads to deposition of insoluble A β aggregates as neurotic plaque in the brain that also induces neuronal dysfunction, in part, by promoting brain inflammatory responses. Bioactive polyphenols from grape-derived polyphenolics from red wine or other dietary products may improve interfere with A β -mediated pathological mechanisms by interfering with the generation (synthesis) of A β peptides from amyloid precursor protein and/or by interfering with assembly (aggregation) of A β peptides into neurotoxic, soluble high-molecular-weight species. This raises the possibility of improving AD dementia with a "combination" of dietary bioactive polyphenolic compounds.

In addition to the Cabernet Sauvignon and the muscadine wine, we recently demonstrated that other grape-derived products, namely, a grape seed polyphenolic extract [33] and a purple grape juice (Ho et al., unpublished observation), also exert bioactivity at the organism level and significantly interfere with the development of A β -related phenotypes in AD mouse models. In light of our observation that multiple dietary grape products with distinct polyphenolic component composition effectively protect against the onset and progression of AD phenotypes, we hypothesize that additional grape-derived products, including other red wines, might also provide beneficial disease-modifying activities in AD.

There is an urgent need for additional studies to identify specific bioactive polyphenolics from red wines or other grape-derived dietary products and to characterize the mechanisms of action of these bioactive polyphenolics. Information generated will provide the rational basis for developing selective bioactive dietary polyphenol(s) as lead compounds for clinical testing in AD. Moreover, this information will promote selection of food sources enriched in targeted bioactive polyphenols that ultimately could be incorporated as key components in the development of potential dietary guidelines for AD prevention and/or management.

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used in a variety of natural health care products (e.g., lozenges, tinctures, ointments, drinks).

17.2.1 History

Propolis, bee glue, a gum gathered by bees from various plants, has been used by humans since early times for various purposes, and especially as a medicine because of its antimicrobial properties [3]. Ancient Greek texts refer to the substance as a "cure for bruises and suppurating sore," and in Rome propolis was used by physicians in making poultices. The Hebrew word for propolis is *tzori*, and the therapeutic properties of *tzori* are mentioned throughout the Old Testament. Records from twelfth-century Europe describe medical preparations using propolis for the treatment of mouth and throat infections and dental care [4].

One of the nonmedical uses of propolis is as a varnish, and it has been suggested that the special properties of Stradivarius violins may be partly due to the type of propolis used, although the claim cannot be substantiated.

17.2.2 Composition

At least 180 different compounds have been identified so far in propolis. The class of compounds present in propolis includes resins, waxes and fatty acids, essential oils, pollen, and minerals. The resins comprise flavonoids, phenolic acids, and esters (45–55%) whereas the essential oil contain 10% volatiles. There are nearly 12 free amino acids in pollen and 14 trace minerals, iron and zinc being the most common. The other organics present are ketones, lactones, quinines, steroids, benzoic acid, vitamins, and sugars. The most important pharmacologically active constituents in propolis are the flavones, flavonols, and flavanones (collectively called flavonoids) and various phenolics and aromatics. Flavonoids play a major role in plant pigmentation. However, the flavonoids present in propolis are not glycosides (they do not have sugar molecules attached to their chemical structure). The majority of flavonoids found in plants are glycosides.

Flavonoids are thought to account for much of the biological activity in propolis [5], although other phenolic compounds are also involved. At least 38 flavonoids have been found in propolis, including galangin, kaempferol, quercetin, pinocembrin, pinostrobin, and pinobanksin [6]. Other phenolics include cinnamic alcohol, cinnamic acid, vanillin, benzyl alcohol, benzoic acid, and caffeic and ferulic acid. The chemical composition of propolis is highly variable because of the broad range of plants visited by honeybees when collecting the substance [7].

At least 67 plant species have been reported to provide propolis material. Important sources include poplars, alders and birches, chestnut, ash, and willows. Variations in the beeswax content of raw propolis also affect the chemical composition. Studies indicate that the plant resins collected by bees are at least partially altered by honeybees before use in the hive.

17.3 HUMAN NUTRITION

Propolis also has nutritive value, because of the presence of small amounts of proteins, amino acids, minerals, and sugars. Vitamins include small amounts of A, B₁, B₆, C, and E [8]. Dihydroflavonoids, like those found in propolis, have been shown to aid the human body in absorbing vitamin C [9]. Propolis and a number of its components exhibit a wide variety of biological and pharmacological activities [6].

17.4 THERAPEUTIC PROPERTIES

Because of its strong antimicrobial activity, propolis is often known as a "natural antibiotic." Propolis is reported to possess anticancer, apoptotic, wound healing, antiinflammatory, antiulcer, antiviral, anesthetic, and immunomodulatory properties.

17.4.1 Antimicrobial Properties

A large number of studies have shown an inhibitory effect on a variety of microorganisms (Table 17.1). Active components of propolis showing an antibacterial effect include pinocembrin, galangin, caffeic acid, and ferulic acid, whereas antifungal components include pinocembrin, pinobanksin, caffeic acid, benzyl ester, sakuranetin, and pterostilbene and antiviral components include caffeine. Propolis has been found to inhibit the synthesis of protein by bacteria, which may account for at least some of its antimicrobial effects [16].

17.4.2 Synergistic Properties

Most studies on the therapeutic properties of propolis have centered on the phenolic constituents (flavonoids and other phenolic compounds such as caffeic acid esters). Research has tended to isolate and test single substances in propolis. However, it is likely that the presence of a large number of compounds in propolis may produce a synergistic effect greater than the sum of the effects of individual components [31]. Studies have shown that the flavonoids in propolis exert significant antimicrobial activity compared to whole product extracts.

propolis modulated cell cycle distribution, increasing p53 levels, without the induced HSPs being able to rescue DU145 from death. Hence chemotherapy based on propolis, alone or in combination with vinorelbine, as a potential useful tool for prostate cancer therapy as it effects increase in cell cycle control and the modulation of HSPs expression, reinforced this suggestion.

17.4.4.2 Propolis-Induced Apoptosis of Carcinoma Cells

The treatment of laryngeal cancer is comprehensive, based on surgery. Chemotherapy is an important component of the treatment for laryngeal cancer, which lacks ideal selectivity. The anticancer active ingredients contained in propolis have been identified as flavonoids, terpenes, sugars, esters, and other compounds, such as a natural combination, their mutual coordination role, given the anticancer effect of propolis [41]. Experimental results show that propolis inhibited chemical carcinogen-induced mutations [42]. Because radiotherapy and chemotherapy kill tumor cells, these would also damage the body's immune and hematopoietic cells, and therefore strengthening the immunity against radiation and chemotherapy-induced side effects, is the important task of cancer prevention and treatment. The results show that propolis on Hep-2 cells significantly inhibited cell proliferation and can induce apoptosis and the cell cycle to a certain extent. It can affect the cell cycle G_1 phase to S phase transition, but also affect the S phase of the transition to the G_2/M leading to tumor cell apoptosis and cell cycle non-specific blocking the whole process. With increase of the concentration of propolis, the killing effect on cancer cells was also enhanced. The study revealed that propolis inhibited the growth of laryngeal cancer cells, and the inhibitory action was through the mechanism of apoptosis [43–45].

In a study two new prenyl flavanones, propolin A and propolin B, isolated and characterized from Taiwanese propolis, induced cytotoxicity in human melanoma A2058 cells and showed a strong capability to scavenge free radicals. In this study, propolin A effectively induced a cytotoxic effect on five different cancer cell lines. The levels of procaspase-8, Bid, procaspase-3, DFF45, and PARP were decreased in dose- and time course-dependent manners. Propolin A and propolin B were also capable of releasing cytochrome *c* from mitochondria to cytosol. The findings suggest that propolin A and propolin B may activate a mitochondria-mediated apoptosis pathway. All these results indicated that propolin A and propolin B may trigger apoptosis of A2058 cells through mitochondria-dependent pathways and also showed that propolin A and propolin B were strong antioxidants [46].

It was demonstrated that the prenyl flavanones propolin A and propolin B, isolated and characterized from Taiwanese propolis, induced apoptosis in human

melanoma cells and significantly inhibited xanthine oxidase activity. Furthermore, the isolation of a third compound called propolin C was reported [47]. The chemical structure of propolin C has been characterized by NMR and HRMS spectra and was identical to nymphaeol-A. Propolin C was found to effectively induce cytotoxic effect on human melanoma cells, with an IC_{50} of about 8.5 μ M. In a study to address the mechanism of the apoptosis effect of propolin C, the effect of propolin C on induction of apoptosis-related proteins in human melanoma cells was evaluated. The findings suggested that propolin C may activate a mitochondrion-mediated apoptosis pathway [47].

17.4.5 Antioxidant Properties

The flavonoids concentrated in propolis are powerful antioxidants. Antioxidants have been shown to be capable of scavenging free radicals and thereby protecting lipids and other compounds such as vitamin C from being oxidized or destroyed. The polyphenolic-rich extracts of beeswax have been reported to exhibit antioxidant property in vivo [48–50]. In this study, the extracts were able to reduce CCl_4 - and paracetamol-induced oxidative stress in rats, as evidenced by the changes in hepatic antioxidant and detoxifying enzymes.

Propolin C is also a potential antioxidant agent and shows a strong capability to scavenge free radicals and inhibit on xanthine oxidase activity with IC_{50} of about 17.0 μ M [47].

17.4.6 Wound Healing Properties

Propolis has been shown to stimulate various enzyme systems, cell metabolism, circulation, and collagen formation, as well as improving the healing of burn wounds [51, 52]. These effects have been shown to be the result of the presence of arginine in propolis [53]. Patients with tibial skin ulcers, aged from 23 to 98 years, were treated with propolis tincture in an ointment. The ointment was applied daily to the ulcerated area, which was also treated on the periphery with antibiotic ointments [54]. The treatment lasted for 4–12 weeks. At the end of treatment, 19 of the 84 treated patients exhibited no clinical signs of the condition, and 19 exhibited an improved condition [54].

Propolis was used in a trial of hospital patients with infected wounds. The propolis improved wound healing rates, while at the same time reducing infection. Over half of infective bacteria were eliminated within 4 days. Propolis did not produce antibiotic-resistant strains of the bacteria [55]. A study of topical application of propolis on wounds, burns, and ulcers showed up to an 80% increase in healing rate compared to control subjects using routine healing regimes.

combination of the propolis, Esberitox N and calcium-magnesium resulted in good improvements in the state of the immune system and the clinical condition of both patients [68].

17.4.12 Antiviral Properties

A clinical trial has shown a prophylactic effect against influenza infection in humans [69], and another clinical trial showed that infections of the common cold were shorter in duration and completely recovered within 3 days in patients treated with propolis, compared to 5 days for recovery for patients not given propolis [70]. A clinical trial conducted on dermatology patients showed that a propolis cream had significant therapeutic effects against recurrent herpes (Herpes simplex type 1) and Herpes zona zoster (shingles). The propolis cream reduced duration of lesions and pain and increased interval between lesion episodes.

Perhaps the most broadly investigated and widely accepted attribute of bee propolis is its immune-boosting activity. It is a natural, broad-spectrum antibiotic that activates the thymus gland. Bee propolis not only prevents infectious diseases but clears them from the system as well. As demonstrated in numerous experiments, propolis has the ability to directly destroy bacteria, viruses, and fungi, even penicillin-resistant *Staphylococcus*.

17.4.13 Dental Care Properties

In rats inoculated with *sobrinus*, about half of fissures were carious, while dental caries were significantly less in rats given water containing propolis extract. No toxic effects of propolis on the growth of rats were observed under experimental conditions in this study [17]. Propolis has also been shown to be effective as a subsidiary treatment for gingivitis (gum infections) and plaque [71]. A 50% propolis extract was found to be antiseptic against pulp gangrene [72]. Propolis has also been shown to inhibit the growth of a range of bacterial organisms found in dental caries [73].

The diverse use of propolis in clinical trials showed that its therapeutic efficiency lies mainly in diseases caused by microbial contamination [74]. The results suggest that a propolis preparation can be a useful subsidiary treatment in oral hygiene.

A double-blind clinical trial showed that a propolis mouthwash (10% tincture diluted 1:5 with water) produced significant improvements in patients with gingivitis and periodontal disease. Patients were evaluated for plaque formation and inflammation of the gums. A clinical study used propolis extract and zinc oxide on 160 teeth with indirect capping or deep cavities and teeth with direct capping. The results showed that the

paste with propolis exerted effects similar to those of zinc eugenate and were superior for healing compared to pastes based on calcium hydroxide.

A clinical study found propolis useful for the treatment of gum inflammation and oral mucosa and also showed antiscarring effects [75]. Another study showed similar results for periodontitis and suggested that propolis be used in root canal fillings because of its bone regeneration and anesthetic properties.

17.4.14 Respiratory and Ear Infections

A total of 260 steel workers suffering from bronchitis were treated for 24 days by various methods including local and systemic regulation of the immune system and local treatment with an ethanol extract of propolis (EEP) in a physiological salt solution [67]. Promising results were obtained with inhalation of the extract, together with propolis tablets. Propolis has also shown positive effects in other otorhinolaryngologic diseases, such as pharyngitis chronic bronchitis [67], rhinopharyngolaryngitis [76], pharyngolaryngitis [77], catarrh [78], and rhinitis.

Patients (126) suffering from external otitis, chronic mesotympanic otitis, and tympan perforation were treated with propolis solutions (5–10%). A positive therapeutic result was reported in most cases. Propolis has also shown positive results in the treatment of acute inflammations of the ear [79].

17.4.15 Effects on Mitosis

Medical researchers (N. Popovici and N. Oita of Rumania) published a report on the effects of bee propolis on mitosis (the process of cell division). They reported that a tissue never becomes entirely malignant; it always contains some normal cells, but the activity of the normal cells is affected and even repressed by malignant cells. Propolis favors the activity of normal cells by repressing malignant cells, which helps the tissue to reestablish its normal condition. Constituents of propolis have a mitodepressive effect (depression of the proliferation of cancerous cells) on cells deranged by malignancy.

17.5 ROLE IN CELLULAR SIGNAL TRANSDUCTION

Signal transduction at the cellular level refers to the movement of signals from outside to inside the cell. The movement of signals can be simple, like that associated with receptor molecules of the acetylcholine class, receptors that constitute channels that, upon ligand interaction, allow signals to be passed in the form of small ion movement either into or out of the cell. These ion movements result in changes in the electrical potential

reported in the literature over the last 70 years [114]. Initial reports were made by beekeepers, who came into daily contact with the raw product. Allergic reactions are now also reported in the general population, because of the more widespread use of products containing propolis. Dermatitis can be produced by skin contact with raw propolis as well as propolis extracts, and products containing caffeic acid and its derivatives have been identified as the major allergenic agent [115]. Cinnamic acid derivatives have also been implicated [65].

Dermatitis is relieved once the skin is no longer in contact with the propolis product. It is therefore recommended that with all preparations intended for human use, usage should be ceased whenever there is an allergic reaction. Very few other adverse reactions to propolis have been documented in the literature, and the product is generally considered not to be harmful [6]. Rare cases of oral inflammation and ulceration, mouth edema (swelling), and stomatitis have been reported as a result of oral ingestion of propolis [116, 117].

17.7 COMMERCIAL USE

Raw propolis is collected by beekeepers and sold in bulk to companies that refine the product and turn it into usable extracts. The main commercial uses of propolis are as a dietary supplement and therapeutic. Propolis is sold in tablets (singularly or in combination with other substances such as pollen, royal jelly and nonhive products). In Japan, the use of propolis is permitted as a preservative in frozen fish.

Tinctures and lozenges are popular treatment for sore throats, and tinctures are often used to treat cuts, mouth sores, and skin rashes. For internal use, a 1- to 3-ml dose three times daily of a 1:10 tincture is typical, but higher doses can be used if necessary. Propolis tincture is normally diluted in water, producing a cloudy liquid. For external use, the 1:10 tincture is diluted in water and used as a lotion.

Propolis is a stable product but should nevertheless be stored in airtight containers in the dark, preferably away from excessive and direct heat. Propolis does not lose much of its antibiotic activity, even when stored for 12 months or longer. Propolis and its extract function as a mild preservative because of their antioxidant and antimicrobial activities and thus may actually prolong the shelf life of some products.

17.8 FOOD SAFETY

Because of its antioxidant and antimicrobial activities, microbial contamination is not considered to be a

problem with propolis, either in the raw form or as extracts. Concentrations of lead above maximum allowable levels for food products have been found in propolis. Studies have shown that lead levels may be reduced by placing the hives away from areas with heavy air pollution and the use of oil-based paints on hive parts [118]. Propolis destined for commercial use should be routinely tested for lead concentration. Brazilian propolis is of the highest quality available, whereas Chinese propolis has been noted for excessive lead.

17.9 CONCLUSION

Propolis, bee glue gathered by bees from various plants, has been used by humans since early times for various purposes, and especially as a medicine because of its antimicrobial properties. At least 67 plant species have been reported to provide propolis material. Important sources include poplars, alders and birches, chestnut, ash, and willows. The class of compounds present in propolis includes resins, waxes and fatty acids, essential oils, pollen, and minerals. The resins comprise flavonoids, phenolic acids, and esters (45–55%), whereas essential oils contain 10% volatiles. There are nearly 12 free amino acids in pollen and 14 trace minerals, iron and zinc being the most common. The other organics present are ketones, lactones, quinines, steroids, benzoic acid, vitamins, and sugars. Propolis also has nutritive value because of the presence of small amounts of proteins, amino acids, minerals, and sugars. Vitamins include small amounts of A, B₁, B₆, C, and E. Because of its strong antimicrobial activity, propolis is often known as a "natural antibiotic." Propolis is reported to possess anticancer, apoptotic, wound healing, antiinflammatory, antiulcer, antiviral, anesthetic, and immunomodulatory properties. Artepillin C, a major component of Brazilian propolis, was reported to act as a neurotropic-like factor and to suppress angiogenesis by inducing apoptosis. With regard to toxicity, propolis has been shown not to be toxic to humans or mammals unless very large quantities are administered.

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ENDOCRINE CONTROL OF OXIDATIVE STRESS IN INSECTS

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18.1 INTRODUCTION

The implications for the toxic properties of oxygen were unknown until Gershman's seminal proposal of the free radical theory of oxygen toxicity in 1954, which suggested that the poisonous nature of oxygen is due to the partially reduced forms of oxygen [1]. In the same year Commoner et al. [2] reported weak but detectable electron paramagnetic resonance (EPR) signal in lyophilized biological materials, and this was attributed to the presence of free radicals. These discoveries triggered intense research into the role of free radicals in biological systems. Since then, a large body of evidence has been accumulated that living systems have not only adapted to a coexistence with free radicals but have also developed various mechanisms for advantageous use of free radicals in various physiological functions. Oxygen free radicals generally termed as reactive oxygen species (ROS) as well as reactive nitrogen species are products of normal cellular metabolism. The harmful effect of ROS causing potential biological damage is called oxidative stress. This occurs in biological systems when there is an overproduction of ROS on one side and a deficiency of enzymatic and nonenzymatic antioxidants on the other. In other words, oxidative stress results from metabolic reactions that use oxygen and represents a disturbance in the equilibrium status of prooxidant and antioxidant reactions in living organisms.

18.2 ENDOGENOUS SOURCES OF OXIDATIVE STRESS

Oxidative stress in insects is an unavoidable by-product of the aerobic lifestyle, because the superoxide anion ($O_2^{\bullet -}$) and H_2O_2 are formed whenever molecular oxygen chemically oxidizes electron carriers. The superoxide anion ($O_2^{\bullet -}$) radical is generated by the one-electron reduction of O_2 . Cellular sources of ROS production include plasma membrane NADPH oxidase and intracellular cytosolic xanthine oxidase, peroxisomal oxidases, endoplasmic reticular oxidases, and mitochondrial electron transport components. ($O_2^{\bullet -}$) production can also be due to autooxidation of catecholamines, ubihydroquinone, hemoproteins, and flavin enzymes [3]. Other endogenous sources of ROS include stress and starvation. In all these cases the generation of free radicals is implied. A free radical is unstable and highly reactive and contains one or more unpaired electrons. It can be formed by any of the processes outlined below:

- a. Homolysis of covalent bonds



- b. Addition of a single electron to a neutral atom



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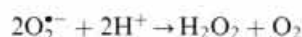
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scavengers designed to remove free radicals generated during reductive processes or during deleterious reactions between excess ROS and cellular macromolecules.

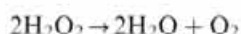
18.4.1 Enzymatic Antioxidative Mechanisms

Insects, like other animals, possess a suite of enzymes that are directed toward the removal of various radicals [12–17]. These include superoxide dismutase, catalase, ascorbate peroxidase, glutathione *S*-transferase peroxidase, etc.

Superoxide dismutase (SOD): Superoxide dismutases are mainly of two main types—the Cu/Zn SOD (located in the cytosol) and the MnSOD (localized in the mitochondria). These enzymes catalyze the following reaction:



Catalase (CAT): Catalase is a 24-kDa homotetrameric enzyme with a heme-iron active center, whose main function is to decompose toxic hydrogen peroxide [18]. This enzyme is localized notably in organelles such as peroxisomes. Hydrogen peroxide is eliminated by catalase as follows:



Ascorbate peroxidase (APOX): This enzyme catalyzes the oxidation of ascorbic acid with the concurrent reduction of hydrogen peroxide and could serve an important function in removal of hydrogen peroxide in insects [19]. Regeneration of reduced ascorbate is achieved by the enzyme dehydroascorbate reductase. Ascorbate by itself is known to scavenge singlet oxygen, superoxide, and hypochlorite as well as water-soluble radicals such as peroxyl, hydroxyl, thiyl, suphenyl, and nitroxide radicals [13,20–25]. Thus, in addition to its peroxidase activity, the efficient recycling of ascorbate itself gives it a powerful antioxidant role.

Glutathione *S*-transferase peroxidase (GSTPx): Vertebrates contain a selenium-dependent glutathione peroxidase (GPOX) that removes both hydrogen peroxide and lipid peroxides [12]. Insects have a glutathione *S*-transferase with peroxidase activity (GST-px). This enzyme can metabolize lipid peroxides but is unreactive toward hydrogen peroxide [12, 15, 26, 27].

Thioredoxin/thioreductase system (Trx/TrxR): To maintain its preferred redox state, the insect cells activate the NADPH-dependent thioredoxin/thioreductase enzyme system, which rapidly restores the modified thiols to their unmodified reduced state [25, 28].

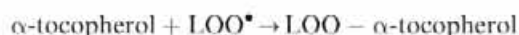
18.4.2 Nonenzymatic Antioxidative Mechanisms

In addition to the classical antioxidant enzyme systems, a number of small molecules also play a significant role

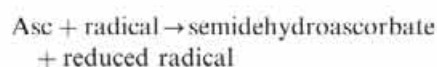
in scavenging ROS and curbing the deleterious effects of oxidative stress. Specifically in insects some of these small molecules are plant derived, while others can be synthesized by insects:

Carotenoids: The carotenoids constitute a family of conjugated polyenes. Carotenoids could be vitamin A precursors and are able to quench singlet oxygen. They may also directly react with peroxyl radicals. In plants their role has been well documented as a free radical scavenger, but the specific role in insect systems remains a speculation.

α -Tocopherol: The natural α -tocopherol is the 2R,4'R,8'R- α -tocopherol. This lipid-soluble vitamin is very reactive in membranes and can react with lipid peroxyl radical LOO to form the relatively stable radical that probably does not react.



Ascorbic acid: Insects cannot synthesize ascorbic acid and depend on dietary sources for their supply of this essential antioxidant. In the presence of reactive species, ascorbic acid (or its conjugated base, ascorbate) is a one-electron donor and is oxidized in semidehydroascorbate radical, a molecule stabilized by the delocalization of the electrons between the three oxygen atoms. The semidehydroascorbate is converted back into ascorbic acid in the presence of glutathione and dehydroascorbate reductase:



Glutathione (GSH): Glutathione is a very important free radical scavenger. This is a tripeptide γ -glutamyl-cysteine-glycine and is one of the most abundant low-molecular-weight thiols present in the cell. Reduced GSH is characterized by its reactive thiol group, and as an effective reductant it plays an important role in a variety of detoxification processes. GSH readily interacts with free radicals and oxidizing compounds such as H_2O_2 , $\text{O}_2^{\bullet-}$, $\text{OH}^{\bullet}$, and carbon radicals including protection against lipid peroxide damage [14, 25, 29]. In the presence of the radicals the reduced form (GSH) is oxidized (GSSG) and can then be recycled back in a NADPH-dependent reaction catalyzed by glutathione reductase or by the thioredoxin reductase systems [30].

In addition to the antioxidant mechanisms and systems described above, insects also possess several water-soluble molecules (uric acid, carbohydrates, polyols) and iron binding proteins (ferritin and transferrin) that also serve crucial antioxidant functions [14].

18.5 REGULATION OF DEFENSES AGAINST OXIDATIVE STRESS

Oxidative stress triggers a range of physiological, pathological, and adaptive responses in insect cells either as a result of cellular damage or through specific signaling molecules. These responses ultimately modulate transcriptional outputs to influence and induce antioxidant systems. In the past couple of decades, a number of transcription factors and signaling pathways have been identified and delineated to mediate critical transcriptional responses to oxidative stress. These signaling pathways include mitogen-activated protein kinases (MAPKs) P13K/Akt, protein kinase C (PKC), protein53 (p53), nuclear factor κ B (NF- κ B), activator protein-1 (AP-1), and redox regulation by redox factor-1 (Ref-1) and the Nrf2-mediated antioxidant response. Nrf2 (nuclear factor erythroid 2 related factor 2) belongs to a group of specialized transcription factors termed xenobiotic-activated receptors (XARs). These recognize specific xenobiotics and coordinate the transcription of batteries of genes. The specifics of activation at the gene level of these signaling pathways are beyond the scope of this particular chapter. Here, we focus on the role that insect hormones play in the regulation of defenses against oxidative stress.

18.6 INSECT HORMONES AND THEIR ROLE IN THE CONTROL OF OXIDATIVE STRESS

The insect endocrine system produces various hormones and biologically active factors that can be divided into three main groups [31] including (a) ecdysteroids, steroid hormones, produced primarily by prothoracic glands and partially also by several other tissues. They control mainly molting, development, metamorphosis, and reproduction and are also involved in a number of diverse processes. (b) Juvenile hormones (JHs), a family of acyclic sesquiterpenoids, the principal products of the corpora allata—a retrocerebral gland—are involved in the regulation of metamorphosis and reproductive processes such as control of gonadal development and vitellogenin synthesis. Additionally, the roles of JH have expanded to include caste determination, behavior, diapause, and various polyphenisms [32]. And finally, insects also produce (c) neurohormones, a large group of peptidergic compounds produced by specialized secretory neurons, which are most abundant in the brain but do occur throughout the whole nervous system. They control a number of biochemical, physiological, and behavioral events in the insect body including those mentioned above for ecdysteroids and JHs [33]. The classification of neurohormones is not uniform, but they are ordinarily

categorized according to their functions. One of the best-defined groups of neurohormones associated with stress responses are metabolic neuropeptides belonging to the AKH/RPCH family (adipokinetic hormone/red pigment concentrating hormone family). A major function of these small peptides (octa-, nona-, or decapeptides), which are synthesized and released from an endocrine retrocerebral gland corpora cardiaca, is the control of insect metabolism. However, they are pleiotropic, with a number of actions in addition to their metabolic role. Generally, they behave as typical stress hormones by stimulating catabolic reactions (mobilize lipids, carbohydrates, and/or certain amino acids), making energy more available, while inhibiting synthetic reactions. They mobilize entire energy reserves to combat the immediate stress problems and suppress processes that are momentarily less important and could, if allowed to continue, even draw on the mobilized energy. These biochemical stress reactions are accompanied by activation of physiological stress response that includes stimulation of heart beat [34], increase of muscle tonus [35], stimulation of general locomotion [36], enhancement of immune response [37, 38], and some others. Recently it has been found that AKHs are also involved in the control of oxidative stress (OS) in insects [see 39].

18.7 ROLE OF ADIPOKINETIC HORMONES IN INSECT OXIDATIVE STRESS

An active role of AKHs in protection of insects against OS was deduced from results of a set of papers published recently (see below). These results reveal that oxidative stressors increase the level of AKHs and, additionally, that exogenous AKHs mitigate OS biomarkers in insect body experimentally enhanced by application of the stressors. These facts indicate that there is a feedback regulation between an oxidative stressor and AKH actions, and that AKHs are involved in the activation of an antioxidant protection mechanism in insects.

Application of the oxidative stressors elevates the titer of AKHs in hemolymph. The effect is reported for paraquat (1,1'-dimethyl-4,4'-bipyridylum dichloride hydrate) [40, 41] a redox cycling herbicide that is commonly used to create conditions of OS in insect body, and also for *Galanthus nivalis* agglutinin and *Bacillus thuringiensis* toxin [40] and for endosulfan and malathion [42]—insecticides with an OS effect. The intensity of AKH level elevation varies depending on insect species, type of oxidative stressor, or time of the stressor incidence. For example, feeding of genetically modified potatoes containing the Cry 3Aa *Bacillus thuringiensis* toxin elevates the AKH level in hemolymph of the Colorado potato beetles *Leptinotarsa decemlineata* up

to 6-fold and treatment by paraquat about 2.7-fold within 4 hours after the treatment [40]. Similarly in the bug *Pyrrhocoris apterus* paraquat increases the AKH level in hemolymph about 5 times (again 4 hours after the treatment) [41] and insecticides endosulfan and malathion about 1.5 times 24 hours after the treatment [42].

Even larger variability was recorded when level of AKH was measured in the corpora cardiaca or the whole CNS (including the corpora cardiaca). In *L. decemlineata* feeding of both the Cry 3Aa toxin and *Galanthus nivalis* agglutinin elevates the level up to 10 times and application of paraquat just 1.9 times [40]. In *P. apterus* the same application has no effect on the AKH level in the CNS (4 hours after the treatment) [41], and treatment with the insecticides endosulfan and malathion elicits only a slight increase in the CNS [42]. On the other hand, the differences between the stress-evoked AKH levels in CNS and hemolymph are not so surprising, because the whole amount of AKH seems to be substantially distinct in those two body parts. It is reported there is about 200 times lower AKH amount in hemolymph than in CNS of *P. apterus* [43]. In addition, no positive correlation is shown for the same species between the AKH level in hemolymph and CNS [44]. This result is in good agreement with the suggestion that a coupling between release and biosynthesis of the AKHs in adipokinetic cells of the corpora cardiaca is very loose or does not even exist [45].

Questions arise on the possible mode of the feedback action on the biochemical pathways by which the enhanced AKH level mitigates the OS. While the mechanisms of AKH action leading to energy mobilization have been documented by a number of publications [46–48, etc.], almost nothing is known about the stress-evoked AKH actions that do not include rapid production and subsequent consumption of energy. Other than OS, stress-induced elevation of AKH titer was demonstrated in the locust *Schistocerca gregaria* and *P. apterus* challenged with an insecticide [42, 49–51], excessive KCl [49], and photophase interruption and exposure to constant darkness [52].

The suggestion that AKHs are involved in the activation of protective antioxidative mechanisms derived from the effect of oxidative stressors on AKH level in the insect body is supported by a series of experiments demonstrating direct involvement of AKHs in the modulation of OS biomarker levels. Only a few biomarkers have been studied in the relationship to AKH actions so far. One of the most convenient is glutathione. It is a low-molecular-weight thiol (see Section 18.4.2) found in the cytosol and other aqueous phases of various living systems [53, 54]. The level of GSH in insect hemolymph is significantly increased (about twice) after AKH injection but significantly depressed (about 2–3 times) after

paraquat treatment both in *L. decemlineata* [40] and *P. apterus* [41] hemolymph. On the other hand, coinjection of AKH together with paraquat results in enhanced GSH content back to the control level. A very similar picture is obtained when the insecticides endosulfan and malathion are used [42]. It appears that AKH is able to directly or indirectly enhance efflux of reduced GSH into the insect hemolymph as demonstrated for the pancreatic vertebrate hormone glucagon that stimulates massive GSH efflux from the liver into the bloodstream [55]. However, it is quite possible that GSH alone (induced by AKH) could not be the one to confer enhanced antioxidant capacity to the hemolymph.

Another important marker of OS is protein carbonylation, whose level illustrates the oxidative damage of the system. Carbonyls are formed from amino groups in the side chain of certain amino acids that are exposed to ROS [56]. Application of paraquat into both *L. decemlineata* and *P. apterus* body, as well as application of Cry 3Aa and *Galanthus nivalis* toxins in the food of *L. decemlineata*, significantly enhance carbonyl contents in hemolymph, but coinjection of paraquat with AKH decreases their levels to those found in control groups [40, 41]. It is interesting that AKH injection alone does not change the carbonyl contents to those below control values. This indicates that possibly a stressor action is needed for AKH to potentiate the response, as in case of phenoloxidase activity [37], lipid store mobilization rate after injection and/or topical application of external AKH [57], and modulation of catalase activity [42] (see also below). It seems that the adipokinetic response is enhanced and/or to some extent modified primarily in the presence of stress, caused by an injection or by an application of insecticides as in the two latter cases.

A lowering of the carbonyl contents also supports the suggestion of an operative role of AKHs in antioxidant action to counter OS in insects. The complex strategy of this action is still unclear; however, it is quite possible that more players, besides GSH, are involved. This suggestion is supported by measurement of the total antioxidant activity in cell-free plasma of *P. apterus* against Trolox (an analog of vitamin E) standards. This procedure relies on antioxidant activity of low-molecular-weight compounds. The results show that paraquat injection alone potentiates an antioxidant response that is significantly enhanced upon coinjection of paraquat with AKH. However, AKH injection alone is not capable of inducing antioxidant activity to the levels produced by paraquat alone or by coinjection with paraquat, although it is more enhanced compared to control groups [41]. Hence, this supports the suggestion that there must be some other additional mechanism(s) by which AKH acts to enhance antioxidant response.

The list of AKH activities involved in the OS control also includes the modulation of activity of catalase that is responsible for decomposition of toxic hydrogen peroxide into water and oxygen [18]. Application of insecticides endosulfan and malathion causes a significant increase in catalase activity in the whole body of *P. apterus* but when coapplied with AKH a significant decrease is recorded compared with insecticides alone. The catalase activity after the coapplication is still significantly higher than that after AKH treatment alone; however, the fall as compared to application of the insecticides is substantial, achieving 2.3- to 3.7-fold lower values. Since free radical-scavenging enzyme complexes like superoxide dismutase, catalase, and glutathione peroxidase are in the first line of cellular defense against oxidative injury [58–60], the induction of catalase after insecticide application is not surprising. Possibly, AKH reduces the production of hydrogen peroxide by an unknown mechanism, and therefore the activity of catalase necessary for reduction of hydrogen peroxide is lower.

AKH activities in the stress elicited by insecticides are also linked to one interesting phenomenon: an enhancement of the insecticide activity by AKH coapplication. This phenomenon is probably not directly connected with OS, because it was first reported for the insecticide permethrin [51], which does not possess the ability to generate OS, but a certain relationship to OS cannot be completely excluded when endosulfan or malathion, known to promote formation of free radicals, is used [61–63]. The coapplication of the insecticides with AKH increases *P. apterus* mortality up to 3-fold [42]. Almost nothing is known about the mechanism of the phenomenon, but it has been suggested that the AKH stimulation of metabolism [33] could enhance the insecticide action. This suggestion is supported by the AKH-induced significant increase of carbon dioxide production [42,51]—an indicator of metabolic rate in the experimental bugs. The increased metabolic rate could intensify the insecticide action by faster penetration of the insecticide into tissues and by a more intensive exchange of metabolites affecting the biochemical pathways, including also the OS reactions. However, in the absence of direct data this statement remains a speculation.

18.8 ROLE OF OTHER INSECT HORMONES IN OXIDATIVE STRESS

Not only have AKHs been implicated to be involved in hormonal control of antioxidative protective reactions in insects, but other hormones such as glucagon, ecdysteroids and JHs have also been suggested to be involved.

Glucagon: It is a 29-amino acid peptide well-known as a vertebrate hyperglycemic hormone. In insects,

immunochemically similar glucagon-like peptides were reported in hemolymph [64], midgut [65, 66], and nervous system [66–69] of several insect species. On the other hand, a role for the glucagon-like peptides in insect body has not been explained satisfactorily. They could play a role in the brain-gut axis [70] and participate also in the control of digestive or other metabolic processes, but direct evidence is still missing. There are some indications that glucagon-like peptides play a role in regulation of insect glycemia, as was shown in the honey bee *Apis mellifera* [71], but this effect is most likely not common in other insects [72]. Analogous to vertebrates [55,73], Alquicer et al. [66] suggested for insects that glucagon could play a role in defense against OS, because injection of porcine glucagon into the *P. apterus* body elicits the antioxidant response by significantly increasing GSH and decreasing protein carbonyl levels in hemolymph, and decreasing both protein carbonyl and protein nitrotyrosine levels in CNS. Moreover, similarly as reported for the AKHs (see Section 18.7 for details), when coinjected with paraquat, glucagon partially eliminates OS markers elicited by this oxidative stressor and returns them to the control levels. Nevertheless, the lack of significant changes of AKH titer in *P. apterus* body after the injection of glucagon suggests that glucagon action is AKH independent.

Ecdysteroids: These are well-known insect steroid hormones playing a crucial role in insect developmental and reproductive events (see also Section 18.6). In vertebrates steroid hormones, namely, estrogens and related compounds possessing a phenolic A-ring, were shown to be involved in the control of OS. They inhibit the oxidation of cholesterol and the peroxidation of polyunsaturated fatty acids (diene conjugation or malondialdehyde formation) in the lipoproteins, microsomes, and other components of biological systems [74]. 20-Hydroxyecdysone, one of the most important member of insect steroid hormones, shows similar features. It has been proven that 20-hydroxyecdysone is a potent antioxidant able to minimize the OS impact of paraquat to *P. apterus* [17]. This ecdysteroid restrains lipid peroxidation and the formation of protein carbonyls, ameliorates changes in microsomal membrane fluidity, enhances the level of reduced glutathione, and upregulates the activity of γ -glutamyl transpeptidase in the brain. However, the protective effect of 20-hydroxyecdysone against the OS has also organismal dimensions. Certain hemolymph proteins, very sensitive to the paraquat treatment, are consistently present in the bugs being injected with 20-hydroxyecdysone despite the paraquat presence. The same injection ameliorates the suppressive paraquat effect on female fertility and in both sexes improves the survival rate curtailed by paraquat.

A similar function of 20-hydroxyecdysone is reported by Roesijadi et al. [75], who show that ecdysone induced methionine sulfoxide reductase A in the fruit fly *Drosophila melanogaster* is associated with enhanced resistance to hydrogen peroxide. Methionine residues in proteins are susceptible to oxidation when subjected to reactive oxygen or nitrogen species [76]. The reduction of the oxidized methionine form is catalyzed by methionine sulfoxide reductase [77]. Expression of the enzyme is regulated via the ecdysone receptor (EcR-UPS) complex [78] controlled by ecdysone. Overexpression of the enzyme is associated with enhanced protection against OS, while its knockdown results in hypersensitivity to OS [79, 80].

The mechanisms of action of these effects are unknown, but their existence indicates the importance of ecdysteroids for the management of OS in the insect body [17].

Juvenile hormones: These are insect terpenoid hormones with two main functions in insect life. As developmental hormones, they prevent premature initiation of insect metamorphosis in juvenile stadia and support reproduction by hormonally controlled gene expression of vitellogenins in adult females. Their role in OS seems to be indirectly mediated through the regulation of biologically active proteins like vitellogenins [81] and/or transferrin [82, 83].

Vitellogenins primarily serve as energetic and building components for a developing embryo in the insect egg. They are synthesized in specialized cells in the fat body or rarely in ovaries by a complicated hormonally controlled process (see 84) in which the JHs in most insect species play a crucial role. In the bee worker vitellogenins protect the organism against the oxidative damage of paraquat [81]. The results of the authors show that vitellogenins are a preferred target of oxidative carbonylation in comparison with other hemolymph proteins, which is a property that is indicative of antioxidant function [85]. There is a direct implication of this finding for bee aging: Treatment of low- and high-vitellogenin level bee phenotypes with paraquat showed that survival was significantly lower for the former group. The data show that vitellogenin activity is causally linked to OS resistance of the bee, and further, they can explain why vitellogenins are synthesized at high levels in honeybee queens and are abundant in long-lived workers [81].

Insect transferrin is known as an iron transporter, regulated by vitellogenin and JH activities. Its essential function is transport of iron that is required for a wide variety of metabolic processes including oxygen transport and electron transfer; iron also participates in the Fenton reaction (see Section 18. 2), in which the very reactive hydroxyl radical is generated [86]. Insect

transferrin is regulated by a lot of diverse processes (87) including hormonal processes. As transferrin is suggested to be a vitellogenic protein, an involvement of the JHs is not surprising. Jamroz et al. [82] showed a strong suppression of the transferrin gene in the fat body by JH treatment of the cockroach *Blaberus discoidalis*. Harizanova et al. [83] demonstrated in the mosquito *Aedes aegypti* that the JH analog methoprene suppresses the increase of transferrin message after blood feeding. The reasons for the suppression are not properly understood: Transferrin gene could be a target of transcription factors of ecdysteroid-induced mosquito homologues, and a negative effect of JH on them could play some role [83].

18.9 CONCLUSION

The past couple of decades have yielded a plethora of literature advancing our understanding of the signal transduction pathways in response to oxidative stress. While these are important in mediating transcriptional response to oxidants at the molecular level, major players in stress response pathways such as stress-responsive hormones are only beginning to be implicated especially in insects. With this has also come an appreciation for the complexity of the endocrine-mediated responses and the awareness that these hormones may not be acting in isolation but intersecting with one another to mediate a myriad of the physiological and adaptive responses to oxidative stress in dose- and cellular context-dependent manners. Emerging from the survey of endocrine actions in stress responses (particularly oxidative stress) is a picture of extraordinary diversity and complexity, whether viewed in terms of the target cells, the metabolic pathways, or the physiological functions that the neurohormones regulate. Although we have tried not to be too elaborate, within our limited goal, we have discussed to the best of our available knowledge the role of insect stress hormones in oxidative stress. Molecular understanding of the interactions between the endocrine system and oxidative stress will continue to yield important insights into mechanisms that trigger such responses. We hope that the present review will stimulate further research within a framework of insect hormone actions to oxidative stress constituting a coherent, albeit complex and heterogeneous, physiological whole.

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OXIDATIVE STRESS IN THE AIRWAY SYSTEM OF THE FRUIT FLY *DROSOPHILA MELANOGASTER*

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19.1 INTRODUCTION

Oxidative stress is a major threat for all organisms living in a high-oxygen atmosphere. Reactive oxygen species (ROS) can be produced either exogenously or endogenously. The major exogenous source of ROS is the atmosphere, which implies that especially those organs or parts of the body that come into close contact with large amounts of air are at risk. This would mean that the airway epithelium is prone to be damaged by ROS and thus needs specialized mechanisms to cope with this situation. Endogenous production of ROS is highest in those tissues with the greatest energy consumption, as it is an inevitable by-product of mitochondrial activity. To cope with this ROS confrontation, animals employ different sets of enzymatic as well as nonenzymatic antioxidants. ROS-mediated damage of tissues and cells is believed to be one major reason for changes associated with aging. The oxidative stress theory of aging that was introduced more than 50 years ago [1] predicts that effective scavenging of ROS may delay aging and thus should increase life span. Free radicals and other ROS induce molecular damage at different levels, leading to increased incidences of mortality as a function of time. These ROS-mediated damages that can occur in all major groups of macromolecules accumulate over time, and, at a certain time-point, the organism is unable to repair all of them. Accumulation of these damages interferes with the normal physiological function of the cells, which may end up in reduced overall performance

of the organism and finally in mortality. A great number of studies have supported this theory, especially those performed with model organisms such as the fruit fly *Drosophila melanogaster*. In general, increasing the antioxidant repertoire of these model organisms tends to increase life span. In particular, two enzymatic antioxidants, superoxide dismutase (SOD) and catalase, tend to mediate life-prolonging effects in *Drosophila* or *Caenorhabditis elegans*, an observation that is still matter of debate.

19.2 OXIDATIVE STRESS SYSTEMS IN *DROSOPHILA*

A number of different tissues show a higher sensitivity toward oxidative stress than others. Among these are the nervous system, but also the airway epithelium, as this structure is constantly exposed to high oxygen flow rates and therefore to ROS. Regarding nonenzymatic antioxidants that are employed by the fly, our knowledge is limited. Interestingly, chronic food supplementation with compounds having antioxidant properties has the potential to increase life span, indicating that nonenzymatic antioxidants are of relevance for various aspects of the fly's biology [2]. In addition to these nonenzymatic antioxidants, a very comprehensive armamentarium of enzymatic antioxidants can scavenge these compounds, thus protecting the organism. Among these compounds are superoxide dismutases, catalase, peroxiredoxins,

thioredoxins, and the plethora of enzymes involved in glutathione metabolism. These enzymes are either constitutively present in different organs of the fly or the expression of the corresponding genes is induced after exposure to various stressors.

A large number of signaling pathways are involved in the response to oxidative stress and are therefore necessary to mount an adequate antioxidative response. In most of these cases, these cellular responses comprise not only oxidative stress resistance but also a general stress resistance. Among these signaling pathways that are of central importance for a proper response to oxidative stress is the PI3-kinase pathway, which converges onto activation of the transcription factor FoxO [3]. FoxO activation leads to expression of for example, 4E-BP, which is required for a proper and efficient expression of other target genes, mainly those associated with an increase in oxidative stress resistance. An alternative way of signaling is represented by the Nrf2/Keap1-pathway (Fig. 19.1). This is a system that appears to be tailored to sense oxidative stress and to induce an adequate cellular reaction. In unstressed conditions, Nrf2 is kept in the cytoplasm by its inhibitor Keap1. Keap1 has also the potential to act as a sensor for oxidants, by reacting with these compounds via redox-sensitive cysteine residues [4].

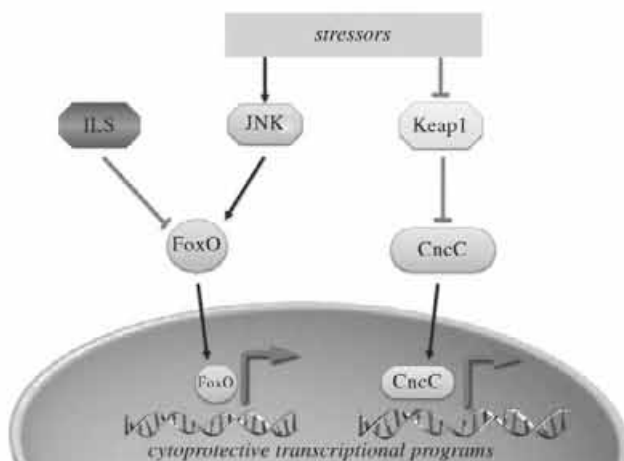


Fig. 19.1 The two transcription factors FoxO and Nrf2 (CncC) are activated by stressors and induce a cytoprotective transcriptional program operative in the airway epithelial cell. FoxO can be activated by a variety of stressors via the JNK pathway, whereas it is inhibited by the insulin-like signaling pathway (ILS). After activation, FoxO translocates into the nucleus, where it initiates transcription of various genes, e.g., those coding for enzymatic antioxidants. On the other hand, Nrf2 (CncC in *Drosophila*) is usually inhibited by Keap1 and held back in the cytoplasm. After stress application, Keap1 is inhibited, thus releasing CncC and allowing it to translocate into the nucleus, where it also starts a cytoprotective transcriptional program (e.g., transcription of glutathione-S-transferases).

Thus oxidants lead to dissociation of the complex between Nrf2 (CncC in *Drosophila*) and Keap1, allowing Nrf2 to translocate into the nucleus, where it binds to the antioxidant response element (ARE), an enhancer sequence present in the target genes of Nrf2 activation. Expression of these target genes represents the so-called electrophile counterattack comprising a whole battery of enzymatic antioxidants of all flavors. Among these are thioredoxins, enzymes of the glutathione metabolism, but also chaperones and parts of the proteasome [5]. In the fly, the Nrf2/Keap1 system fulfils all the tasks described above, and persistent activation of Nrf2 signaling via depletion of Keap1 increases resistance against oxidative stress and life span.

Other signaling systems known to be relevant for the resistance against stressors in general are also known to have an impact on the resistance against oxidative stress. The *Drosophila* p38 ortholog, which is an essential part of the p38 mitogen-activated protein kinase (MAPK) cascade, is essential for a reaction and the resistance against oxidative stress, but also for the resistances against heat stress and starvation [6]. p38, as one of the major parts of the MAPK pathway, has a high potential as a target for intervention in chronic inflammatory diseases including rheumatoid arthritis or asthma, but the side effects caused by chronic interference with its normal function are still not well understood.

19.3 REACTIVE OXYGEN SPECIES AND AGING

A direct connection between aging and oxidative stress induced damages was the idea behind one of the first theories of aging [1]. Accumulation of these damages over time leads to impairment of cellular function and finally to death of the organism. Studies from different fields show a connection between aging and ROS-mediated impairments of various different features. Aging and the reaction to oxidative stress share a number of identical transcript signatures. In particular, genes coding for enzymatic antioxidants and immune relevant genes are upregulated under these two different conditions [7]. Nevertheless, it must be kept in mind that the correlation between oxidative stress-mediated impairments and the physiological decline of various abilities during aging is not necessarily direct [8]. Decline in sensory perception occurs at the same rate in control flies and those showing a high oxidative stress resistance. Although the contribution of single parts of the antioxidant armamentarium to life span is hard to quantify, it is common sense that a highly effective antioxidant response is beneficial. A relatively large number of enzymatic antioxidants appear to have a positive effect on longevity if present in the organism. Among these

are, for example, the thioredoxins; corresponding knockout flies lacking a functional Thioredoxin-2 have a shortened life span [9]. A comparable effect on life span has been attributed to a peroxiredoxin of type II, named Jafrac1, whose overexpression in the nervous system appears to increase life span whereas its down-regulation has the opposite effect [10]. Interestingly, Jafrac1 is a target gene of the JNK pathway that is activated after stress and cellular damage to restore the cellular homeostasis. Other peroxiredoxins, especially those present in mitochondria, are also relevant for life span, as their downregulation has a significant impact on this aspect of life [11].

Regarding the role of enzymes involved in glutathione metabolism, the picture is relatively complex because of the large number of gene products being part of this machinery. Glutathione reductase increases resistance under hyperoxic conditions, whereas no effects on life span could be observed at normoxia [12]. The hypothesis that glutathione metabolism is involved in life span regulation was further supported by the observation that overexpression of the glutamate-cysteine ligase, which is the rate-limiting enzyme in glutathione de novo synthesis, increases life span by more than 20% [13].

Two other sets of enzymatic antioxidants have been implicated in life span extension, the SODs and catalase. In particular, the role of SODs in this context is still a matter of debate. Overexpression of a human SOD1 in motoneurons only was shown to be sufficient for a 40% life span extension [14]. As mentioned above, overexpression of SODs was shown to increase life span significantly, but this effect appeared to be restricted to short-living strains of *Drosophila melanogaster* [15]. A combined overexpression of SOD and catalase appears to represent a more robust way of life span extension compared with overexpression of either of the two components [15, 16]. This observation is in line with the enzymatic activities of both enzymes that act in common.

Hyperoxia is a relatively simple way to induce oxidative stress. Although very high oxygen concentrations are lethal to naive flies, it was possible to select for survivors under these hostile conditions. These flies show phenotypical differences compared with control flies, and their gene expression profile was altered accordingly. Very interestingly, genes belonging to the family of antimicrobial peptide genes are obviously relevant for this adaptation to extreme oxygen concentrations and therewith to higher levels of oxidative stress [17, 18].

The plethora of signaling and effector molecules involved in the response to oxidative stress indicates that this response is of vital importance for the organism. Apparently, this response is tightly controlled and obviously of vital importance, especially regarding life span-associated aspects.

19.4 OXIDATIVE STRESS IN THE NERVOUS SYSTEM

Most important among these is obviously the nervous system, where ROS-mediated decline of neuron survival can be observed. As could have been expected, one class of neurons shows highest sensitivity to oxidative stress, namely, the dopamine-producing cells. Obviously, the dopamine production machinery is inevitably linked to an endogenous production of relatively high amounts of ROS, thus making these cells prone to damages caused by "extra doses" of ROS. This high sensitivity is the major reason why dopamine-producing neurons die whereas other neurons are almost unaltered [19]. This highly interesting observation enables us to link high ROS concentration with the development of Parkinson disease-like phenotypes. One major reason for the development of this disease is believed to be chronic confrontation with very high ROS concentration in conjunction with the sensitivity of dopamine-producing cells in general [19]. Consequently, confrontation with ROS doses is a suitable mechanism to induce neurodegenerative processes as typically seen in Parkinson disease. One of the most reproducible ways to do this is hyperoxia, which consequently is able to induce these phenotypes [20]. Especially based on these approaches, it was possible to verify hypotheses that ROS-scavenging enzymes should reduce the sensitivity to these high ROS levels in corresponding *Drosophila* models. SOD overexpression was sufficient to protect the dopaminergic cells, whereas catalase was not [20]. In addition, increasing the glutathione-S-transferase activity in the nervous system protects these dopaminergic neurons in another *Drosophila* model of Parkinson disease [21].

In an Alzheimer disease model based on tau-activation in the nervous system, ROS have been shown to modulate the sensitivity of the animals, thus demonstrating that oxidative stress plays a major role in the development of Alzheimer disease [22]. The positive effects of enzymatic and nonenzymatic antioxidants on disease progression appear not to hold true for all neurodegenerative diseases. In a well-established model for Huntington disease, neither overexpression of enzymatic antioxidants nor supplementation with nonenzymatic antioxidants decreased the lethality in this model [23].

19.5 OXIDATIVE STRESS IN THE DIGESTIVE SYSTEM

The intestinal immune system is characterized by a very special situation. It faces one of the most dense

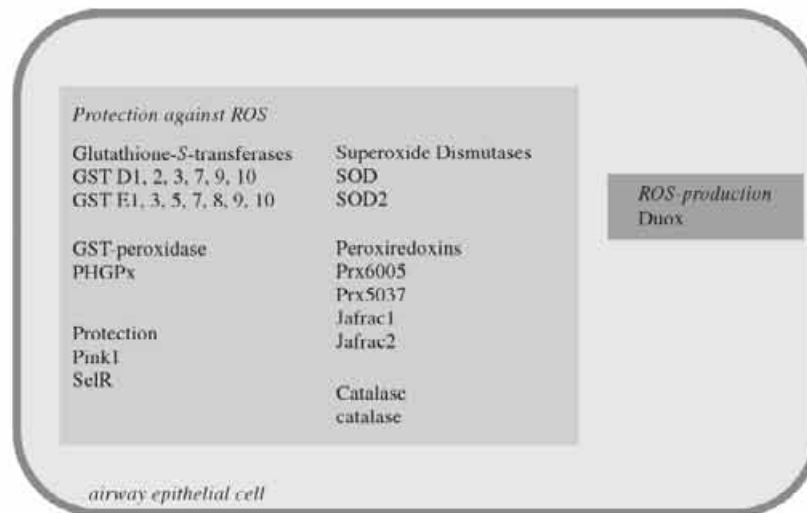


Fig. 19.3 Schematic drawing of reactive oxygen species (ROS)-producing enzymes and those protecting from ROS in the airway epithelial cells of the fly.

A recent study was performed with the aim of determining the relationship between oxygen concentration, oxidative stress, and life span [33]. Counterintuitively, the relation between these parameters is not linear, and reduced life spans were observed at maximal and minimal oxygen concentrations in the atmosphere.

It has been shown that all major enzymatic antioxidants are expressed in the airway epithelium. Among these, superoxide dismutase (SOD), catalase (cat), and all molecules involved in glutathione metabolism are of greatest importance. These enzymatic antioxidants are not only expressed in the airway epithelium but are also part of the reaction to stressful situations. The only well-studied situation is the reaction to airway infection [34]. In addition to classical aspects of an antibacterial response including of antimicrobial peptide genes, some of these enzymatic antioxidants are strongly upregulated.

The complex armamentarium of enzymatic antioxidants comprises both SODs (SOD and SOD2), the sole catalase, four different peroxiredoxins (Jafrac 1 and Jafrac 2, Prx 6005 and Prx 5037), a big consortium of glutathione-S-transferases (GST D1, 2, 3, 7, 9, 10, GST E1, 3, 5, 7, 8, 9, 10), the glutathione-peroxidase PHGPx, and molecules involved in protection such as *PINK1* and *SelR* (Fig. 19.3). A very potent producer of reactive oxygen species, the dual oxidase Duox, which is known to be very effective against invading bacteria, supplements this armamentarium. Thus the antioxidant system of the airway epithelium contains both producers of ROS and protectors against them. This architecture of oxidant and antioxidant systems allows the organism to cope with the very peculiar system in the airway, namely, that very high ROS concentrations have to be tolerated by these very peculiar cells.

19.8 CONCLUSION

ROS are an omnipresent threat for almost all cells in the body. In particular, those organs with highest metabolic rates and those directly exposed to the atmosphere are in danger. Thus the airway epithelium especially has to cope with this situation. To overcome problems associated with ROS-mediated damage, corresponding cytoprotective systems must be installed. In particular, enzymatic antioxidants including SOD, catalase, peroxiredoxins, thioredoxins, and those involved in glutathione metabolism are relevant. Although the vast majority of the corresponding genes are transcribed constitutively, different stressors can induce their expression further via the JNK-FoxO axis or the Nrf2 system. Our knowledge regarding their physiological role under different conditions is limited, although we know the almost complete armamentarium of antioxidants in the airway epithelium. With the experimental advantages of the *Drosophila* system, it should be possible to obtain an in-depth understanding of this very important regulatory system.

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other phylogenetic groups, genes encoding glutathione reductase (GR) and glutathione peroxidase (GPX) are absent. Their functions are performed by homologous genes encoding thioredoxin reductase (TrxR) [2] and thioredoxin peroxidase (TPX) [3]. In addition, insects have genes that encode enzymes of antioxidant defense that act as peroxidases: phospholipid-hydroperoxide GPX homologs with TPX activity (GTPX) [4] and glutathione *S*-transferases (GST) [5, 6]. Thus the group of secondary antioxidant enzyme in insects acting on ROS indirectly includes TrxR, which converts both TRX and GSH, and also methionine sulfoxide reductases (MsrA and MsrB), which are involved in protein reparation by catalyzing the TRX-dependent conversion of methionine sulfoxide to methionine [7, 8].

Insects have an open circulatory system, so a free flow of ROS in the body can not only lead to foreign agent destruction but also can be very dangerous for the insect organism. Simultaneous functioning of the systems producing and neutralizing ROS allows on the one hand stopping the development of infection and on the other hand preventing the insect organism from total intoxication.

20.2.2 Honeybee as Species

Coding of the complete genomes of several species of insects allows us to make a preliminary comparison. The honeybee genome has been found to evolve more slowly than the genomes of the fruit fly and mosquito. In this case, the honeybee genome with respect to genes

involved in circadian rhythm, RNA interference, and DNA methylation has more similarities to the genomes of vertebrates than to the genomes of the fruit fly and mosquito. At the same time, compared to *Drosophila melanogaster* and *Anopheles gambiae*, there are fewer genes presented in the genome of *Apis mellifera* associated with innate immunity, detoxification proteins, and gustatory receptors but more genes associated with the odorous substance receptors [9–11]. There are absolutely unique genes responsible for collecting and processing nectar and pollen. New microRNAs have been discovered that are expressed depending on the stage of development and specialization of bees; this means that these microRNAs participate in the honeybee social diversification [12]. To date, 39 genes coding 10 groups of antioxidant proteins have been identified in the honeybee genome (Table 20.1).

Special attention is paid to vitellogenin, the glycolipoprotein egg yolk precursor, involved in the development of reproductive functions of an insect female and a nutrient of the bee brood, which performs an antioxidant function in the honeybee organism [23–26]. Antioxidant properties of vitellogenin are stipulated by its Zn-binding capacity [22] and preferential oxidative carbonylation under oxidative stress in bees [23]. With respect to these properties, vitellogenin is compared to Cu/ZnSOD, key metal binding antioxidant enzymes also undergoing preferential carbonylation [27], and serum albumin, metal binding proteins that can function as free radical acceptors and reduce levels of oxidative markers such as protein carbonylation [28].

TABLE 20.1 Antioxidant proteins of the honeybee

Antioxidant proteins	Antioxidant Functions	References
Superoxide dismutase: mitochondrial MnSOD cytoplasmic Cu/ZnSOD	Reduction of $O_2^{\bullet -}$ formed in mitochondria to O_2 and H_2O_2	1, 13, 14
Catalase: cytoplasmic extracytoplasmic (in honey)	Reduction of $O_2^{\bullet -}$ to O_2 and H_2O_2	1, 13, 15
TPX (peroxiredoxin)	Reduction of H_2O_2 with the use extracytoplasmic (in honey) of TRX as a donor of e^-	1, 3, 16, 17
GTPX	Reduction of H_2O_2 and organic hydroperoxides	1, 4
TrxR	Transfer of reducing equivalents from NADPH to TRX and GSH disulfide with formation of powerful intracellular antioxidants—thiol-based reductants	1, 2, 18
TRX	Preservation of redox homeostasis homologs of TRX of the cell	1, 2, 18
Glutaredoxin (GRX) homologs	Preservation of redox homeostasis of the cell	1, 2, 18
GST	Metabolism of xenobiotics and protection against peroxidative damage	1, 5, 6, 13, 15, 17, 19–21
MsrA and MsrB	TRX-dependent reduction of methionine sulfoxide to methionine, participation in protein repair	1, 7, 8
Vitellogenin	Predominant carbonylation	22, 23

20.3 ROS AS COSTS OF AN AEROBIC METABOLISM

The main sources of ROS in any organism are the mitochondria (respiratory chain), microsomal oxidation of xenobiotics, and phagocytosis.

20.3.1 Mitochondria

ROS as by-products of aerobic metabolism are being continuously formed in the cells of honeybees under normal physiological conditions, and they necessitate constant monitoring of the antioxidant system. Two main reactions take place with the formation of ROS in the mitochondrial respiratory chain: the formation of $O_2^{\bullet-}$ from O_2 and its dismutation (disproportionation) under the influence of MnSOD with the formation of H_2O_2 . In addition to the reactions occurring in the electron transport network of the inner mitochondrial membrane, the source of ROS is the oxidative deamination of biogenic amines with the formation of H_2O_2 under the influence of monoamine oxidases localized in the outer membrane of mitochondria. This reaction contributes significantly to the creation of a stable concentration gradient of ROS between the mitochondrial matrix and cytosol [29, 30]: The concentration of $O_2^{\bullet-}$ in the mitochondria in the norm is 5–10 times higher than in the cytosol and nucleus. Exceeding this level, and hyperproduction of H_2O_2 , being relatively a long-lived molecule that easily diffuses through the mitochondrial membranes, creates the preconditions for the oxidative damage to mitochondrial matrix. According to some researchers, the intensive formation of H_2O_2 in mitochondria leads to disruption of intermolecular interactions and damage of the mitochondria inner membrane, as has been shown in studies of mitochondria isolated from muscle tissue of synanthropic flies in model systems generating ROS [31, 32]. Mitochondrial DNA is the most vulnerable to the damaging effect of ROS because it is in close proximity to the ROS-generating sites and is not protected, like the nuclear DNA, by histone proteins. The extreme sensitivity of the mitochondrial DNA to the damaging effect of ROS may lead to an increase in the number of mutations and the consequent suppression of aerobic respiration, since mitochondrial DNA encodes carrier proteins of the electron transport network.

20.3.2 Microsomes

Another source of ROS in the honeybee organism is the microsomal oxidation of xenobiotics. Microsomes contain enzymes of the system of cytochrome *P*450 (CYP), which catalyze polyvalent oxidation of xenobiotics with

simultaneous generation of $O_2^{\bullet-}$ and other ROS. Two phases are distinguished in the process of xenobiotic clearance: the introduction of polar groups with the help of the CYP hydroxylase system and the conjugation of molecules with water-soluble ligands. Both processes are used to eliminate foreign components from the internal environment of an organism. The CYP hydroxylase system includes flavoproteins and a family of hemoproteins, which are localized on the cytoplasmic side of membranes of the endoplasmic reticulum. Different isoforms of CYP are involved in metabolism of various xenobiotics [33, 34]. Two groups of CYP are distinguished: The first is involved in the metabolism of endogenous substances; the second is induced by exogenous agents. The conjugation processes are often catalyzed by UDP-glucosidase, sulfotransferase, and GST. Glucuronidation is the major form of conjugation for agent detoxification. Sulfation usually provides a lowering of toxicity and acceleration of xenobiotic clearance. The GST reaction is important for neutralization of unstable electrophilic molecules. Microsomal GST is closely linked with the CYP system, which contributes to rapid inactivation of active metabolites produced during the metabolism of xenobiotics.

20.3.3 GST of Honeybee

The genome of the honeybee, in contrast to other insects, contains significantly fewer genes of xenobiotic detoxification protein. This may be due to the evolution of the hormonal and chemosensory processes and is a genetic payment for the highly organized eusociality of the honeybee. The most notable features of the honeybee are manifested in the coding of three important families of xenobiotic detoxification enzymes: Honeybees have only about half the genes of GST, CYP, and carboxyl/cholinesterases as other insects [35–37]. In particular delta and epsilon GST and CYP4, which contribute significantly to the binding of insecticides in other species, are insufficient. This deficit can contribute not only to honeybees' increased sensitivity to insecticides but to insufficient resistance to oxidative stress caused by incomplete microsomal oxidation of xenobiotics [38]. Foragers suffer from it more, because other members of the colony are relatively isolated from external influence in the hive.

Other insect GSTs have been assumed to play a minor role in protection against insecticides, only attenuating the effects of oxidative stress. However, lately it has been shown that GST, belonging to the Delta and Epsilon classes, are directly involved in the disposal of insecticides. The complete absence of class Epsilon enzymes in the honeybee and the presence of only one enzyme of class Delta GSTD1 [19, 39] may partly explain the

extreme sensitivity of this species to certain insecticides. Perhaps GSTD1 can serve as a universal mechanism for the protection of bees against harmful xenobiotic action. This enzyme is also involved in protection against oxidative stress. It is available in the spermatheca of the queen bee, and it is assumed to protect the sperm from oxidants.

In contrast to the lack of genes Delta and Epsilon GST, the honeybee has more genes of class Sigma. These enzymes were initially assumed to play an important role in the structural organization of muscle tissue [20], but enzymes of class Sigma GST turned out to have a high affinity to the products of lipid peroxidation and are localized in metabolically active tissues, such as those in the thorax muscle of flies. They are hypothesized to play an important role in protecting these tissues against oxidative stress by-products [21]. Omega, Theta, and Zeta classes of GS, are presented in bees as well as in other organisms. Probably, they play a key role in metabolic processes, in contrast to a predominantly detoxifying role of the Delta and Epsilon GST. This hypothesis is supported by the participation of the first in the degradation of products of tyrosine and phenylalanine metabolism and the importance of Omega GST in removing of S-thiol adducts from proteins [36]. Despite the fact that microsomal GST and cytosolic enzymes may play a similar role in the overall reaction of detoxification and protection from oxidative stress, they do not have structural similarities and are different in genetic origin [40].

Increase of activity of microsomal mixed-function oxidases and GST is recorded as the initial response of foragers to the action of pesticides and allelochemicals contained in toxic nectar, and the increase of the detoxifying activity depends on the age of worker bees [41, 42].

Thus the features of the cytochrome oxidase spectrum in the honeybee are mostly connected with the limitation of their function in xenobiotics metabolism and a great accentuation on hormones metabolism. As noted above, this aspect has a particular evolutionary significance for the honeybee.

20.3.4 ROS as Component of Immunity

Protection of insects against pathogenic microorganisms is carried out by phagocytic cells of hemolymph. The unlocked type of circulatory system promotes development of rapid generalized response to any influence. Metabolic activity, the so-called respiratory burst of phagocytic cells, is a source of formation in the NADPH oxidase reaction of $O_2^{\bullet-}$, triggering a cascade mechanism of ROS generation (Fig. 20.1). The latter have expressed microbicidal activity and are part of the cytotoxic

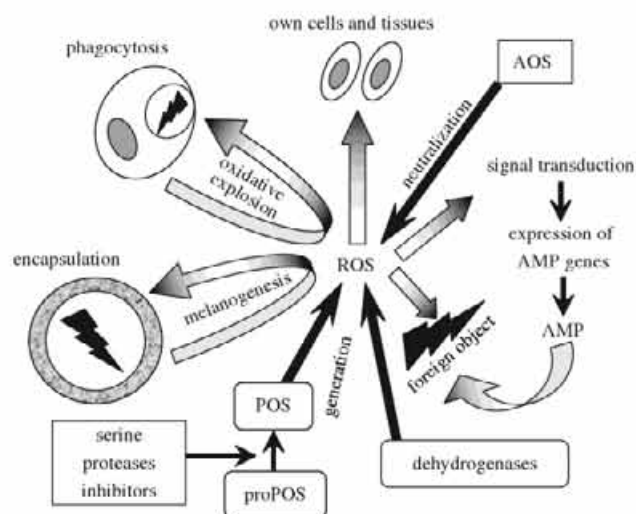


Fig. 20.1 Immune processes of the honeybee with ROS generation and neutralization. POS, phenoloxidase system; AOS, antioxidant system.

arsenal of the humoral immune system of honeybees [43, 44]. ROS are generated in the insect hemocytes with the participation of several enzyme systems—a phenoloxidase system (POS), a respiratory cascade, and a complex of dehydrogenases.

20.3.5 ROS and Phenoloxidase Cascade

Phenoloxidase (PO) is one of the oxidases responsible for the main part of the oxygen uptake during initiation of immune responses, as well as in morphogenetic processes of insects. The defeat of the honeybee by pathogens is accompanied by an increase of levels of proPO gene expression and PO activity [45–48]. In general, the power of the honeybee immune response and its ability to resist infection directly depend on the level of PO activity [49]. Quinone intermediates of melanin, $O_2^{\bullet-}$, H_2O_2 , hydroxyl radical ($\bullet OH$), nitric oxide ($\bullet NO$), and peroxynitrite ($ONOO^-$), involved in the cytotoxic reactions of insects in granule formation and encapsulation, are formed in large numbers during the realization of the PO cascade and melanogenesis in the hemolymph of insects [50–52].

From the immunological point of view it should be noted that biogenic amines (as mediators of stress reaction), as well as quinones, melanin, and other highly reactive metabolites formed during the activation of PO directly or indirectly participate in such defense reactions of insects as hemolymph coagulation and wound healing, encapsulation, granule formation, and destruction of the pathogen penetrated into the body by ROS. Components of the POS are also involved in the process

of immune recognition, carrying out cross-links of the foreign cell surface with the corresponding phagocyte receptors [53]. Uptake of foreign material by phagocytes is accompanied in both insects and vertebrates by an increase in oxygen consumption with active formation of ROS, which is analogous to the oxidative explosion [54, 55].

In addition, ROS generated during activation of PO and phagocytosis act as mediators of signal transduction, inducing gene expression of antimicrobial peptides (AMP) [51, 56, 57]. The presence of genes encoding proteins of the Toll-pathway of signal transduction in *A. mellifera* [58] suggests the existence of the same process in the honeybee.

However, if killer mechanisms of AMP are directed exclusively against bacterial and fungal cells, ROS have cytotoxic effects on all living cells, including cells of the insect organism itself. To reduce the cytotoxicity of ROS to the insect organism itself, its level is regulated by inhibitors of the proPO cascade, such as serine protease inhibitors [58] and components of the antioxidant system (AOS) [59, 60].

20.3.6 Interaction of Phenoloxidase and Antioxidant Systems

Correlation between functioning of the POS and antioxidant mechanisms in the defensive response of insects has been shown in a number of studies. The initial stage of infection in the honeybee has been established to be accompanied by activation of a PO cascade and inhibition of antioxidant enzymes activity [61]. The activity level of PO, catalase, and peroxidase in the honeybee imago changes under the action of the immunomodulator chitosan [62]. An analogous reaction of PO and AOS

has been found under bacteriosis in lepidopterous larvae [52] and adults of Colorado potato beetle [63].

In the above-mentioned papers it is assumed that the temporary inhibition of the organism's own AOS may play an important role in antimicrobial immunity of insects through the production of ROS generated in the implementation of the PO cascade (see Fig. 20.2). However, a different explanation of these changes in enzymatic activity can be supposed. The PO cascade may act mainly on the pathways of the formation of quinones, serving as a trap for oxygen radicals and reducing the concentration of ROS in hemolymph and, therefore, the activity of enzymes neutralizing them [64].

Study of the interaction between different antioxidant enzymes under the action of honeybee pathogens has revealed a similar pattern. A marked differentiation between catalase and peroxidase functions has been shown in the hemolymph and gut [65, 66]. Utilization of H_2O_2 at the early stage of infection development is carried out by catalase, whereas in the later stages of pathogenesis peroxidase gets involved. An inverse correlation is observed in the development of these reactions.

20.3.7 Social Immunity

The above defense responses are components of individual immunity of the honeybee and are encoded by genes, being only one-third of the number of immune genes of nonsocial insects [58]. This payment for eusociality is compensated by effective social immunity, including health behavior and the secretion of antiseptic substances in the larval food and honey, as well as in the internal environment of the hive.

Glucose oxidase, catalyzing the oxidation of β -D-glucose to D-glucose with the formation of H_2O_2 and

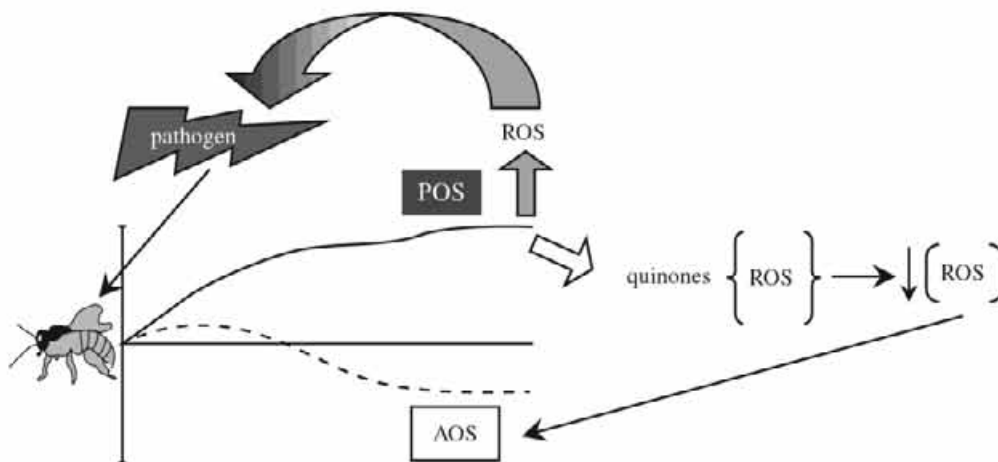


Fig. 20.2 Correlation of phenoloxidase and oxidative systems in honeybee defensive responses.

being a biochemical indicator of the level of social immunity of honeybees, plays an important role in providing of antiseptic properties of bee products [67]. Its activity is inhibited by the reaction product. The presence of this enzyme in honey with a large amount of substrate creates a relatively constant and self-regulating bactericidal system [68]. Importance of glucose oxidase in the group protection of honeybees is accentuated by the direct dependence of the colony strength on the level of this enzyme activity: H_2O_2 stipulates the antiseptic properties of the larval food and honey, inhibiting pathogen development [69, 70].

However, catalase activity, supporting H_2O_2 concentrations less toxic to the honeybee are found in honey. It is noteworthy that the genome of the honeybee contains only the gene of the intracellular catalase. The fact that the gene of catalase contained in honey is not integrated into the genome of the honeybee and extracellular catalase is found only in some bacteria and fungi suggests the expression of extracellular catalase of honey by endosymbiotic organisms [1].

Another component of the larval food—royal jelly—also contains highly active antioxidant substances that can reduce cadmium-induced genotoxicity and oxidative stress in mice significantly [71].

20.4 ONTOGENESIS AND LIFE SPAN

Oxidative stress and the molecular mechanisms of antioxidant protection of the honeybee motivate a particular research interest in connection with aging and longevity of animal organisms. The honeybee, because of the caste subdivision of the family, age-related functional specialization, and different life spans of the castes, is a natural model for gerontological research. According to the free radical theory of aging, the main reason for this process is the accumulation of ROS, oxidizing proteins [72], lipids, and DNA [73] and thus leading to DNA damage, degradation of membrane proteins, disruption of signaling pathways, and ultimately necrotic or apoptotic cell death [74].

Ontogenesis of the honeybee, which is a holometabolous insect, is characterized by a change of stages that are significantly different in morphology, physiology, and functioning. The transition from one stage to another is provided by major morphogenetic transformations occurring during metamorphosis and accompanied by the development of cellular and humoral reactions characteristic of anticontagious response [75]. Change in hormonal balance during periods of metamorphosis of the larvae and pupae, the appearance of the macrophagocytes in the hemolymph which are involved in the processes of histolysis, is also

accompanied by the analog of the oxidative explosion and the induction of molecular mechanisms to restrain it. Changes in the activity of SOD, catalase, and GST in the development of different insects implies that the cellular antioxidants are involved in protecting cells against damage and regulation of redox levels in the process of insect morphogenesis and are hormone dependent [76–78]. In honeybees during the first 3 days of embryonic development the level of thioredoxin peroxidase and GST expression significantly increases, which indirectly confirms the assumption of an active generation of ROS due to the high oxygen demand in the developing embryo [17]. Catalase activity reaches its highest values in the intestine of fertilized queens, which is associated with their more active feeding and significantly greater life span [13].

Ontogenetic changes in the activity of immune factors of the honeybee are under hormonal control [22, 25, 79] and can be related to the varying ability of the honeybee for anti-infective protection at different stages of development. Evolutionarily established social relationships in a colony of honeybees exhibit age-related features of cellular protection in individuals. In the honeybee the total number of functionally active hemocytes increases at the larval and pupal stages of development [49], which confirms their active involvement in morphogenetic processes. During the maturation of adults immunocompetence of the hemocytes decreases regardless of bees' sexual and caste identity [80], and levels of POS and AOS activity increase [15, 49].

Relatively low resistance to pathogen action and short duration of life are beneficial in terms of viability of the bee colony. In accordance with the "law of 40 days," bees of the second summer generation (May–June) are grown in large quantities on a relatively poor diet for mass participation in the honey harvest. According to the Institute of Beekeeping in Russia, from June 20 to August 1 bees collect 90% of all feed stocks [81]. At the end of the main honey harvest the existence of worn-out "unemployed" foragers becomes unprofitable for the colony. Drone removal from the colony takes place at the same period.

In the period when worker bees start forage conservation, the intensive death of hemocytes is accompanied by increase of juvenile hormone titers and a simultaneous decrease in the vitellogenin level [23, 79] (see Fig. 20.3). It is remarkable that an active cell proliferation, accompanied by a fall in titer of endogenous juvenile hormone and increasing of vitellogenin levels, starts in the hemolymph of foragers that are forced to return to hive work [82].

In general, an increase in ROS generation and enhancement of antioxidant protection is associated with aging and transition of worker bees to foraging.

inactivated and JNK and SIRT1 is induced. Dephosphorylated transcription factor FOXO moves from the cytoplasm to the nucleus, which stops cell growth and, in turn, leads to increase of resistance to oxidative stress (FOXO-regulated genes include genes encoding SOD and catalase) and life expectancy [85, 88].

The molecular bases of complex social behavior of social insects are still vague. The gene encoding vitellogenin required for the formation of insect eggs performs a number of other functions in the honeybee that are related to social labor organization. Vitellogenin protein affects the age at which a worker bee stops working inside the colony and starts foraging, when it will collect nectar or pollen, as well as its duration of life [79]. It is possible that such behavior could occur because of desynchronization of the work of genes responsible for reproduction and care for the brood. Thus parental instincts of worker bees appear before their reproduction period starts. With no brood of their own, such insects have been forced to take care of closely related members of the colony [91].

Gene regulators initially associated with reproductive function, for example, influencing the production of gonadal hormones, can be expected to play an important role in the regulation of labor organization in the honeybee [92]. Vitellogenin gene in insects, both social and nonsocial, is closely linked to female reproductive function: Protein encoded by this gene is necessary for the formation of eggs in the ovaries of females. However, this gene is also active in worker bees that do not oviposit. Moreover, its activity in worker bees has been observed to decline with age. This means that vitellogenin serves as a singular behavioral "switch": Decrease of its concentration forces a bee to leave household chores and to start foraging outside the hive at some moment of life. Thus the same gene that was originally associated with female reproductive function started to perform a number of new functions in the honeybee such as regulation of labor organization and social structure.

It has been found that in worker bees a wide range of enzymes involved in the processes of glycolysis, ATP synthesis, and generation of free radicals depends on the changes of tasks performed by the bees: from functions inside the hive to collecting of nectar or pollen [14, 93]. Evolution of the insect social castes with different reproductive potential is also associated with changes in metabolism that affect the life span [94]. From this point of view, evolutionary development of the honeybee supposes a positive connection of vitellogenesis and life span, which is not regulated only for the queen bee. This occurred because the fertility and longevity of the queen bee determines the viability and development of the colony [95]. Thereby, the honeybee is an example of how socialization in the process of evolution and changes

in the scheme of reproductive potential control in the colony may be interrelated with life prolongation [94, 96–98]. This assumption is based on analysis of the interdependence of vitellogenin levels and life expectancy of the queen bee, as well as the dynamics of reproductive protein and structure of longevity in worker bees.

In addition, use of vitellogenin for stress resistance management of worker bees has been evolutionary confirmed. In worker bees vitellogenin begins to synthesize when health functions in the nest are performed prior to the function of feeding [99]. However, the level of synthesis of vitellogenin in worker bees is lower in comparison with that of the queen [100]. The protein content is depleted in the period from the performance of health functions by the working bee to larvae feeding [24]. Thus the absence of brood is a necessary but not sufficient condition for the emergence of long-living worker bees [79, 82]. Modulation of regulatory mechanisms of vitellogenin on the reproduction and maintenance of homeostasis may be recombined in queens and worker bees during the migration of the species to temperate regions [101, 102]. This recombination of functions between longevity increase and stress resistance contributed to the emergence of the phenotype of long-living worker bees, which promoted the maximum survival of the colony during the winter, when the worker bees could not be replaced because of environmental restrictions on brood raising.

20.5 WINTER GENERATION OF HONEYBEE

Before human intervention the species *A. mellifera*, having mastered West Asia, Africa, and Europe, was scattered so widely that its further evolution was in radically different natural climatic conditions. This factor led to the formation of 25–30 subspecies [103], differing in a number of features, having a specific reaction to the external environment, and often not even able to exist under artificial latitudinal displacement within the species area [104].

It is known that honeybee defensive reactions are characterized by clear interspecific differences [65, 66]. Honeybee subspecies differ in expression of antimicrobial peptide genes, the dynamics of PO activity, and hemocyte response [67, 105–107]. Interspecific differences are manifested in the reactions of the defensive systems of the honeybee to bacterial contamination, expressed in increased phagocytic reaction and an earlier activation of redox processes in *A. m. mellifera* in comparison with *A. m. caucasica* [108]. These results confirm that during the honeybee's evolution modification of adaptive properties did not concern individuals, but a colony in general as an integral biological unit [91].

A phenomenon of the winter generation of bees, which appeared (or was preserved) in the evolution in the subspecies living in conditions of pronounced change of seasons, winter to summer, is of particular interest in the issue of interspecific differentiation. This feature is characteristic of subspecies of temperate climates, such as *A. m. mellifera* and *A. m. carnica*, but is absent in the subtropical subspecies *A. m. scutellata* and others [79, 95]. It was established that worker bees can accumulate levels of vitellogenin up to 50–60% of the hemolymph protein fraction because of limited brood raising [99, 110] and live for 6–10 months [87, 111].

Winter generation is characterized by a high life span and delayed organism aging, which is associated with influence of the protein vitellogenin and hormonal balance on the process of free radical oxidation. This feature allows the bee colony to survive the harsh long winter and to cope with many pathogens. An additional tension in the honeybee organism occurs during the long winter season in conditions of the northern part of the species habitat, for example, in Russia where the non-flight winter period lasts 5–7 months. During this time the intestines of the honeybee collect from 20 to 70 mg of undigested food residues. In this situation, the balance in the system ROS-antioxidants is especially important for the survival of the colony. First of all it relates to neutralizing putrefactive processes in the intestine. Otherwise, these residues are allocated by the bee inside the hive and can lead to the outbreak of nosemosis and other diseases.

The European dark honeybee subspecies (*A. m. mellifera*) is the most evolutionarily adapted to northern conditions. Intensive studies of features of the honeybee adaptation to a long, cold winter conducted by MV Zhrebkin (1979) [112] showed that life span varies between bees appearing by (before) the warm season and focused on honey gathering during the entire plant vegetative period and bees appearing in the colony by the cold season for wintering provision. These differences are associated with the larval diet. The most winter-hardy subspecies, the European dark honeybee is characterized by a relatively large body mass of individuals and a greater volume of stored reserve nutrients, as well as a significant number of high-quality food stocks in the nest for successful wintering of the colony. The latter aspect largely determines the potential ability of different strains (types) of the honeybee to produce the total volume of honey in beekeeping. In the body of winter bees the amount of unstructured water gradually decreases and pharyngeal glands, ovaries, and fat body remain developed for a long time. Thus MV Zhrebkin showed that in the process of winter preparation the majority of bees in the colony acquired characteristics of physiologically young bees with

well-developed pharyngeal glands that produced large amounts of protein.

Subspecific differences include the specific functioning of the antioxidant defense systems in the gut and hemolymph of the honeybee and its distinctions in adaptation to climatic factors and exposure to the pathogens [66]. Comparative analysis in the climatic conditions of the Southern Urals revealed significant differences in the character of the stress reaction of bees of native subspecies *A. m. mellifera* and subspecies *A. m. caucasica*, introduced from the Black Sea coast. *A. m. mellifera* is characterized by a lower level of redox processes in the norm, a higher reactivity of the antioxidant enzymes, glucose-6-phosphate dehydrogenase and enzymes of PO cascade under the action of pathogens, but high and stable levels of glycosaminoglycans. This level of metabolic processes is justified in these natural climatic conditions, since a higher level of protective system response is necessary in case of threatened breach of homeostasis, ensuring response adequacy. A higher metabolic rate is constantly observed in introduced honeybees, which negatively affects the viability of the individuals if there appears excessive functional stress associated with any negative factor. These and other features of the protective reactions of *A. m. mellifera* reflect the stability of the European dark honeybee to climatic factors of the northern part of the species range [66].

Experimental data showed exhibition of normal peroxidase activity in *A. m. mellifera* honeybees predominantly in brain tissues, fat body, and the thorax muscles and mainly in the tissues of the midgut and large intestine in *A. m. caucasica* bees. Catalase activity in *A. m. mellifera* is much higher in the brain and hemolymph in comparison with other organs and tissues, and in the large intestine catalase activity increases only at the end of winter (prolonged nonflight period) in order to protect the bee organism from putrefactive processes in the intestine. This feature is also functionally fair for *A. m. mellifera*. In *A. m. caucasica* catalase activity is localized mainly in the tissues of the fat body and gut, organs that provide the highest level of metabolism [108]. This creates the conditions for maximum antioxidant protection, in particular, the catalase-peroxidase system [113, 114]. The high metabolic rate that is a characteristic of the southern subspecies as a whole will contribute to the emergence of stronger pathogenetic processes during development of stress reaction and reduce viability under the natural climatic conditions of the northern part of the species area.

During wintering not only behavior and metabolic rates of the honeybee individuals but also similar characteristics of the whole colony change, which is associated with changes in both external environmental factors

and the microclimate in the nest. Increase in the temperature in the nest during the winter is accompanied by a change in the ratio of CO₂ and O₂. In winter there is a transition of bees from aerobic to predominant anaerobic respiration in conditions of a high CO₂ content.

In conditions of reduced oxygenation, when the intensity of the Krebs cycle is reduced, in the mitochondrial respiratory chain substrates supplied by AOS (GSH, TRX, ascorbate) can be oxidized. Production of the alternative substrates of oxidation by AOS is conjugated with NADPH₂-generating mechanisms that are resistant to hypoxia and anoxia. A high activity of MnSOD is noted in the mitochondria. In intact cells in situ significant concentration of O₂^{•-} is not detected and leakage of electrons cannot serve as a sufficient ground for the destructive changes in mitochondria [115, 117]. In addition, in mitochondrial cycles the main constriction factors are catalase and enzymes with functions of GPX. Still-unknown functions of mitochondrial catalase and GPX-like enzymes have been proposed. These functions can be reduced to the transformation of H₂O₂ to O₂ and the subsequent intensification of respiration and ATP synthesis. Therefore, inhibition of catalase leads to a sharp decrease in the rate of respiration and oxidative phosphorylation in the mitochondria in vitro and in vivo. Flavoproteins possessing peroxidase activity play a major role in the process of linked oxidative phosphorylation [117]. TPX may also be such a flavoprotein, exhibiting peroxidase activity. The enzyme is multifunctional: It is involved in the peroxide metabolism of oxygen, restoring TRX and ascorbic acid and formation of deoxyribonucleotides in the metabolism of selenium-containing compounds. An important characteristic of the enzyme is its high resistance to respiratory toxins, which greatly distinguishes it from catalase and GPX [118].

20.5.1 Regulation of ROS in Honeybee Intestine

Features of climatic zones potentially affect honeybee behavior, physiology, and metabolism, as well as the development of interaction between the honeybee and pathogens [119]. In this regard, the intestine of the honeybee, which is one of the key points of the honeybee's interaction with its environment, is of great interest. In the first place, the intestine is the main organ of digestion, where nutrients and toxins, as well as enteric pathogens first come. The intestine of the honeybee in many respects determines resistance to disease at the level of individuals and the colony as a whole [120, 121]. The intestine was not randomly chosen as the main object for studying of the gene expression of different nature in the search for the causes of the Colony Collapse Disorder phenomenon [122].

TLR 3, Vanin-1, Ferretin 2, and chitinase expressed in the intestine and forming the basis of innate immunity in the honeybee differ in activity in different subspecies of the honeybee and determine the features of the confrontation of pathogenic microorganisms in different climatic conditions. Tool-like receptors are involved in the recognition of viral pathogens and also activate necrosis of the intestinal tissue under inflammatory reactions [123, 124]. Vanin-1 mediates oxidative burst in the intestinal epithelium, which occurs through the activation of the Tool/cytokine signaling pathway [125, 126]. Ferretin 2 also participates in the oxidative stress reactions; in this case it sequesters the excess ferric iron, reduces the generation of H₂O₂, and decreases the effects of oxidative stress in response to the activity of such proteins as Vanin-1 [127]. Chitinases are involved in the inflammatory response in the intestine induced by cytokines [128]. In addition, insect chitinases modulate the peritrophic membrane thickness, which forms a structure that separates the undigested nutrients from the intestinal epithelial cells [129].

The functioning of the basic biochemical mechanisms of the honeybee has been found to express caste and subspecific differences. Differential expression of the proteins involved in response to pathogens proves that various subspecies of the honeybee may have different levels of susceptibility to disease. These local adaptive responses may take place in the levels of protective protein expression, which may occur because of genetic or epigenetic changes in different subspecies of the honeybee. Metabolism in the honeybee subspecies is adapted to conditions in the place of their origin, so that the same climatic conditions have different effects on the biosynthesis, stability, and activity of proteins. All this contributes to the adaptation and conservation of the ecotypes. Therefore, measures should be taken for the study and preservation of species diversity of bees to prevent the loss of this rich genetic material that is so valuable to the world's biodiversity.

20.6 CONCLUSION AND PERSPECTIVES

The development of a unique ecological niche in the evolution of the honeybee defined a special role of oxidative stress in the vitality of the species.

One of the leading factors in the progressive evolution in the number of Hymenopteran species is the development of social instincts. In eusocial species there is a close contact with grown offspring, constant care of it at all stages of development. Communication means, maintenance of the optimal conditions of microclimate in the colony, and protection against enemies and adverse conditions have been significantly improved. Within

the colony there is a morphological and functional differentiation of individuals. In the individual actions of each individual the hereditary program that activates the mechanisms of organism alteration at certain times, physiological processes, and certain types of behavior plays a major role.

In the genome of the honeybee there are relatively few genes associated with innate immunity and detoxification proteins. In the evolution of the honeybee adaptation did not change the attributes of individuals but features of the bee colony as an integral biological unit. In accordance with this, the functioning of a large part of the genome is refocused on solving problems related to ensuring eusociality. Nevertheless, the reaction of phagocytosis in the hemolymph to pathogen introduction, the effect of a toxicant or other negative environmental impact, is accompanied by oxidative burst. It is traditionally appeared in the ROS generation and changes in antioxidant enzyme activity. Oxidative stress is a universal high-performance reaction of the honeybee organism to the impact of negative factors. Functional expression of hemocyte activation is the formation of quinone intermediates of melanin, $O_2^{\bullet-}$, H_2O_2 , and other ROS with high cytotoxic activity. Change in hormonal balance during the metamorphosis of the honeybee larvae and pupae and the appearance of macrophagocytes participating in processes of histolysis in the hemolymph are also accompanied by oxidative burst and induction of the molecular mechanisms to restrain it. Hormone-dependent changing of the functions performed by the working bee in the colony and control over longevity are also worthy of thorough study, including gerontology. Thus the honeybee is not only a subject economically and socially important to the human but also a very interesting model for studying of the mechanisms of memory, behavior, immunity, etc. In a complex system of protective reactions of the honeybee, oxidative stress and molecular mechanisms of its regulation demonstrate a high efficiency in solving a wide range of tasks with strictly limited resources. The honeybee genome [12] and the interpretation of its operating principles are expected to give a better understanding of the features of *A. mellifera* species.

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MOLECULAR BASIS OF IRON-INDUCED OXIDATIVE STRESS IN THE HONEYBEE BRAIN: A POTENTIAL MODEL SYSTEM OF OLFACTORY DYSFUNCTION IN NEUROLOGICAL DISEASES

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21.1 INTRODUCTION

It is well known that all living organisms are exposed to environmental stress. Many organisms are exposed to harsh environmental conditions such as extreme temperatures (freezing and heating), anoxia, desiccation, cross-tolerance, and oxidative stress, which may impair the operation of vital neuronal circuits and put animals in danger before these conditions directly cause cell and tissue death. Oxidative stress is defined as a condition that is produced by a disturbance in the cellular prooxidant-to-antioxidant ratio [1]. Reactive oxygen species (ROS) is a collective term that includes both oxygen radicals (such as superoxide, hydroxyl, peroxy, and hydroperoxyl radicals) and nonradical oxidizing agents such as hydrogen peroxide (H_2O_2), hypochlorous acid, and ozone that can be converted into radicals. ROS are mainly produced by the mitochondrial electron transport chain and oxidation of polyunsaturated fatty acid (PUFA) [2].

In eukaryotic systems, reduction of molecular oxygen by one electron yields superoxide radical that has limited reactivity with some proteins but is not reactive with lipids or DNA (Fig. 21.1). Under the influence of superoxide dismutase (SOD), H_2O_2 is formed by the addition of $1 e^-$ and $2 H^+$. H_2O_2 is not a free radical because it does

not have an unpaired electron, but it is an effective nonradical oxidizing agent for many biological molecules because reduction of H_2O_2 yields hydroxyl radicals ($OH^\bullet$) [2]. Both superoxide ($O_2^{\bullet-}$) and hydroxyl ($OH^\bullet$) radicals are continuously generated during oxidative metabolism in biological systems. $O_2^{\bullet-}$ reacts with some proteins; however, $OH^\bullet$ radicals react with carbohydrates, proteins, lipids, and DNA (Fig. 21.1).

The level of oxidative stress is determined by the balance between the rate of induction of oxidative damage (depending on how fast ROS are generated) and the rate of efficient repair and/or removal processes (endogenous defense system including levels of repair enzymes and antioxidants) for such damage (Fig. 21.2). In response to low levels of ROS, the nuclear factor-erythroid-2-related factor 2 (Nrf2) translocates to the nucleus and regulates expression of genes involved in cell survival. At higher ROS concentrations, reaction between ROS and proteins or unsaturated lipids in the plasma membrane leads to a chemical cross-linking of membrane proteins and lipids and a reduction in membrane unsaturation, resulting in reduction of membrane fluidity and decreased activity of membrane-bound enzymes, ion channels, and receptors [3]. Furthermore, high ROS level prevents translocation of Nrf2 to the nucleus but stimulates nuclear factor- κ B (NF- κ B), a

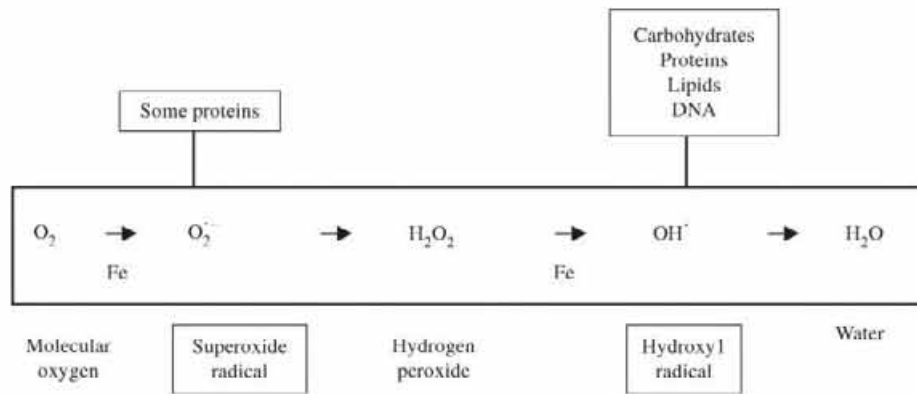


Fig. 21.1 Iron-induced reactive oxygen species (ROS) formation.

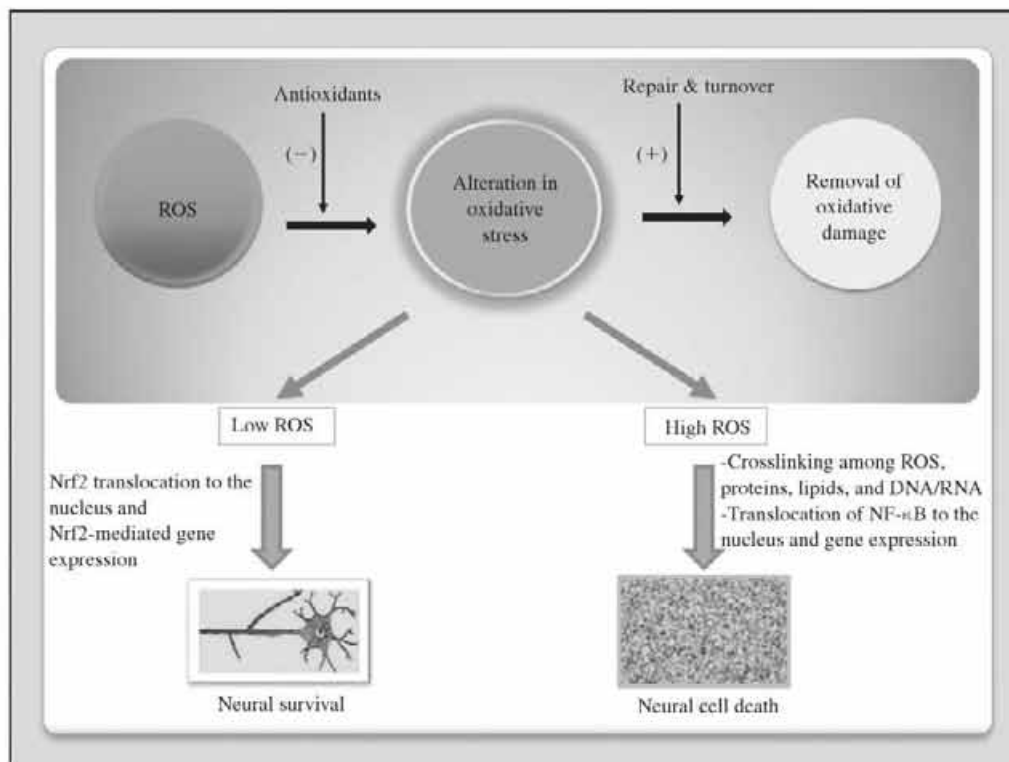


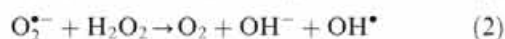
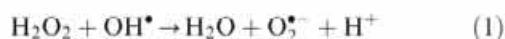
Fig. 21.2 Connection between reactive oxygen species (ROS)-mediated oxidative stress and abnormal functions. Increase in oxidant level, high ROS, decrease in antioxidants, and failure in repair and turnover result in functional abnormalities. Repair and turnover of oxidative damage, the so-called cellular defense mechanism, include DNA excision, resynthesis, and rejoining of DNA strands; repair of oxidized methionine residues in proteins; and normal membrane turnover releasing damaged lipids. Low ROS allow the translocation of nuclear factor-erythroid-2-related factor 2 (Nrf2) to the nucleus to regulate the expression of surviving genes for neural survival, whereas high ROS prevents translocation of Nrf2 to the nucleus. The cross-linking of high ROS occurs with proteins, lipids, DNA/RNA, which promotes neurodegeneration. (See color insert.)

transcription factor that, after translocating into the nucleus, induces expression of many genes involved in inflammation and oxidative stress and therefore promoting cell death (Fig. 21.2) [3]. The imbalance between

ROS production and cellular defense mechanism is also implicated in destruction of neural cells in a wide variety of pathological conditions such as abnormal functions, aging, and diseases.

Iron, a transition metal ion, plays a crucial role in oxygen transport. Iron is a basic requirement for electron transport and cellular respiration and serves as a key element in most of the cytochrome enzymes involved in the oxidative phosphorylation of the Krebs cycle. Its overloading is potentially deleterious because of its involvement with lipid peroxidation in biological systems. Iron can induce generation of ROS through reduction of H_2O_2 to $\text{OH}^\bullet$ via the iron-catalyzed Haber–Weiss/Fenton reactions [4, 5]:

Haber–Weiss reaction:



The Haber–Weiss cycle consists of the above two reactions. The Haber–Weiss reaction can form the hydroxyl radical ($\text{OH}^\bullet$) in an interaction between superoxide radical ($\text{O}_2^{\bullet-}$) and H_2O_2 in the presence of ferrous iron (Fe^{2+}) or ferric iron (Fe^{3+}).

Fenton reaction:



Fe^{2+} is oxidized by H_2O_2 to Fe^{3+} and produces the $\text{OH}^\bullet$ and a hydroxyl anion in the Fenton reaction. Fe^{3+} is reduced back to Fe^{2+} , a peroxide radical ($\text{O}^\bullet\text{HH}$), and a proton. The $\text{OH}^\bullet$ is highly reactive. It can abstract a hydrogen atom from PUFA to initiate lipid peroxidation [6]. Increased Fe^{2+} directly initiates additional lipid peroxidation with accumulated lipid hydroperoxides, resulting in changes in membrane structure and functional damage [6]. The $\text{OH}^\bullet$ attacks and damages every category of macromolecules such as lipids, proteins, carbohydrates, and DNA [7].

Brain is highly susceptible to oxidative damage. High rate of oxygen consumption, high lipid content, and relative scarcity in antioxidant enzymes compared to other tissues may account for the vulnerability in brain [8]. Several studies have demonstrated altered iron metabolism participating in the generation of ROS, resulting in marked increase in protein oxidation [9, 10], lipid peroxidation [11, 12], and DNA/RNA oxidation [13, 14] in the Alzheimer disease (AD) brain. Because of the excess of ferrous iron, oxidative stress has been shown to play a significant role in neurotoxicity associated with a variety of neurodegenerative and neuropsychiatric diseases (Table 21.1), affecting olfactory neuroepithelial cells at an early stage [15–33]. Increased oxidative stress is a prominent feature of vulnerable neurons in AD. Oxidative damage is shown to be increased in olfactory epithelium in biopsy

TABLE 21.1 Olfactory dysfunction frequently observed in patients suffering from brain diseases

Disease	Reference
Alzheimer disease	14–16
Alzheimer disease, Idiopathic Parkinson disease, parkinsonism-dementia complex of Guam	14–16, 18–21, 24, 29, 30
Huntington disease	22, 23
Amyotrophic lateral sclerosis	24, 25
Multiple sclerosis	26
Neuropsychiatric disorders	17
Schizophrenia	27, 28
Idiopathic REM sleep behavior disorder	30
Tauopathies	31
Open-angle glaucoma	32

specimens of AD [15]. ROS-mediated oxidative damage is markedly increased in olfactory epithelium from biopsy specimens of AD [15]. PAN-811 (3-aminopyridine-2-carboxaldehyde thiosemicarbazone, or Triapine), a novel neuroprotectant, not only effectively suppresses intracellular ROS accumulation but also reduces ROS-mediated oxidative damage in both AD-derived and age-matched olfactory neuroepithelial cells [16]. It has also been shown that ROS-mediated olfactory deficit may be a characteristic feature of several neurological disorders including AD [15–17, 23], idiopathic Parkinson disease (PD), parkinsonism-dementia complex (PDC) of Guam [19, 20, 25], Huntington disease (HD) [23, 24], Guamanian amyotrophic lateral sclerosis (G-ALS), several forms of dementia [25, 30, 31], multiple sclerosis (MS) [27], schizophrenia [28, 29], idiopathic REM sleep behavior disorder [31], tauopathies [32], and open-angle glaucoma [33], supporting the view that there is a link among ROS, olfactory dysfunction, and neurodegenerative and neuropsychiatric diseases (Table 21.1).

Very little information is available on ROS-mediated oxidative stress in the insect brain. Although honeybees are a well-known model system for behavioral studies for olfactory learning and memory because they can be conditioned to respond with feeding movements of the mouthparts (proboscis) to a variety of floral odors, which is called proboscis extension reflex (PER) conditioning [34–37], only a few studies have been attempted to demonstrate a correlation between ROS-mediated oxidative stress in the brain and its effect on the learning behavior in individual restrained honeybees [38–40].

The purpose of the present overview is to discuss our iron-induced oxidative stress model system in honeybee *Apis mellifera* brain, created by injecting ferrous ammonium citrate (FAC) in the antennal lobes, which results in impairment of olfactory learning and memory [40] suggesting that oxidative stress plays a major role in

olfactory dysfunction. Olfactory deficits have been reported in many neurological disorders (Table 21.1). A similar mechanism has been proposed for olfactory abnormalities in patients of AD and PD. Because of the similarities in cellular and molecular processes that govern neuronal plasticity in humans and honeybees [38], the author proposes that the honeybee can be used as a potential and relatively simple model system for understanding human olfactory dysfunction in neurological diseases.

21.2 A COMPARISON BETWEEN GENERAL PHYSIOLOGY OF OLFACTORY PROCESSING IN HONEYBEES AND HUMANS

The honeybee *Apis mellifera* olfactory system is well adapted to detect and discriminate a diverse array of odors [41]. The perceptual qualities of odors vary depending on several factors such as carbon chain length in the drug, shape, functional group, as well as concentration [42–49]. The honeybee olfactory system possesses olfactory sensory neurons inside the cuticle-covered sensillae along the antennae, which are equivalent to olfactory epithelia within the nasal cavity in vertebrates [38, 50]. The olfactory receptors are located on the olfactory sensory neurons in antennae. The recognition and discrimination of odor molecules starts at the antenna via binding of odor with olfactory receptor. From the antenna, information is carried directly to the antennal lobe via axons of olfactory sensory neurons. The antennal lobe is the structural and functional analog of the olfactory bulb in vertebrates that is subdivided into identified glomeruli. It processes incoming signals from broadly tuned olfactory sensory neurons [51, 52]. The axons of olfactory sensory neurons converge on two types of antennal lobe neurons: (1) local interneurons communicate within the antennal lobe, and (2) outgoing projection neurons mediate the signal information from the antennal lobe to the protocerebrum [53]. The synaptic contact between olfactory sensory neurons, local interneurons, and outgoing projection neurons takes place within the glomeruli arranged in a single layer around the antennal lobe [54, 55]. The glomerular layer in the antennal lobe probably contains the summarized representation of the receptor types activated by a given odorant. Thus olfactory information received from the antennae is processed in the antennal lobe and relayed to the mushroom body and the lateral protocerebrum via projection neurons, leaving the antennal lobe in three different antenno-cerebral tracts. The small mediolateral antenno-cerebral tract contains pluriglomerular cells, whereas lateral and median antenno-cerebral tracts contain axons of uniglomerular projection neurons [55, 56]. A synaptic response to focal electrical stimulation recorded in the mushroom

body of the honeybee brain suggests involvement of the mushroom body in memory consolidation [57]. The synaptic plasticity exhibited in the mushroom body of the honeybee brain is similar to the synaptic plasticity occurring in the mammalian hippocampus [58]. Neuroplasticity in the honeybee brain, existing in the α lobe of the mushroom body, contributes to the associative long-term potentiation linked with olfactory learning and memory [59, 60]. This is in agreement with findings in *Drosophila* that all forms of olfactory learning (such as aversive, appetitive, and extinction) confined to the output sites of the mushroom body [61]. Moreover, GABAergic inhibitory interneurons in the antennal lobe of the honeybee brain modulate the overall activity and compute olfactory information by forming an odor-specific topographic map, relaying information from the antennal lobe to other brain regions via projection neurons [62–64].

Similar to the antennal lobe in honeybees, each glomerulus of the olfactory bulb in humans contains the axons of several thousands of olfactory sensory neurons, each expressing the same odorant receptor and the dendrites of mitral and tufted cells, which are the main input and output neurons of the olfactory bulb [65]. These neurons are activated by olfactory sensory neurons, but odorant information is further processed by lateral and feedback inhibitory pathways via activity of inhibitory interneurons, periglomerular cells, and granule cells [64–66]. During air inhalation, many volatile molecules reach the nasal cavity and interact with the odorant receptors located on the cilia of olfactory sensory neurons in the olfactory epithelium, eliciting an electrical signal that is transmitted to the second-order neurons in the olfactory bulb, which is then projected to pyramidal neurons in the olfactory cortex and to other brain regions such as thalamus and neocortex of the brain [38, 65]. Thus it is suggested that the peripheral olfactory systems involved in sensory transduction and early synaptic processing in the antenna and the antennal lobe of honeybees show a vast array of similarities to the olfactory epithelium and the olfactory bulb of humans [38, 68, 69].

21.3 OLFACTORY NEURONAL NETWORK IN HONEYBEES

In honeybees, the olfactory system is comprised of three major components: (1) antennae, (2) antennal lobes, and (3) mushroom bodies [38]. Honeybees use antennae to detect odors by making use of olfactory receptors that are located on olfactory sensory neurons in the antennae. Olfactory sensory neurons project their axons into the antennal lobes. The pathway involving olfactory sensory neurons is called the conditioned stimulus (CS) pathway, where CS represents an odor. Honeybees have

taste receptors located on olfactory sensory neurons in the antennae that project their axons into the antennal lobes. This pathway is called the unconditioned stimulus (US) pathway, where US represents nectar or sucrose. The primary sensory afferents (CS and US pathways) converge into the glomeruli of the antennal lobe.

In the honeybee *Apis mellifera*, an identified ventral unpaired medial cell (VUMmx1; an octopaminergic neuron) produces an associative link between US and CS pathways in the antennal lobe. The VUM interneuron releases octopamine into most if not all glomeruli of the antennal lobe innervating antennal lobe, mushroom body, and lateral protocerebrum. Electrical stimulation of VUM neuron substitutes for the unconditioned stimulus (sucrose) in an associative olfactory learning paradigm, supporting its role in olfactory learning and memory [70]. Local injection of octopamine into defined areas of brain increases honeybee's learning ability as well as recall, suggesting that octopamine is involved in regulation of learning and memory [71]. Actually, exactly how VUM interneuron couples the CS and US pathways is not clear except that this neuron has a reinforcing property. The convergence of two types of stimuli suggests that the antennal lobe is a site where part of olfactory memory consolidates [72]. The release of octopamine from VUM neuron or acetylcholine from olfactory sensory neuron in the synaptic cleft may induce GABA release from GABAergic local interneuron in the antennal lobe [38]. GABA-mediated inhibitory response is considered to be important for shaping and tuning the output from the antennal lobe [73].

21.4 ROS, OLFACTORY DYSFUNCTION, AND AGING

Increased oxidative stress is a prominent and early feature of aging and neurodegenerative diseases; therefore, oxidative damage has been considered an important factor in the progression of pathological and nonpathological age-related functional declines including olfaction. Again, this harmful condition occurs when there is either an excess of free radicals or a decrease in antioxidant levels, resulting in variety of changes in cells and tissues. Free radicals cause oxidative damage by attacking PUFA in neural cell membranes and generate metabolic waste products that interfere with cell-cell communication, damaging cellular proteins, lipids, and DNA, lowering energy levels and vitally impeding biochemical processes. The source of the devastating actions of ROS is mainly the oxygen molecule's unpaired electron, which makes it unstable and electrically charged [74]. Although sufficient ROS accumulation makes an organ system unable to function, endogenous defense

mechanisms can bring ROS levels back down to a normal level consistent with organ function. Increased levels of oxidative stress markers have been observed in cultured human olfactory neurons in AD patients compared to control subjects [75]. Oxidative stress in the aging murine olfactory bulb has shown significant changes in steady-state levels in olfactory bulbs of 20- versus 1.5-month-old mice, demonstrating greater carbonylation and nitration of specific proteins with aging [76]. Nitric oxide (NO) and derived nitrogen species in CNS may interact with catecholamines, thus modifying not only NO regulatory actions but also producing oxidants and free radicals that are likely to trigger toxic effects. Peroxynitrite, a potent oxidizing agent formed by the interaction between superoxide anion and nitric oxide, produces nitration of tyrosine groups present in the proteins and inactivates several enzymes [77]. Peroxynitrite appears to be involved in the pathophysiology of many neurodegenerative diseases. Increased oxidative stress in olfactory epithelium of AD patients causes modification in olfactory receptor proteins, resulting in olfactory impairment [78]. Moreover, abnormal processing of the proteins in AD supports contribution of RNA oxidation [14]. In humans, olfactory function deteriorates progressively with increasing age, even in the absence of overt medical problems [79]. Similarly, a marked deficit has been observed in olfactory learning and recall in healthy aged honeybee workers (30 days old) compared to young workers (4 days old), indicating that olfactory learning and memory processing in the honeybee brain appear to be vulnerable to aging [38]. Collective evidence supports the notion that ROS-mediated oxidative stress may have a strong impact on olfactory dysfunction with aging.

21.5 IRON INDUCES OXIDATIVE STRESS IN THE HONEYBEE BRAIN

Iron, an important ion used for biological electron transfer in aerobic systems, also exhibits a chemical dark side with side reactions that form ROS, resulting in oxidative stress. ROS-mediated lipid peroxidation has been suggested to be an important factor in posttraumatic neuronal degeneration [80]. Addition of FAC allows evaluation of a quick onset of oxidative stress that is free from other conditions, such as neuroinflammation, which are produced by chronic levels of iron injury during neurodegeneration [80, 81]. By applying *in vivo* injections of FAC into the antennal lobes of the honeybee brain to produce $\text{OH}^\bullet$ radical, and monitoring the subject's responses to odorants by proboscis extension reflex conditioning assay, we have examined whether oxidative stress can be induced into the antennal lobes of the honeybee brain and whether or not ROS-associated alterations in brain can modulate olfactory

response of honeybees [40]. We have used different concentrations of FAC in each antennal lobe to evaluate its dose-dependent response and showed that the level of acquisition response in FAC-treated groups (0.5 mM to 5 mM range) significantly decreases with increasing dose (Fig. 21.3A), suggesting that iron interferes with olfactory learning in a dose-dependent manner. Testing of subjects in each group with conditioned odor (C), molecularly similar odor (S), and molecularly dissimilar odor (D) for retention and generalization responses show that iron interferes with retention in a dose-dependent manner but impairs generalization gradient at all FAC concentrations tested (Fig. 21.3B).

Reduced glutathione (GSH), an intracellular antioxidant, is known to prevent the oxidation of protein sulfhydryl groups and to help in maintaining cellular thiols in reduced state (-SH) [82]. To evaluate the reversal of FAC-mediated inhibitory effect on olfactory learning and memory, we injected GSH in each antennal lobe before injecting FAC, with an assumption that presence

of excess thiol compound in the brain may efficiently protect honeybees against toxic effects of ROS on olfactory learning and memory. We observed that the FAC-mediated inhibitory effect on acquisition response is fully reversed in presence of GSH (Fig. 21.4A). Pretreatment of antennal lobes with GSH before injection of FAC also results in complete reversal of iron-mediated inhibition of retention response as well as showing a characteristic generalization gradient (Fig. 21.4B). This suggests that increased concentration of antioxidant forms an important component of the defense by reversing the oxidative stress created by free radicals [40].

Several novel brain-permeant and multifunctional iron chelators (such as HLA20, M30, VK-28, and M32) have been tested for their ability to inhibit iron-dependent lipid peroxidation in rat brain homogenates [83]. The brain-permeant iron chelator VK-28 has been shown to prevent *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced neurotoxicity in mice [84] and 6-hydroxydopamine (6-OHDA) lesion in rats [85], without showing

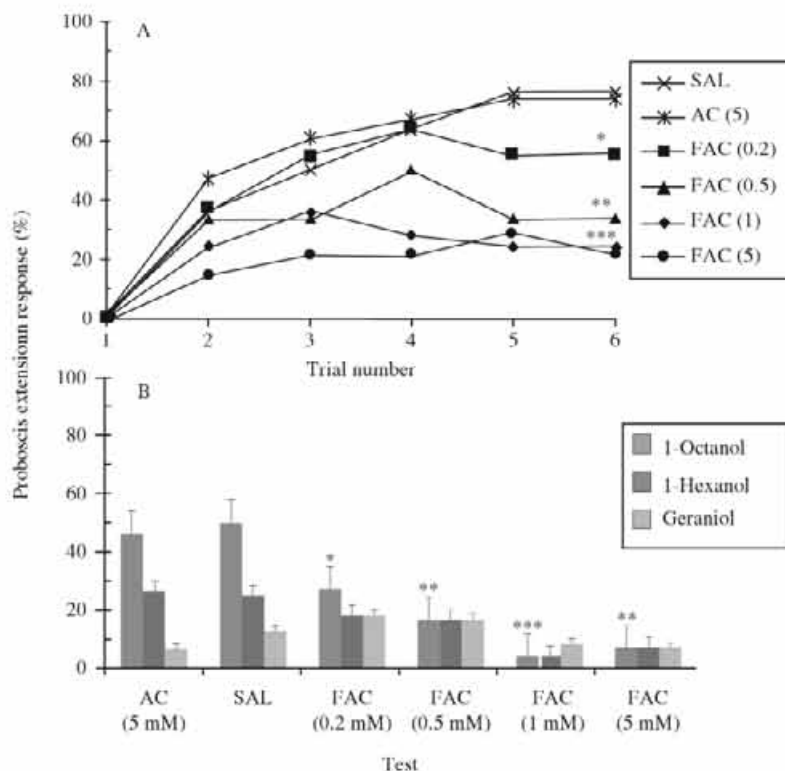


Fig. 21.3 Dose-response effect of ferrous ammonium citrate (FAC) on olfactory learning and memory. (A) Acquisition of the conditioned odor C: In this experiment, honeybees received 4 nl of saline (SAL), ammonium citrate (AC), or FAC at 0.2 mM, 0.5 mM, 1 mM, and 5 mM in each antennal lobe. Twenty-four hours later, subjects in each group were conditioned with 1-octanol (odor C). (B) Test with odors C, S, D: Ninety minutes after conditioning, subjects in each group were tested with C, molecularly similar (S), and molecularly dissimilar (D) odors in randomized order. *ns*, Not significantly different from controls (SAL, 5 mM AC). Asterisks indicate significant differences of respective points from control group: * $P = 0.05$, ** $P = 0.005$, 0.001, *** $P = 0.0001$). This figure is modified from reference [40]. (See color insert.)

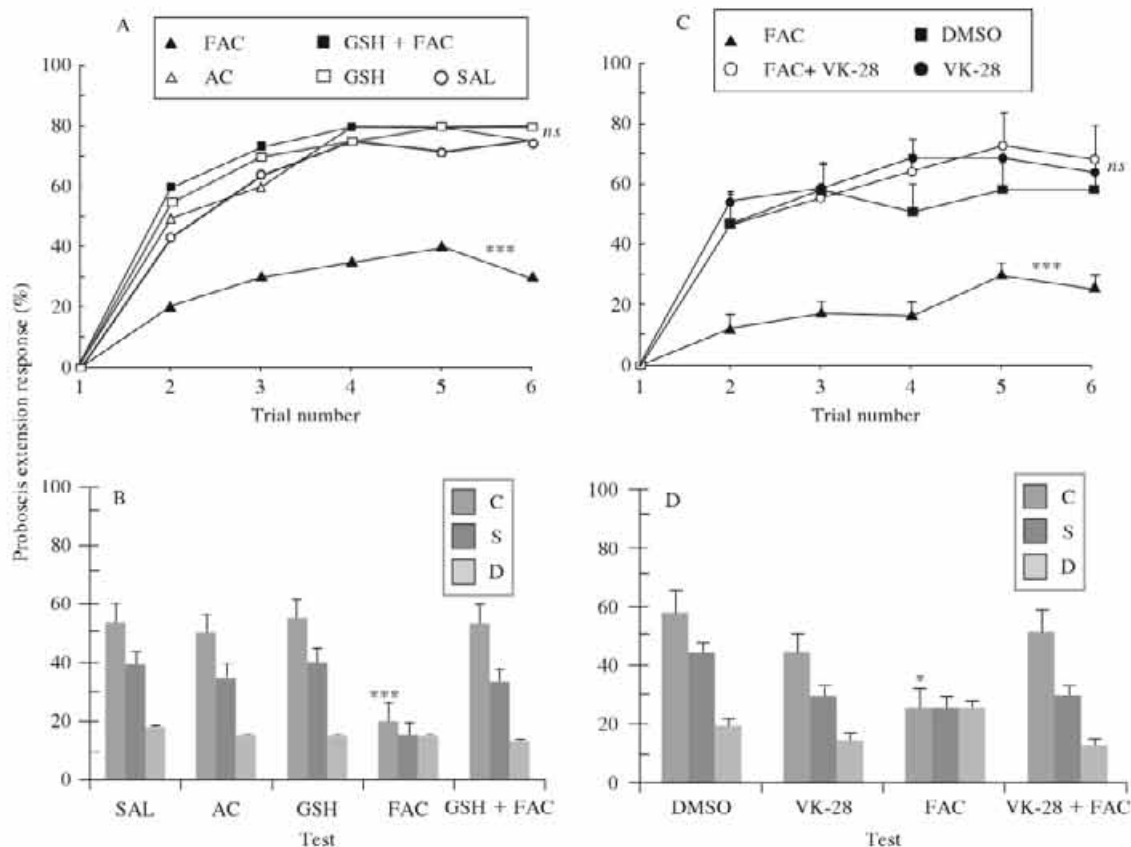


Fig. 21.4 Glutathione and VK-28 can reverse ferrous ammonium citrate (FAC)-mediated oxidative stress. (A) Acquisition of the conditioned odor C: In this experiment, subjects received 4 nl of either saline (SAL) or 2.5 mM reduced glutathione (GSH) in each antennal lobe. Two hours later, 4 nl of 500 μ mol/l ammonium citrate (AC) or 500 μ mol/l FAC were injected in each antennal lobe. Twenty-four hours later, subjects were conditioned with 1-octanol. (B) Test with odors C, S, D: Ninety minutes after conditioning, subjects in each group were tested with different odors (1-octanol, C; 1-hexanol, S; and geraniol, D) in randomized order. (C) Acquisition of the conditioned odor C: In this experiment, subjects received 4 nl of either dimethyl sulfoxide (DMSO) or VK-28 or DMSO + VK-28 in each antennal lobe 30 min before injection of FAC. Twenty-four hours later, subjects were conditioned with 1-octanol. (D) Test with odors C, S, D: Ninety minutes after conditioning, subjects in each group were tested with C, S, and D odors in randomized order. Asterisks indicate significant differences of respective points from control group (* $P = 0.05$, *** $P = 0.001$). *ns*, Not significant. This figure is modified from reference [40]. (See color insert.)

any appreciable inhibitory effect on monoamine oxidase (MAO) [86]. VK-28 inhibits both basal and Fe/ascorbate-induced mitochondrial membrane lipid peroxidation [85]. To confirm that FAC-mediated inhibitory effect on learning and memory is due to the addition of a high concentration of iron, we injected VK-28 into the antennal lobes of the honeybee brain before injecting FAC. We observed that the combination of FAC and VK-28 treatment restores learning response to 100% (Fig. 21.4C), implying that the FAC-mediated inhibitory effect on learning is due to increased ferrous iron. Subjects in the FAC-treated group show 40–50% reduction in retention response compared to controls and do not show a characteristic generalization gradient (Fig. 21.4D). However, FAC + VK-28-treated group overcomes

these inhibitory responses due to iron-chelating effect (Fig. 21.4D). Collectively, FAC-induced oxidative stress in the antennal lobes of the honeybee brain markedly inhibits olfactory learning and memory, supporting a major role of iron in olfactory dysfunction [40].

The inhibitory patterns of olfactory learning have also been observed by disruption of octopamine receptor function by either receptor antagonism or gene silencing [72]. However, addition of GSH into the antennal lobes prior to mianserin/dsRNA treatment does not reverse octopamine receptor disruption-mediated inhibitory responses of olfactory learning and memory [40], suggesting that octopamine receptor disruption and iron-mediated oxidative stress confer two independent mechanisms that impair olfactory learning and memory in honeybees.

21.6 MOLECULAR BASIS OF IRON-INDUCED OXIDATIVE STRESS

The brain is susceptible to iron-induced oxidative stress in both vertebrates and invertebrates. Oxidative stress in the olfactory system is a major factor associated with age- or disease-related olfactory impairment. Patients of AD at the early stage of the disease exhibit perceptual deficits in odor identification, although the molecular mechanisms are not completely understood. Protein oxidative modification in aging murine olfactory bulb has shown the presence of the specific carbonylated proteins in astrocytes and mitral/tufted neurons but nitrated proteins in the vasculature, as molecular substrates of age-related olfactory dysfunction [87]. Studies on redox-competent copper and iron indicate that redox activity in AD resides exclusively within the cytosol of vulnerable neurons, and chelation with deferoxamine or diethylenetriaminepentaacetic acid (DTPA) removes this activity [88].

The role of mitochondria as a potential source of redox-active metals and oxygen radical production is assuming more prominence. Increased mitochondrial DNA and cytochrome *c* oxidase activity but reduction in the number of mitochondria in AD brain indicates accelerated mitochondria turnover [15]. Neurons, and also the surrounding epithelial cells, in biopsy specimens have been observed with increased lipid peroxidation and heme oxygenase-1, whereas no increase in nucleic acid or protein oxidation was observed in vulnerable neurons in AD [15], suggesting that the abnormality in array of oxidative stress markers may occur depending on different cell types in AD.

A hypothetical scheme is shown in Fig. 21.5 to present molecular mechanism(s) involved with olfactory dysfunction in the honeybee brain. In the iron-induced oxidative stress model, phospholipase A_2 (PLA $_2$) peroxidizes PUFA that are present in phospholipids into peroxidized phospholipids, which are better substrates for phospholipase A_2 than native phospholipids in the honeybee brain (Fig. 21.5). This process results in the formation of free peroxidized arachidonic acid (ARA). The oxidation of ARA ultimately results in the generation of 4-hydroxynonenal, which impairs mitochondrial electron transport and produces ROS. Increased ROS production causes oxidative stress that damages proteins, DNA/RNA, and lipids, causing olfactory dysfunction (Fig. 21.5). Reduced glutathione protects brain against oxidative stress by modulating the redox state of specific thiol residues of target proteins, whereas VK-28 chelates iron from the system and therefore slows down the formation of ROS. Oxidation of catecholamines may form quinones, which can form ROS, leading to olfactory dysfunction (Fig. 21.5). Furthermore, octopamine receptor function is disrupted by mianserin (octopamine receptor antagonist),

which competes with octopamine in binding to octopamine receptors and therefore blocks receptor function, resulting in olfactory dysfunction (Fig. 21.5). Octopamine receptor function disruption by octopamine receptor dsRNA (AmOA1-dsRNA) silences octopamine receptor gene expression, which inhibits its protein synthesis, resulting in olfactory dysfunction (Fig. 21.5). The receptor functional disruption mechanisms (mianserin/AmOA1-dsRNA) seem to be independent of ROS formation. The receptor functional disruption is also not a random process but appears to be associated with increased oxidation of specific proteins [89]. Similarly, disruption of noradrenergic receptor or production of dopamine quinones in the olfactory bulb of vertebrates (including humans) may contribute to increased levels of oxidative stress, resulting in olfactory dysfunction.

21.7 HONEYBEE MODEL SYSTEM FOR OLFACTORY DYSFUNCTION

The olfactory system is phylogenetically highly conserved among invertebrates and vertebrates. Honeybees have the ability to learn about the association of odor with reinforcement. There are well-established behavioral paradigms, such as proboscis extension reflex, for monitoring olfactory learning in honeybees. They have a short life span, which makes them suitable for study of aging. They are straightforward to propagate and easy to manipulate in the lab. They have short gestation periods that produce large numbers of offspring, and their genome sequence is available. Their brains can be easily dissected out to focus on the relevant neurons in a specific region such as antennal lobe from the honeybee brain because of its larger brain size compared to other insect models such as *Drosophila*. Finally, the antennal lobe of the honeybee brain is a functional analog to the mammalian olfactory bulb.

Oxidative damage has been demonstrated to be increased in the olfactory system (including neurons and the surrounding epithelial cells) in the brains of AD patients [15]. Neuropathological studies in the brains of PD patients also suggest neuronal damage in the olfactory system (including olfactory bulb and the anterior olfactory nucleus) [90]. These findings support our findings in the honeybee brain, where oxidative stress induced in the antennal lobe of the brain results in olfactory dysfunction [40]. All of the criteria mentioned above for a better model system for olfactory research, neuropathological studies performed in AD and PD brains [15,90], and our findings associating oxidative stress and olfactory dysfunction in the honeybee brain [38, 40] support the honeybee brain as a potential model system for olfactory dysfunction during aging and neurodegenerative diseases.

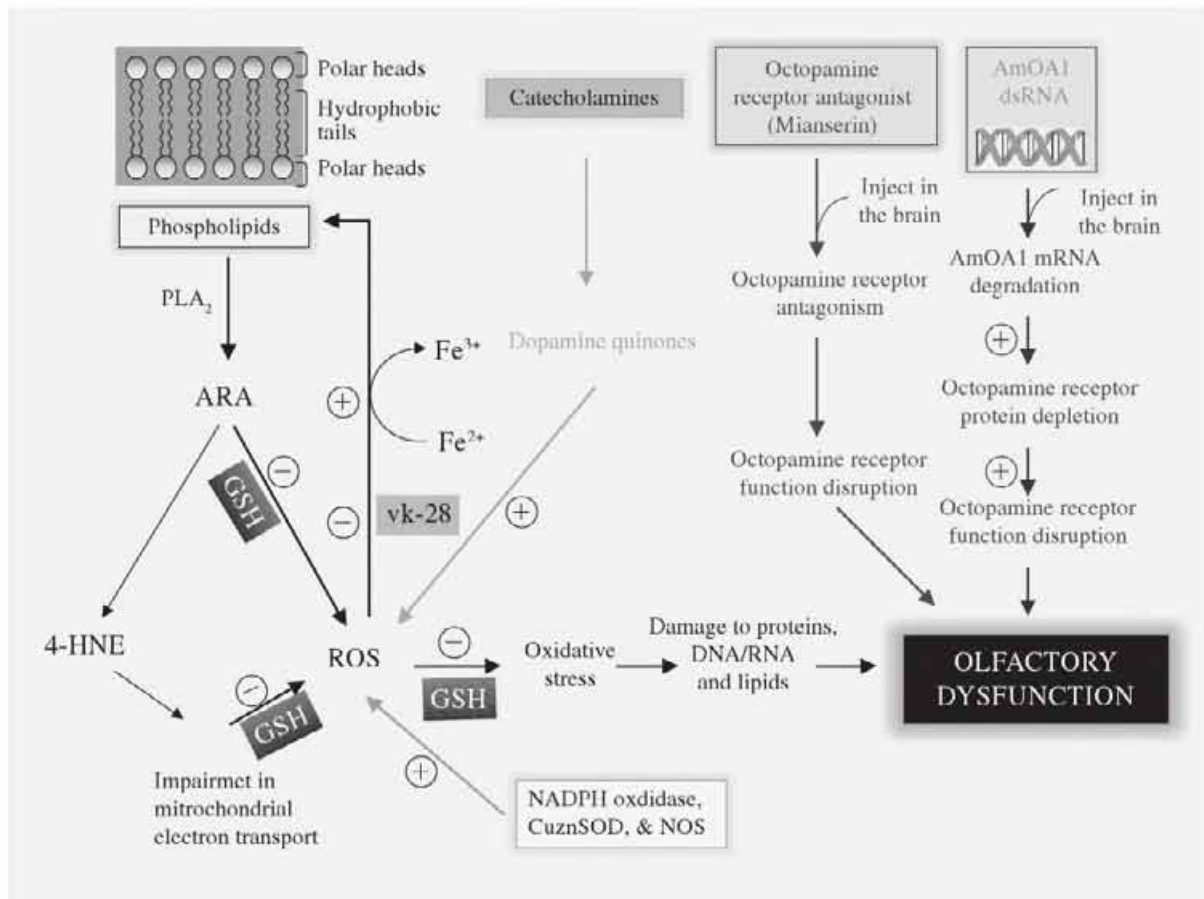


Fig. 21.5 Hypothetical molecular mechanism underlying reactive oxygen species (ROS)-mediated olfactory dysfunction in the honeybee brain. Iron-induced oxidative stress: Iron peroxidizes polyunsaturated fatty acids into peroxidized phospholipids, which are considered better substrates for phospholipase A_2 (PLA_2) than native phospholipids. PLA_2 catalyzes this reaction, forming arachidonic acid (ARA). The nonenzymatic oxidation of ARA results in the generation of 4-hydroxynonenal (4-HNE) that impairs mitochondrial electron transport, producing ROS. Increased ROS produces oxidative stress that damages proteins, DNA/RNA, and lipids, impairing olfactory processes (encoding, consolidation, and/or retrieval processes). Both octopamine receptor antagonism by mianserin (MAS) and octopamine receptor protein depletion by octopamine receptor double-stranded RNA (AmOA1-dsRNA) result in functional disruption of octopamine receptor, which leads to olfactory dysfunction. Oxidation of catecholamines forms quinones, which results in formation of ROS, leading to olfactory dysfunction. GSH protects brain against oxidative stress by modulating the redox state of specific thiol residues of target proteins. VK-28 chelates excess iron from the system. Monoamine oxidase inhibitor (MAOI) inhibits monoamine oxidase enzyme and therefore prevents oxidation of catecholamines. ROS can also be produced by activation of NADPH oxidase. (See color insert.)

21.8 CONCLUSION AND FUTURE PERSPECTIVE

Iron plays a role as a catalyst in free radical generation. Increase in ferrous iron catalyzes $OH^\bullet$ radical production in the antennal lobes of the honeybee brain, which causes impairment in learning and memory. The inhibitory effect of iron-induced oxidative stress on learning and memory can be reversed by antioxidant as well as iron chelator in the antennal lobes, suggesting that the honeybee brain is highly susceptible to iron-induced oxidative damage and can be used as a model of olfactory dysfunction.

Iron is not regarded as the underlying cause of olfactory dysfunction in neurodegenerative diseases, but it does play an important role in progression of neurodegenerative diseases, such as AD and PD, by participating in redox reactions that catalyze the formation of ROS that produces increased oxidative stress, leading to olfactory abnormalities. Thus newly developed antioxidants and brain-permeant iron chelators (such as VK-28, HLA20, M30, and M32) can be screened for their rescuing effect on olfactory dysfunction in honeybees during aging and iron-induced oxidative stress. The efficacy of these drugs may depend on their ability to penetrate the subcellular

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MODULATION OF OXIDATIVE STRESS BY KEAP1/NRF2 SIGNALING IN *DROSOPHILA*: IMPLICATIONS FOR HUMAN DISEASES

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22.1 INTRODUCTION

The signaling module comprising the transcription factor Nrf2 (NFE2-related factor 2) and its cytoplasmic inhibitor Keap1 (Kelch ECH-associated protein 1) is a master mediator of ubiquitous antioxidant and detoxification responses to cellular stressors as well as of various cell type-specific homeostatic functions. Nrf2 belongs to the cap'n'collar (cnc) family of leucine zipper transcription factors named after the *cnc* locus of *Drosophila melanogaster*, which was originally characterized as a regulator of fly development. Even though the sequence similarity between Nrf2 and Cnc was evident early on, it took no less than a decade after Nrf2 was cloned to demonstrate experimentally its functional homology with a protein product of *cnc* (isoform C, CncC). This chapter recounts the story of these recent discoveries and discusses ongoing and future studies thereby made possible in flies on the Keap1/Nrf2 system as well as their implications for understanding and combating human diseases.

22.1.1 The Keap1/Nrf2 System Safeguards Homeostasis and Mediates Antioxidant Defense with Implications for Preventing and Treating Human Diseases

To defend themselves against the deleterious effects of oxidative stress, cells possess antioxidant defense networks that can sense prooxidant and electrophilic reactive

species and launch adaptive responses [1]. Since it was cloned in the mid-1990s [2], the vertebrate transcription factor Nrf2 has been established as a ubiquitously expressed master regulator of the cellular redox status and mediator of cell-protective antioxidant and detoxification responses [3–8]. Numerous studies have shown that in mice and in human cultured cells the expression of many protective genes increases in an Nrf2-dependent manner in response to oxidative and electrophilic chemical challenges [reviewed in 3, 4]. Target genes of Nrf2 include those encoding a broad range of redox regulators and so-called phase II detoxification enzymes, such as glutathione *S*-transferases, glutathione-synthesizing enzymes, thioredoxins, peroxiredoxins, NAD(P)H quinone oxidase 1 (NQO1), heme oxygenase 1 (HO1), and many others [9–12]. This battery of antioxidant and detoxifying genes are transcriptionally induced in a coordinated manner through the binding of Nrf2 to antioxidant response element (ARE) sequences in their regulatory regions (Fig. 22.1). The predominant dimerization partners of ARE-bound Nrf2 are the members of the small Maf (musculo-aponeurotic fibrosarcoma oncogene) family of proteins, which are themselves devoid of transcriptional activation potential [13–15].

The abundance and transcriptional activity of Nrf2 increase markedly when cells are exposed to oxidative stressors or electrophilic chemicals, and several of the mechanisms involved have been elucidated over the last

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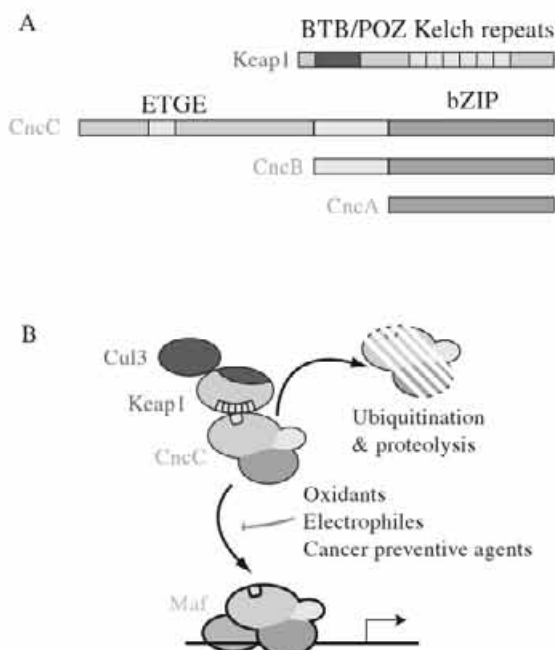


Fig. 22.1 Conservation and simplified illustration of the Keap1/Nrf2 pathway in *Drosophila*. (A) Nrf2 and Keap1 homologs are present in *Drosophila*. The fly *keap1* gene is predicted to encode a protein with high sequence similarity to its vertebrate Keap1 counterparts. Conserved domains include the BTB/POZ domain required for dimerization and 6 Kelch repeats for binding to Nrf2 and anchoring to actin. The *cnc* locus encodes three protein products, which all contain the bZIP region that mediates dimerization and DNA binding. The Nrf2 homolog is the longest isoform, CncC, which contains domains predicted to bind Keap1 such as the ETGE motif. (B) In basal conditions, Keap1 binds to CncC and inhibits its activity, likely through Cul3-mediated ubiquitination and proteasomal degradation. Oxidative stressors, electrophilic xenobiotics, and cancer chemopreventive agents relieve this inhibition. Stabilized CncC then accumulates in the nucleus and transcriptionally activates a battery of cell-protective genes, likely in a dimer with the single small Maf protein of *Drosophila*. This figure is adapted from Sykietis GP and Bohmann D, Keap1/Nrf2 signaling regulates oxidative stress tolerance and lifespan in *Drosophila*, *Dev Cell*, vol. 14, p. 76–85, Copyright 2008, with permission from Elsevier. (See color insert.)

decade [reviewed in 16–19]. Notably, the activation of Nrf2 requires its posttranslational stabilization, because in nonstressed conditions Nrf2 undergoes rapid proteasomal degradation in the cytoplasm. This proteolysis is effected by a Cul3-based ubiquitin ligase system and facilitated by an interaction between Nrf2 and its cognate substrate adaptor, Keap1 (Kelch-like ECH-associating protein 1), a protein tethered to the actin cytoskeleton (Fig. 22.1) [3, 20–26]. The inhibitory effect on Nrf2 is abolished when specific redox-sensitive cysteine side chains of Keap1 undergo oxidative

modifications in response to oxidants and electrophiles, which likely triggers a conformational change in Keap1 structure that interferes with Nrf2 ubiquitination [27–32]. Additional mechanisms involved in Nrf2 activation include its phosphorylation by stress-activated kinases and the redox-sensitive regulation of its nuclear import and export [reviewed in 8, 16–8]. In addition to regulating adaptation to acute cellular stress via the induction of ubiquitous antioxidant and detoxification genes, the Keap1/Nrf2 system contributes to the homeostasis of diverse tissues by regulating the expression of corresponding sets of tissue-specific target genes with distinct roles in the specialized function of the respective cell types [reviewed in 4]. Its generic and tissue-specific homeostatic properties have established Nrf2 as an important “multiorgan protector” [4] and a mediator of “programmed cell life” [33].

By damaging proteins, lipids, and DNA, oxidative stress causes or exacerbates various diseases in model organisms and in humans [34, 35]. Conversely, by ameliorating oxidative stress or reversing its sequelae, Nrf2 protects against such pathologies. In mice, a protective role of Nrf2 has been demonstrated in diverse models of disease, including chemically induced cancers [3, 4, 6, 8, 36–39]; pulmonary diseases, such as asthma [40], emphysema [9], pulmonary fibrosis [41], hyperoxia [42], and acute lung injury [43]; neurodegenerative disorders, such as Parkinson disease [44], Alzheimer disease [45], and amyotrophic lateral sclerosis [46]; inflammatory disorders, such as inflammatory bowel disease [47]; liver toxicity, including toxin- and alcohol-induced liver damage [48, 49]; atherosclerosis [50]; and insulin resistance [51], among others [4, 6]. Importantly, compounds that activate Nrf2 have been shown to be effective in several of these disease models, highlighting Nrf2 as a promising target in preventing and treating various disorders [36, 52–57]. In humans, inherited DNA polymorphisms that reduce the expression of Nrf2 [58, 59] have been associated with diseases such as skin vitiligo [60], chronic gastritis [61], peptic ulcer [62], ulcerative colitis [63], and adult respiratory distress syndrome [59]. Moreover, impaired Nrf2 signaling has been implicated in human respiratory and neurodegenerative disorders [64–69]. Thus there is intense interest in elucidating the mechanisms by which Nrf2 activity is induced or impaired during the pathogenesis and progression of various human diseases, with the goal of identifying pharmacological or nutritional strategies to safely activate Nrf2 signaling for disease prevention and treatment. Although the model organism *Drosophila melanogaster* (commonly known as fruit fly) could help to obtain new insights relevant to these pursuits, it had not been utilized in the study of Nrf2 signaling until quite recently.

22.1.2 Cnc as a Developmental Locus of *Drosophila melanogaster*

Nrf2 is a member of the cap'n'collar (*cnc*) family of transcription factors, which are conserved from worms to mammals. Cnc factors are defined by the presence of a conserved 43-amino acid Cnc domain located amino-terminally to the DNA binding domain. In addition to Nrf2, the Cnc family comprises the vertebrate p45 NFE2 (nuclear factor erythroid-derived 2), which is present only in hematopoietic progenitor, erythroid, megakaryocytic, and mast cells and is required for proper development of platelets [70–72]; the NFE2-related factors Nrf1 and Nrf3 [73, 74], which have broad expression patterns partly overlapping that of Nrf2 and also function as stress-activated transcription factors [2, 73–75]; the *Caenorhabditis elegans* SKN-1 (Skinhead family member 1) [76], which is critical for the formation of the digestive system during worm embryogenesis [76] and also regulates a phase 2 detoxification response in the digestive tract [77]; and the *Drosophila* Cnc [78], after which the family was named.

The *Drosophila cnc* locus was originally identified 20 years ago as a gene expressed in the primordia of three segments of the developing fly embryo's head: the labral segment, where *cnc* expression is detectable as an anterior "cap," and the intercalary and mandibular primordia, where *cnc* expression is detectable as a more posterior 3–4 cell-wide "collar"—hence the name of the locus and of the gene family [78]. The anterior domain of *cnc* expression (corresponding to the labral segment primordium) was found to be activated by the *bicoid* and *torso* maternal pathways, independently of known zygotic gap genes, and sequentially constricted to its final size by repression from neighboring region-specific genes [79]. Control of the posterior domain (corresponding to the intercalary and mandibular segment primordia) involved combinatorial regulation by zygotic gap genes: activation by *buttonhead* and repression by *orthodenticle* anteriorly and *snail* ventrally [79]. Thus *cnc* is expressed and regulated as is typical for a second-tier region-specific gene. It was further shown that *cnc* is a homeotic gene that defines labral and mandibular segment identity: *cnc* mutants show absence of labral and mandibular head structures, and their mandibular tissues are transformed to a maxillary fate, which is a clear example of classical homeosis [80].

The *cnc* locus was subsequently found to give rise to three RNA isoforms, designated *cncA*, *cncB*, and *cncC*, each of which encodes a different protein [81]. CncA, CncB, and CncC share their carboxy-terminal regions, which include the DNA-binding domain, but have distinct amino-termini. CncC is the longest protein and encompasses CncB, which in turn encompasses the

shortest, CncA [81]. The isoform that mediates the role of *cnc* in embryonic head development was shown to be CncB: In mandibular cells, CncB antagonizes the ability of Deformed (Dfd), another homeotic transcription factor, to transcriptionally activate response elements of its downstream genes [81]. In the absence of CncB, unchecked Dfd activity transforms mandibular segment cells to cells of a maxillary fate [81]. A genetic interaction between *dfd* and *cnc* had been previously discovered in a genetic screen for modifiers of Dfd function [82], and these two genes were also found later to jointly regulate the initial embryonic expression pattern of the gene *proboscipedia*, a member of the Antennapedia complex that is required for the proper specification of the adult mouthparts of *Drosophila* [83].

In the specification of embryonic head structures, the CncB-mediated suppression of Dfd was found to require the *Drosophila* homolog of the mammalian small musculo-aponeurotic fibrosarcoma (Maf) proteins, named Maf-S [84]. In fly embryos, multimers of simple CncB/Maf-S heterodimer sites were transcriptionally activated in response to CncB, and in tissue culture cells the amino-terminal domain of CncB showed properties of a strong transcriptional activation domain [84]. Because the known elements that are activated in response to Dfd and are repressed in a CncB-dependent fashion do not match the CncB/Maf binding consensus sites, the suppressive effect of CncB/Maf-S proteins on Dfd activity must be exerted either indirectly or via direct binding to as yet uncharacterized Dfd response elements [84]. Similar to *Drosophila* CncB, the worm SKN-1 is required for the developmental specification of the pharynx, indicating phylogenetic conservation of this role of cnc transcription factors [76]. In contrast to *cncB* mutants, flies harboring mutations that selectively affect the *cncC* isoform develop normal larval head structures, indicating that CncC is not required for this developmental process [84]. However, *cncC* mutant flies are unable to complete larval development, suggesting a later developmental requirement for CncC [84]. The precise developmental role of CncC is an issue of substantial biological interest that awaits elucidation.

In addition to its role in head development, *cnc* is important for establishing the dorsoventral (DV) polarity of the oocyte's follicular epithelium during oogenesis [85]. Body axis establishment in *Drosophila* requires an ordered progression of events with determination of oocyte polarity, establishment of the anterior-posterior (AP) axis through localization of *bicoid* (*bed*) and *oskar* (*osk*) mRNA at the anterior and posterior poles, respectively, and asymmetric movement and positioning of the oocyte nucleus in the anterior cortex, which defines the dorsal side of the egg chamber and initiates the DV patterning of the eggshell and the embryo. In developing

gstD-GFP reporter activity was induced when the flies were exposed to various oxidants, including hydrogen peroxide, the free radical generator Paraquat, the glutathione-depleting agent diethyl-maleate, and the heavy metal arsenic. In addition, the reporter was activated by the known Nrf2-activating compounds oltipraz and *tert*-butylhydroquinone. The knockdown of CncC suppressed *gstD-GFP* reporter inducibility by such conditions, and no induction was detected in transgenic reporter flies bearing a version of the enhancer in which the ARE had been mutated (*gstD Δ ARE-GFP*) [88].

To test whether an ARE sequence is sufficient to mediate transcriptional activation downstream of Keap1/CncC, we employed reporter flies containing a lacZ transgene driven by a synthetic sequence comprising a concatamer of binding sites that conformed to the consensus ARE and had been shown to respond to CncB (which has the same DNA binding site as CncC) [84]. Indeed, lacZ was markedly induced in CncC gain-of-function or Keap1 loss-of-function conditions, further supporting the functional conservation of ARE-mediated transcriptional responses in *Drosophila* [88]. Finally, we examined the contribution of Keap1 and CncC to oxidative stress resistance at the level of the intact organism. The conditional overexpression of CncC significantly increased the survival of flies that had been exposed to a semilethal dose of Paraquat; in contrast, CncC knockdown significantly decreased survival (Fig. 22.2) [88]. Taken together, these results demonstrated the functional homology of *Drosophila* Keap1 and CncC to vertebrate Keap1 and Nrf2, respectively. Much like their mammalian counterparts, *Drosophila* CncC and Keap1 regulate ARE-mediated transcription and detoxification gene expression and are crucial for the organism's defense against oxidative stress. Thus this work established *Drosophila* as a new genetically accessible model system for the study of this important homeostatic and cell survival pathway.

22.1.4 Advantages and Limitations of *Drosophila* as a Model Organism to Study the Nrf2 Pathway

22.1.4.1 Advantages For a century *Drosophila* has been one of the workhorses of genetic research [94, 95]. Therefore, the characterization of Keap1 and Nrf2 in this organism offers the possibility to use flies as a genetic platform for deriving new insights on the regulation and functions of this pathway. Before specific opportunities for such endeavors are presented, it is important to note that as a model organism *Drosophila* has several advantages over other models like worms, zebrafish, and mice. Naturally, it also has certain limitations. Although a general comparison of flies to other model systems is beyond the scope of this chapter, it is

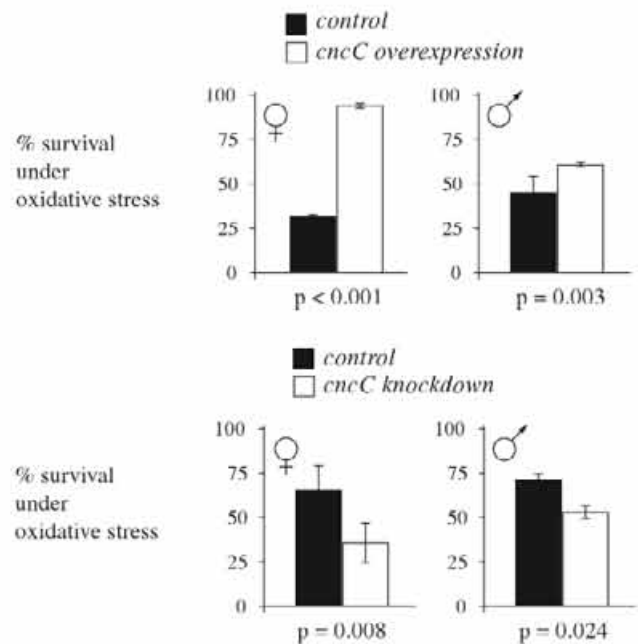


Fig. 22.2 CncC regulates oxidative stress resistance in *Drosophila*. (Top) Conditional ubiquitous overexpression of CncC before exposure to the free radical generator Paraquat significantly increases the flies' survival rate compared to genetically identical controls. Data are shown as means $\pm$ SE of three experiments performed in triplicate. (Bottom) RNAi-mediated conditional ubiquitous knockdown of CncC before exposure to Paraquat significantly decreases the flies' survival rate. This figure is adapted from Sykiotis GP and Bohmann D, Keap1/Nrf2 signaling regulates oxidative stress tolerance and lifespan in *Drosophila*, *Dev Cell*, vol. 14, p. 76–85, Copyright 2008, with permission from Elsevier.

useful to highlight those advantages and limitations of flies that are relevant for the study of the Nrf2 pathway. Some of these features may be specific to the study subject. For example, the relatively short life span of the fly compared to mice makes it a very useful model for investigations of the genetic mechanisms that regulate aging and longevity, especially since such mechanisms are known to involve increased resistance to oxidative stress (see next section).

Other features of *Drosophila* that impact on its utility as a model organism for studies on Nrf2 signaling are directly related to its genetic properties. One major genetic feature of *Drosophila* is its low degree of genetic redundancy. Whereas higher organisms can have multiple genes encoding functionally related and even redundant proteins, this is rather uncommon in flies. At the same time, the large majority of human genes have known or presumed homologs in the fly genome. Thus flies strike a very useful balance of good conservation and low redundancy. This is true also in the case of the

Nrf2 pathway: Since CncB and CncA lack the Keap1-binding domain, CncC is assumed to be the only Nrf2 homolog. Flies, like mice and humans, have a single locus encoding Keap1, whereas worms have none and zebrafish have two [96]. Notably, the fly genome also carries only a single gene encoding a small Maf (CG9954), while none has been identified in worms; and anyway Skn-1 lacks the leucine zipper domain that mediates the dimerization of Nrf2 homologs in higher organisms with Mafs, and thus this aspect of Nrf2 function does not appear to be conserved in the worm [97]. Conversely, the small maf gene family is represented by no fewer than three members in vertebrates [98]. The genes encoding components of the Nrf2 ubiquitination and degradation machinery (Cul3, Rbx1, Candel1, and others) are also well conserved in *Drosophila*. This combination of conservation with little genetic redundancy greatly facilitates investigations of Nrf2 signaling in flies, including studies that might be impossible or very difficult in organisms with either an incomplete Nrf2 module or a high degree of redundancy. At the same time, this property of *Drosophila* suggests that the results of fly studies might be readily extrapolated to higher organisms, in which the pathway is similarly constructed, to form the basis for experimental validation of insights derived from flies.

Another major advantage of *Drosophila* is its exquisite amenability to genetic manipulation. Various genetic technologies have been developed that allow the up- or downregulation, inactivation, or ectopic expression of a chosen gene either ubiquitously or in a tissue-specific and/or temporally controlled manner. With these methods, *Drosophila* offers increased efficiency, higher speed, and lower cost of experimentation compared to vertebrates. Thus, now that the principal role of CncC in oxidative stress protection of *Drosophila* has been established, flies can be used to investigate the tissue-specific requirement for Nrf2 signaling in defending various organs and sustaining their specialized functions [4]. Moreover, the phylogenetic conservation of Nrf2 signaling in flies offers the opportunity to identify new genes that regulate the pathway through unbiased genetic screens. The critical requirement for conducting such screens is to establish an appropriately sensitive *in vivo* or cell-based assay reflecting the activity status of the pathway, such as the one shown in Fig. 22.3. It then becomes possible to identify pathway components and modulators either *in vivo*—by utilizing available large collections of flies harboring chemically induced mutations, transposable elements disrupting gene function, or transgenes expressing gene-specific double-stranded RNAs (dsRNAs) that trigger gene knockdown via

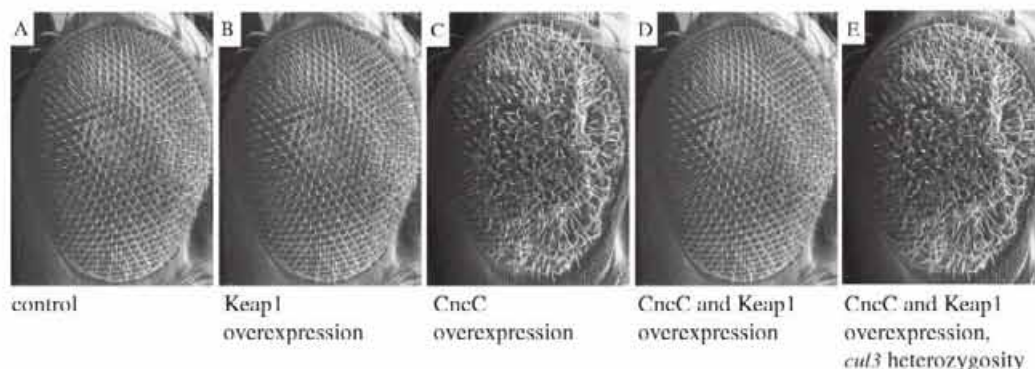


Fig. 22.3 A genetically sensitized *in vivo* *Drosophila* assay of Keap1/Nrf2 system activity. (A) The compound eye of *Drosophila* comprises about 800 individual ommatidia (meaning “small eyes”) arranged in a regular pattern. This pattern can be disrupted by the genetic manipulation of cell signaling during eye development, generally without causing lethality. Thus the *Drosophila* eye is a convenient system for the analysis of regulatory interactions between genes. (B) The wild-type pattern is unaffected by the eye-specific overexpression of Keap1. (C) In contrast, eye-specific overexpression of CncC results in a rough-appearing eye due to loss and disrupted arrangement of ommatidia. (D) Coexpression of Keap1 and CncC results in wild-type eye appearance. The complete suppression of the CncC-induced phenotype by Keap1 is consistent with its role as an inhibitor of Nrf2 signaling. Thus the interaction of overexpressed Keap1 and CncC in the *Drosophila* eye is an *in vivo* assay reflecting Nrf2 pathway activity. (E) The eye interaction of Keap1 and CncC is sensitive to genetic manipulation, as demonstrated by the reemergence of the rough eye phenotype in the presence of either one of two *cul3* alleles (the chemically induced mutation *cul3*², and the transposable element-induced mutation *cul3*^{064.30}). Therefore, this eye interaction assay, or other similarly sensitized *in vivo* or cell-based readouts, could be used to test whether specific candidate genes are regulators or components of the Nrf2 pathway, as well as to identify new such genes through unbiased genetic screens. This figure is adapted from Sykiotis GP and Bohmann D, Keap1/Nrf2 signaling regulates oxidative stress tolerance and lifespan in *Drosophila*, *Dev Cell*, vol. 14, p. 76–85, Copyright 2008, with permission from Elsevier.

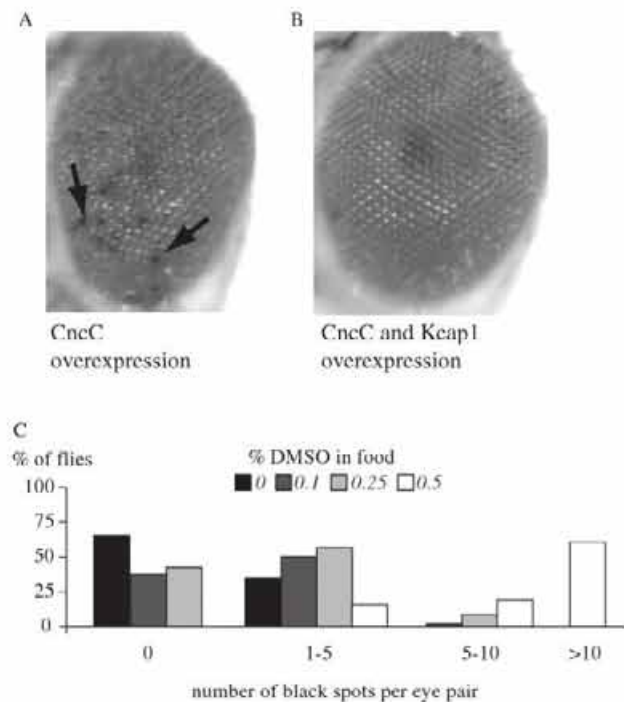


Fig. 22.4 The commonly used solvent DMSO can affect Nrf2 pathway activity in *Drosophila*. (A) In addition to missing and disarranged ommatidia, the eyes of flies overexpressing CncC show black spots (indicated by arrows) that probably represent necrotic and/or melanotic ommatidia. The number of these spots per eye pair correlates with the severity of the phenotype. (B) When Keap1 is coexpressed with CncC in the eye and the flies develop in normal food, the presence of black spots in the eye is suppressed, consistent with the inhibitory function of Keap1. (C) When flies coexpressing CncC and Keap1 in the eye develop in food containing DMSO, which is an oxidant, black spots are detected, and their number correlates positively with the concentration of DMSO. More than 300 flies were scored at each DMSO concentration.

Ethanol may be a more appropriate solvent in such cases, at least in cultured cells, as it most likely evaporates from the medium that flies grow in/on. We have also found lipid-based solvents to be problematic because of their high viscosity: As they are excreted from the flies' guts on the container walls, they tend to trap adult flies—such solvents may, however, be suitable for studies on larvae.

Finally, there is no escaping the fact that *Drosophila* is a latecomer to the Nrf2 arena, in which mammalian studies have been ongoing for more than a decade and worm studies for several years. Thus the cost-benefit ratio of investing in flies to address a research question on the Nrf2 system should be carefully weighed. The study topics ought to be carefully selected to take advantage of *Drosophila*'s unique assets and relative strengths, so as to facilitate real breakthroughs and not mere replications or extensions of mammalian studies. Thus a high degree of strategic planning will be required over the short term to "catch up" with worms and vertebrates in characterizing the pathway, while at the same time proving the value of the fly system for Nrf2 research by deriving unique insights not readily available from these historically leading experimental platforms. Table 22.1 lists some of the fundamental questions that should be addressed as soon as possible to better characterize the pathway in flies, and highlights research directions that should be pursued in parallel because they have the potential to lead to contributions of major impact in the field of Nrf2 biology.

22.1.5 New Insights Derived from *Drosophila* on the Functions of the Nrf2 System

By establishing the phylogenetic conservation of Nrf2 signaling in *Drosophila* and exploiting the experimental

TABLE 22.1 Prospects for Nrf2-related research in the *Drosophila* system.

Fundamental questions that need to be addressed to better characterize the Keap1/Nrf2 system in <i>Drosophila</i>	Questions of Nrf2 biology for which <i>Drosophila</i> could be used to derive novel insights of potentially major impact
• Is the single small Maf of <i>Drosophila</i> the interaction partner of CncC in activating transcription via the ARE? • Do CncA and CncB have roles in regulating ARE-mediated protective responses? Is CncA, which lacks transcriptional activity, an ARE repressor? Is CncB, which lacks a Keap1-binding domain, a constitutive ARE activator? • Is CncC degraded via the action of Cul3, similarly to vertebrate Nrf2? • Which is the set of CncC target genes, and does it include genes with tissue-specific homeostatic roles like vertebrate Nrf2? • Which are the naturally-occurring oxidative stressors that CncC has evolved to protect flies against? • What are the reasons for the developmental lethality of flies homozygous for <i>cncC</i> and <i>keap1</i> mutant alleles?	• Which genes are required for the activation of Nrf2 signaling by oxidants and other inducers? • Which genes encode targets for the pharmacological activation or inhibition of Nrf2 signaling? • By which mechanism(s) does the relative abundance of ARE-binding factors determine the transcriptional status of Nrf2 target genes? • How is Nrf2 signaling involved in promoting longevity and extending the healthy life span? • How does Nrf2 prevent the degeneration of dopaminergic neurons in vivo? • What are the mechanisms by which Nrf2 affects and is affected by the metabolism of sugars and lipids?

advantages of flies, new insights have already been gleaned on the functions of the Nrf2 system; albeit few in number, these advances are of substantial impact, as they pertain to the roles of Nrf2 in the regulation of longevity [88], the degeneration of dopaminergic neurons in Parkinson disease [104], and the resistance to the environmental pollutant methylmercury [105].

22.1.5.1 The Keap1/Nrf2 Pathway as a System Controlling Longevity One of the most interesting biological questions is whether and how the aging process is regulated and how longevity and youthfulness might be extended. According to the oxidative stress theory of aging, oxidative damage to biological macromolecules is a key driver of aging; conversely, delaying the accumulation of oxidation products in the cells and tissues of an organism could promote longevity [106]. Studies in model organisms have supported this hypothesis by showing that life span extension by various genetic or dietary manipulations correlates with increased oxidative stress resistance, as well as that the overexpression of antioxidant genes can not only augment oxidative stress tolerance but can also promote longevity [107–110]. As a master regulator of antioxidant and detoxification responses, Nrf2 was a plausible regulator of the aging process. Indeed, SKN-1 was shown to be required not only for normal life span in *C.elegans* [77] but also for the life span-extending function of caloric restriction through its function in a pair of specialized neuroendocrine cells in the worms' brain [111]. Having characterized the Nrf2 system in flies, we undertook a study to address whether activation of Nrf2 signaling could promote longevity under normal laboratory growth conditions [88].

For this test we employed flies heterozygous for either of two independent *keap1* alleles. These flies showed elevated mRNA levels of the CncC target gene *gstD1* (Fig. 22.5), suggesting that loss of one copy of the *keap1* gene can result in a gain of CncC function in vivo [88]. Male heterozygous *keap1* mutant flies showed increased resistance to Paraquat and also had a median life span significantly longer than that of their otherwise genetically identical siblings (Fig. 22.5) [88]. These findings demonstrated that partial loss of function of the Nrf2 pathway's negative regulator can have significant beneficial effects on the oxidative stress tolerance and longevity of male *Drosophila*. This was consistent with the oxidative stress hypothesis of aging and also provided the first evidence for a role of Keap1 in life span regulation. Interestingly, female *keap1* heterozygotes did not show significant differences in either Paraquat resistance or longevity (Fig. 22.5). Although we have not yet thoroughly investigated the underlying reasons for this sexual dimorphism, we suspect that it reflects

hormonal or metabolic differences between the sexes. A subsequently published study in *C. elegans* showed that SKN-1 mediates the life span extension associated with reduced insulin/IGF-like signaling and that its transgenic overexpression at certain levels can also confer longevity [112]. Taken together, these studies in invertebrates revealed a new role for Nrf2 signaling as an antiaging and life span-extending factor that warrants detailed characterization in invertebrate and vertebrate models [further discussed in 8].

22.1.5.2 Protective Role of Nrf2 Against Parkinson Disease Oxidative stress in the nervous system is associated with neuronal cell death during the pathogenesis of multiple neurodegenerative disorders, including Parkinson disease (PD) [113, 114]. PD is an age-related disorder in which severe oxidative damage occurs in the substantia nigra, resulting in the degeneration and loss of dopaminergic (DA) neurons [115]; this manifests clinically as progressive functional impairment with ultimate mortality. Effective treatments for PD are lacking, and thus a better understanding of the mechanisms regulating DA neuron survival and death is urgently warranted. A series of recent studies have implicated the Nrf2 signaling pathway as an important factor for DA neuron survival during PD pathogenesis. Notably, compounds that are approved treatments for PD, such as bromocryptine and selegiline, were found to activate Nrf2 [116, 117]. Genetic and pharmacological studies also documented the importance of Nrf2 as a protective factor against PD in mouse models of the disease in which DA neuronal toxicity is elicited through administration of the prooxidant compounds 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 6-hydroxydopamine (6-OHDA) [44, 118–120]. Furthermore, a recent human study documented that the few surviving DA neurons of postmortem brains from PD patients showed increased abundance and nuclear localization of Nrf2 [68]. Consistently, other studies have shown that the expression or function of p62 and DJ-1, which are both proteins modulating Nrf2, is compromised in neurodegeneration, likely leading to impaired antioxidant response. p62 is a cytoplasmic protein that mediates the formation of ubiquitinated aggregates that are removed by autophagy [121, 122], and it is localized to intracellular aggregates in various neurodegenerative diseases [123]. p62 binds directly to Keap1 and increases the protein stability of Nrf2 [124–127]; moreover, Nrf2 upregulates the transcription of *p62* [126, 128–130]. The *p62* promoter shows increased oxidation in PD leading to reduced *p62* gene transcription, which may account for Nrf2 dysfunction [131]. Regarding DJ-1, inherited loss-of-function mutations in its gene (*PARK7*) are associated with early-onset

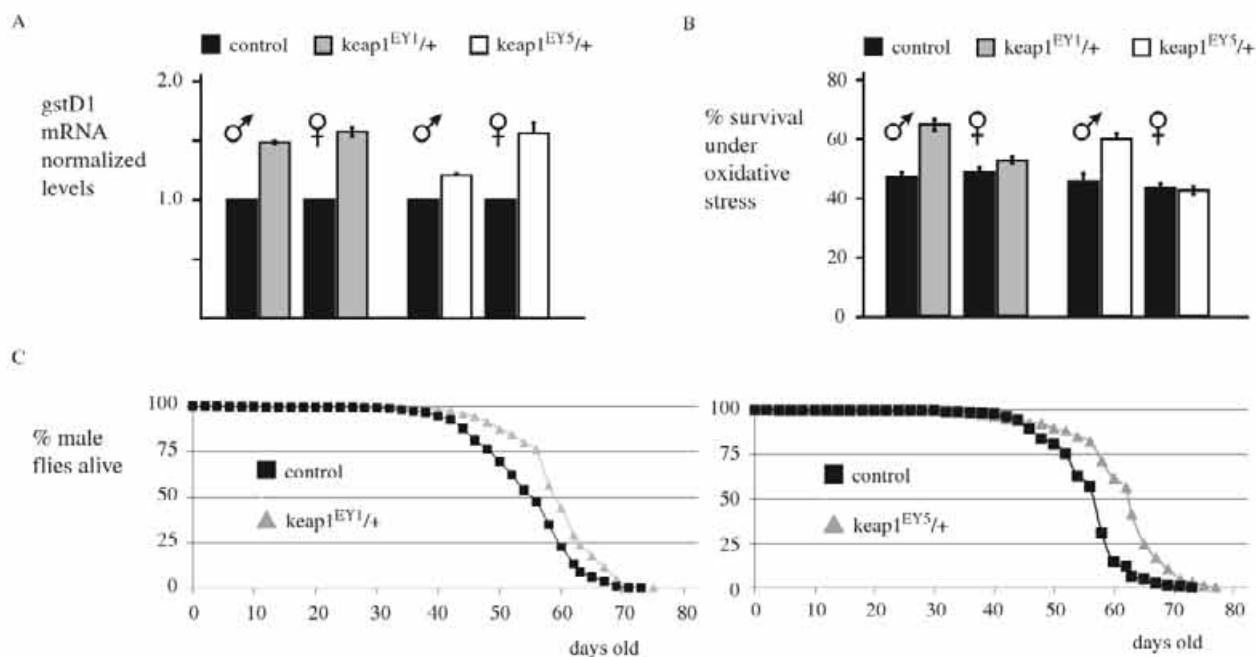


Fig. 22.5 Heterozygosity for *keap1* is associated with increased oxidative stress resistance and extended longevity in male *Drosophila*. (A) The *keap1^{EY1}* and *keap1^{EY5}* alleles were created by the reinsertions of mobilized transposable elements in the coding sequence of the *keap1* gene. Both alleles cause larval lethality and are presumed nulls. One-day-old male and female *keap1* heterozygous flies have significantly elevated *gstD1* mRNA expression levels (normalized to *actin* mRNA levels) as quantified by real-time RT-PCR. Control flies were siblings of the *keap1* heterozygotes and were genetically identical with them except that they were homozygous wild-type for *keap1*. Data are shown as means $\pm$ SE of three experiments performed in duplicate. (B) Male, but not female, heterozygous *keap1* flies show a significantly higher survival rate after exposure to Paraquat than their otherwise genetically identical wild-type sibling controls. Data are shown as means $\pm$ SE of four experiments performed in triplicate. (C) Under standard culture conditions, male heterozygous *keap1* flies live significantly longer than their otherwise genetically identical wild-type sibling controls. Data are shown as percentage of flies alive at each age; 500–700 flies of each genotype and sex were assayed. This figure is adapted from Sykietis GP and Bohmann D, Keap1/Nrf2 signaling regulates oxidative stress tolerance and lifespan in *Drosophila*, *Dev Cell*, vol. 14, p. 76–85, Copyright 2008, with permission from Elsevier.

Parkinsonism [132, 133]. DJ-1 protects neurons from oxidative stress, but its functionality is lost upon oxidation [134]. Because DJ-1 stabilizes Nrf2 by preventing its association with Keap1, the oxidation of DJ-1 in PD could compromise Nrf2 signaling and precipitate the collapse of the antioxidant response system [135]. In summary, multiple independent lines of evidence highlight Nrf2 as an important factor that protects against PD.

In an effort to elucidate PD pathogenesis, several *Drosophila* models of the disease have been created by manipulating genes implicated in rare hereditary forms of early-onset parkinsonism. These include flies harboring loss-of-function mutations in the genes encoding parkin or transgenically overexpressing human α -synuclein in the DA neurons [136–138]. The resulting phenotypes are reminiscent of human PD and include premature loss of DA neurons, locomotor deficits, and

increased sensitivity to oxidative stress [136–143]. As genetic models of PD, these flies facilitate the in vivo discovery of genes, pathways, and compounds that may be useful for the prevention and/or treatment of this debilitating disorder. Loss-of-function *parkin* mutations lead to the degeneration of a cluster of DA neurons termed protocerebral posterior lateral 1 (PPL1). This neuronal loss was exacerbated by a loss-of-function allele of the detoxification gene *gstS1*; conversely, overexpression of *gstS1* specifically in DA neurons reduced *parkin*-associated DA neuronal loss [139]. Significant neuronal loss in the PPL1 cluster is also observed when human α -synuclein is expressed in DA neurons [138]. Loss-of-function mutations in *gstS1* or in *gclm*, which encodes the rate-limiting enzyme in the synthesis of the major antioxidant glutathione, further exacerbate the loss of DA neurons due to α -synuclein toxicity [138]. Conversely, overexpression of either of these enzymes

organisms are ideally suited. The protective role of SKN-1 against MeHg toxicity to DA neurons [156] also prompts testing whether CncC has a similar role in flies, which would be consistent with the recent findings in the fly PD models reviewed in the previous section.

22.2 CONCLUSION

Because Nrf2 has emerged as a critical transcription factor for the maintenance of tissue homeostasis; the protection from oxidative and electrophilic stressors; and the prevention of diverse pathologies, it holds substantial promise as a therapeutic target for human diseases. To date, most progress in the Nrf2 field has resulted from studies in mice and cultured mammalian cells. Flies have recently been established as an invertebrate genetic model organism in which Nrf2 signaling is functionally conserved [88]. Thus, together with worms, they have the potential to complement vertebrates in this exciting research area. Through their unique experimental strengths, flies could also facilitate breakthroughs of potentially major impact on human health issues, such as the extension of the healthy life span, the prevention of neuronal degeneration, and the treatment of metabolic disorders [157], which are also generally accompanied by elevated levels of oxidative stress.

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existing motifs in the fruit fly motif database FLYREG database-Bergman and Pollard v2 with TOMTOM. The motifs were compared by using the Sandelin-Wasserman similarity function, and only statistically significant matches ($P < 0.001$) with a low false discovery rate (q value) less than 50% were obtained.

Three of the four novel motifs had significant alignments with existing motifs in the database. Motif 1 aligned with the existing *hb* motif (P value = 0.00012, q value = 0.018) (Fig. 23.5A). No significant match was obtained for motif 2. Motif 3 produced significant alignments with three existing motifs in the database, *Med* (P value = 0.0038, q value = 0.021), *Mad* (P value = 0.0038, q value = 0.021), and *brk* (P value = 0.0096, q value = 0.035) (Fig. 23.5B). Motif 4 aligned to the *Med* (P value = 0.0014, q value = 0.041) and *Mad* (P value = 0.0021, q value = 0.3) motifs (Fig. 23.5C).

23.3.3 Predicting Functions of Novel Motifs

GO terms and functions associated with the novel motifs in the promoter regions were searched with GOMO v4.3.0. Interestingly, motif 4 was predicted to be a transcription factor binding site for the regulation of transcription, locomotor rhythms, and the entrainment of the circadian clock (Table 23.2). Further, motif 2 was identified to be a potential site for the binding of casein kinase 2 (CK2), a serine/threonine protein kinase that is known to phosphorylate clock proteins, resulting in their circadian rhythmicity [22, 23]. Motifs 1 and 3 were predicted to have general housekeeping functions like triplet codon-amino acid adaptor activity and regulation of biosynthetic processes, respectively (Table 23.2).

23.4 DISCUSSION

The consensus E-box bHLH transcription factor binding site has been shown to mediate rhythmic expression of several core clock genes [7, 11]. The presence of this consensus sequence in the promoter regions of six antioxidant genes in *Drosophila* suggests likely circadian control in their regulation.

Keap1 and *cnc* both revealed the presence of the typical E-box in their promoters. Members of the CNC (cap 'n' collar)-basic leucine zipper family of transcription factors are principal mediators of defensive responses to redox stress [24]. The *cncC* isoform transcribed off the *Drosophila cnc* locus has been suggested to be the counterpart of mammalian *Nrf2* (NF-E2-related factor 2), which contains the KEAP1 binding ETGE motif and an upstream hydrophobic region [25, 26]. The *Drosophila* genome also harbors a homolog of vertebrate *Keap1* genes. This gene (CG3962) and its protein

product (dKeap1) have been shown to have striking similarity with mammalian and zebrafish homologs. The physical association between dKeap1 and CncC has previously been suggested by a genomewide yeast two-hybrid (Y2H) experiment [27]. This interaction predicts that dKeap1 should act as a negative regulator of CncC in vivo. It has been demonstrated that dKeap1/CncC signaling regulates oxidative stress responses in *Drosophila* [26] and could also function in xenobiotic stress responses. Circadian expression profiling of drug-processing genes and transcription factors in mouse has revealed that both *Keap1* and *Nrf2* are rhythmic with a 50% increase in *Nrf2* expression at 2 P.M., whereas the expression of its cytoplasmic repressor *Keap1* was shown to be higher (40%) during the dark phase than during the light phase [28]. A similar rhythmic pattern of these genes remains to be identified in the case of *Drosophila* because of circadian binding elements in their promoters.

While diurnal rhythms in the activity of various antioxidant enzymes have been described from various phylogenetically distant organisms [29], not many studies have actually been able to demonstrate rhythms at the mRNA level. In this study, the canonical E-box was not detected in any of the classical antioxidant enzyme genes. The circadian clock has been reported to gate the expression of two *Catalase* genes (*CAT2* and *CAT3*) in *Arabidopsis* to distinct opposite circadian phases [30]. Rhythms in catalase activity has been reported in mouse brain, kidney, and liver [31], while epigenetic inactivation of circadian clock gene *BMAL1* in hematologic malignancies in human cell lines has been reported to disrupt circadian expression pattern of *catalase* [32]. However, we have not discerned any circadian expression pattern in *Cat* gene expression in *Drosophila* [15]. Neither *Cu/Zn Superoxide dismutase (Sod)* nor *Mn Superoxide dismutase (Sod2)* revealed any of the putative circadian binding elements in their promoter regions.

Sniffer protein (encoded by *sni*) is an NADPH-dependent carbonyl reductase belonging to the enzyme family of short-chain dehydrogenases/reductases (SDRs). *sni* has been directly implicated in the cellular defense mechanism against oxidative stress in *Drosophila* [33], and not much is known about its circadian regulation. *Trxr1* has been reported to substitute for the glutathione reductase system in *Drosophila* and plays a crucial role in the thiol-based glutathione recycling system [34], which is essential to combat oxidative stress. Similarly, *Trx2*, *msrA*, *msrB*, and *GstS1* have also been reported to participate in oxidative stress responses in *Drosophila* [35–37]. While some of these genes participate directly in elaborating an antioxidant defense response, there are others such as methionine sulfoxide reductases (*msr*) that perform a role in repair of oxidatively damaged proteins.

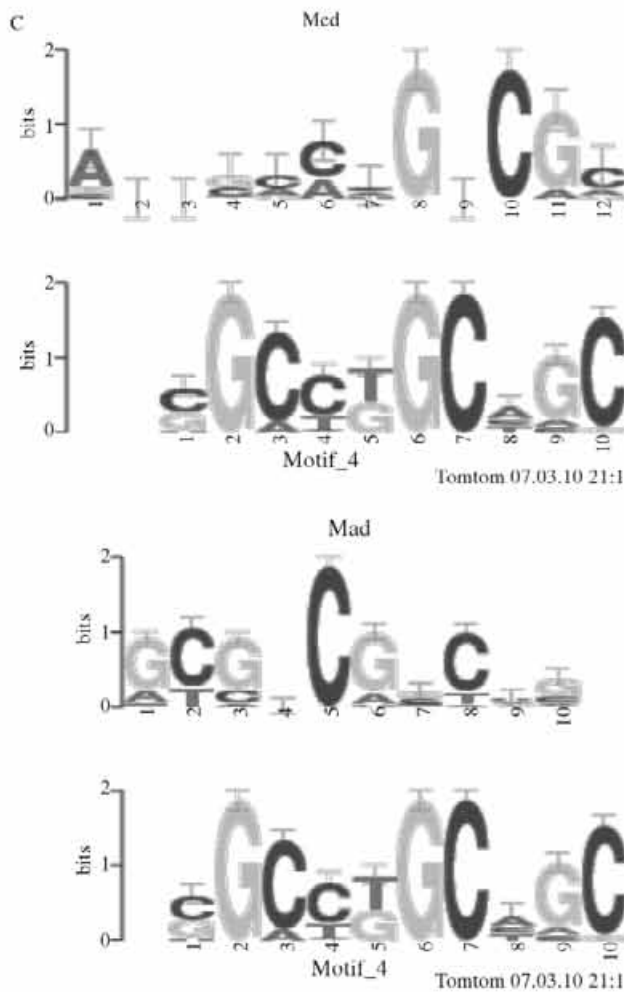


Fig. 23.5 (Continued)

Additionally, the tripeptide L- γ -glutamyl-L-cysteinyl-glycine, that is, glutathione (GSH), is the most abundant cellular non-protein thiol and has multiple physiological roles in vivo, acting as a cellular redox buffer, a potent nucleophile, and an antioxidant against ROS, and recent

evidence also suggests that intracellular GSH is a key regulator of stress-activated signal transduction pathways [38]. GSH is synthesized by glutamylcysteine ligase (GCL), which is the primary rate-limiting reaction in the pathway. GCL is heterodimeric, having a catalytic subunit (*Gclc*) and the modulatory subunit (*Gclm*). Both of these genes showed the canonical E-box consensus sequence.

The E-box is a widely used DNA control element. It affects many different genetic programs, including proliferation, differentiation, tissue specific responses, and cell death. The circadian clock is also one among many pathways that employ the E-box for establishing the robust waves of gene expression characteristic of circadian transcription. However, presence or absence of E-box alone in the promoter regions of genes may not be sufficient to guarantee control by the clock. The regulatory flexibility of the E-box hinges on the sequence ambiguity allowed at its core, the strong influence of the surrounding sequences, and the recruitment of spatially and temporally regulated E-box binding factors [39]. However, lack of an E-box in the promoter region of a gene does not necessarily mean that the gene may not be under circadian control. Hence, other motifs that are conserved in the promoter regions of genes under likely circadian control may serve as binding sites for clock transcription factors (TFs).

TF binding sites in the promoters of clock genes and oxidative stress responsive genes used in this study revealed novel conserved motifs that had significant matches with existing *Drosophila* motifs in the database (Fig. 23.4, Fig. 23.5). One of these motifs (motif 4) had a predicted functional role in the entrainment of circadian rhythms and locomotor function, while another (motif 2) was a predicted binding site for casein kinase 2 (CK2), known to phosphorylate clock proteins [23, 40], playing an important role in their stability and function (Table 23.2). However, the presence of a variety of circadian elements that suggest control of promoter activity is not the only manner of control of rhythmic activity. In

TABLE 23.2 Gene Ontology (GO) functions associated with the novel MEME motifs in the promoter regions of candidate genes using GOMO v4.3.0

Motif	GOMO Score	P Value	Q Value	Accession No.	GO Definition
1	2.09e-10	1.42e-06	1.73e-03	GO:0000499	<i>molecular_function</i> : base pairing with mRNA
	2.09e-10	1.42e-06	1.73e-03	GO:0030533	<i>molecular_function</i> : triplet codon-amino acid adaptor activity
2	2.39e-05	1.42e-06	9.44e-03	GO:0005956	<i>cellular_component</i> : protein kinase CK2 complex
3	5.16e-04	1.37e-04	2.57e-02	GO:0034984	<i>biological_process</i> : cellular response to DNA damage stimulus
	5.26e-04	1.42e-04	2.57e-02	GO:0009889	<i>biological_process</i> : regulation of biosynthetic process
4	9.82e-05	3.13e-05	4.47e-02	GO:0045449	<i>biological_process</i> : regulation of transcription
	2.02e-04	5.55e-05	4.47e-02	GO:0045475	<i>biological_process</i> : locomotor rhythm
	3.58e-04	1.10e-04	4.70e-02	GO:0009649	<i>biological_process</i> : entrainment of circadian clock

THE PROTECTIVE ROLE OF SESTRINS AGAINST CHRONIC TOR ACTIVATION AND OXIDATIVE STRESS

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24.1 INTRODUCTION

Oxidative stress is an undesirable consequence of oxidative respiration, which our cells utilize to produce the required levels of energy for life processes. Reactive oxygen species (ROS), a side product of mitochondrial respiration, can damage many important macromolecules, including DNA, lipids, and proteins. Therefore, complex mechanisms have evolved to defend cellular architecture and genomic information against damage produced by ROS. One well-characterized defense mechanism is performed by redox regulating enzymes, including catalase, peroxidase, and superoxide dismutase, which can directly scavenge or eliminate ROS [1]. Another defense mechanism is the stress-induced signal transduction pathway, which can block cell growth and promote repair of damaged macromolecules such as DNA under conditions of oxidative stress [2, 3]. During oxidative stress, ROS-induced damage of mitochondrial DNA, lipids, and proteins can decrease respiration efficiency, provoking mitochondria to compensate and produce more ROS. To avoid this destructive cycle, cells eliminate their damaged mitochondria through a mechanism called mitophagy—mitochondria-specific autophagy [4, 5].

In this chapter, we discuss a unique family of proteins, the Sestrins, that is critically involved in the cellular defense system against ROS (Fig. 24.1) [6, 7]. Sestrins are induced by oxidative stress-induced

signaling pathways, and they protect cells and organisms from the detrimental consequences from oxidative stress by several means [8]. One activity of Sestrins is promotion of recycling of peroxiredoxins, which are important ROS-scavenging small proteins [1]. This effect of Sestrins contributes to elimination of ROS during oxidative stress [9]. A separate activity of Sestrins is inhibition of signaling mediated by the target of rapamycin complex 1 (TORC1). This activity of Sestrins can stop cell growth and funnel the saved energy expenditure into macromolecular repair machinery [10]. In addition, by inducing autophagy that eliminates damaged mitochondria, Sestrins contribute to mitochondrial quality control [11]. Sestrin deficiency therefore leads to cellular ROS accumulation, which can cause diverse ROS-associated pathologies such as muscle degeneration and cardiac dysfunction [12].

24.2 SESTRIN—A UNIQUE GENE FAMILY

The first discovered Sestrin family protein was Sestrin 1 (Sesn1), which was identified as a p53 target gene in a differential display screen using a cell line expressing a temperature-sensitive mutant of p53 [13] and was originally named PA26 (p53-activated gene 26). The *cis*-regulatory sequences in the *Sesn1* genomic locus contain p53 binding sites, and *Sesn1* is induced by virtually every p53-activating stimulus. However, the function of

phosphorylates the autophagy-initiating protein kinase complex [74–77], composed of ATG1/ULK1, ATG13, and ATG17/FIP200. ATG1 is the catalytic subunit of the complex, which is inhibited by TORC1-mediated phosphorylation [78–83]. ATG1 activity is also critical for mitophagy [84]. Therefore, TORC1 hyperactivation can cause chronic downregulation of ATG1 activity, subsequently reducing mitophagy, which then leads to the accumulation of damaged, ROS-producing mitochondria. Conversely, inhibition of TORC1 by starvation or rapamycin treatment induces autophagy including mitophagy [85, 86] and improves mitochondrial quality and hence energy conservation [4].

Autophagic defects cause mitochondrial dysfunction and oxidative stress in diverse model organisms. Depletion of ATG1, as well as ATG6, ATG8, or ATG12, causes accumulation of dysfunctional mitochondria and oxidative stress in the yeast *Saccharomyces cerevisiae* [87]. Depletion of ATG5 or ATG7 in mouse cardiac cells, skeletal muscle, and insulin-producing pancreatic beta cells induces accumulation of damaged mitochondria, which ultimately leads to oxidative stress and then organ dysfunction [88–92]. In *Drosophila*, ATG1 ablation causes severe degeneration of cardiac and skeletal muscles, which is associated with accumulation of damaged mitochondria and oxidative stress [12]. The neurodegenerative phenotypes observed in mice with autophagy-deficient neurons [93, 94] may be also associated with mitochondrial dysfunction and oxidative stress commonly observed in brains of Alzheimer and Parkinson disease patients [95]. Such chronic TORC1 activation and reduced autophagy can result in degenerative phenotypes [4]. Indeed, obesity, which is associated with chronic TORC1 activation, is also associated with cardiac dysfunction and neurodegeneration [50, 96–99]. Conversely, inhibition of TORC1 by caloric restriction or rapamycin reduced the incidence and severity of cardiac and neuronal dysfunction upon aging and stresses in various model organisms [100–103].

24.7 SESTRIN AS A SUPPRESSOR OF TORC1

Sestrins were identified as an inhibitor of TORC1, which can suppress TORC1-dependent cell growth [10] and anabolic processes [12]. Upon induction, Sesn1/2 completely blocked the TORC1-dependent phosphorylation of S6K and 4E-BP in cells and caused cell size reduction [10]. Sesn1/2 is a part of large protein complex that contains AMPK and TSC2. In the complex, Sesn1/2 induces activating phosphorylation of AMPK and potentiates AMPK-induced phosphorylation of TSC2, which in combination silence TORC1 activity [10]. Sesn2-mediated inhibition of TORC1 can also induce

autophagy [11]. Given that Sestrins are induced upon diverse stresses, including genotoxic and oxidative stresses as well as hypoxia, they may mediate the stress-dependent silencing of TORC1 activity, which eventuates in inhibiting cell growth and induction of autophagy under more extreme conditions [8, 12, 104].

There is ample documentation of the TORC1-suppressing role of Sestrins in diverse cell lines, as well as in animal models [6]. Both in cells and in *Drosophila* tissues, overexpression of Sestrins caused cell size reduction mediated via inhibition of TORC1 [10, 12]. Since Sestrin is induced upon chronic TORC1 activation, Sestrin acts as a feedback inhibitor of TORC1 signaling. Indeed, loss of Sestrins enhanced clonogenic growth of cancer cells [10] or hyperplastic growth of *Drosophila* wing tissue caused by TORC1 hyperactivation [12]. Sesn2-dependent TORC1 silencing was also observed in the mouse lung [105], while in the liver it mediated DNA damage-induced silencing of TORC1 [10]. Similarly, in fibroblasts, Sesn3 mediated oxidative stress-induced silencing of TORC1 [106]. Collectively, Sestrins can mediate stress-induced silencing of TORC1, ultimately attenuating cell growth and funneling the saved energy into cellular repair and protecting cells from damage-induced apoptotic or necrotic cell death.

24.8 SESTRIN DEFICIENCY RESULTS IN AGE-ASSOCIATED PATHOLOGIES

Although TORC1 is a critical regulator of cell growth [47] and Sestrin potently inhibits TORC1 activity when overexpressed [10, 12], we were unable to detect any gross developmental defects in cell size and growth regulation in *Sestrin*-null mutant flies [12], implying that Sestrin-dependent control of TORC1 is not critical for normal development and cell growth under standard laboratory conditions. Therefore, the role of Sestrins may only be important in the context of homeostatic regulation rather than developmental cell growth, although there may be combinations of circumstances in nature in which developmental events are also dependent on Sestrin regulation. Supporting a primarily homeostatic role for Sestrins, expression of *Drosophila* Sestrin increases with maturation and aging [12].

Sestrin-null mutant adult flies showed significant elevation of triglyceride level in the fat body [12], which provides functions similar to the mammalian liver [107]. In the fat body, Sestrin deficiency reduced AMPK activity while increasing that of TORC1 [12]. Fat accumulation was found to be dependent on AMPK-TORC1 regulation, since pharmacological activation of AMPK or inhibition of TORC1 relieved the accumulation

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CURRENT ADVANCES IN THE STUDIES OF OXIDATIVE STRESS AND AGE-RELATED MEMORY IMPAIRMENT IN *C. ELEGANS*

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25.1 INTRODUCTION

Memory impairment is one of the diverse manifestations of the aging process. It can occur either as a part of normal aging or in association with a pathologic process. Age-related memory impairment (AMI) is a normal result of aging, which is observed in a wide variety of species from the nematode *Caenorhabditis elegans* to human. Recent studies suggest that aging causes heterogeneous effects: Some types of learning and memory are decreased, but other types are increased or show no change. AMI is not a gradual decay but rather an aspect of age-related alterations in the nervous functions. Recently, the studies of AMI in *C. elegans* have been emerging. However, there are misconceptions that remain to be clarified. The mechanism of AMI is thought to be related, at least in part, to oxidative stress, which is caused by an imbalance between the generation and removal of reactive oxygen species (ROS) and reactive nitrogen oxygen species (RNOS). Oxidative stress damages intracellular and extracellular components, including proteins, lipids, and DNA, and therefore it is predicted that aging and oxidative stress interfere with normal function of learning and memory. Detailing the mechanism of AMI will allow development of methods for identifying, delaying, and preventing both AMI and disease-associated memory impairment. It requires rigorous analysis of both aging neurons and the aging processes that affect learning and memory.

This chapter describes the emerging field of cognitive aging and discusses current understanding of oxidative stress and AMI, while referencing the insights from mammalian systems.

25.2 MEMORY IMPAIRMENT DURING AGING

Age-related changes in learning and memory can be seen as a form of “forgetfulness” in our daily life. This section provides basic information about AMI that is applicable from *C. elegans* to humans. We define terms and clarify major misconceptions.

25.2.1 Terminology

Age-related memory impairment (AMI), also called age-associated memory impairment (AAMI), includes mild impairment of the ability to learn new information and to recall previously learned information. The term age-associated cognitive impairment (AACI), is also used to describe memory impairment during aging. Similar to humans, we define AMI (or AAMI) as memory impairment in comparison with young normal controls and AACI as in comparison with age-matched normal counterparts. In this chapter, we use AMI but not AAMI, since AAMI is often confused with the transition state, MCI.

There is also confusion about the definition of short-term memory (SST), long-term memory (LTM), and

working memory. The confusion is mainly due to various definitions from different investigators [1]. In this chapter, we define the terms as follows: Short-term memory (SST) is a transient memory, which lasts for a short time (ranging from seconds to hours) and decays rapidly; long-term memory (LTM) lasts days to decades. How long do SST and LTM last in *C. elegans*? In a typical associative learning assay, SST typically lasts for several hours. LTM lasts more than a day in *C. elegans*. Additionally, SST is sensitive to cold shock and other conditions. More details are described in Section 25.3.2, "Associative Learning and Memory." Importantly, SST and working memory are not totally different [1]. Working memory uses SST as a buffer of memory for maintaining and manipulating memory or for using to affect behavioral outputs [1, 2]. Typically, working memory uses multiple SST buffers. Working memory is more complex than SST as working memory assumes more functions. The term working memory was originally described in Miller *et al.* (1960) [3]. An early model of working memory has been developed in order to describe STM more accurately [4] than the classical modal model [5].

Memory can be classified into two classical forms, including explicit memory (or declarative memory) and implicit memory (or nondeclarative memory) [summarized in Ref. 2]. When you can determine whether you remember or not, memory is called "explicit" (you know that you know) [2]. Explicit memory includes memory for event (episodic memory) and memory for fact (semantic memory). In contrast, memory other than explicit memory is generally classified as implicit memory. Implicit memory includes memory for skills, habits, and behaviors (procedural memory) and classical conditioning. Classical conditioning associates a stimulus to predict important events, and therefore it is a form of associative learning and memory. Recent studies suggest that associative learning and memory are vulnerable to aging from *C. elegans* to humans (see below).

25.2.2 Age-Related Memory Impairment

AMI creates an increasing number of concerns in elderly patients [6, 7]. AMI is the first step toward mild cognitive impairment (MCI; transition state) and dementia (disease state) [8]. Alzheimer disease (AD) is the major type of dementia. Progression to dementia occurs at a high rate in patients with AMI (42% within 3 years) [9]. In humans, it appears likely that memory impairment occurs in the order of:

AMI → MCI → Dementia

The current understanding of AMI is that a wide variety of associative learning and memory are altered (impaired or increased) during aging from *C. elegans* to

humans [7, 10–14] (also see Section 25.3.2). Aging selectively affects working memory and some tasks of short-term memory and long-term memory [10, 13, and this Chapter]. It seems to be generally agreed that implicit memory is reduced in learning new information and procedures (procedural learning) and remains intact in old memory and well-learned procedures (procedural memory for long term) during aging [2]. Other types of implicit memory, including classical conditioning, are differentially reduced in a wide variety of species, including *C. elegans* [reviewed in Ref. 13; also see this chapter], in fruit flies [15], in bees [16], in snails [17], in rodents [18–20] and in humans [21]. Similar to implicit memory, explicit memory shows a decline in learning new information but retains existing memory well. It appears that plasticity to new environments is reduced. Our current model is that aging causes a reduced ability to refresh the memory previously formed. More details of mammalian AMI are described elsewhere [2, 22]. Cognitive aging in *C. elegans* is described below.

A key question in cognitive aging is how aging affects cognitive functions. It has been suspected that aging does not occur at the same speed [2] (also see Section 25.7.1). Cognitive aging is presumably caused by age-related processes and by the results of aging (i.e., age-related changes) in neurons and by modifiers of learning and memory. The model systems, including *C. elegans*, are useful to reveal the mechanisms underlying cognitive aging. Excitingly, the study of cognitive aging has recently begun in *C. elegans*.

25.2.3 Common Misconceptions

In the field of AMI, it is useful to know misconceptions that remain to be or have been clarified. We list up some examples we have encountered through communication with the experts. First, AMI is not a simple decrease in learning and memory. Recent studies suggest that aging causes not only declines but also increases in learning and memory [e.g., Refs. 10, 23]. AMI is not a gradual decay but rather an aspect of age-related alterations in the nervous functions. A good example is seen in visual memory. Older patients require briefer stimulus time than younger patients in motion discrimination [23]. Aging may reduce the function of the interneurons, which is inhibitory to motion discrimination, and improve motion discrimination in senescent neurons [23]. In humans, some but not all forms of short-term memory and procedural memory show age-related declines [10]. Therefore, it is essential to describe which type of learning and memory is altered and how it is altered.

Secondly, AMI is not a disease. AMI is a normal state prior to the disease state. What distinguishes AMI from

dementia besides cognitive deficits? A few lines of evidence from functional magnetic resonance imaging (fMRI) analysis suggest that reduced metabolic activity may be a hallmark of AMI in two hippocampal subregions (the subiculum and the dentate gyrus) [24]. More detailed analysis of aging neurons and aging processes is essential for the understanding the mechanism, prevention, and treatment of AMI.

Finally, AMI cannot be independent of aging. There is an argument that aging should be separated from AMI. However, aging affects learning and memory, which results in AMI. More precisely, AMI is caused by age-related processes that affect learning and memory. The age-related processes should include age-related changes in neurons as well as elsewhere, including modifiers and pathways for learning and memory. In fact, a type of AMI can be suppressed by serotonin inhibitors [25], which are good examples of such modifiers. It is unlikely that aging neurons are the sole cause of AMI. Thus, focusing only on neurons would miss important aspects of the mechanisms for AMI. Although the study of aging neurons has been emphasized, it is equally essential to investigate the aging processes.

25.3 COGNITIVE AGING IN *C. ELEGANS*

C. elegans shows non-associative and associative learning and memory. Both forms of memory show alterations during aging. This section overviews the studies of aging of learning and memory that have been emerging recently.

25.3.1 Non-associative Learning and Sensory Functions

Several studies have investigated aging of learning and memory in *C. elegans*. Similar to mammals, not all functions of learning and memory decline with increasing age. A form of non-associative learning, habituation (i.e., a simple reduced response after a repeated stimulus) [26], increased in old animals, while recovery from habituation was slower in the old compared to the young [27]. Glenn *et al.* (2004) showed that chemotaxis, including attraction to benzaldehyde and avoidance of octanol, declines during aging [28]. However, it is not clear whether the declines were caused by reduced motor activity or by reduced sensory functions.

25.3.2 Associative Learning and Memory

C. elegans shows implicit memory that includes classical conditioning. By classical conditioning, non-associative can learn to associate food [unconditional stimulus(US)]

with a stimulus [conditional stimulus(CS)] and use the stimulus to look for food. The stimulus is associated with food (US), forming US-CS association. Thus it is a form of associative learning and memory. CS can be temperature or a chemical stimulus (i.e., smell and taste); US can be starvation (absence of food) or noxious stimulus (e.g., electric shock, heat, some chemicals and heavy metals). There are three types of associative learning behaviors known in *C. elegans*. They include thermotaxis, chemotaxis (with conditioning), and basal/enhanced slowing response.

25.3.3 Thermotaxis Learning and Memory

The study of thermotaxis has provided the first evidence for aging of associative learning and memory in *C. elegans* [11]. In thermotaxis, *C. elegans* can learn to associate a temperature with food (food-temperature association) [29, 30]. Food-temperature association leads to the tracking of the given temperature, using short-term memory (STM). It has been shown that peak performance of temperature-food association declines with increasing age [11]. It is likely that STM for temperature-food association is declined during aging, since old animals that have normal locomotion show impaired thermotaxis.

Thermotaxis can be assessed either in a single-animal assay (isothermal tracking) or in a thermotaxis population assay. The single-animal assay uses a thermal gradient as shown in Figure 25.1A. Worms can learn to move toward a given temperature (16°C) when conditioned with food (temperature-food association). When a worm is placed in a thermal gradient created on an assay plate, it moves toward the given temperature and tracks the isotherm. Thermotaxis can also be assessed with multiple worms. In this case, conditioned worms are placed on a thermal gradient plate. The fraction of worms in the area that covers the given temperature is assessed. In contrast, when worms are conditioned with no food (starvation), they avoid the temperature (temperature-starvation association). Worms conditioned with starvation are placed on thermal gradient near the area of the given temperature.

25.3.4 Chemotaxis/Olfactory Learning and Memory

Naive *C. elegans* moves to an attractant odor. When *C. elegans* is conditioned with the attractant, such as benzaldehyde, in the absence of food, it is no longer attracted by the odor and exhibits avoidance behaviors (starvation-odor association). The type of chemotaxis avoidance is a function of associative learning and is not related to exhaustion of sensory or motor functions [31]. STM and LTM can be assessed with a single

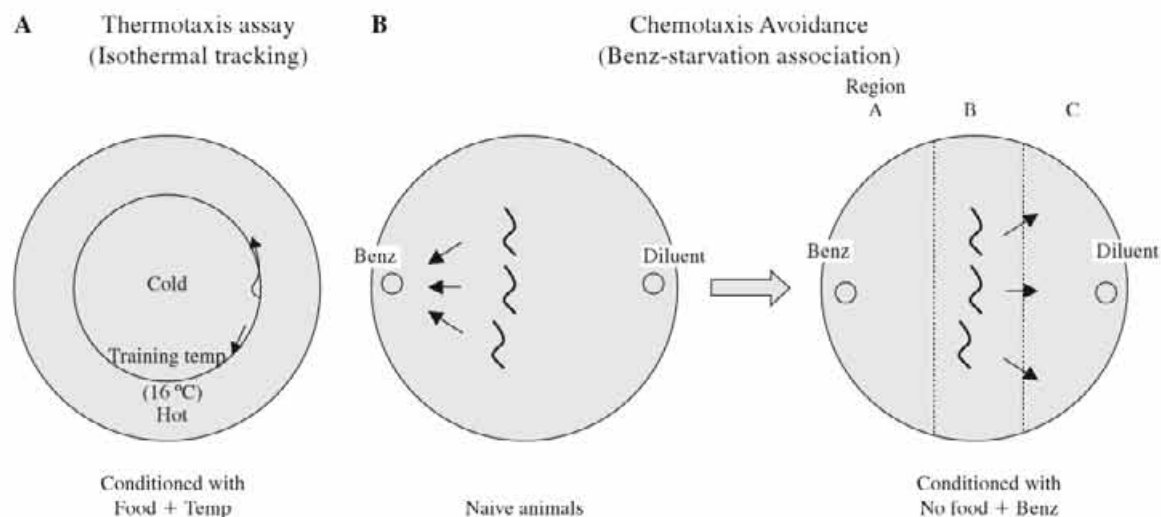


Fig. 25.1 Assay for learning and memory in *C. elegans*. **A**, Assay for thermotaxis learning and memory. The diagram represents an assay plate with a thermal gradient. A learning animal moves to and tracks the given temperature (16 °C) created on the plate. **B**, Assay for chemotaxis learning and memory. Shown is a classical assay system for chemotaxis assay. Animals are placed in B and allowed to crawl. The chemotaxis index (CI) is calculated as follows:

$$CI = (A - C)/(A + B + C)$$

where A, B, and C are the numbers of the animals in each area. The learning index (LI) for avoidance assay is calculated as follows:

$$LI = (CI_{\text{naive}} - CI_{\text{conditioned}})/CI_{\text{naive}}$$

When CI conditioned is less than 0, LI becomes more than 1. To avoid this overestimation, CI conditioned = 0 when CI conditioned has a negative value Benz, benzaldehyde.

conditioning and repeated conditionings, respectively. It has been shown that starvation-odor association declines during aging [32, 33] (Murakami *et al.*, unpublished). Although both STM and LTM decline, it appears that age-related decline in LTM occurs slightly earlier than that of STM (Murakami *et al.*, unpublished). Since LTM uses repeated conditioning with starvation, LTM may be more sensitive to age-related changes in starvation response than STM. Therefore, the results should carefully be validated.

There are several systems for chemosensory avoidance and attraction using different combinations of odor and taste [e.g., Refs. 11, 33–35]. Figure 25.1B describes a typical example of the assay system. Naive animals move to the attractant (benzaldehyde) (Figure 25.1B, left). When animals experience starvation with benzaldehyde, they avoid and move away from benzaldehyde (Figure 25.1B, right). The chemotaxis index (CI) and the learning index (LI) are defined in the Figure 25.1B legend.

25.3.5 Experience-Dependent Modulation of Locomotory Rate

The earliest change in associative learning behavior has been observed in another type of associative learning

and memory, called basal and enhanced slowing response [25]. Basal and enhanced slowing responses are experience-dependent locomotion behavior with associative learning properties [36]. When well-fed animals enter the presence of food, their movements slow to minimize the risk of moving away from the food area. This is called basal slowing response, and is regulated by dopamine signal [37]. Starved animals have a greater slowing response, called enhanced slowing response, than that of well-fed animals. This response is dependent on serotonergic signal [37, 38]. In normal aging, basal slowing response is increased, leading to a diminished difference between basal and slowing response [25]. It has been shown that the age-related change in slowing response is regulated by the serotonin pathway [25].

25.3.6 Neurons and Genes Relevant to Associative Learning and Memory

The neuronal circuits for thermotaxis associative learning consist of several types of neurons (Fig. 25.2). A pair of interneurons, AIY and AIZ, mediate thermal inputs from the thermosensory neuron AFD. These AIY and AIZ interneurons are required for associative learning; the thermosensory circuit shows conservation with the

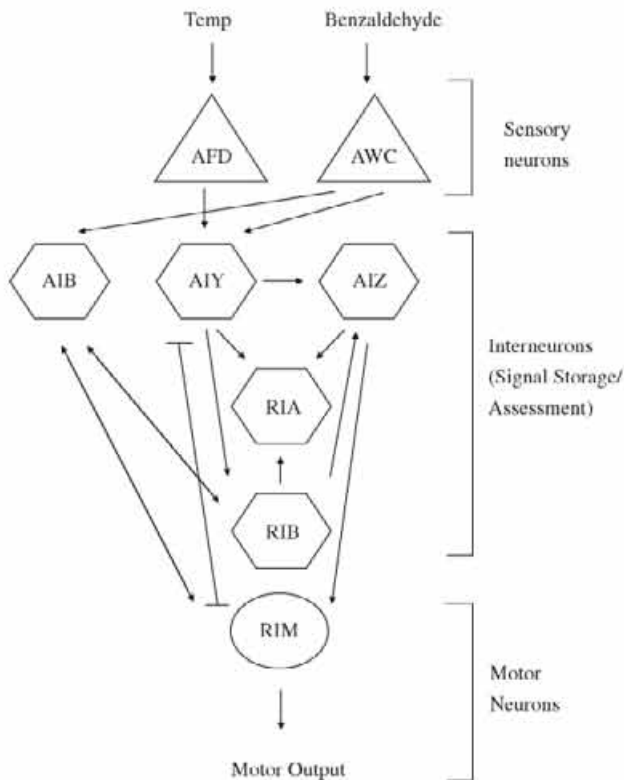


Fig. 25.2 Neurons relevant to thermotaxis learning and memory. Arrows, synapses; H-shaped bars, Gap junctions.

visual sensory circuit in vertebrates [39]. Thermotaxis learning requires the *ncs-1* neuronal calcium sensor gene [40] and the genes in the insulin/IGF-1 pathway (Fig. 25.3). A few modulators of thermotaxis learning are known. Serotonin mediates food responses associated with thermotaxis and olfactory associative learning [41, 42]. The *ncs-1* gene encodes an EF hand-containing calcium sensor protein, which is well conserved in a variety of species [40]. Expression of a neuronal calcium sensor protein gene, *ncs-1*, in AIY can increase isothermal tracking performance, while *ncs-1* knockout reduces the performance [40]. Another modulator, *hen-1*, encodes a secretory protein with an LDL receptor motif gene and is expressed in AIY and a chemosensory neuron ASE [43]. Mutations in *hen-1* abolish thermotaxis and chemotaxis learning behavior.

Olfactory learning requires sensory neurons for odors and some genes including *glr-1* (AMPA-like glutamate receptor gene). Benzaldehyde is primarily sensed by the AWC sensory neurons, which are chemosensory neurons for volatile odors (Fig. 25.2). Also of interest, mutations in *glr-1* impair olfactory associative learning when diacetyl is paired with acetic acid [44]; AMPA-type glutamate receptor mediates fast excitatory neurotransmission in the vertebrate brain. The *hen-1* LDL receptor gene is also involved in chemotaxis learning behavior when Cu^{2+} and diacetyl are paired [43]. There are four mutants that affect chemotaxis associative learning, including *lrn-1*, *lrn-2*,

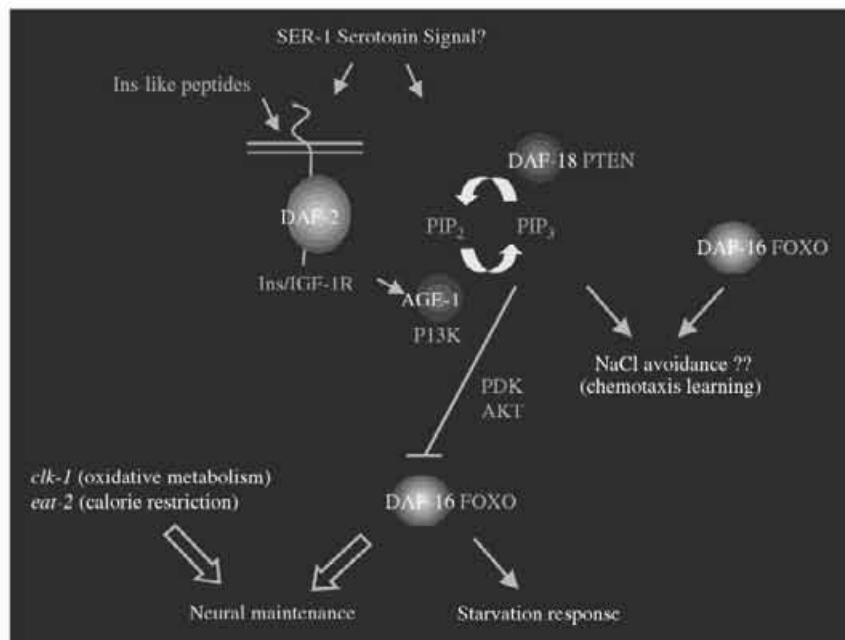


Fig. 25.3 A model for memory regulation by the insulin/IGF-1 pathway. It appears likely that the effects of life extension, or improved neuronal maintenance, lead to an increase in temperature-food association. (See color insert.)

JN603, and JN683 [34, 35]. Associative learning and memory are known to involve cAMP, calcium signaling and CREB in model systems including fruit flies, *Aplysia*, and rodents [45–47]. Locomotory associative-learning behaviors require dopaminergic neurons and serotonin-secreting neurons [37]. Basal slowing response uses the dopaminergic neurons, including CEP, ADE, and PDE, to sense mechanosensory stimuli. In contrast, enhanced slowing response uses serotonin-secreting neurons, including NSM.

25.3.7 *C. elegans* AD Models

The field is rapidly growing (196 publications by NCBI search, 2010) [reviewed in Refs. 48, 49]. Genes similar to those involved in human AD have been identified in *C. elegans*. They include App (*apl-1*), presenilin (*sel-12*), and Tau (*ptl-1*). *C. elegans* models of AD have been available as a form of transgenics expressing amyloid- β 1–42 in muscle [50]. Recently an improved version has been generated that express A- β in neurons [51]. Importantly, the life-extending mutants of the insulin/IGF-1 pathway can delay behavioral deficits in *C. elegans* and mouse models of AD [52, 53]. AD appears to be caused by proteotoxicity rather than aggregation of the proteins.

25.4 WHAT CAUSES AMI?

This section and the next section summarize age-related changes (and processes) that affect AMI. Important questions are how the changes are related to AMI and which changes are causes of AMI. This area of the study still remains to be explored rigorously.

25.4.1 Functional Alterations in Normal Aging

Age-related changes that affect AMI include (but are not limited to) increased oxidative stress, physiological alterations, neuroinflammation, reduced metabolic functions, and changes in endocrine function. Interestingly, microarray analysis has revealed two well-conserved processes, including reduced mitochondria metabolism (see Section 25.5.3, Reduced Mitochondrial Functions) and stress response in mice, monkeys and humans [reviewed in Ref. 54]. Stress response may be triggered by increased oxidative stress, which is consistent with the multiplex stress resistance model of aging [55]. Since microarray analysis determines relative abundance of mRNA, functional analysis should be performed to confirm the results. In addition, a large-scale functional analysis should be performed in order to overview the genetic basis of AMI.

25.4.2 Limited Neural Loss in Normal Aging

It was thought that cognitive aging is caused by a loss of neurons. However, it is becoming clear that a loss of neurons plays a relatively minor role. The number of principal neurons remains relatively intact during aging in *C. elegans*, rodents, and humans [56, 57]. *C. elegans* has a simple nervous system with a small number of neurons compared to other model animals [58], providing an ideal system to keep track of the fate of neurons. The structures of *C. elegans* neurons are relatively well preserved during aging, including the number of synapses [59].

In humans and monkeys, there is reduced brain volume that may be linked to cognitive aging [60, 61]. MRI studies suggest that healthy brains show a shrinkage in volume in humans [e.g., Ref. 61] and in monkeys [62, 63]. Neuronal loss plays a limited role in the shrinkage of the brain. The brain shrinkage is probably caused by shrinkage of neurons, reductions of synaptic spines, and lower numbers of synapses [61]. It seems consistent that brain shrinkage is observed in the region called white matter, which is rich in myelinated nerve fibers [64]. The integrity of myelin may be important for cognitive aging. The most affected areas in the brain include the frontal cortex and the temporal cortex. The frontal cortex is required for working memory, which is sensitive to aging. In contrast, the hippocampus remains relatively intact in the numbers of neurons and synapses. The hippocampus is critical for storing memory in classical conditioning; neural loss in the hippocampus is a warning sign of a disease state, including early AD [65]. Although the volumetric variable has been frequently linked to cognitive aging, there is little evidence for volume-cognition relations because of technical difficulties [22].

It is known that spatial memory can be impaired without neural loss [66]. In primates, neural loss occurs in the frontal cortex and in the cerebellum, including Purkinje neurons [67]. Purkinje neuron loss is well correlated with eye-blink conditioning deficits [66]. However, Purkinje neuron loss can be induced by various factors not necessarily specific to aging, including development, toxins, and autoimmune diseases [66, 68, 69]. Taken together, neural loss appears to play a limited role in AMI.

25.4.3 Oxidative Stress in Normal Aging

Oxidative stress occurs *in vivo* when ROS generated by normal metabolism are not fully scavenged. The free radical theory of aging, also called the oxidative stress theory of aging, assumes that there is an accumulation of oxidative damage in macromolecules during aging, which eventually results in dysfunction of biological functions [70, 71]. Although the role of oxidative stress

in life span specification has been challenged (see Section 25.8, Role of Oxidative Stress in Aging and AMI), it remains convincing that increased oxidative stress has an impact on age-related functional declines in biological processes. It has been shown that an indicator of oxidative damage on DNA [8-oxo-2-deoxynucleotide (oxo8dG)] increases with aging in rodent strains including F344 rats, B6D2F1 mice, and C57BL/6 mice [72]. Another indicator of oxidative damage, protein carbonyl, increases sharply in the last third of life span in a variety of species from *C. elegans* to humans [73, 74].

The central nervous system consumes a great deal of oxygen and is rich with components susceptible to oxidative stress, such as polyunsaturated fatty acids (PUFA, often referred to as “fish oil”) [75, 76]. In the rodent strains, brain is one of the tissues that show the greatest increases in oxo8dG levels (ranging from 167% to 340%) in 24-month-old compared to 6-month-old animals [72]. The increases in oxo8dG may be caused by increased sensitivity to oxidative stress.

In humans, gene expression is reduced in the frontal cortex, including the genes involved in synaptic plasticity, including NMDA and AMPA receptor function, calcium-mediated signal, and synaptic vesicle release and recycling [77]. The reduced expression occurs in the relatively early phase of normal aging (after the age of 40; mid-preproduction period).

25.4.4 Oxidative Stress in Neurodegenerative Diseases

There are extensive studies that suggest an association between increased oxidative stress and neurodegenerative diseases, including AD, Parkinson disease, and Huntington disease [e.g., Refs. 78–80]. Although the mechanism is to be determined, oxidative stress has been implied as a factor that promotes the initial phase of neurodegenerative diseases; other factors include glutamate signaling, altered calcium homeostasis, decreased growth factors, and genetic mutation [81]. For example, AD patients show increased levels of protein carbonyls, oxo8dG, and lipid peroxidation, especially in the brain area with amyloid plaques and neurofibrillary tangles [82]. In cultured neurons and in rat synapses, lipid peroxidation has been observed after exposure to amyloid- β , a component of amyloid plaques [e.g., Refs. 81, 83].

25.4.5 Mutations with Increased Oxidative Stress in *C. elegans*

Experimental evidence for the role of oxidative stress in AMI comes from a study using two mutational defects in the mitochondrial respiratory chain. The *mev-1* mutation has a defect in a cytochrome *b* large subunit (SDHC) in complex II in the mitochondrial electron

transport chain) [84]. The *gas-1* mutation has a defect in a subunit of the mitochondrial NADH:ubiquinone-oxidoreductase in complex I of the respiratory chain [85]. Both the *mev-1* SDHC mutation and the *gas-1* mutation cause overproduction of superoxide (O_2^-) and precocious aging [reviewed in Ref. 84].

It has been shown that thermotaxis is impaired by *mev-1* and *gas-1* mutations with increased oxidative stress [86]. Treatment of a natural pro-antioxidant, lipoic acid, can partially restore impaired thermotaxis in the *mev-1* mutant. It is plausible that oxidative stress plays a role in the performance of thermotaxis. Perhaps more convincing evidence for the role of oxidative stress has been provided by the studies of mutants and drugs that can reduce oxidative stress or oxidative metabolism (see Section 25.6.2).

25.5 OTHER FACTORS THAT MAY CAUSE AMI

This section continues on from Section 25.4, which describes potential causes of AMI. Listed below here are the factors that may affect AMI in *C. elegans* and mammals.

25.5.1 Physiological Alterations

AMI is in part caused by physiological changes in normal aging of the nervous system in mammals [7, 14]. It seems that AMI is caused by impairment of the ability to renew recent acquired information [87; this chapter]. Although neural loss is minimal, there is disruption of myelinated fibers, which may affect physiology of synapses and communication among the brain regions in normal aging [88]. fMRI studies suggest that coordination between brain regions is significantly weakened in normal aging [88]. The elderly use a broader region of the brain (i.e., delocalization) than younger counterparts, which may be a compensatory response in the elderly.

25.5.2 Neuroinflammation

In normal aging, there is an imbalance between inflammation and antiinflammation, shifting toward a proinflammatory state. In humans and rodents, there is an increase in reactivity of microglia and astrocytes, which is indicative of increased innate immunity [89]. However, in humans, upregulation of genes involved in inflammatory is seen in the disease state, AD, while upregulation is weak in normal patients [90]. It is known that neuroinflammation is involved in an early phase toward AD and the diseases of aging [91] rather than AMI. It appears that patients with AMI are in a reactive but pre-inflammation state. Upon exposure to an inflammatory stimulus, including infection and stress, the

reactive state should trigger an overreaction of inflammation, leading to increased decrements in learning and memory [92]. Interestingly, anti-inflammatory drugs, including nonsteroidal anti-inflammatory drugs (NSAIDs), can reduce age-related cognitive decline in the elderly [93].

25.5.3 Reduced Mitochondrial Functions

The central nervous system relies on mitochondrial metabolism for energy production. Organismal aging in *C. elegans* shows reduced metabolic function [94], although energy production is uncoupled from life span extension in the Clk mutations that reduce oxidative metabolism [Ref. 94; also see Section 25.6.2, "Update"]. Microarray analysis has shown that mitochondrial genes show progressive declines during aging in humans, monkeys, and rats [reviewed in Ref. 95]. Importantly, proteomics analysis identified oxidatively modified proteins involved in energy metabolism and ATP production [96]. The modified proteins showed reduced activity. Oxidative stress also modifies the promoters of the mitochondrial genes [95], possibly leading to reduced expression of the genes. More detailed discussion about energy metabolism is available elsewhere [95].

25.6 IMPROVED LEARNING AND MEMORY

An exciting aspect for the studies of AMI is to identify genetic and pharmacological manipulations that rescue deficits in learning and memory. This section explores possibilities from previous findings.

25.6.1 Exploring Genetic and Pharmacological Interventions

Understanding of AMI will include neurological variables: (1) variables that cause AMI (e.g., modifiers) and (2) variables that are secondary or compensatory changes in response to the factors that causes AMI. The variables that cause AMI are reasonable targets for intervention, which is being explored. In *C. elegans*, such intervention includes mutations and drugs that reduce oxidative stress, alter neuroendocrine function (the insulin/IGF-1 pathway and the serotonin/octopamine pathway), and cause calorie restriction.

25.6.2 Mutants and Drugs that Can Reduce Oxidative Stress or Oxidative Metabolism

Mutants with deficits in mitochondrial transport chains are known to alter oxidative metabolism. The *isp-1* (iron sulfur protein gene) mutant decreases oxygen consumption

by half and therefore reduces oxidative metabolism [97]. Mutations in *clk-1* (mitochondrial di-iron carboxylase gene) lack an endogenous isoform of a redox-active lipid, coenzyme Q (Q) [98, 99]. The *clk-1* mutant accumulates a biosynthetic intermediate, demethoxy-Q₉ (DMQ₉) [99], that has a strong ROS scavenging activity. The mutants are Clk mutants and show increased longevity [100], stress resistance [101], delayed development, and extended rhythmic behavior cycles [97, 98].

A metabolic antioxidant, lipoic acid (LA), can improve the performance of thermotaxis [86]. LA is reduced to a potent antioxidant, dihydrolipoic acid, which can recycle other antioxidants such as vitamins C and E [103]. LA can decrease oxidative damage in the brains of older rats and partly restore age-related declines in nervous functions [104]. LA, along with statins (inhibitors of HMG-CoA reductase), can reduce LDL oxidation, which is also a risk factor of neurodegenerative disease and cardiovascular disease [105]. In mice, SOD/catalase mimetics (EUK-189 and EUK-207) can reduce oxidative damage (lipid peroxidation, nucleic acid oxidation, and ROS levels) and improve age-related decline in performance in the fear-conditioning task [106].

Update: During the review process of this chapter, a manuscript has been published that reverses the original claim that the *clk-1* and *isp-1* mutations reduce oxidative stress [97, 98, 100] to a new claim that the mutants modestly increase mitochondrial superoxide [102]. Importantly, the mutants show reduced mitochondrial functions, including oxidative metabolism, and, therefore, it is still reasonable to claim that the *clk-1* and *isp-1* mutations show reduced oxidative metabolism. Although the manuscript provides an interesting model that mitochondrial superoxide plays a role as a protective signal to mediate life extension [102], no data are presented to support the model, which remains to be tested.

25.6.3 Serotonin Pathway

Increased serotonin appears to alter behaviors during aging [25]. It is suggested that mRNA and expression of the serotonin biosynthesis gene, *tph-1* (tryptophan hydroxylase-1), are increased during aging [Ref. 25 and Murakami *et al.*, unpublished]. Consequently, reducing serotonin signal by serotonergic inhibitors and by the serotonin/octopamine pathway genes rescues age-related changes in basal/slowing response and chemotaxis avoidance [Refs. 25, 107, and Murakami *et al.*, unpublished]. Importantly, serotonin pathways also regulate life span [108].

25.6.4 Insulin/IGF-1 Pathway

It has been shown that the insulin/IGF-1 pathway modulates the late phase of age-related changes in

thermotaxis (temperature-food association) [11]. Figure 25.3 summarizes the current model; alternative models have been discussed elsewhere [13]. The insulin/IGF-1 pathway includes *daf-2* (insulin-like receptor gene) and *age-1* (phosphatidylinositol-3 OH kinase gene), which negatively regulate the Forkhead transcription factor, encoded by *daf-16* (Fig. 25.3). The *age-1* gene functions in the AIY, AIZ, or RIA interneurons [11, 109], suggesting that interneurons may be the core site of modulation by the insulin/IGF-1 pathway. Of note is the fact that proteotoxicity in the AD models is also suppressed by the mutations in the insulin/IGF-1 pathway (See Section 25.3.7, *C. elegans* AD Models).

Update: Several groups have confirmed that the insulin/IGF-1 pathway controls associative learning and memory in various *C. elegans* systems in young adults and during aging [33, 110–112].

25.6.5 Calorie Restriction

Interpretation of previous studies on calorie restriction (CR) requires careful consideration. CR can improve associative learning and memory in thermotaxis (food-temperature association) [86] and in chemotaxis avoidance (starvation-odor association) [33] in *C. elegans*. In contrast, CR in fruit flies does not: CR extends life span but does not suppress aging of an aversive learning task (odor-mechanical shock association) [113]. This may suggest a difference between the species. Alternatively, it may suggest a difference in experimental assay systems. The *C. elegans* studies use food (absence or presence of food) as a UC, while the fruit fly study uses mechanical shock as a UC. Thus an alternative model is that CR improves responses to food or starvation.

25.7 WHEN DOES COGNITIVE AGING BEGIN?

Although each form of learning and memory shows alterations at a different speed, current studies indicate two major time points that alter learning and memory in *C. elegans*. These include the early-mid reproduction period and around the end of the preproduction period, which appear consistent with human studies.

25.7.1 Multiple Phases of AMI

The studies of *C. elegans* suggest at least two phases of AMI. The early phase of AMI includes age-related decline in basal and slowing response and olfactory learning and memory (STM and LTM) [33, 86, 112]. The timing is at the early to mid-reproduction period. The late phase, in contrast, is around the end of the reproduction period. In the late phase, AMI includes disturbances

of various behaviors, including reduced thermotaxis associative learning behaviors and locomotion. It is unlikely that reduced locomotion at the late phase affects thermotaxis, as there is no correlation between reduced locomotion (by up to 40%) and thermotaxis in some mutations [86]. For example, the *clk-1* and *isp-1* mutations with reduced mitochondrial metabolism show reduced locomotion but an increased performance of thermotaxis [86].

Similarly, there are intriguing observations in humans. The cross-sectional studies suggest that some aspects of age-related cognitive decline begin in healthy, educated adults when they are in their 20s and 30s [114]. In contrast, the longitudinal data suggest around 60 years of age [115]. Although there seems to be a discrepancy, the two methods may be effective to reveal different phases of AMI in humans. It is notable that the standard cognitive tests use age-related adjustment, which artificially minimizes age-related changes [22]. The timing of the two AMI phases in humans is relatively similar to *C. elegans*. It is worth noting that there is an increase in oxidative damage around 40 years of age [77]. It is also possible that there are more than two phases of AMI. This line of experiments will be promising and should be performed for future studies.

25.8 ROLE OF OXIDATIVE STRESS IN AGING AND AMI: A THEORY

Oxidative stress increases with aging. However, the free radical theory of aging does not appear to explain aspects of aging. In this section, we propose a theory that incorporates evolutionary and biological aspects of aging.

25.8.1 Midlife Crisis Theory

Oxidative stress increases during aging and can damage macromolecules. The free radical theory of aging assumes that oxidative stress causes aging of biological functions [70]. Recent studies suggest that mutants in the superoxide dismutase (*sod*) genes show increased oxidative damage but fail to accelerate aging, providing evidence against the theory [116–118].

It is puzzling that ROS scavenging activities are relatively well retained, although oxidative damage increases during aging [73, 119]. In *C. elegans*, enzymatic activities of SOD (superoxide dismutase) and catalase do not decline until late in the life span (summarized in Fig. 25.4) [120]. This is consistent with mRNA levels of *sod* and *ctl* (catalase) genes, except for *sod-2*, declines with aging at mRNA levels [121].

Our midlife crisis theory assumes that oxidative stress is a part of an age-related crisis that occur in the middle

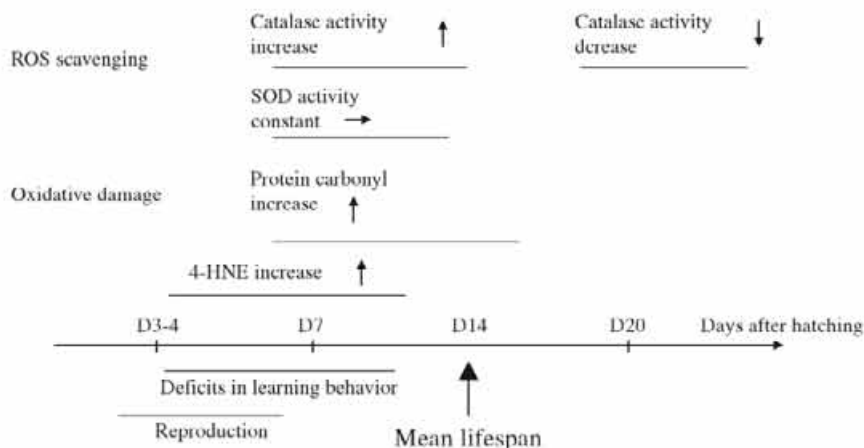


Fig. 25.4 A summary of *C. elegans* ROS and oxidative damage results during life span. Days are adjusted to the growth at 20 °C.

of the life span. It has been thought that normal metabolism in mitochondria is the primary source of oxidative stress [70]. Previous studies suggest that activities of mitochondria gradually change during aging [121]. If normal mitochondrial metabolism is the primary site of ROS generation during, ROS levels should gradually change during aging. However, an indicator of oxidative damage, protein carbonyl formation, increases in the middle of the life span [73]. The mechanism that causes age-related oxidative stress may be more complex than originally predicted by Harman (1956) [70].

The timing of increased oxidative stress is similar to that in which mortality increase, altered learning behaviors, and deteriorations of reproductive tissues are observed. We are inclined to propose a “midlife crisis” theory in which age-related increase in ROS is associated with the age-related deteriorations. In this theory, age-related ROS is associated with a wide variety of crises ranging from minor to major deficits. The midlife deficits occur around the end of reproduction, at which there is a diminishing force of natural selection. The deficits are unlikely to affect fitness of the species since they have a low impact on prosperity of the species. Therefore, the theory has an aspect of evolutionary and biological theory of aging. Importantly, in the theory, the *sod* mutations should have a limited impact on aging.

25.9 CONCLUSION AND PERSPECTIVE

AMI and neurodegenerative diseases impact more than 6.9 million people in the US [8, 123, 124]. Oxidative stress increases during aging and is a major risk factor for AMI and neurodegenerative diseases. Increased oxidative stress reduces expression of a specific set of neural genes during human brain aging [77] and is a

component of the transition state prior to the onset of neurodegenerative diseases such as AD [81]. It appears that aging neurons are sensitive to stress, including infections, oxidative stress, and environmental toxins. Such intrinsic and environmental stress may be partially rescued by nutritional approaches that include foods rich in antioxidants or anti-inflammatory compounds (e.g., berries, nuts, and grapes) [125, 126].

Despite the recent investigations, the genetic basis of AMI still remains to be determined. How does aging cause AMI? Can general aging be genetically delineated from tissue-specific aging that occur in neurons? Model systems, such as *C. elegans*, will provide useful systems to overview the mechanisms of AMI. Current understanding of AMI and oxidative stress appears to point out that oxidative stress and other age-related deteriorations occur in the mid to late phase of AMI. In the early phase of AMI, it seems that there is endocrine disturbance, as mutations in the insulin/IGF-1 pathway and serotonin pathway can rescue early markers of AMI. The emerging field of AMI is waiting to reveal its mechanisms.

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The nature of the hypoxia response element triggered by HIF in anoxia-tolerant mollusks is probably partly specific to these organisms, but the relative similarity in sequence and functional domains of oyster HIF- α with those of mammals suggests that some comparable genic responses such as NO synthase (NOS) expression could also be expected [7].

Second Messengers NOS activity has been detected in several groups of mollusks and has been particularly studied in the nervous system, where NO acts as a neuromodulator [34]. As an ancient and general biological regulator, NO is nonetheless involved in several functions of which are mediation of O_2 sensing and redox balance [34, 35]. NO is able to inhibit mitochondrial functions, and regulation of NO production is an important feature of hypoxic response [26, 35, 36]. However, there is a paradox in the fact that NO synthesis by NOS requires molecular oxygen and is consequently limited in severe hypoxia [35]. NO is a radical that is alternatively considered as a pro- or antioxidant according to the reaction in which it is involved and consequent products [38]. Hence, it can react with superoxide anion to form peroxynitrite ($ONOO^-$), a highly cytotoxic radical, which is probably the major way of NO disappearance. This oxidation depends on O_2^- production and consequently on O_2 concentration. It has been shown that at low pO_2 NO half-life is longer than at physiological pO_2 ; hence, during hypoxia, NO degradation is lowered [36]. NO can also form nitrites NO_2^-/NO_3^- , which could constitute a form of storage of NO molecules. Mitochondria are sites of NO production, and this could be achieved by the reduction of nitrites when hypoxia occurs, probably thanks to cytochrome *c* oxidase [39, 40]. It is intriguing to note that cytochrome oxidase can both generate NO (at low pO_2) and have its oxidase activity inhibited by NO (particularly at phosphorylated and active state 3).

NO is also involved in hypoxia response through its role as activator of guanylyl cyclase and consequent influence on the production of cGMP [41], which most probably mediates O_2 sensing in anoxia-tolerant mollusks [26, 37]. Recent experiments suggest that guanylyl cyclases may actually not be activated by NO itself but rather by a complex of NO with glutathione, called *S*-nitrosoglutathione (GSNO). Indeed, it has been shown that GSNO, which has a hypoxia-mimetic activity, can activate guanylyl cyclase and that GSNO reductase, which reduces GSNO to restore reduced glutathione (GSH) and degrade NO in NH_4^+ form, affects significantly NO-mediated hypoxic response [35]. Regulation of the GSNO pool could constitute a link between cellular redox status and signal transduction insofar as GSNO can generate nitrosothiols by transnitrosylation of protein sulfhydryl groups that may participate in cell

signaling. In addition, a relationship between the phosphorylation state of glycolytic enzymes and GSH content and synthesis was found in *Mytilus galloprovincialis* [42] that may be due to GSNO-induced cGMP production and subsequent activation of metabolic regulators. Indeed, the involvement of cGMP during hypoxia in mollusks seems to be linked to the cGMP-dependant protein kinase PKG, a key signaling protein that is known to mediate both phosphorylation of metabolic enzymes and anoxia genic response [26, 43]. Among numerous substrates, PKG activates the stress-activated kinase p38 MAPK that regulates the activity of transcription and translation factors, these functions being highly modulated in anoxia-tolerant animals [44]. In *Mytilus galloprovincialis*, p38 MAPK activation is apparently involved in response to different stresses (oxidative stress, osmotic stress, hyperthermia, exposure to diverse pollutants) [45–47]. Such stimuli also activate other stress-activated kinases (SAPK/JNK) at the same time as p38 MAPKs [47–48]; however, this is not the case for ischemia/reperfusion phenomenon in rat heart, where p38 MAPKs are activated during both anoxia and recovery but SAPKs only at reoxygenation [49]. This suggests that p38 MAPK could be a specific contributor to the response to anoxia-reoxygenation. Gaitanaki and co-workers found that anoxia exposure induced biphasic changes in phosphorylated rate of p38 MAPK in *M. galloprovincialis*: maximal values occurred at 1 h and 8 h of anoxia [45]. Interestingly, levels of phosphorylated p38 MAPK also increased transiently in the first 5 min of recovery after a 15-min air exposure. In *Littorina littorea*, an increase in phosphorylated p38 MAPK as well as phosphorylated 27-kDa stress protein (HSP27), which is regulated by the p38 MAPK downstream element MAPK-activated kinase 2 [51], was found in hepatopancreas after 12 h of aerial exposure [50]. HSP27 is a chaperone-like protein that increases cell resistance to heat shock or osmotic stress as well as short-term oxygen deprivation and myocardial ischemia-reperfusion, providing this protection in the latter situations by a role of the phosphorylated form in F-actin stabilization [52, 53]. Interestingly, 2-DE analysis on *Mytilus edulis* gills showed a downregulation of gelsolin, an efficient actin filament-severing factor, after 2 h of emersion on the shore [54], which is consistent with the idea of a reinforcement of the polymerization rate and stabilization of the cytoskeleton during hypoxic stress. However, activation of the p38 MAPK pathway is also involved in mediating apoptosis or necrosis during ischemia-reperfusion in myocardium and kidney [55, 56]; further experiments should help us to understand whether its role in the tolerance of mollusks to anoxia-reoxygenation is related to an enhanced cell resistance or an elimination of insulted cells.

26.2.3.2 Cellular Response to Oxygen Decline—Preparation for Oxidative Stress In addition to metabolic adjustments, signaling cascades and transcription factors activated by hypoxia most probably control the regulation of actors involved in the fight against oxidative stress. Evidence of such anticipated regulation is provided at the genomic level and through the modulation of antioxidant enzymatic activities during hypoxic stress.

Gene Transcription Contrary to mechanisms observed in anoxia-sensitive species, which consist mainly of compensatory mechanisms (expression of glycolytic enzymes and proteins involved in oxygen transport and vascularization), the genic response triggered in hypoxia-tolerant animals like certain mollusks generates products that are rather involved in altering metabolism, which allows them to withstand long-term oxygen deprivation [58]. Upregulation of certain transcripts has been shown in *Littorina littorea* during hypoxia, but most of them remain as latent mRNAs during this stressing period [37, 57]. These transcripts, which probably correspond to functions that are important for recovery, accumulate in the course of metabolic depression until normal conditions enable their translation. cDNA microarrays performed in hepatopancreas and foot of *L. littorea* allowed identification of a metallothionein [59] and a ferritin heavy chain [37] as upregulated genes. Both transcripts showed quick and important increase of expression and a maintenance of high levels throughout the hypoxia stress before declining at recovery. Resulting proteins are able to sequester metals and presumably participate in antioxidant defenses when free radical production is enhanced at recovery. Indeed, metallothioneins are shown to provide protection against oxidative stress in vertebrates [60, 61] and in invertebrates [62] due to metal binding but also to an inherent antioxidant function as ROS scavenger. Ferritin regulates Fe storage when its translation is not prevented by the binding to iron-regulatory proteins (IRP) that occurs when iron is low. This interaction is regulated by oxygen concentration [63]; in *L. littorea*, hypoxia probably triggers the dissociation of IRP-ferritin mRNA complexes, allowing ferritin translation. Management of the Fe pool and especially sequestration of free iron ions is a crucial aspect of coping with oxidative stress for mollusks [64]. In similar experiments in *C. gigas*, upregulation of glutathione peroxidase (GPx) and metallothionein transcripts were also observed in gills and mantle, which is consistent with the results described above [65]. At the proteic level, 2-DE experiments showed that a decrease of a thioredoxin peroxidase and Cu/Zn superoxide dimutase (Cu/Zn SOD) occurred between the end of emersion and 2 h of recovery in gills of *M. edulis* [54]. Both of these are antioxidants, and these decreases may indicate that they

are partially degraded after having contributed to the protection against oxidative stress at immediate recovery. Surprisingly, measurement of Cu/Zn SOD activity in the same study showed that it remained stable during hypoxia and increased after 2 h of recovery [54]. This discrepancy between expression and activity underlines that enzymatic antioxidant activities are regulated to a great extent at the posttranslational level. In this way, specific revelation of mussel Cu/Zn SOD activity after isoelectrofocusing reveals that the total activity visible on gel results from the contribution of three isoforms with different isoelectric points. The relative contribution of isoforms to total activity is different according to the tidal height of animals and reversibly modulated during the tidal cycle and by the pollution status of the environment [66, 67]. Authors suggest that these changes, which are clearly linked to cellular redox status, could correspond to interconversion of isoforms through oxidative modifications, perhaps as a functional response.

Regulation of Antioxidant Activities Earlier studies on the antioxidant response in mollusks exposed to air showed that antioxidant activities are modulated during hypoxia as well as at recovery according to species and tissue [6]. Most observations suggest that a spread strategy is an increase of certain antioxidant forces during hypoxia as a preparation to recovery, considering the probable adaptation to cyclic emersion or aestivation of anoxia-tolerant mollusks [68-70]. In *L. littorea*, antioxidant activities diminished during a 6-day exposure to N₂ and increased at recovery in hepatopancreas and foot muscle, but total glutathione increased during anoxia [69]. These changes in molecular antioxidant that are attributed to an anticipated adaptation to ROS generation seem efficient, since a decrease in lipid hydroperoxides was observed in hepatopancreas throughout the experiment. Almeida and co-workers exposed *Perna perna* mussels to air for 24 h and found that only glutathione transferase activity increased at the end of the anoxia period and remained high after recovery; on the other hand, increase in oxidative damages on lipids and DNA was reported during air exposure, with a return to basal values at recovery [29]. This suggests that oxidative stress occurs also during emersion for aquatic mollusks. In addition to the ROS generation by mitochondria due to electron flow lowering, another possible cause is gaping behavior. This consists in opening briefly the shell in order to extract aerial oxygen and increase pO₂ in water of the pallear cavity [71]. This behavior that significantly participates in energy production during emersion implies a sudden exposure to air of tissues that are oxygen depleted and consequently the generation of oxidative stress [29, 30]. In another experiment where mussels were exposed to air

exposed to a mixture of potentially toxic substances. Oxidative stress is a common consequence of xenobiotic presence responsible for biomolecule alteration. Some of these alterations constitute probable links with signaling processes that bring about cell death or, conversely, increase of resistance capacities.

26.3.1 Free Radical Production by Pollutants in Mollusks

As soft-bodied organisms, xenobiotics can enter in molluscan organisms through different ways (digestive tract by ingestion of water and food, respiratory surfaces, and across integumentary system) and accumulate because of low degradation capacities. Many compounds are implicated in the production of free radicals in mollusks, the main compounds being metals, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), and other organohalogenated molecules [38]. This toxicity is either due to the metabolization of xenobiotics in the case of organic molecules, although such capacities are limited in invertebrates [4], or the catalysis of redox reactions in the case of transition metals and depletion of thiol-containing antioxidants for other metals [75]. The ROS produced as a result of Haber–Weiss reactions or incomplete redox cycle, as well as organic peroxides that can be generated by cytochromes, can interact with biomolecules such as lipids, proteins, and DNA and, as in other organisms, generate damage that can be irreversible. Measurement of this damage (mainly products of lipid peroxidation and, to a lesser extent, DNA oxidation and fragmentation and protein damage) as well as induction of defense and repair mechanisms are used as biomarkers of oxidative insult in ecotoxicology [76]. Among the diverse oxidative attacks, protein and glutathione oxidations as well as DNA alteration probably play a role in the activation of pathways controlling cell response.

26.3.2 Oxidation of Proteins: Sensing Redox Changes?

Free radicals induce different kinds of oxidative modifications on proteins that can be reversible—and possibly involved in regulatory functions—or not [77]. A dramatic modification is the carbonylation of proteins, which consists of irreversible modifications of lateral chains into ketones or aldehydes and leads to protein aggregation and consequent inactivation and degradation. Measurement of carbonyl groups in soluble proteins is a tool used in ecotoxicology for evaluating oxidative damages to proteins induced by contamination [78]; new approaches now allow study of the specific patterns of proteins that are affected by these modifications [79]. In 2-DE experiments coupled with blotting

and specific immunorevelation of carbonylated groups, it was shown that levels of carbonylation increased in parallel to the intensity of oxidative stress in *M. edulis* and in *Ruditapes decussatus* but with tissue-specific and pollutant-specific patterns [80, 81]. In particular, these studies revealed that a major target of such oxidation in *M. edulis* is actin, which is known as one of the most sensitive cytoskeletal component to oxidant attacks [82]. Interestingly, revelation of ubiquitinated proteins in similar experiments done in *M. edulis* and *R. decussatus* showed that the print is different from that of carbonylated proteins, suggesting that the latter are degraded independently from ubiquitin-proteasome pathway [83–85]. Other modifications generally involve alteration of sulfhydryl groups, which are preferential targets of free radicals; oxidation of thiol functions gives rise to cleavage or formation of disulfide bonds that can be intra- or interproteins or occur between proteins and cysteines, glutathione, or even lipids. Disulfide bonds were thought to lead to the loss of protein function and inactivation of enzymes [86], but these bonds are reversible and could play regulatory roles in mild stress situations [87]. Glutathionylation of proteins, for instance, is a post-translational modification whose level increases in response to prooxidant treatment in diverse models. It allows the regulation of protein function and consequent modulation of cellular processes, protects cysteines during oxidative stress by preventing irreversible oxidation, and could play an essential role in diverse signaling pathways [88, 89]. It notably regulates the ubiquitin-conjugating activity and therefore controls the protease activity in mammalian cells [90]. An equivalent role of glutathionylation in the modulation of signal transduction can be expected in mollusks, but no evidence has been provided until now. Study of the glutathionylated proteome in gills and digestive gland of *M. edulis* showed that actin is again a preferential target of this modification [80]. This protein was slightly affected by inter- or intraprotein disulfide bond formation in redox proteomics experiments performed on tissues of mussels exposed to H_2O_2 [91]. A modified protocol helped to identify proteins touched by sulfhydryl oxidation in response to menadione treatment and revealed that proteic response to oxidative stress in *M. edulis* principally involves disulfide isomerases and cytoskeletal and chaperone proteins [92]. The role and mechanisms of these specific proteic modifications in cellular signaling and processes of mollusks have yet to be elucidated.

26.3.3 Pollutant-Induced Apoptosis

There is a correlation between apoptosis levels in mollusks and environmental stresses (salinity or temperature changes, pollution) whose common denominator is

response to Cu^{2+} exposure [46]. Stress-induced HSP70 overexpression is generally observed at the same time as p38 MAPK activation in bivalves, probably because of induction of HSPs by MAPK prosurvival pathways in addition to protein damage, but it is notable that HSPs could themselves regulate signaling cascades [115].

Hence, tissues present a differential sensitivity to chemical stress, as suggested by the higher levels of chaperones in gills than in mantle [116], the diverse p38 MAPK pathways apparently activated by oxidant stimuli [46], and the differences in global phosphorylation levels observed in response to pollutant exposure [110]. This divergence of vulnerability could be due to a higher level of oxidative stress undergone by mantle cells, since mantle is more sensitive to lipid peroxidation [117] and gills possess higher levels of enzymatic antioxidant defenses [4]. The disparity in the response between tissues may also be due to a different "threshold" of stress level that activates proapoptotic pathways; indeed, it has been shown that MAPK activation involves different pathways and responses according to the severity of oxidative insult [118]. For instance, in human lymphoid cells, a high level of oxidative stress induced apoptosis, whereas

a low level induced mitotic arrest and probable transcription of antioxidant actors to balance redox status. Gills are the first tissue exposed to adverse factors, to which they probably exhibit greater tolerance than other tissues because of differential sensitivity and capacities to cope with a higher level of insult. Effects of diverse pr-oxidant contaminants on MAPKs in *M. galloprovincialis* gills were studied recently [47]. Low concentrations of TBT activated p38 MAPK and JNK but not ERK, and higher doses activated only p38 MAPK; in both cases DNA damages decreased, suggesting that the concentrations used in this study induced protective pathways of MAPKs, which contrasts with previous results obtained with very high doses of TBT directly introduced in the pallial cavity of mussels [100]. p38 MAPK/JNK activation and reduction of DNA damage were also observed in gills of mussels exposed to soluble fraction of diesel oil, indicating a probable role of MAPK activation in DNA repair [47].

Figure 26.2 proposes a schematic view of the hypothetical induction of apoptotic or resistance responses to oxidative stress in mollusks according to the severity of the insult, with p38 MAPK as a central regulator.

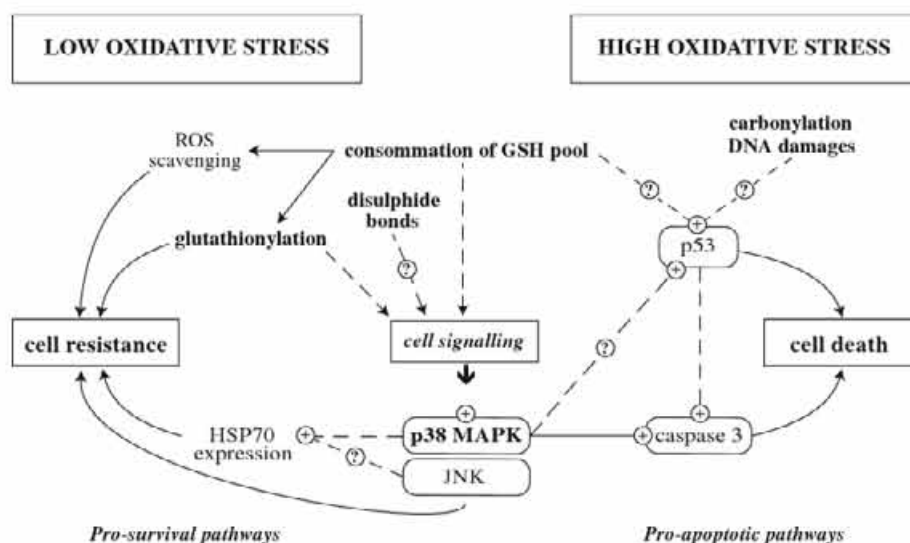


Fig. 26.2 A hypothetical model of the induction of apoptotic or resistance responses to oxidative stress according to the severity of insult. Oxidative stress induces the oxidation of thiol groups of proteins. Resulting disulfide bridges could play a role in signaling, while oxidation of free thiol is generally deleterious except in some cases (metal binding protein) where it allows scavenging of ROS. Glutathione is also oxidized or conjugates to proteins (glutathionylation), protecting them from irreversible modifications; in addition, glutathionylated proteins are also probably involved in signal transduction. Stress kinases, namely, p38 MAPK and to a lesser extent JNK, are activated probably in correlation with GSH levels. p38 MAPK induces the expression of HSP70 that protects proteins from damages and misfolding. These features allow enhancement of cell tolerance, which becomes capable of resisting oxidative stress if not too high. If oxidative stress is too severe, which is a relative notion depending on cell type sensitivity, p38 MAPK activates proapoptotic pathways by the cleavage of caspases, perhaps through p53 activation. Activation of p53 could also be regulated by GSH level more directly and by proteins or DNA damage. p38 MAPK plays a central role in the modulation of cell response to oxidative stress in mollusks.

26.4 IMMUNE SYSTEM

The molluscan immune system is less complex than that of mammals and involves humoral (lectins, hydrolytic enzymes, antimicrobial peptides) and cellular (hemocytes) mechanisms that recognize invaders and destroy them by various means [11]. Hemocytes are crucial components of this system and capable of migration, phagocytosis, aggregation, and secretion of antibacterial substances and ROS. One of the common features of molluscan host reaction against pathogen infection is the generation of an oxidative burst by hemocytes to kill microbes [119]. NADPH oxidase catalyzes the production of singlet oxygen, superoxide O_2^- , hydroxyl radical OH^- , as well as hydrogen peroxide H_2O_2 that forms hypochlorous acid in a reaction with chloride catalyzed by myeloperoxidase, then enhancing antibacterial activity. It is nonetheless proposed that H_2O_2 could also originate from the Fenton reaction and hydroxyls result from interaction of H_2O_2 and superoxides. In addition, the amount of the different ROS produced depends on the cellular subtype as well as the stimulation [119, 120]. Oxidative burst is triggered by exposure to pathogens [119] but also carbohydrates [121], bacterial extracellular products [122], and cytokines [123]. Then, whereas it was thought that the phagocytosis itself activates a ROS-forming system associated with NADPH oxidase [119], it seems probable that ROS production is mediated by cGMP-dependent protein kinase PKC [124] and stress-activated MAPKs like p38 MAPK, as observed in *M. galloprovincialis* challenged with strains of *Vibrio* sp [120]. However, pathways triggered by a bacterial challenge depend on the pathogen as well as the extent and duration of the stimulation [125].

ROS production by hemocytes as a defensive reaction has the disadvantage of potentially inducing cytotoxicity to the host itself, which exhibits antioxidant forces to counteract this deleterious aspect of microbial killing. In addition to cellular antioxidants, circulating antioxidants are detected that could help to protect host cells from ROS insults. For instance, an extracellular superoxide dismutase has been characterized in oyster plasma [126]. This Cu/Zn form is exclusively expressed by hemocytes and is able to bind lipopolysaccharides (LPS) exhibited by bacteria such as *Escherichia coli* as well as integrin-like receptors on the surface of the oyster's hemocytes. In addition to putative roles in oxidative burst modulation and host protection against oxidative injury, this extracellular SOD could thus be involved in the recognition of LPS and subsequent triggering of the immune response by interacting with integrin receptors [126]. In the bay scallop *Argopecten irradians*, an extracellular SOD that does not possess

the LPS-binding motifs has been characterized, whose expression was enhanced in hemocytes when animals underwent *Vibrio anguillarum* challenge, probably as a consequence of ROS accumulation during oxidative burst [127].

If not balanced, ROS surproduced for bacterial killing may generate irreversible damage to host cells, even death. Apoptosis is observed as an important component of molluscan immune reaction to degrade infected or phagocytic cells [10, 128]. The mechanisms ruling this immunomodulatory process are still unclear, but oxidative burst probably participates in its activation, as observed for other environmental stress involving ROS formation [11, 95]. Hence, *C. gigas* hemocytes challenged in vitro with the Gram-positive marine bacterium *Planococcus citreus* phagocytized and killed invading cells within a few hours; then phagocytizing hemocytes, mainly hyalinocytes, underwent cell death that probably corresponds to apoptosis as suggested by preceding membrane blebbing, cell shrinkage, and chromatin condensation [11]. Besides, treatment with antioxidant agents suppressed phagocytizing-hyalinocyte death, whereas treatment of nonchallenged hemocytes with a prooxidant failed to induce apoptosis, suggesting that hyalinocyte apoptosis triggered by *P. citreus* exposure is actually induced by ROS produced within cells. Curiously, other experiments showed that granulocytes generally present higher levels of apoptosis than hyalinocytes, possibly due to higher phagocytic and respiratory burst activities [10].

In addition to ROS *sensu stricto* themselves, NO is also produced as a defense mechanism by molluscan hemocytes, where NO synthase activity has been detected, and different isoforms of NOS, some of them unique to mollusks, have been characterized [11]. NO synthesis occurs apparently after phagocytosis and would be involved in bacterial clumping [34, 123]. In the clam *R. decussatus*, NO synthesis seems to be independent from phagocytosis and constitutes an alternative method to kill pathogens [129]. In *C. virginica*, infection by the protozoan parasite *Perkinsus marinus* also induced NO production that was correlated with a decrease in parasite loads at early time points after infection [130]. In parallel, it has been shown that *P. marinus* increased hemocyte apoptosis at early and later stages of infection, with differential profiles according to the virulence of the strain [128]. Hence, the role of NO in the triggering of hemocyte apoptosis in mollusks is under question [10, 11]. Outside the immune system, the roles of NO in cell death are investigated in only one study that suggests an antiapoptotic role of NO in larval development [131]; on the other hand, data on mammalian macrophages show that stimulated NO production is associated with apoptosis of host cells [132, 133].

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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 ($\text{NO}^\bullet$) to produce the peroxynitrite anion (ONOO^-), a nonradical product, which is as toxic as $^\bullet\text{OH}$ in terms of its ability to oxidize and destroy by standard molecules. $\text{NO}^\bullet$ 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 peroxidase, 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

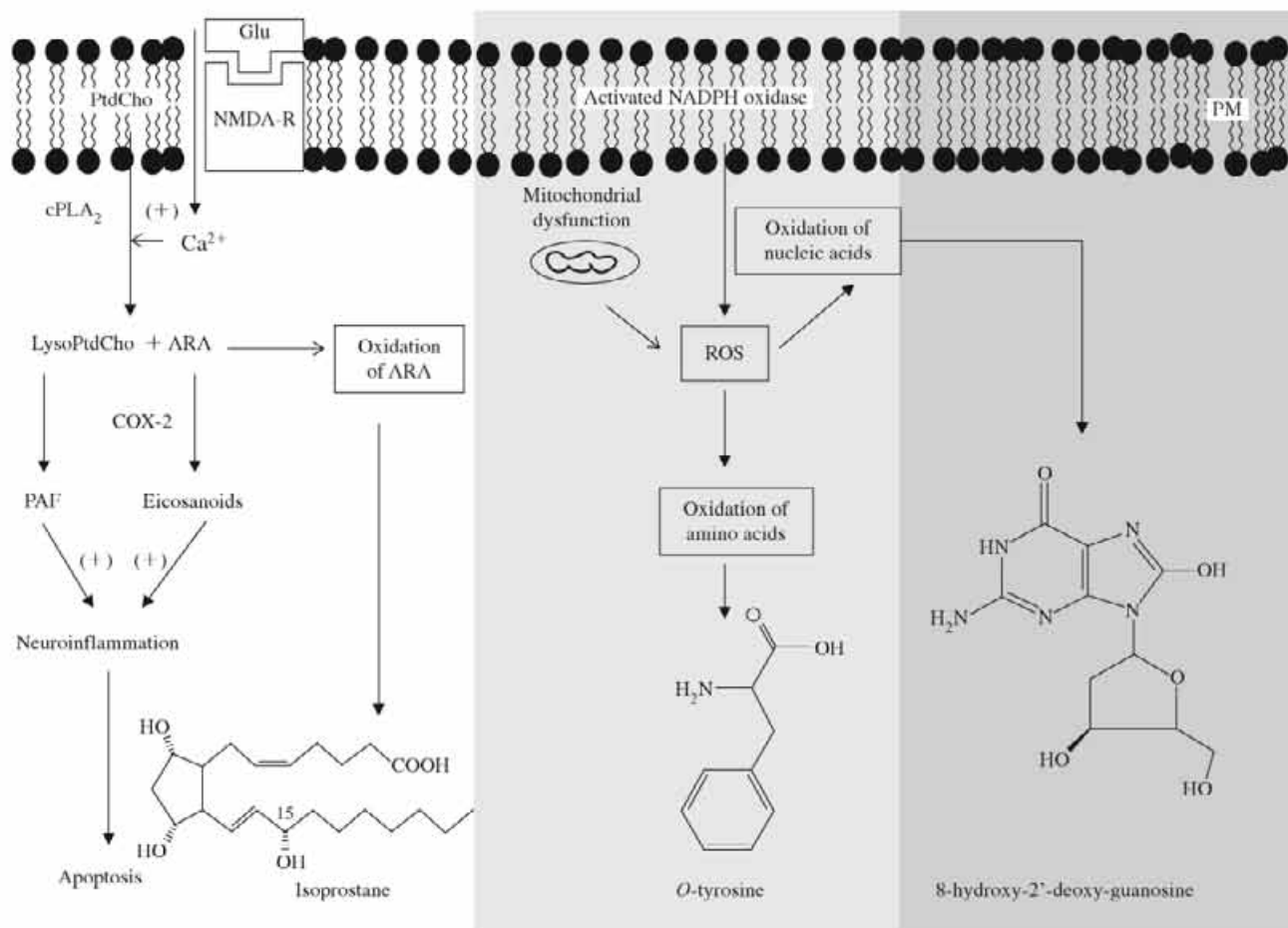


Fig. 27.2 Generation of biomarkers for oxidative stress in vertebrates. PM, plasma membrane; NMDA-R, *N*-methyl-D-aspartate receptor; Glu, glutamate; PtdCho, phosphatidylcholine; lyso-PtdCho, lyso-phosphatidylcholine; cPLA₂, cytosolic phospholipase A₂; COX-2, cyclooxygenase; ARA, arachidonic acid; PAF, 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 function. 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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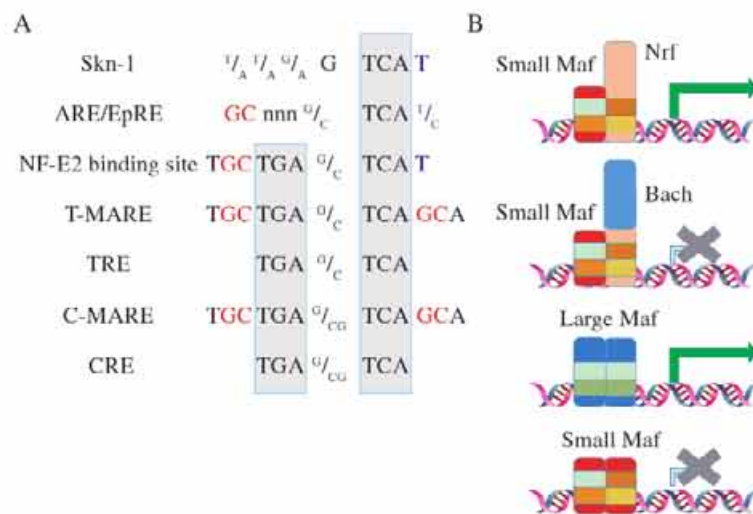


Fig. 5.2 DNA recognition sequences and the transcriptional activities of Maf-containing dimers. (A) Various MARE-related sequences are shown. The dinucleotide “GC” (marked in red) is essential for recognition by Maf proteins. The nucleotide “T” or “T/C” (marked in blue) enhances the binding of CNC proteins. Trinucleotides (boxed in gray) with the central G and GC sequence consist of the TRE and CRE, respectively, which make up the core region of the MARE. (B) Nrf1, Nrf2, Nrf3, and NF-E2 activate transcription by forming heterodimers with small Maf, while Bach1 and Bach2 repress transcription. A large Maf homodimer, possessing a *trans*-activation domain, activates transcription, while a small Maf homodimer, lacking a *trans*-activation domain, represses transcription. All of these Maf-containing dimers bind to T-MARE with high affinity.

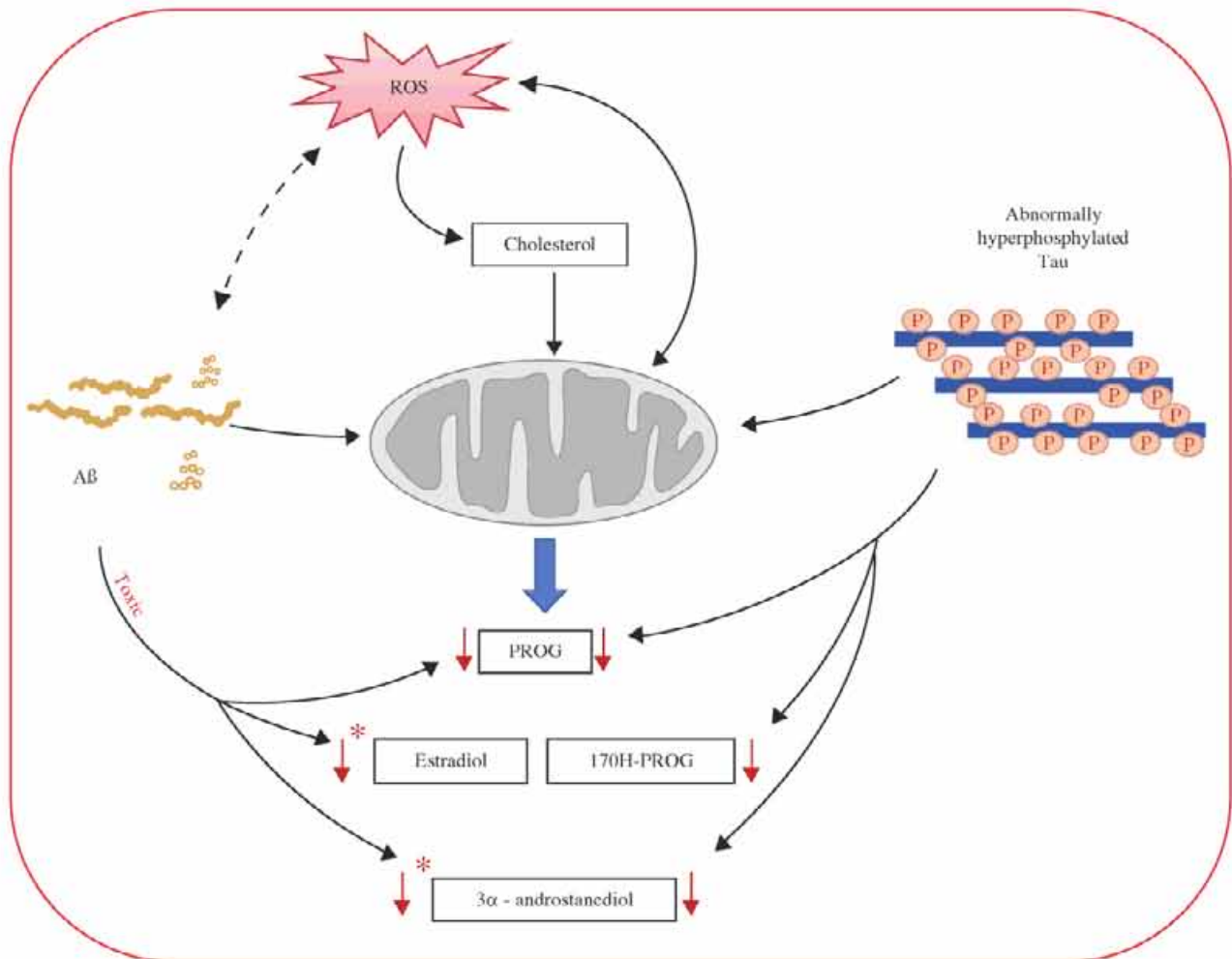


Fig. 8.3 Effect of toxic concentrations of Aβ peptides and abnormally hyperphosphorylated Tau protein on neurosteroid biosynthesis. Aβ induced a drop of the level of progesterone (PROG), estradiol, and 3α-androstanediol by acting on reactive oxygen species (ROS) formation and mitochondrial function and/or directly on steroidogenesis. The presence of abnormally hyperphosphorylated Tau protein had the same effect by inducing a decrease of progesterone, 17-hydroxyprogesterone (17OH-PROG), and 3α-androstanediol. On the other hand, it has been shown that nontoxic concentrations of Aβ induced an increase in estradiol and 3α-androstanediol levels (this pathway is marked by *).

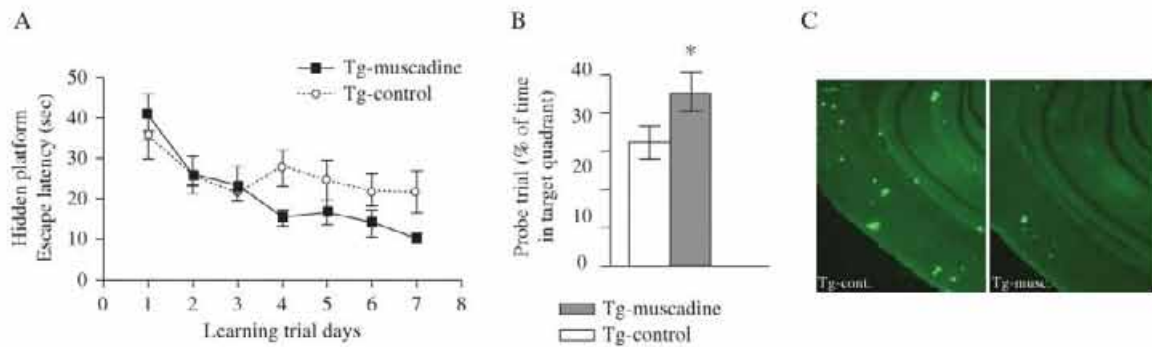


Fig. 16.4 Muscadine treatment improves spatial memory function and A β neuropathology in Tg2576 mice. (A,B) Assessments of spatial memory behavioral functions of 14 month old muscadine-treated (Tg-muscadine) and control, gender- and age-matched non-treated (Tg-control) Tg2576 mice using the Morris water maze protocol. (A) Learning trial hidden-platform acquisition curves. Tg-muscadine group performed significantly better than the control, non-treated group (Tg-control) [2-way ANOVA analysis of Tg-muscadine vs. Tg-control groups for muscadine treatment ($p < 0.05$, $F = 4.24$, $DF_n = 1$, $DF_d = 84$) and for training days ($p < 0.05$, $F = 6.43$, $DF_n = 6$, $DF_d = 84$)]. (B) Probe trial conducted 24 hours after completion of hidden-platform training. Muscadine-treated Tg2576 mice exhibited a significantly higher preference for the target platform compared to control, non-treated Tg2576 mice ($p < 0.05$, 2-tailed Student t test). In (A,B) Values represent group mean (+SEM); $n = 7-9$ mice per group. (C) Assessments of A β neuropathology reflected by amyloid neuritic plaque density in cerebral cortex and in the hippocampal formation of brain specimens from muscadine-treated and control, non-treated Tg2576 mice. Representative micrograph of brain specimen stained for amyloid neuritic plaques in muscadine-treated (Tg-musc.) or in control, non-treated (Tg-cont.) Tg2576 mice.

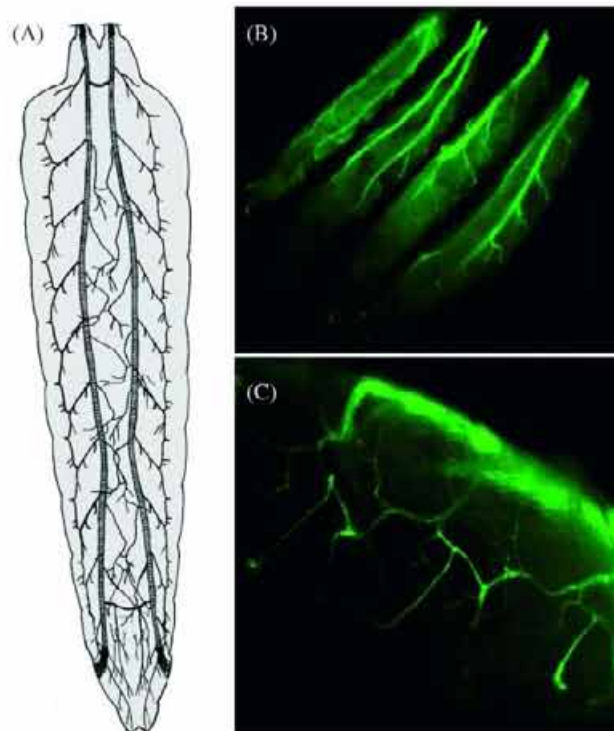


Fig. 19.2 Structure of the airway system of *Drosophila*. The airway system (trachea) of a larval fly is made up of interconnected tubes that deliver oxygen to almost every cell in the body (A). Upon stimulation with different stressors including infection, the airway epithelium launches a very effective response, comprising the expression of antimicrobial peptide genes (B and C, the latter at higher magnification).

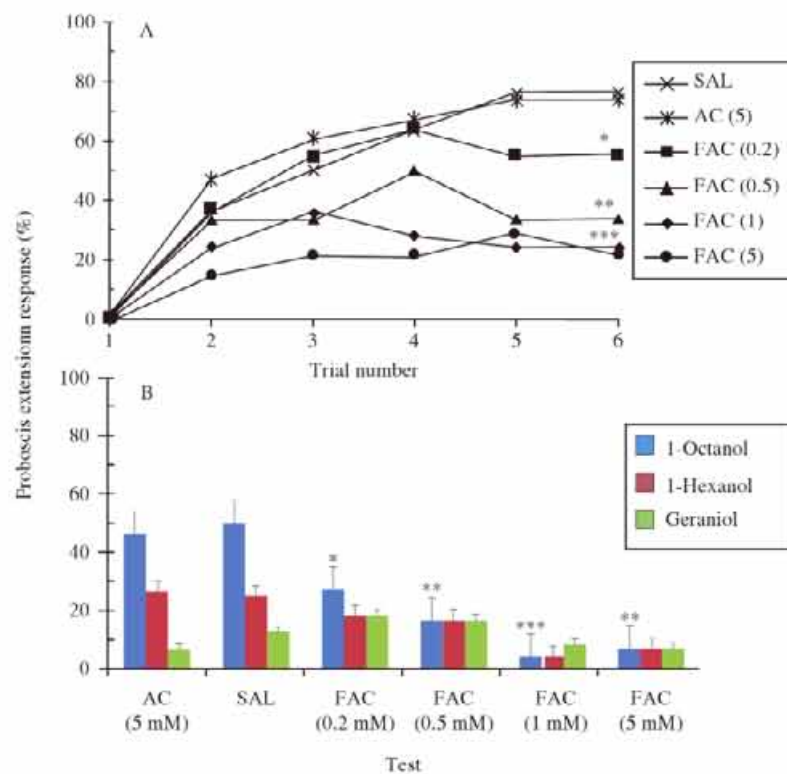


Fig. 21.3 Dose-response effect of ferrous ammonium citrate (FAC) on olfactory learning and memory. (A) Acquisition of the conditioned odor C: In this experiment, honeybees received 4 nl of saline (SAL), ammonium citrate (AC), or FAC at 0.2 mM, 0.5 mM, 1 mM, and 5 mM in each antennal lobe. Twenty-four hours later, subjects in each group were conditioned with 1-octanol (odor C). (B) Test with odors C, S, D: Ninety minutes after conditioning, subjects in each group were tested with C, molecularly similar (S), and molecularly dissimilar (D) odors in randomized order. *ns*, Not significantly different from controls (SAL, 5 mM AC). Asterisks indicate significant differences of respective points from control group: * $P = 0.05$, ** $P = 0.005$, 0.001, *** $P = 0.0001$). This figure is modified from reference [40].

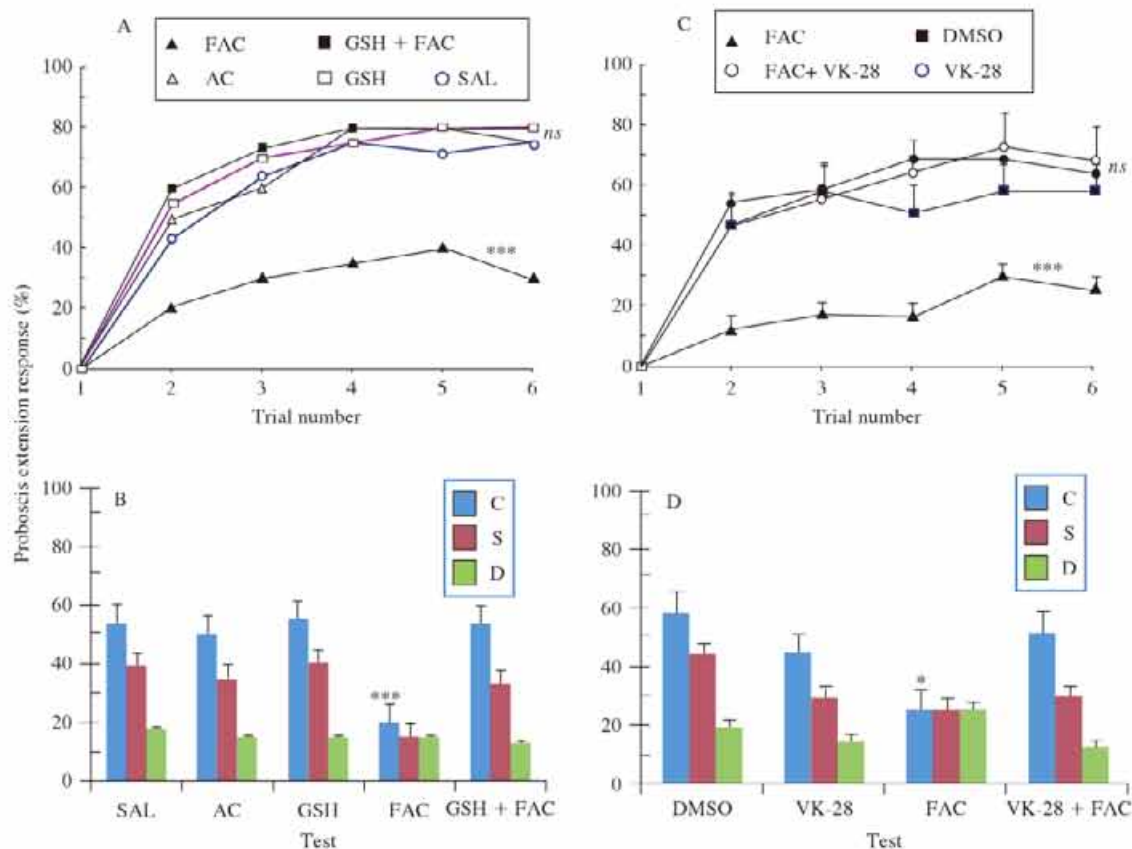


Fig. 21.4 Glutathione and VK-28 can reverse ferrous ammonium citrate (FAC)-mediated oxidative stress. (A) Acquisition of the conditioned odor C: In this experiment, subjects received 4 nl of either saline (SAL) or 2.5 mM reduced glutathione (GSH) in each antennal lobe. Two hours later, 4 nl of 500 μ mol/l ammonium citrate (AC) or 500 μ mol/l FAC were injected in each antennal lobe. Twenty-four hours later, subjects were conditioned with 1-octanol. (B) Test with odors C, S, D: Ninety minutes after conditioning, subjects in each group were tested with different odors (1-octanol, C; 1-hexanol, S; and geraniol, D) in randomized order. (C) Acquisition of the conditioned odor C: In this experiment, subjects received 4 nl of either dimethyl sulfoxide (DMSO) or VK-28 or DMSO + VK-28 in each antennal lobe 30 min before injection of FAC. Twenty-four hours later, subjects were conditioned with 1-octanol. (D) Test with odors C, S, D: Ninety minutes after conditioning, subjects in each group were tested with C, S, and D odors in randomized order. Asterisks indicate significant differences of respective points from control group (* $P = 0.05$, *** $P = 0.001$). *ns*, Not significant. This figure is modified from reference [40].

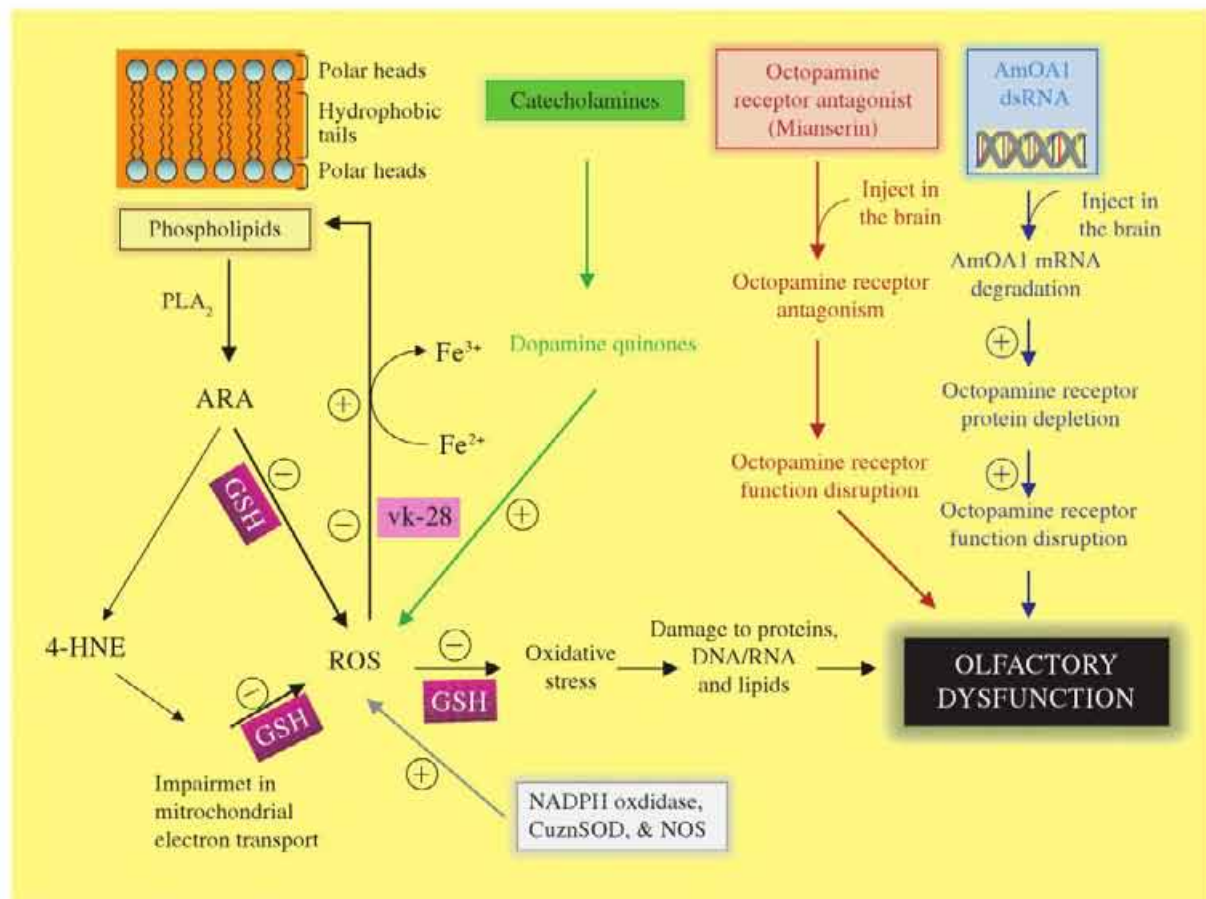


Fig. 21.5 Hypothetical molecular mechanism underlying reactive oxygen species (ROS)-mediated olfactory dysfunction in the honeybee brain. Iron-induced oxidative stress: Iron peroxidizes polyunsaturated fatty acids into peroxidized phospholipids, which are considered better substrates for phospholipase A_2 (PLA_2) than native phospholipids. PLA_2 catalyzes this reaction, forming arachidonic acid (ARA). The nonenzymatic oxidation of ARA results in the generation of 4-hydroxynonenal (4-HNE) that impairs mitochondrial electron transport, producing ROS. Increased ROS produces oxidative stress that damages proteins, DNA/RNA, and lipids, impairing olfactory processes (encoding, consolidation, and/or retrieval processes). Both octopamine receptor antagonism by mianserin (MAS) and octopamine receptor protein depletion by octopamine receptor double-stranded RNA (AmOA1-dsRNA) result in functional disruption of octopamine receptor, which leads to olfactory dysfunction. Oxidation of catecholamines forms quinones, which results in formation of ROS, leading to olfactory dysfunction. GSH protects brain against oxidative stress by modulating the redox state of specific thiol residues of target proteins. VK-28 chelates excess iron from the system. Monoamine oxidase inhibitor (MAOI) inhibits monoamine oxidase and therefore prevents oxidation of catecholamines. ROS can also be produced by activation of NADPH oxidase.

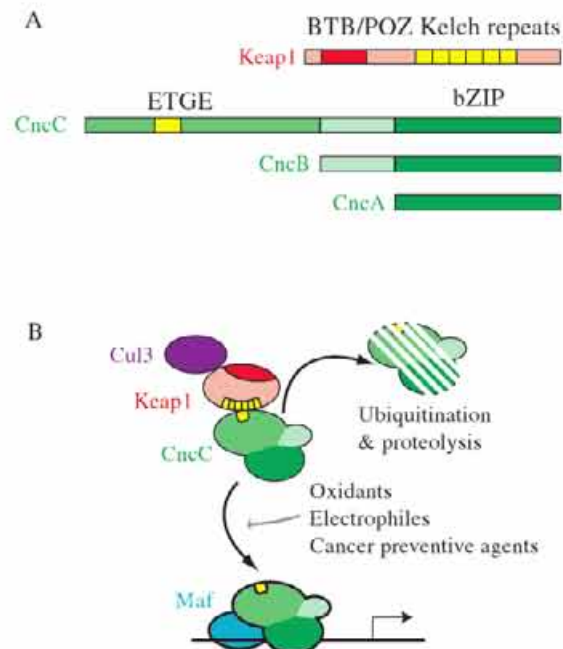


Fig. 22.1 Conservation and simplified illustration of the Keap1/Nrf2 pathway in *Drosophila*. (A) Nrf2 and Keap1 homologs are present in *Drosophila*. The fly *keap1* gene is predicted to encode a protein with high sequence similarity to its vertebrate Keap1 counterparts. Conserved domains include the BTB/POZ domain required for dimerization and 6 Kelch repeats for binding to Nrf2 and anchoring to actin. The *cnc* locus encodes three protein products, which all contain the bZIP region that mediates dimerization and DNA binding. The Nrf2 homolog is the longest isoform, CncC, which contains domains predicted to bind Keap1 such as the ETGE motif. (B) In basal conditions, Keap1 binds to CncC and inhibits its activity, likely through Cul3-mediated ubiquitination and proteasomal degradation. Oxidative stressors, electrophilic xenobiotics, and cancer chemopreventive agents relieve this inhibition. Stabilized CncC then accumulates in the nucleus and transcriptionally activates a battery of cell-protective genes, likely in a dimer with the single small Maf protein of *Drosophila*. This figure is adapted from Sykietis GP and Bohmann D, Keap1/Nrf2 signaling regulates oxidative stress tolerance and lifespan in *Drosophila*, *Dev Cell*, vol. 14, p. 76–85, Copyright 2008, with permission from Elsevier.

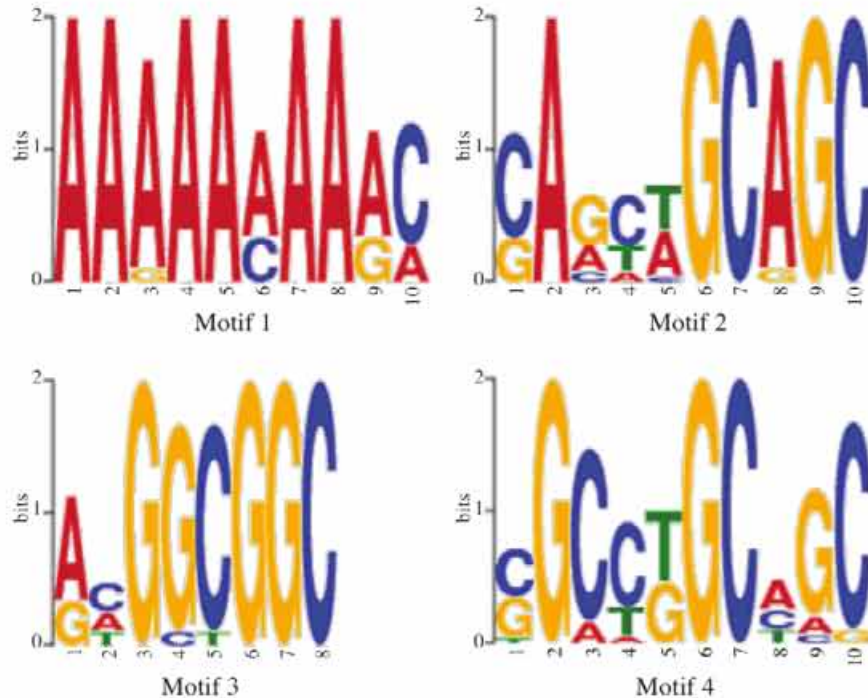


Fig. 23.4 Sequence LOGOS of the MEME motifs 1–4 displaying the probability of each base appearing at every possible position in the motif. The total height of the stack is the information content of that position in the motif in bits. The height of the individual letters in a stack is the probability of the letter at that position multiplied by the total information content of the stack.

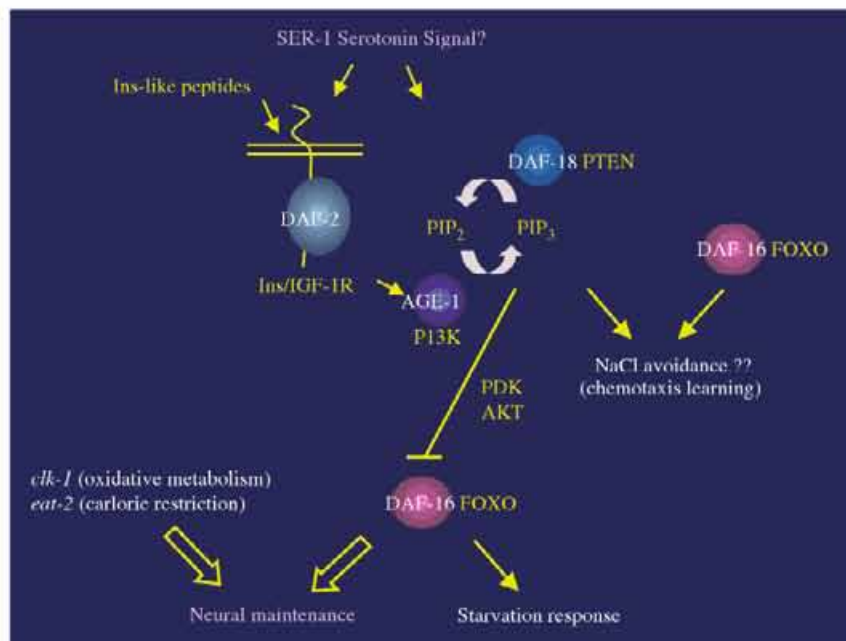


Fig. 25.3 A model for memory regulation by the insulin/IGF-1 pathway. It appears likely that the effects of life extension, or improved neuronal maintenance, lead to an increase in temperature-food association.

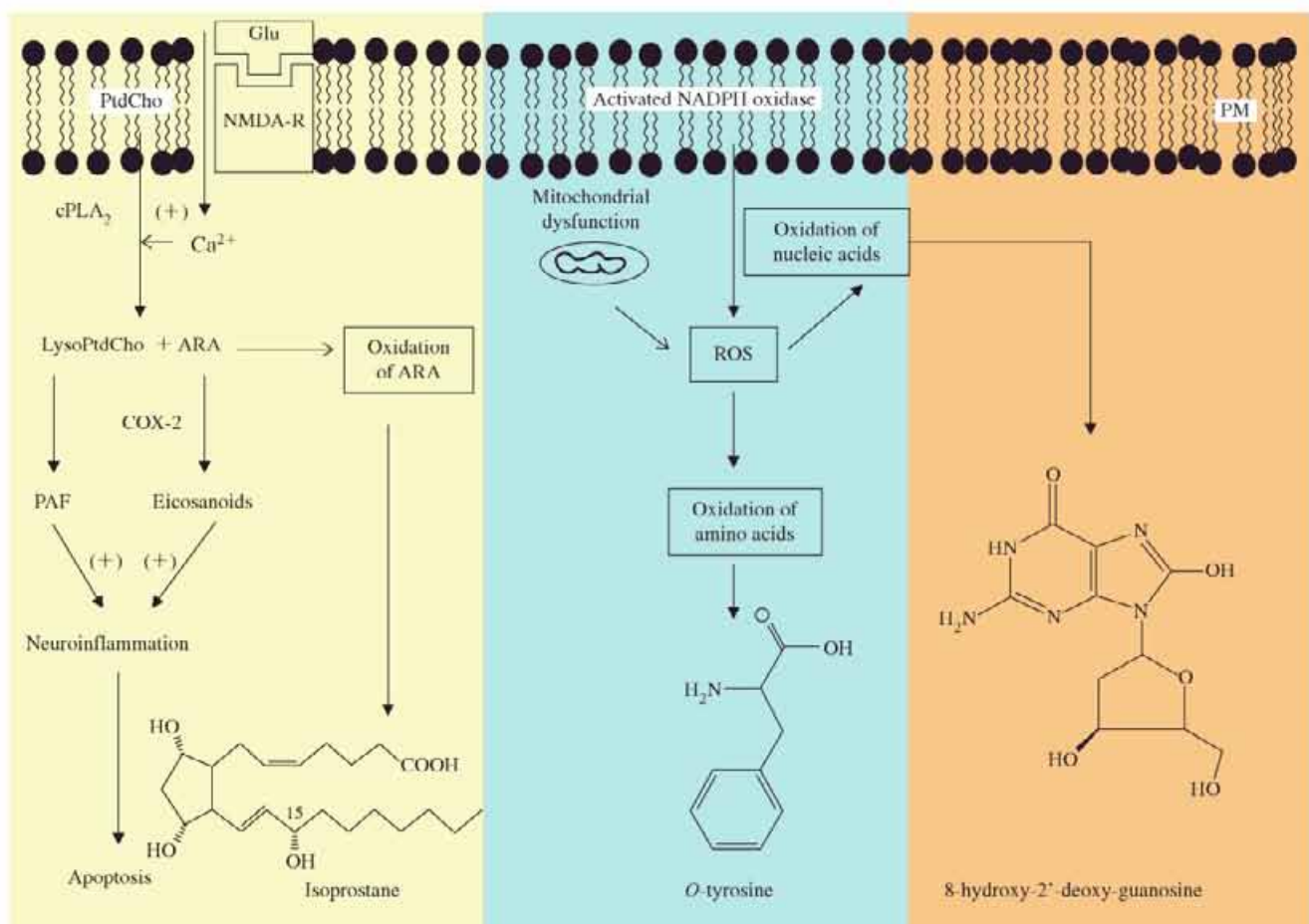


Fig. 27.2 Generation of biomarkers for oxidative stress in vertebrates. PM, plasma membrane; NMDA-R, *N*-methyl-D-aspartate receptor; Glu, glutamate; PtdCho, phosphatidylcholine; lyso-PtdCho, lyso-phosphatidylcholine; cPLA₂, cytosolic phospholipase A₂; COX-2, cyclooxygenase; ARA, arachidonic acid; PAF, platelet-activating factor; ROS, reactive oxygen species.