13

OXIDATIVE STRESS AND MEDICAL SCIENCES

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13.1 OXIDATIVE STRESS IN BIOLOGY AND DISEASE

Helmut Sies, from Dusseldorf, Germany, originally proposed the concept of oxidative stress in the mid-1980s. He defined oxidative stress as "an imbalance between oxidants and antioxidants in favor of the oxidants, potentially leading to damage" (Sies, 2000, p. 102). Under normal circumstances there is an equilibrium between free radical generation (and/or their concentration) and their removal by antioxidants (including the ability of cells to repair oxidative damage). Many pathological conditions and diseases are associated with either an increase in the generation of free radicals or a decrease in antioxidant capacity; sometimes with both conditions. The balance between oxidants and antioxidants lies at the border between health and many human diseases and pathological conditions, including neuronal diseases, inflammation, atherosclerosis, reperfusion injury (these four conditions/diseases are addressed below), iron overload (see Text Box 12.1), carcinogenesis (see Section 12.5), porphyria (see Text Box 13.1) and diabetes (see comments in Chapter 12).

The concept of oxidative stress is not only applicable to humans, laboratory animals, or mammalian cells. A huge number of studies have focused on invertebrate and lower vertebrate free radical toxicology, as well as on plants, fungi, and bacteria. Increased free radical formation prompts an adaptive cell response by mobilizing or augmenting the biosynthesis of endogenous antioxidants. However, under certain circumstances, the antioxidant apparatus is overwhelmed by excess free radical formation. This leads to an oxidative stress condition.

Moreover, oxidative stress is not only the result of a pathologic state. There are several conditions that cause physiological oxidative stress, that is, situations where oxidative damage is part of a natural life cycle of an organism (in some cases, life cannot go on without stress!). Examples are muscle exercise, fertilization, bacterial killing by phagocytes (see Section 13.3), natural aging (addressed in Chapter 12), and arousal from hibernation or dormancy in small rodents and a variety of other animals. Furthermore, as addressed in Chapter 12, reactive oxygen species (ROS) and reactive nitrogen species (RNS) are also of key relevance in signal transduction pathways, in both normal and pathological conditions.

13.2 FREE RADICALS AND NEURONAL DISORDERS

Many studies have correlated oxidative stress with a number of neuronal disorders, including brain damage in Wilson's disease, Friedreich's ataxia, amyotrophic lateral sclerosis (ALS), Parkinson's disease, Alzheimer's disease, and brain ischemia (see Section 13.6.4 for general discussions on ischemia). In all these cases there is evidence for increased levels of biomarkers of free radical damage, provoked by oxygen and nitrogen reactive species. However, each case differs as to how free radicals are generated and how/where they affect the normal cell metabolic homeostasis. Mitochondrial dysfunction may also be linked to neurodegenerative diseases through a variety of different pathways, including increased free-radical generation, impaired calcium buffering, release of apoptotic mitochondrial factors (e.g., cytochrome *C* and apoptosis inducing

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TEXT BOX 13.1 ABOUT PORPHYRINS, MADNESS, WEREWOLVES, AND FREE RADICALS[†]

Acute intermittent porphyria (AIP) and porphyria erythropoietica are two inborn disorders of human metabolism where reactive oxygen species are implicated: oxygen radicals in the former disease and singlet oxygen in the latter. Both diseases prevail in Northern Europeans and result from deficient biosynthesis of enzymes of the heme biosynthetic pathway, porphobilinogen deaminase and protoporphyrin-IX ferrochelatase, respectively. Consequently, 5-aminolevulinic acid (5-ALA) plus porphobilinogen (PBG), two heme precursors, accumulate in tissues of AIP patients and are excreted in their urine. The PBG present in urine gives it a wine color, hence the term "porphyria" (derived from *porphurus*, Greek for *purple*). A high amount of protoporphyrin IX (PP-IX) is deposited in the skin of porphyria erythropoietica patients.

Beginning in the late 1980s, results from Bechara's research group have shown that 5-ALA undergoes iron-catalyzed oxidation by molecular oxygen to yield 4,5-dioxovaleric acid (DOVA), ammonium ions, and H_2O_2. This reaction is propagated by O_2^{-}, and $^{\bullet}OH$ is co-produced via the iron-catalyzed Haber–Weiss reaction. The first evidence connecting 5-ALA with cellular oxidative stress was the observation that ROS generated from 5-ALA aerobic oxidation caused injury to isolated liver mitochondria [see Hermes-Lima et al. (1991). *Biochim Biophys Acta* **1056**:57–63).

$$\underset{\textbf{5-ALA}}{H_2C(NH_2)CO(CH_2)_2CO_2^{-}} + O_2 \rightarrow \underset{\textbf{DOVA}}{HCOCO(CH_2)_2CO_2^{-}} + NH_4^{+} + H_2O_2$$

5-ALA oxidation also induces *in vitro* and *in vivo* oxidative damage to red muscle and liver mitochondria, to liver DNA, and to γ-butyric acid (GABA) receptors of cortical synaptic membranes. 5-ALA may also be intimately involved in iron metabolism since, *in vitro*, it releases iron from ferritin and activates the iron regulatory protein (IRP-1). Also, in liver biopsy samples of AIP patients, the elevated 5-ALA is correlated with iron deposits in hepatocytes. These effects of 5-ALA may produce some of the typical symptoms of AIP syndrome: muscular weakness, primary liver cancer, and neurological dysfunctions (behavior alterations, hallucinations, intense abdominal pain). Interestingly, two famous bloody wars took place in the reign of symptomatic AIP carriers—the American War of Independence during the reign of King George III, the Mad King (1738–1820) of Britain, and World War I, led by the last German Emperor Wilhelm II (1859–1941).

In individuals with porphyria erythropoietica, dermal accumulation of PP-IX causes dramatic cutaneous reddening, inflammation, and blistering in areas exposed to the sun, as well as facial hirsutism. The biochemistry behind these manifestations is attributed to PP-IX-photosensitized formation of singlet oxygen ($^1O_2$), a highly oxidant form of molecular oxygen (see Section 12.1). Porphyrins as well as chlorophyll, rose Bengal, methylene blue, and chlorpromazine act as photoreceptors in this process. Photon absorption by these "dyes" excites them to the singlet (fluorescent) state, which is followed by intersystem crossing to the triplet (phosphorescent) state and energy transfer to colliding oxygen molecules. The acceptor, ground state triplet oxygen, is then promoted to the very electrophilic singlet state. Biological damage driven by the triad of oxygen, dye, and light is called *photodynamic action*. This same principle can be harnessed in methods to kill viruses, fungi, and tumor cells (photodynamic therapy) or to design insecticides and herbicides.

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PP-IX (ground state singlet) + $h\nu$ → PP-IX^{1*} (excited singlet) → PP-IX^{3*} (excited triplet)

PP-IX^{3*} + O_2 (ground state triplet) → PP-IX + $^1O_2$ (excited singlet)

$^1O_2$ + biomolecule targets → chemical and biological damage

People carrying porphyria erythropoietica develop nocturnal habits to avoid sunlight, and a reddish and hairy semblance are thought to be responsible for the creation, in Medieval times, of werewolf and vampire myths. Ironically, in folklore, vampires can be frightened by garlic, a vegetable that is exceptionally rich in thiols, which can offer protection from peroxidation reactions. Perhaps, the immortal vampires don't want to be "cured"!

factor, AIF), and the induction of mitochondrial permeability transition (opening of a calcium-dependent "megapore" recognized years ago as a major player in mitochondrial damage). This can lead to both apoptotic and necrotic cell death.

It is relevant to point out that several discrepancies appear when comparing specific results on neuronal oxidative stress from different laboratories. This may be due to factors such as differences in the length of postmortem intervals before removal of brain samples, or different techniques for sample processing and analysis.

13.2.1 Oxidative Stress in Wilson's Disease, Friedreich's Ataxia, and ALS

13.2.1.1 Wilson's Disease An autosomal-recessive disorder of copper metabolism Wilson's disease results from the absence or dysfunction of a copper-transporting P-type adenosinetriphosphatase (ATPase) that leads to impaired biliary copper excretion and disturbed synthesis of holoceruloplasmin (ceruloplasmin is a blood protein involved in copper storage and detoxification). In addition, copper overload occurs in several organs, including liver and brain. Oxidative stress caused by increased copper levels arises due to Fenton chemistry (see Section 12.1.2) and leads to dysfunction of mitochondrial energetics and cell damage. Plasma copper concentration is also increased in Wilson's disease, affecting the levels of plasma antioxidants. Copper chelation therapy by means of *d*-penicillamine or triethylenetetramine administration is the current form of clinical management of Wilson's disease [other chelators have been proposed, including pyridoxal isonicotinoyl hydrazone (PIH); see Text Box 12.1] and antioxidant-based therapy has been proposed as a complementary form of treatment.

13.2.1.2 Friedreich's Ataxia The most common form of autosomal recessive spinocerebellar ataxia, with an incidence of 1 : 50,000 in the European population, Friedreich's ataxia is often associated with a cardiomyopathy. Friedreich's ataxia derives from a defect in frataxin, a protein involved in regulating mitochondrial iron metabolism. This leads to problems in Fe–S cluster formation in aconitase (a Krebs cycle enzyme) and mitochondrial complex I, as well as faulty cellular bioenergetics, mitochondrial iron overload, and increased free-radical-mediated damage. A recent study has shown significant improvement in muscular and cardiac levels of adenosine 5′-triphosphate (ATP) in patients treated with both coenzyme Q and vitamin E for 6 months.

13.2.1.3 Amyotrophic Lateral Sclerosis (ALS) Neurodegeneration in ALS is characterized by the specific loss of central and peripheral motor neurons. There are several different mutations associated with familial ALS, but about 20% of the cases have a defect in the gene encoding CuZn–SOD, located on chromosome 21. Several mutated forms of CuZn–SOD have been described and, in these cases, neuronal degeneration correlates with loss of CuZn–SOD activity. Increased carbonyl protein, nitrotyrosine and 8-OH-dGua, biomarkers of oxyradical and $ONOO^-$ attack (see Sections 12.4 and 12.5), have been observed in ALS spinal cords. Moreover, there are mitochondrial abnormalities in liver and skeletal muscle biopsies, as well as a decrease in cytochrome oxidase activity in motor neurons, that could be associated with oxidative damage.

The partial loss of CuZn–SOD activity in ALS was formerly thought to be due to increased neuronal oxidative stress caused by a general decrease of antioxidant capacity. This view began to be challenged when CuZn–SOD-deficient mice were found to suffer no neuronal abnormalities, at least when they are young (see Text Box 12.3). However, there is new evidence that several mutant CuZn–SOD isoforms in ALS induce free radical generation due to a peroxidase-like activity (as well as reduced "normal" SOD activity). Thus, this could partly explain the origin of increased free radical formation in ALS. Indeed, recent experiments with mice expressing a

CuZn–SOD mutant of ALS show motor neuron degeneration and mitochondrial damage.

13.2.2 Parkinson's Disease (PD) and Oxidative Stress

Parkinson's disease is a chronic, progressive disorder of the central nervous system that belongs to a group of conditions called motor system disorders. PD is the direct result of the loss of cells in the substantia nigra, an area of the brain (the name of this region is due to the presence of neuromelanin, a black pigment). Usually, loss of 50 to 75% of the substantia nigra's dopaminergic cells triggers PD symptoms.

Parkinson's disease has been known since ancient times. An English doctor, James Parkinson, first described it extensively in 1817. The thoroughness of his analysis is such that researchers and clinicians are still urged to read his original notes on the condition. At least one million people in the United States are estimated to have PD. Many of them, perhaps half, are thought to be undiagnosed (for more information visit www.michaeljfox.org).

13.2.2.1 Involvement of Free Radicals in PD Etiology Much evidence links PD with oxidative stress. Damage to the brain's substantia nigra has been correlated with iron accumulation, increased lipid peroxidation and deoxyribonucleic acid (DNA) oxidation (as determined by 8-OH-dGua), and depletion of GSH. Increased carbonyl protein and advanced glycation end products (AGE, see Section 12.4) were also observed recently in the substantia nigra of PD patients. Elevated iron levels may cause increased free radical formation, which possibly accounts for the decrease in antioxidant capacity and increase in oxidative damage to neuronal cell components. However, the increase in iron concentration in the substantia nigra has been connected recently to the advanced stages of the disease, suggesting that this phenomenon may be a secondary, rather than a primary initiating event. In any case, the loss of iron homeostasis in PD and in other neurodegenerative disorders is a relevant event for neuronal dysfunction. The autoxidation of iron–neuromelanin complexes is proposed to be an important source of ROS in advancing PD.

Other evidence has indicated that L-DOPA (3,4-dihydroxyphenylalanine; a precursor of dopamine) therapy, which is beneficial at the onset of PD, may ultimately be a further cause of oxidative stress and worsening of PD. This might happen because dopamine is a well-known H_2O_2 generator via monoamino oxidase B [MAO-B; see reaction (13.1)]. In the presence of transition metals, H_2O_2 will lead to $^{\bullet}OH$ formation [reactions (13.2)]. L-DOPA and dopamine also react with O_2^- and peroxyl radicals, in the presence of iron, and initiate chains of events leading to oxidative stress. It is possible that the abnormal intracellular iron distribution in substantia nigra cells, when they are still active in dopamine production, could set up a condition for increased free radical formation and oxidative stress. Later events would be cell dysfunction and death, and an overall decrease in dopamine formation and secretion by substantia nigra.

$$\text{Dopamine} + O_2 + H_2O \rightarrow \text{L-DOPA} + H_2O_2 + NH_3 \quad (13.1)$$

$$H_2O_2 + \text{iron} \rightarrow {}^{\bullet}OH \rightarrow \text{cell damage} \quad (13.2)$$

If the oxidative stress hypothesis proves to be right, it is possible that antioxidant-based therapies and the use of effective iron chelators could be of relevance as alternative forms of PD management. Indeed, a different treatment uses deprenyl (a specific inhibitor of MAO-B), which inhibits MAO-B-mediated H_2O_2 formation without effects on dopamine biosynthesis.

13.2.2.2 Toxic Agents, Oxidative Stress, and PD Another hypothesis that links PD with oxidative stress is related to free radical generation by xenobiotic drug metabolism, which could promote (chronically or acutely) oxidative damage to substantia nigra's neurons. One example is 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a heroin substitute of the 1970s, which causes severe substantia nigra destruction and PD symptoms. Moreover, MPP^+, a MPTP metabolite, causes inhibition of mitochondrial ATP synthesis, loss of mitochondrial calcium homeostasis, and increased formation of O_2^- radical (and consequently $ONOO^-$ generation, via reaction with nitric oxide; see Section 12.1.3). Iron and nitric oxide are also involved in the toxic effects of MPTP. Interestingly, many neuroscientists use MPTP to produce animal models of PD.

Thus, several environmental toxins and pesticides could be involved in neuronal damage, which could also target substantia nigra, and produce PD symptoms. Proving these links is a complex process because they not only involve free radical/neuronal biochemistry but also epidemiological surveys correlating PD and toxin exposure. As pointed out by Halliwell and Gutteridge (1999), a currently studied candidate is *n*-hexane, widely used in modern society, which is metabolized to aldehydic neurotoxins (remember that many aldehydes can promote alterations in DNA and proteins; see Section 12.3.2.2 in Chapter 12). Rotenone, a pesticide and a classic inhibitor of mitochondrial electron transport, was recently shown to provoke PD symptoms in rats.

13.2.3. Alzheimer's Disease: The Free Radical Connection

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by severe loss of memory and cognitive functions that affects approximately 4 million persons in the United States alone, with a prevalence of 3 and 18.7% in persons 65 to 74 and 75 to 84 years old, respectively. Between one-third and one-half of the population over 85 years is affected by AD. With an aging society, it is expected that 9 million people will be affected by AD in the United States by the year 2040.

The German doctor Alois Alzheimer made the first clinical and pathological description of an AD case in 1907 when examining a 51-year-old woman suffering from dementia. Upon autopsy, Alzheimer observed massive cerebral cortical neuron loss, the presence of silver-positive neurofibrillary tangles, and alterations subsequently known as senile plaques. All these observations are typical of postmortem analysis of AD brains.

13.2.3.1 Involvement of Oxidative Stress in AD Etiology The search for the molecular etiology of AD has been intense and has included a search for genetic defects, altered amyloid precursor protein (APP) processing (inducing accumulation of amyloid β precipitates; see Text Box 13.2), glutamate neurotoxicity, trace metal toxicity, calcium influx and inhibition of $Ca^{2+}-$ATPase-mediated calcium efflux) and an increase in intracellular calcium. This event causes calcium-dependent activation of nitric oxide synthase (bringing about $ONOO^-$ formation upon reaction of nitric oxide with O_2^-) and uncontrolled activation of calcium-dependent proteases (such as calpain). The loss of calcium homeostasis also contributes to mitochondrial dysfunction, which may increase the normal rates of mitochondrial O_2^- production (leading eventually to $^\bullet OH$ and/or $ONOO^-$ formation). This chain of events could explain the process of neuronal cell dysfunction and death in AD brain [reaction (TB13.2)]. Butterfield's proposal is based on investigations of the oxidizing effects of Aβ peptides in membrane preparations, isolated enzymes, and cell cultures. It was also observed that certain Aβ peptides generate ESR-detectable ROS in simple *in vitro* incubations [see Varadarajan et al. (2000), *J Struct Biol* **130**:184–208].

$$A\beta + O_2 \rightarrow\rightarrow \text{oxidized-}A\beta + \text{ROS}$$

(Possibly a process mediated by transition metals) (TB13.1)

Plasma membrane + ROS

→ damage to membrane protein and lipids

→ loss of Ca^{2+} homeostasis

→ mitochondrial dysfunction → necrosis (TB13.2)

The key question to ask of this oxidative stress hypothesis is: Why is only a certain percentage of aged persons subjected to Aβ-induced toxicity? This leads to other questions: Are there Aβ isoforms (mutant or not) that are more prone to free radical generation? Are there genetic and/or environmental factors contributing to differential neuronal cell vulnerability to Aβ toxicity? Is it possible that if we live long enough, the natural processes connected with aging (including increased free radical brain damage) would make us all AD patients?

George Perry and Mark A. Smith, from Case Western Reserve University, Ohio, proposed a totally different view for the role of the senile plaques (composed mostly of amyloid-β deposition) in AD. They observed, for example, that the percentage of amyloid burden (i.e., the area of Aβ deposition, obtained from autopsy of 22 AD cases) is inversely proportional to the neuronal levels of RNA oxidative damage, measured as cytoplasmic 8-OH-Gua. The authors proposed that Aβ deposition may be a compensatory response against oxidative stress. In other words, Aβ deposition could be regarded

TEXT BOX 13.2 ALZHEIMER'S DISEASE, Aβ, AND OXIDATIVE STRESS

The most common explanation for the onset of AD is the precipitation of amyloid beta peptides (Aβ), leading to neurotoxic fibril formation. Several types of extracellular Aβ are formed from the processing of the amyloid precursor protein (APP). Numerous experimental efforts have been made to understand and inhibit fibril formation. Several researchers have also connected Aβ deposits with the oxidative stress hypothesis of AD. In the mid-1990s D. Allan Butterfield, from the University of Kentucky, proposed an interesting explanation that Aβ itself, independently of its precipitation, may be directly responsible for the free-radical formation [reaction (TB13.1)] and consequent damage to neuronal membrane systems, leading to lipid peroxidation and oxidation of membrane proteins. The presence of increased iron levels may further contribute to the propagation of the peroxidation process, causing release of toxic aldehydes and lipid hydroperoxides to the cytoplasm.

Oxidative damage to the neuronal plasma membrane may lead to a loss of ionic homeostasis (increase in

as a protective response of the brain. This is also supported by the fact that many aged and even middle-aged individuals (with intact cognition) often show extensive Aβ deposits. According to Perry and co-workers, when talking about the current pharmacological approach to AD, "we are playing a dangerous game in focusing efforts on the removal of amyloid-β, which could quite likely have the opposite effect to that promised: of a return to cognition." [Perry et al. (2000). *Lancet* **355**(9205):757, 2000].

In the radical approach to AD by Perry and Smith, aging could be at the center of a system of causes leading to a dysfunctional and damaged brain. Amyloid would be just one of these causes (or even a consequence!). Others include: (a) increased sensitivity to oxidative stress that may be the result of alterations in membrane constituencies, reductions in the synthesis of endogenous antioxidants, and increases in membrane lipid peroxidation and redox active iron; (b) increases in inflammation (a process that also produces ROS and RNS) possibly mitigating the effects of anti-inflammatory agents; and (c) regional alterations in lipid membrane composition, which could have consequences in the membrane fluidity and function of ion pumps and channels (also causing loss of calcium homeostasis). Thus, aging could influence one or more of the causes of AD, predisposing people for AD as they age. For a more complete view of these radical ideas see Joseph et al. [*Neurobiol Aging* **22**:131–146 (2001)] and Perry et al. (*Comp Biochem Physiol C* **133**:507–513 (2002)].

deficit of mitochondrial ATP synthesis, free-radical-induced oxidative stress, and neuronal cell death. The evidence that links AD with free-radical-mediated cell damage derives from increased levels of biomarkers of oxidative stress [thiobarbituric acid reactive substances (TBARS), 4-hydroxynonenal, isoprostanes, carbonyl protein, AGE, nitrotyrosine, 8-OH-dGua, and DNA strand breaks; see Sections 12.3 to 12.5] in brain areas that are typically affected by AD, as compared with age-matched controls. Both mitochrondrial DNA (mtDNA) and nuclear DNA are affected in AD. Decreased levels of several polyunsaturated fatty acids (PUFAs), such as arachidonic and docosahexaenoic acids (which are highly susceptible to lipid peroxidation), are also found in AD brain.

Moreover, glutathione *S*-transferase (GST) activity is reduced in AD brains, suggesting that the capacity to deal with aldehydes and lipid hydroperoxides (via GST-Px activity; see Section 12.2.1.4) is compromised. Mitochondrial energy metabolism is also affected in AD, with reduction in the activity of various components of the respiratory chain, such as cytochrome oxidase (COX activity is decreased by 16 to 38% in the cerebral cortex of AD subjects).

Increased levels of iron were observed in different regions of AD brain (amygdala, hippocampus, and olfactory pathway), in comparison with age-matched controls. The regions where iron was elevated are the ones that show severe degenerative changes in AD. Elevated levels of iron could increase the potential for free radical generation and neuron damage. Increased aluminum ions have also been found in AD brain, even though there is not a consensus among neuroscientists and epidemiologists as to their relevance. Aluminum ions can accelerate lipid peroxidation reactions and thus could be an additional player in the oxidative stress process.

13.2.3.2 Formation of Free Radicals in AD brain What would cause increased free radical formation in AD? The increase in iron concentration could be just part of the story. A great number of studies have focused on the role of amyloid β peptide (Aβ) in AD etiology. It is a soluble component of the plasma and cerebrospinal fluid, derived from APP processing. It has also been recognized that Aβ is the major protein constituent of the senile plaques, present in virtually all AD cases. Genetic studies of early-onset familial AD (FAD; which is a rare disease) offer some evidence for the central role of Aβ in the pathogenesis of AD. Several FAD mutations have been found in the APP gene, which is encoded by chromosome 21. Individuals with Down syndrome also have Aβ deposits, possibly due to overexpression of APP (these persons have three copies of chromosome 21).

There is considerable amount of evidence linking free radical production by Aβ (either soluble or in the fibril precipitated form) and the development of oxidative stress and neuronal damage in AD (see Text Box 13.2). On the other hand, acute inflammatory response (a common phenomena in AD brain), which could be caused by fibrillar Aβ, prompts respiratory bursts of activated phagocytes (see Section 13.3) and generation of nitric oxide and O_2^-. This leads to $^{\bullet}OH$ radical formation via Fenton chemistry, as well as $ONOO^-$ formation. The generation of ROS and RNS may trigger oxidative damage to neuronal membranes, loss of calcium homeostasis, mitochondrial degeneration, and cell dysfunction.

It is likely that AD is associated with multiple etiologies and pathogenic mechanisms (see Text Box 13.2). However, there is solid evidence showing that oxidative stress is an important part of the mechanism of neurodegeneration in AD brain. If so, the use of brain-accessible antioxidants and nontoxic iron chelators could be useful as therapeutic strategies. Indeed clinical trials with vitamin E administration in AD patients resulted in some attenuation of disease development.

13.3 INFLAMMATION AND OXIDATIVE STRESS: AN OUTLOOK

It has long been known that phagocytes are key components of the immune defense system. They are used to kill potentially pathogenic microorganisms such as bacteria and fungi using free radicals as weapons. However, under certain conditions, phagocytes may produce too many free radicals and "unintentionally" cause oxidative stress and damage to nontarget cells. Many disorders have been connected with free radical production by phagocytes.

13.3.1 "Manufacture" of Free Radicals by Phagocytes

Formation of free radicals by activated phagocytes is associated with a sharp and transitory increase in oxygen uptake well known as a "respiratory burst." The respiratory burst was first observed in the 1930s and was associated with increased mitochondrial respiration. Two decades later, it was shown that mitochondria were not directly involved with the respiratory burst because the phenomenon occurred in the presence of cyanide. In 1961, it was shown that activated phagocytes produced H_2O_2 during the respiratory burst, which could be involved in bacterial killing. Subsequently, in the early 1970s (after the discovery of SOD) Bernard Babior and co-workers, from La Jolla, California, demonstrated that activated phagocytes produce mainly O_2^-; hydrogen peroxide formation was just the result of O_2^- dismutation.

Babior also demonstrated that O_2^- formation during the respiratory burst was catalyzed by the FAD-containing enzyme reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase [see reaction (13.3)]. Under resting conditions in phagocytes the enzyme is in a dissociated form that includes both membrane-bound subunits (such as the flavo-cytochrome b_{558}), which are present in vesicles, and cytosolic subunits including p47 and p67. During phagocyte activation, p47 is phosphorylated and this triggers the association of the membrane-bound subunits with the cytosolic ones. The next step is the fusion of the vesicle containing the assembled enzyme with the membrane of the phagocytic vesicles that have engulfed bacterial pathogens, followed by O_2^- production. Patients with chronic granulomatosus disease have phagocytes with dysfunctional NADPH-oxidase and cannot produce O_2^-. These patients are highly susceptible to infections caused by bacteria and fungi.

Formation of O_2^- and H_2O_2 in the phagocytic vesicles triggers $^\bullet OH$ production due to iron-catalyzed Haber–Weiss reactions, and this is part of the weaponry to kill the engulfed bacteria [reaction (13.4)]. Other oxidants are also key players in the bactericide action of phagocytes. Nitric oxide is formed by the induction of nitric oxide synthase (NOS) when phagocytes are activated. The reaction of O_2^- with nitric oxide leads to $ONOO^-$ formation, whereas decomposition of $ONOO^-$ yields $^\bullet OH$ and nitrogen dioxide [$^\bullet NO_2$, also very reactive; reaction (13.5)]. Finally, the heme-containing enzyme myeloperoxidase, which is secreted into phagocytic vesicles, catalyzes the reaction of H_2O_2 with chlorine ions to produce highly reactive hypochlorous acid (HOCl) or the unprotonated form OCl^- [see reaction (13.6)]. Br^- and I^- are also substrates of myeloperoxidase but of much less physiological significance. Moreover, nonenzymatic reactions of HOCl with H_2O_2 or with ammonia yield, respectively, singlet oxygen [$^1O_2$; see reaction (13.7)] and chloramine (NH_2Cl), which is an oxidizing agent more toxic than HOCl:

$$2O_2 + NADPH \rightarrow NADPH^+ + H^+ + 2O_2^- \quad \text{(catalyzed by NADPH-oxidase)} \qquad (13.3)$$

$$2O_2^- + 2H^+ \rightarrow H_2O_2 \,(\text{plus iron}) \rightarrow {}^\bullet OH \qquad (13.4)$$

$$^\bullet NO + O_2^- \rightarrow ONOO^- \,(\text{plus } H^+) \rightarrow ONOOH \rightarrow {}^\bullet OH + {}^\bullet NO_2 \qquad (13.5)$$

$$H_2O_2 + Cl^- \rightarrow H_2O + OCl^- \quad \text{(catalyzed by myeloperoxidase)} \qquad (13.6)$$

$$HOCl + H_2O_2 \rightarrow H_2O + Cl^- + O_2{}^1 + H^+ \qquad (13.7)$$

In summary, an array of oxidizing agents are produced by activated phagocytes, including O_2^-, H_2O_2, $^\bullet OH$, $^\bullet NO$, $ONOO^-$, $^\bullet NO_2$, HOCl (or OCl^-), $^1O_2$, and NH_2Cl. These are the oxidizing agents that should kill bacteria and pathogenic fungi.

13.3.2 Oxidative Stress Induced by Phagocytes

When too many oxidants are produced by phagocytes during inflammatory events (not necessarily related to microbial infection), they can instead have damaging effects. For example, chronic inflammatory processes are linked with an increased risk of carcinogenesis. Several laboratories found that the products of activated neutrophils can induce mutations in the DNA of nearby cells; such mutations can give rise to carcinogenic processes. Generation of several oxidants by inflammatory cells has been linked to oxidation of low-density lipoproteins (LDL) particles and arterial damage in atherosclerosis (see Section 13.4). Oxidative stress in postischemic processes, for example, in heart, has also been linked with increased free radical formation by neutrophils (see Section 13.5). Moreover, the pathophysiology of rheumatoid arthritis (RA), acute respiratory distress syndrome (ARDS), emphysema, and cystic fibrosis is also connected to phagocyte-induced free radical formation. In all these cases, antioxidant-based therapy could be a relevant alternative experimental procedure.

13.3.2.1 Rheumatoid Arthritis (RA) This disease is characterized by chronic inflammation of the synovial tissue, affecting up to 3% of the population in most countries. The increased presence of activated macrophages and neutrophils in the synovial fluid suggests that oxidants formed by those cells could partly account for damage to the joint tissues. Indeed, neutrophils from the synovial fluids of RA patients have augmented *ex vivo* O_2^- production. Moreover, levels of carbonyl protein, nitrotyrosine, and products of lipid peroxidation are increased in the synovial fluid of RA patients, as well as levels of urinary 8-OH-dGua and breath pentane (indicators of systemic increases in DNA oxidation and lipid peroxidation, respectively; see Chapter 12). Furthermore, "free iron" levels are augmented in the synovial fluids of RA patients, creating an environment for increased $^{\bullet}OH$ formation.

13.3.2.2 Acute Respiratory Distress Syndrome (ARDS) A clinical condition characterized by leakage of fluid into the alveoli, mostly caused by shock, ARDS results in damage to the pulmonary endothelium. There is evidence to suggest that oxidants produced by activated neutrophils could be partly responsible for the damage. Indeed, in patients with ARDS, the lungs are full of neutrophils. Phenomenological data support the role of oxidative stress in ARDS, including increased plasma levels of 4-hydroxynonenal and carbonyl proteins and decreased levels of GSH (and increased GSSG; as discussed in Chapter 12, this is evidence for peroxide-mediated oxidative stress) in the alveolar lung fluid.

13.3.2.3 Emphysema In emphysema there is excessive release of elastase (and other proteases) by active neutrophils and inactivation of α1-antiproteinase, a protein that inhibits protease activity. The greatly increased activity of alveolar phagocytes in smokers could cause oxidative inactivation of α1-antiproteinase, which could make the tissue more susceptible to proteolytic damage. Indeed, alveolar fluids from smokers have higher elastase activity and lower α1-antiproteinase activity when compared to controls. A critical methionine residue of α1-antiproteinase is a relevant target for oxidation. This is in agreement with the observation of high levels of methionine sulfoxide (see Section 12.5) in alveolar fluids from smokers.

13.3.2.4 Cystic Fibrosis An an inherited disease, affecting 1 per 2500 Caucasians, cystic fibrosis is caused by a defect in a single protein involved in chlorine transport across epithelial surfaces. The defect has consequences that affect many organs but is seen most dramatically in lung, where it results in severe accumulation of mucus and chronic lung infection. Production of free radicals by activated phagocytes seems to participate in lung tissue destruction. Indeed, neutrophil counts are increased in the lung. Moreover, patients with cystic fibrosis have (i) an augmented plasma concentration of TBARS and dityrosine, (ii) increased carbonyl protein, nitrotyrosine, and free iron in the sputum, (iii) decreased levels of antioxidants in the plasma (ascorbate and β-carotene) and sputum (GSH), and (iv) increased breath pentane levels and augmented urinary excretion of 8-OH-dGua. Furthermore, similar to emphysema, α1-antiproteinase activity is decreased in the lung of cystic fibrosis patients.

13.3.3. More on Microbial Killing: A Radical New Hypothesis

Anthony W. Segal and coworkers, from the University College London, published in March 2002 a new hypothesis for the mechanism of bacterial killing by neutrophils (see Reeves et al. *Nature* **416**: 291–297, 2002) that may change totally our view about this matter. They observed that mice deficient in neutrophil-granule proteases but normal in respect of O_2^- formation are unable to resist staphylococcal and candidal infections. They proposed that O_2^- production in the phagocytic vacuole (leading to other ROS) of activated neutrophils is not the main factor for bacterial killing. The highly increased anionic charge in the vacuoles (caused by massive O_2^- formation) is compensated for by a pH-dependent surge of K^+ ions that cross the vacuole membrane. In their words "the consequent rise in ionic strength engenders the release of cationic granule proteins including elastase and cathepsin G." Under these conditions the proteases are activated, causing bacterial destruction.

13.4 ATHEROSCLEROSIS AND FREE RADICALS: TO BE OR NOT BE?

Cardiovascular diseases (CVD) are the major source of morbidity and mortality in the Western word, claiming nearly 1 million lives per year in the United States alone of an estimated 60 million Americans that have CVD. The principal manifestations of CVD are heart attack and stroke, which represent the clinical end result of a systemic vascular process known as atherosclerosis. This is usually considered a disease of aging. However, excess LDL cholesterol, diabetes, hypertension, and cigarette smoking can provoke premature atherosclerosis.

Thrombosis and lipids have been known to be involved in atherosclerosis since the nineteenth century, based on autopsy studies. This condition is manifested in three stages known as early, developing, and mature lesions. Early lesions are characterized by the presence of nodular areas of lipid deposition, the "fatty streaks," composed of cholesterol-filled cells, as well as smooth muscle cells.

13.4.1 Free Radicals, LDL Oxidation, and Atherogenesis

The free radical hypothesis for atherosclerosis begins with the oxidation of LDL particles, forming oxidized LDL particles (ox-LDL). Some evidence indicates that binding of LDL particles to proteoglycans of the arterial wall precedes LDL modification. The accumulation of ox-LDL particles stimulates the production of proinflammatory cytokines from local arterial cells and leukocytes. Various cytokines and growth factors can activate mononuclear phagocytes that accumulate within the nascent atheroma to produce further cytokines and growth factors that amplify the process. Other protein factors, induced by ox-LDL particles, augment the expression of scavenger receptors on the surface of macrophages, which promote ox-LDL endocytosis (this is actually considered a defense mechanism!) and the further transformation of lipid-filled macrophages to foam cells, the hallmark of the early atheroma. Moreover, primary formation of O_2^- and $^\bullet NO$ by inflammatory cells (see Section 13.3) may prompt $^\bullet OH$ and $ONOO^-$ generation, which induces a "second round" of oxidative stress within the early lesion phase. Foam cells eventually die (possibly by apoptosis), and extracellular lipids may accumulate in the lesions, which are at this point forming fibrous dome-shaped plaques (the *developing lesions*). Later events include calcification and physical disruption of the plaque leading to thrombosis (the *mature lesions*).

The big questions for the hypothesis that oxidative stress participates in atherosclerosis are: Is oxidation of LDL particles *in vivo* indeed a relevant process of early lesions? Which oxidizing agents are involved in LDL modifications? Can antioxidants both inhibit LDL oxidation and minimize atherogenesis?

13.4.2 What Is to Blame for Primary LDL Oxidation?

There is very little doubt that ox-LDL accumulates in human atheromas. However, the precise mechanisms that trigger LDL oxidation are still a matter of intense debate. Aldehyde conjugation in lysine residues has been immunologically detected in lipoproteins (mostly in ApoB, which makes about 95% of the apoprotein content in LDL) in early lesions. These modifications in ApoB make LDL particles recognizable by scavenger receptors in macrophages. As previously discussed (see Section 12.3), aldehydes can be formed as end products of peroxidation of phospholipids, which are present at the LDL surface (the molar ratio PUFA : LDL is about 1300, so there is plenty of oxidizable substrate in LDLs), and react with ApoB. Moreover, biomarkers of lipid peroxidation and protein oxidation, such as isoprostanes, MDA, carbonyl protein, and di-tyrosine, are increased in early lesions.

13.4.2.1 Metal-Catalyzed LDL Oxidation It has been long proposed that oxidation of LDL could be initiated *in vivo* by metal-catalyzed processes. Copper ions are the most active metals in inducing LDL peroxidation *in vitro*, and the reaction is inhibited by chain-breaking antioxidants such as vitamin E, BHT, and probucol (a cholesterol-lowering drug). However, plasma copper is highly regulated and plasma "free copper" concentrations may not be high enough to prompt LDL peroxidation or ApoB oxidation. "Catalytic" iron and copper have been detected in advanced human atheromas, but this may not account for LDL oxidation in early lesions. Moreover, O_2^- can also be formed from NADPH oxidase activity of phagocytes (see Section 13.3) and induce metal-catalyzed $^\bullet OH$ formation and LDL oxidation. The problem with this hypothesis is that the initial oxidation of LDL starts before accumulation of macrophages and other phagocytes. Thus, LDL oxidation might be initiated by oxidizing agents formed from other sources; vascular/endothelial cells seem to be good candidates.

In agreement with the putative role of transition metals in the oxidation of LDL components, the research group of Jukka T. Salonen recently showed a positive correlation between serum ferritin concentration (as a marker of body iron) and plasma levels of cholesterol oxidation products (oxysterols) in 669 Finnish men (see Tuomainen et al. *Free Radic Biol Med* **35**: 922–928, 2003).

13.4.2.2 Nitric Oxide and LDL Oxidation There is some new evidence showing NADPH oxidase activity and O_2^- formation in vascular cells. Thus, the formation of too much O_2^- may prompt $ONOO^-$ formation [$ONOO^-$ degradation also yields $^\bullet OH$ and $^\bullet NO_2$ radicals; reaction (13.5)] due to the reaction of O_2^- with endothelium-generated nitric oxide ($^\bullet NO$). This could promote LDL peroxidation and oxidation/nitration of tyrosine residues in ApoB. Indeed, nitrotyrosine is accumulated in early lesions.

On the other hand, endothelium-generated $^\bullet NO$ is inhibited by atherogenesis and by lipid peroxidation, which causes decreased $^\bullet NO$-mediated control of arterial relaxation and vascular homeostasis. This can be an escalating step toward arterial dysfunction. Probucol, vitamin E, and high levels of ascorbate (as well as L-arginine, a precursor of $^\bullet NO$ formation) seem to increase $^\bullet NO$ bioactivity in vascular endothelium in animal models of atherosclerosis. The use of competitive inhibitors of nitric oxide synthase (such as L-NAME) in rodents can also induce atherosclerotic lesions.

13.4.2.3 Effect of Other Agents on LDL Oxidation Hypochlorous acid may also participate in LDL modification. Phagocyte myeloperoxidase produces the highly oxidizing agent HOCl (see Section 13.3.1), which can attack many protein targets including ApoB. There is evidence for increased 3-chlorotyrosine, a marker of HOCl attack in protein, in early lesions. Another type of LDL modification

that may be atherogenic and proinflammatory is glycation, followed by formation of advanced glycation end products (AGE). This may be especially relevant in diabetic patients.

Oxidation of cholesterol molecules during LDL peroxidation yields several products, including cholesterol hydroperoxides (Ch-OOH) and epoxy-cholestanols, which are toxic to arterial cells. However, the actual *in vivo* role of this process in atherogenesis is still uncertain.

13.4.3 Vitamin E and Atherosclerosis

Several epidemiological studies were designed to verify the role of vitamin E in CVD, but they produced conflicting results. Recently, the double-blind placebo-controlled trials HOPE (with 9541 patients at high risk for myocardial infarction) and GISSI (with 11,324 patients with myocardial infarction) showed that intake of vitamin E supplements for several years had no positive effects in men and women in relation to CVD. On the other hand, the well-known CHAOS study determined that vitamin E supplements reduced the incidence of nonfatal heart attacks in 2000 British patients with established coronary artery disease by 77%. Prospective cohort studies published in 1993, the Nurses' Health Study (with 87,000 female subjects) and the Health Professionals' Follow-up Study (39,000 male subjects) verified an inverse association between coronary artery disease and intake of vitamin E. (Keaney Jr. 200; listed in the Selected References).

There is no clear explanation for the discrepancies among different studies. It is possible, as proposed in 2000 by John Keaney, Jr., from Boston University School of Medicine, that lipid peroxidation plays a modest role in atherogenesis in humans. This radical proposal would explain discrepancies in the effects of vitamin E (dosages of 0.1 to 5 g/kg diet) in many animal studies, where it can inhibit LDL oxidation without affecting the outcome of atherogenesis. On the other hand, probucol usually inhibits both LDL peroxidation and the development of early lesions in animal models.

Another explanation for the disagreements in the epidemiological studies is that in well-nourished populations most individuals are already receiving the maximum benefit of antioxidants through a healthy diet. So, only individuals at "heightened oxidative stress" would benefit from "extra" vitamin E intake. However, this explanation does not account for the positive effects of vitamin E intake detected in the 1993 cohort studies.

13.5 ROLE OF FREE RADICALS IN ISCHEMIA AND REPERFUSION

Ischemia is defined as an arrest of blood flow to a tissue or organs. Of major importance is the disruption of oxygen delivery, but during ischemia substrates are no longer delivered by the blood and waste products cannot be removed. When the interruption of blood flow is prolonged, it can have devastating effects on metabolism and organ functioning. Well-known examples of ischemic damage are myocardial infarction and stunning, stroke, and organ removal for transplantation (see Chapter 12). Certain organs are more susceptible to oxygen-rich blood deprivation than others. For example, a few minutes of ischemia causes irreversible brain damage in mammals, whereas skeletal muscle can recover after much longer episodes.

In the case of heart, coronary occlusion resulting from atherosclerotic plaques or vasospasm results in severe reduction in myocardial blood flow, leading to myocardial cell injury or necrosis. About 1.5 million Americans develop myocardial infarction per year, leading to nearly 200,000 deaths. The severity of the myocardial damage is proportional to the period under ischemia, bringing about diminished and/or fatal cardiac function. Treatment of acute myocardial ischemia, with the aim of restoring blood flow, involves the use of antithrombolytic agents or coronary balloon angioplasty. Although the reestablishment of perfusion is a necessary intervention, and reduces infarct size and overall mortality, it causes a chain of events that produces additional myocardial cell dysfunction, a phenomenon termed *reperfusion injury*. Depending on the length of the ischemic period, reperfusion can be even more damaging than ischemia itself. Interestingly, however, reperfusion injuries were not seen in laboratory animals if deoxygenated perfusate was used (even though such perfusion does not restore organ function!). This "oxygen paradox" remained a mystery to medicine until the free radical ideas appeared in the 1970s.

In the case of brain, stroke is a major cause of serious long-term disability, with about 600,000 Americans suffering a new or recurrent stroke per year. The annual direct and indirect cost of stroke in the United States is beyond $40 billion. Ischemic brain injury is caused not only by a regional incomplete ischemia (ischemic stroke), but also by the consequences of a transient cardiac arrest, leading to global brain ischemia. The high ATP turnover demanded by neuronal tissues and normally supplied by the aerobic oxidation of glucose leads to a very rapid energy depletion when both oxygen and glucose delivery by the blood is disrupted. This is associated with membrane depolarization (loss of Na^+ and K^+ intracellular/extracellular gradients needed for propagation of action potentials) and disruption of calcium homeostasis (loss of calcium gradients), leading to increased cytoplasmic calcium and calcium-activated phospholipases. The restoration of oxygen-rich blood flow may restore aerobic bioenergetics but also brings about neuronal reperfusion injury.

13.5.1 Postischemic Free Radical Generation

It is now recognized that reperfusion injury is associated with the overgeneration of ROS (and RNS as well) result-

TEXT BOX 13.3 MITOCHONDRIAL ROS FORMATION IN PHYSIOLOGICAL AND PATHOLOGICAL CONDITIONS*

Among the many physiological sources of ROS (and RNS), such as eicosanoid metabolism, nitric oxide production, cytochrome P-450 systems, and phagocytosis processes, the mitochondrial respiratory chain is considered to be the most important. In this organelle, it has been estimated that 1 to 4% of the oxygen consumed is deviated into ROS production. However, very recent determinations indicate that under approximate physiological conditions this number may be only about 0.1%.

Early experiments by Alberto Boveris (from Argentina) and Britton Chance (from the United States) suggested that complex I from isolated mitochondria generates H_2O_2 during reoxidation of the complex's flavin mononucleotide (see *Biochem J* **134**:707–716, 1973). In addition, they observed that H_2O_2 production increased with increasing oxygen tension. Superoxide is actually the primary species formed by mitochondria, and it is then dismutated to H_2O_2 either spontaneously or via a SOD-catalyzed reaction. A second proposed site for O_2^- production during mitochondrial respiration is the ubiquinone–cytochrome *bc*1 segment of complex III, which transfers electrons from ubiquinol to cytochrome *c*. Electron transfer within the ubiquinone–cytochrome *bc*1 complex involves a ubisemiquinone radical intermediate [see reaction (12.2.24)], which has been shown to be reductant for O_2^- generation in complex III [see Turrens et al. (1985). *Arch Biochem Biophys* **237**:408–415]. It has also been proposed that other flavin-containing enzymes and iron–sulfur centers in the respiratory chain are sites of ROS production. Superoxide production from mitochondria is regulated by the metabolic state, pO_2, and ADP availability and the rate of ATP formation. Nitric oxide also modulates mitochondrial O_2^- formation by regulating electron flow in the respiratory chain.

The phenomenon of ischemia reperfusion in mammalian organs (see Section 13.5) is associated with a considerable rise in postischemic ROS production. This can be explained by the relatively high oxygen tension during reoxygenation interacting with a fully reduced respiratory chain, leading to increased O_2^- and H_2O_2 formation. Moreover, there is also evidence that iron, and perhaps copper, is released as a free metal during the ischemic phase. Thus, increased postischemic H_2O_2 formation, in the presence of augmented free iron levels, leads to Fenton-mediated $^{\bullet}OH$ formation and oxidative damage to mitochondria and other cell constituents. Oxidative damage to mitochondrial proteins (such as those of the electron chain, F_0F_1-ATPase, aconitase, and the adenine nucleotide translocase), leading to defective ATP formation, has also been linked to neurodegenerative disorders such as Parkinson's and Huntington's diseases.

For many years, mitochondrial ROS formation was considered to be a "physiological defect" in the normal process of electron transfer that results in oxygen reduction to water. This was because mitochondrial ROS formation has been found in all species tested including yeast, plants, invertebrates, and vertebrates. Alterations in aerobic metabolic activity has also been correlated with mitochondrial ROS formation; organisms with high metabolic rates usually produce more mitochondrial ROS than organisms with low metabolic rates. A balance between mitochondrial ROS formation, antioxidant capacity, and membrane phospholipid composition (particularly the unsaturation index) has also been linked with aging.

However, since the mid-1990s, mitochondrial ROS production has been considered to be a relevant source of O_2^- and H_2O_2 for physiological redox signaling pathways and also for apoptotic processes. The release of cytochrome *c* from mitochondria is a primary step in apoptosis, and this release may create a more reduced state of the respiratory electron chain, prompting increased mitochondrial O_2^-/H_2O_2 formation, which is one of the many steps toward apoptotic cell death. Elegant experiments from a University of São Paulo research team showed that isolated mitochondria from yeast mutants that lacked cytochrome *c* show higher H_2O_2 production than wild types. Addition of exogenous cytochrome *c* to isolated mitoplasts significantly diminished H_2O_2 formation [see Barros et al. (2003), *Free Radic Biol Med* **35**:179–188]. It is possible that the "antioxidant" action of cytochrome *c* addition is due to a decrease in electron accumulation at earlier steps of the respiratory chain, thus minimizing O_2^-/H_2O_2 formation from those sites.

For more information on mitochondrial ROS formation (and their effects) we recommend the recent reviews by Enrique Cadenas and Kelvin J.A. Davies (*Free Radic Biol Med* **29**:222–230, 2000) and by Gustavo Barja (*Free Radic Biol Med* **33**:1167–1172, 2002), from Spain.

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TEXT BOX 13.4 POSTISCHEMIC FREE RADICAL DETECTION BY ESR

An elegant demonstration of free radical overproduction during reperfusion of ischemic heart was made in the late 1980s by Garlick and co-workers (*Circ Res* **61**: 757–760). By analyzing the reaction of free radicals with the spin-trap PBN (added to the perfusion solutions), the authors showed no increase in the basal levels of ESR signals of PBN adducts (see information on ESR techniques in Section 12.1) during 15 min of ischemia in isolated rat heart. However, ESR signals increased dramatically within 4 min of reperfusion before decreasing again within several minutes. This study clearly demonstrated that a burst of free radical production takes place during early reperfusion. Interestingly, reperfusion using deoxygenated solutions caused no increase in ESR signal. Many other studies have demonstrated that an increase in lipid peroxidation occurs immediately after reperfusion of ischemic organs.

Direct demonstration of free radical production just after reperfusion was first published in 1987 by Jay Zweier and co-workers, from The Johns Hopkins Medical Institutions (Baltimore, Maryland) (*Proc Natl Acad Sci USA* **84**:1404–1407, 1987) from analysis of ESR signals from isolated rabbit hearts that were deep-frozen under three conditions: control, ischemic (10 min), and postischemic (10 s following 10 min ischemia). The ESR analysis was made in samples that were pulverized under liquid nitrogen (at $-196^{\circ}C$ such a low temperature was used to "freeze" the radical molecules). In samples taken at 10 s following reperfusion, they observed highly increased signals as compared to control and ischemic samples. The ESR

ing in free-radical-mediated damage. Mitochondria are a main cellular site of free radical generation under normal conditions. It is estimated that 1 to 4% of all oxygen consumed by mitochondria is converted into reactive oxygen species. This is caused by "electron leak" at the respiratory chain, primarily leading to O_2^- generation. The greater the reducing state of the respiratory chain, the greater the availability of electrons that can leak from the chain, and the greater the production of O_2^- radicals. This process is also dependent on the oxygen concentration and tension. During ischemic events, the respiratory chain is in a reduced state because there is little or no oxygen that can be converted to H_2O by cytochrome oxidase. During reperfusion, the quick influx of oxygen to ischemic tissues causes overgeneration of ROS (see Text Box 13.3) and overall damage to cell constituents. Mitochondria from several organs/tissues (including brain and heart) seem to be the main site of ROS generation at the onset of reperfusion [see Text Box 13.4 on electron spin resonance (ESR) studies of free radical detection in postischemic heart].

The mitochondrial burst of ROS production during reperfusion can overwhelm existing cellular antioxidant defenses and cause damage to macromolecules including DNA, proteins, and membrane lipids. Moreover, postischemic peroxidation of endoplasmic reticulum causes a further increase in cytoplasmic calcium concentration (as discussed above for the brain, partial loss of calcium homeostasis takes place during ischemia due to disruption of aerobic ATP supply) that can lead to uncontrolled activation of phospholipases and proteases. Calcium activation of nitric oxide synthase (NOS) may also provoke increased formation of $^{\bullet}NO$ and consequently of $ONOO^-$. In mammalian systems undergoing reoxygenation or reperfusion, these free-radical-induced events can lead to severe cell damage, apoptosis and organ failure.

Other important sources of ROS and/or RNS formation in postischemic tissues are the enzyme xanthine oxidase (producing O_2^- radicals, which can be converted to other reactive species) and NADPH oxidase in activated phagocytes (see Section 13.3). There is evidence that allopurinol, an inhibitor of xanthine oxidase, improves functional recovery of the stunned myocardium and reduces infarct size in dogs. Even though this enzyme may be responsible for ROS generation in the canine heart (as well as in mammalian gut and liver, other organs that can be subjected to ischemia and reperfusion), its activity is just too low in human heart to be a relevant player in postischemic oxidative stress. The role of xanthine oxidase in reperfusion injury was originally proposed for intestinal ischemia in the mid-1980s. During ischemia, the breakdown of ATP (due to hypoxic conditions) causes accumulation of hypoxanthine and xanthine, which are substrates of xanthine oxidase. When oxygen is reintroduced to the system, xanthine oxidase may produce higher concentrations of O_2^- and, consequently, of $^{\bullet}OH$ radicals.

There is evidence that activated neutrophils do contribute to ROS/RNS generation and necrosis in postischemic myocardium (but possibly not in myocardial stunning, which is not accompanied by neutrophil accumulation). Moreover, the vascular endothelium can also be a relevant source of ROS/RNS in events of ischemia and reperfusion. As stated by David Lefer and Neil Granger (see Selected References), from Louisiana State University, the vascular endothelium can adopt an inflammatory phenotype that promotes the recruitment and activation of leukocytes in postischemic tissue. This may promote a second and

TEXT BOX 13.5 NATURE'S FACULTATIVE ANAEROBES—HOW DO THEY DEAL WITH REOXYGENATION-INDUCED OXIDATIVE STRESS?*

Although alien to mammalian life, many invertebrates and cold-blooded vertebrates are excellent facultative anaerobes that experience natural situations where oxygen availability to their tissues varies widely. Many can live without oxygen for days, weeks, or even months at a time. As discussed in Chapter 15, this includes various freshwater turtles that hibernate underwater, some fish that experience seasonal oxygen depletion of the waters in which they live (including cold temperate waters or in warm ponds in the Amazon jungle), and many gill-breathing intertidal marine mollusks that must endure oxygen deprivation during each aerial exposure at low tide. Other organisms experience not just anoxia but also ischemia. With each freezing exposure, freeze-tolerant animals endure extended periods of ischemia due to the freezing of blood plasma and all other extracellular body fluids (see Chapter 17). Hundreds of species of insects, many intertidal marine invertebrates, and various amphibians and reptiles living in seasonally cold climates have developed freeze tolerance. These anoxia- and ischemia-tolerant animals express a variety of biochemical adaptations that sustain their survival during oxygen deprivation, one of these being antioxidant protection.

In the early 1990s, at Carleton University, Ottawa, we hypothesized that hypoxia/anoxia-tolerant species should also have effective antioxidant defenses to deal with the oxidative stress that might occur with the reintroduction of oxygen into their tissues. After days, weeks, or months without oxygen, its sudden reintroduction should create a danger of ROS overgeneration and oxidative damage to cells, much like the reperfusion stress experienced by mammalian organs. The big difference was that these animals readily survive the stress of oxygen reperfusion, and, hence, they constitute good model systems for trying to assess the factors that prevent or minimize reperfusion stress.

We observed that in several cases, the activity of antioxidant enzymes (usually catalase, SOD, or selenium-dependent GPx) and levels of GSH were increased in the organs of anoxia- or freeze-tolerant species while under oxygen deprivation or freezing exposure. That was the case in (i) red-sided garter snakes *Thamnophis sirtalis parietalis* and wood frogs *Rana sylvatica* during freezing (at −2.5°C for 5 and 24 h, respectively), (ii) goldfish *Carassius auratus*, garter snakes, leopard frogs *Rana pipiens*, freshwater snails *Biomphalaria tenagophila*, and marine snails *Littorina littorea* under anoxia (for 8, 10, 30, 24, and 144 h, respectively), and (iii) leopard frogs under dehydration stress at 5°C (50% loss of body water after 92 h, a condition that imposes great restriction on blood flow to internal organs). We also observed that red-eared turtles *Trachemys scripta elegans* increase the activity of alkyl hydroperoxidase reductase (an enzyme that decomposes lipid hydroperoxides) and GSH-synthetase in some organs after 20 h of underwater anoxia. Moreover, the activity of tissue antioxidant enzymes (and levels of GSH) was generally higher in the freeze-tolerant wood frog when compared to freeze-intolerant frogs; anoxic-tolerant turtles also showed high constitutive tissue levels of antioxidant enzymes and GSH when compared to nontolerant vertebrates. Similar observations were made in the mid-1990s by Margaret Rice (New York University) when comparing ascorbate concentration in the brain of anoxic-tolerant and nontolerant vertebrates: The anoxic-tolerant species showed higher ascorbate levels. [Rice et al. (1995) *J Neurochem* **64**:1790–1799].

These data on increased antioxidant content during anoxia/freezing exposure [see for example: Hermes-Lima and Storey (1993) *Am J Physiol* **265**: R646–R652; Lushchak et al. (2001) *Am J Physiol* **280**: R100–R107] could be considered an apparent paradox. Why would antioxidant defenses be improved (apparently by enzyme synthesis) in tissues under conditions of low or zero oxygen when ATP availability for protein synthesis is at a premium? Under these situations there would be too little ROS formation to turn on signal transduction mechanisms that normally lead to the biosynthesis of antioxidant enzymes and GSH. We still do not know the answers to this paradox, but we have a reasonable physiological explanation. It appears that anoxia/freeze-tolerant species prepare for the eventual return of oxygen and the oxidative stress that it will cause by up-grading their antioxidant defenses while under low oxygen conditions. This prepares organs with enhanced protection against potential ROS overgeneration that can occur immediately when oxygen-rich blood flow resumes (i.e., during reoxygenation after anoxia, thawing after freezing, or rehydration after dehydration). Indeed, measurement of lipid peroxidation products during these recovery processes generally show very little sign of the production of damage products as a result of oxidative stress. Thus, although there is still much to be learned, it appears that well-developed antioxidant defenses are an integral part of natural anoxia and ischemia tolerance. For a more

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comprehensive view of the role of ROS and antioxidants in anoxic/hypoxic-tolerant species, read Hermes-Lima and Zenteno-Savín (2002) listed in the Selected References.

more prolonged phase of free radical generation (the first phase being the free radical burst right after reoxygenation) and consequently of oxidative damage.

13.5.2 Antioxidants versus Reperfusion Injury

Several studies have shown that addition of antioxidants to perfusion solutions can have beneficial effects against reperfusion injury in heart, kidney, and brain. This is a relevant strategy because endogenous antioxidant capacity can be overwhelmed by oxyradical overgeneration during reoxygenation. Moreover, in some cases, the activities of certain endogenous antioxidant enzymes are actually decreased during ischemia in mammalian organs. On the other hand, there are many invertebrates and cold-blooded vertebrates that are highly tolerant of extended anoxia exposure (Chapter 15) and reoxygenation. Interestingly, these typically display high antioxidant defenses, either constitutively or induced by anoxia exposure (see Text Box 13.5).

Addition of antioxidants after the onset of reperfusion is, however, without much effect. This is because overproduction of free radicals has already occurred. Several antioxidants are capable of reducing postischemic oxidative stress in heart, brain, and kidney; examples are SOD, polyethylene glycol bound SOD (PEG-SOD has longer plasma half-live than free SOD), SC-52608 (a low-molecular-weight SOD mimetic), U74006F (a chain break antioxidant), the oxyradical scavengers dimethylurea and *N*-2-mercaptopropionyl glycine, and the iron chelator deferoxamine. The efficacy of deferoxamine demonstrates the involvement of transition metal ions in free radical production during reperfusion, possibly via Fenton reactions. Delocalization of iron during ischemia in several organs, such as kidney (the cause is still under debate), seems to be of relevance for iron-mediated oxidative stress and cell damage during reperfusion.

Many studies that have administered exogenous antioxidants for myocardial infarction have had disappointing results. Similarly, not all studies of antioxidant effects on brain ischemia/reperfusion have shown neuroprotection. One reason for the variability may be the ability (or not) of the antioxidants to gain access to the sites of free radical production during reperfusion. On the other hand, the use of exogenous antioxidants and allopurinol has been beneficial in improving the efficiency of experimental organ transplants, especially kidney, after cold (0 to 5°C), hypothermic (5 to 30°C), or warm ischemic storage (37°C) (see Chapter 19). Cold or hypothermic storage are known to prolong kidney metabolic viability by delaying ATP depletion and the loss of calcium homeostasis.

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