ROS

REVIEW ARTICLES

Free Radicals: From Health to Disease

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ABSTRACT | Over the past 40 years, there has been a tremendous amount of research on the dual role of free radicals as both toxic and beneficial species. Free radicals are produced as by-products of normal cellular metabolism or generated by chemicals in our external environment (e.g., cigarette smoke, air and water pollution, exposure to sunlight, gamma-irradiation, and certain chemotherapeutic drugs). At low to intermediate concentrations, free radicals exert their effects through regulation of cell signaling cascades. At high concentrations, they damage all macromolecules, inducing DNA damage, lipid peroxidation, protein modification, and eventually cell death. Free radicals have been implicated in the pathogenesis of a number of conditions, such as aging, atherosclerosis, ischemic heart disease, cancer, and Alzheimer's disease. Aerobic organisms have evolved sophisticated antioxidant systems to protect themselves from cellular damage and death caused by free radicals. The ability to estimate chemical biomarkers of free radical damage in body fluids and tissues is an important step in understanding the mechanisms contributing to disease processes. This review of a large amount of research studies discusses formation of free radicals in normal cells, their basic properties, toxic effects on cellular processes, potential beneficial role in signaling and phagocytosis, and the part the oxidative stress plays in some major disease processes.

KEYWORDS | Antioxidants; Free radicals; Oxidative stress; Reactive oxygen species; Redox signaling

ABBREVIATIONS | CVD, cardiovascular disease; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, reduced form of glutathione; GSSG; glutathione disulfide; HNE, 4-hydroxy-2-nonenal; MDA, malondialdehyde; MPO, myeloperoxidase; NO, nitric oxide; NOS, nitric oxide synthase; 8-OH-dG, 8-hydroxy-2-deoxyguanosine; PUFA, polyunsaturated fatty acid; RNS, reactive nitrogen species; ROS, reactive oxygen species; SOD, superoxide dismutase

CONTENTS

1. DEFINITION OF FREE RADICAL

A free radical is any chemical species that contains a single (unpaired) valence electron in the outermost electron orbital. Free radicals are produced when the covalent bond is broken and one electron from each pair remains with each atom. A free radical is an unstable configuration and therefore highly reactive, owing to the tendency of electrons to pair. Free radicals can either donate an electron to or accept an electron from other molecules, therefore behaving as oxidants or reductants. A free radical lasts just for a

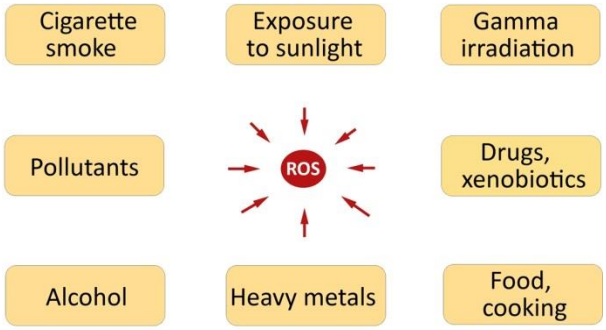

FIGURE 1. Exogenous sources of free radicals/ROS. As illustrated, a number of environmental factors contribute to the production of free radicals and ROS in biological systems.

few milliseconds, because it immediately reacts with adjacent molecules, such as proteins, nucleic acids, or lipids. When a free radical reacts with a non-radical molecule, a chain reaction is initiated, whereby the non-radical molecules are themselves converted into free radicals. The chain reaction is terminated when two free radicals react with each other and cross-link two unpaired electrons forming a covalent bond, with each radical contributing its single unpaired electron [1, 2].

2. OXYGEN: A DANGEROUS FRIEND

Free radicals of most concern in biological systems are derived from oxygen. Oxygen is an element indispensable for aerobic life. Cells require oxygen to generate energy in the mitochondrial electron transport chain. This need for oxygen obscures the fact that oxygen is a toxic gas that produces oxygen radicals during the ATP synthesis in mitochondria. About 90% of the oxygen taken up in the lungs is utilized by the mitochondria to produce ATP. The reminder (~10%) of oxygen is used in metabolism by various oxidizing enzymes, which catalyze oxidation of diverse chemical compounds, i.e., combination of compounds with oxygen. It is worth mentioning that some anaerobic bacteria survive by hiding in environments into which oxygen does not penetrate (e.g., pockets of gums, in deeper layers of dental plaque, or in colonic fecal content). These bacteria have insufficient antioxidant defenses to protect against oxygen free radicals. They can often be killed by exposure to 21% oxygen, the atmospheric level [2, 3].

3. PRODUCTION OF FREE RADICALS IN CELLS

Free radicals are generated from either endogenous or exogenous sources. Exogenous free radicals result from cigarette smoke, air and water pollution, pesticides and herbicides, exposure to sunlight, alcohol, heavy metals, certain chemotherapeutic drugs (e.g., doxorubicin, cyclosporine, tacrolimus), industrial solvents, cooking, and gamma-irradiation (**Figure 1**). But the biggest source of free radicals is our own bodies. Free radical formation occurs continuously in the cells as a consequence of both enzymatic and nonenzymatic reactions. Enzymatic reactions, which serve as sources of free radicals, include those involved in the respiratory chain in mitochondria, cytochrome P450 system in endoplasmic reticulum, oxidative reactions in peroxisomes and during phagocytosis, as well as transition metal ion-catalyzed reactions (**Figure 2**).

3.1. Mitochondria

The most important source of free radicals is the mitochondrion. During the energy transduction in the electron transport chain, a small number of electrons (about 1–3%) leak to oxygen prematurely forming the radical superoxide. Superoxide is released into mitochondrial matrix. The production of superoxide by mitochondria may contribute to damage to mitochondrial proteins, lipids, and DNA. Mitochondrial lipids damaged by free radical reactions form cross-linked compounds that accumulate in the form of yellow-brown lipofuscin pigment granules [4, 5].

3.2. Endoplasmic Reticulum

Cytochrome P450 mixed function oxidase enzymes of endoplasmic reticulum are involved in the oxidation of a wide range of substrates at the expense of oxygen. Oxidation is any chemical reaction in which a material gives up electrons, as when the material combines with oxygen. The result is a compound called oxide. Oxidases are capable of reducing oxygen to superoxide before using it in oxidizing operations. Some superoxide always diffuses away from

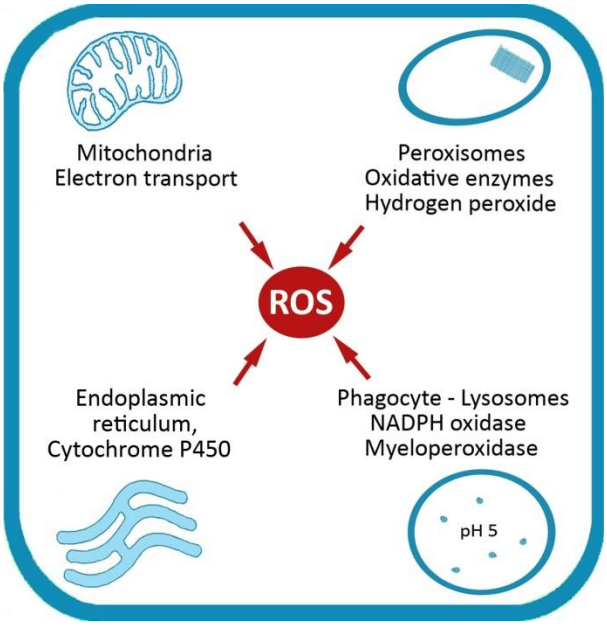

FIGURE 2. Endogenous sources of free radicals/ROS. As depicted, the main endogenous sources of free radicals and ROS include mitochondrial electron transport chain, cytochrome P450 enzyme system associated with endoplasmic reticulum, peroxisomes, and oxidases in phagocytic cells.

the enzyme before it is used in the chemical reaction to oxidize the substrate [6, 7].

3.3. Peroxisomes

Peroxisomes are membrane-enclosed organelles that contain enzymes involved in a variety of oxidative reactions. A variety of organic molecules, such as fatty acids, amino acids, and uric acid, are broken down by a process of oxidation to produce hydrogen peroxide. These organelles are called peroxisomes because they produce large amounts of hydrogen peroxide. To scavenge hydrogen peroxide produced by these reactions, peroxisomes possess an appreciable amount of catalase [8] (also see Section 10.2).

3.4. Phagocytosis

Phagocytosis that takes place in neutrophils and macrophages is associated with an oxidative burst that releases large amounts of free radicals. These reactive radical species serve as important components of defense mechanism against invading pathogens. Bursts of free radicals are produced through a controlled reaction and are used to kill the phagocytized bacteria [9, 10].

3.5. Transition Metals

Transition metals, such as iron and copper, are important in biology. Approximately 30% of enzymes use metals because they facilitate enzyme catalysis. Metalloproteins that contain a metal ion cofactor include hemoglobin, myoglobin, transferrin, ferritin, hemosiderin, catalase, and cytochrome c, among many others.

Transition metals, including iron and copper, can undergo autoxidation and change valence. For example iron exists in two oxidation states: ferrous state (Fe^{2+}) and ferric state (Fe^{3+}). Copper also exists in two oxidation states: cuprous (Cu^{1+}) and cupric (Cu^{2+}) states. In normal cells, iron and copper ions are typically not found in a free state, but are tightly bound to proteins. The tightly bound iron is transported (as in transferrin) or stored (as in ferritin) to discourage redox cycling. In this context, free iron is considered a loose cannon, chemically. When ferrous iron is converted into ferric iron, it donates one electron, and vice versa. However, because of its ability to redox cycle between Fe^{2+} and Fe^{3+}, iron may promote the formation of hydroxyl radical via the Fenton reaction ($H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^{\bullet} + OH^{-}$). Transition metal ions in unbound forms are potentially devastating since they catalyze unwanted free radical formation [11–14].

3.6. Nitric Oxide

Nitric oxide (NO) is a free radical, i.e., its structure includes an unpaired electron. Cells produce NO from the amino acid L-arginine by the enzymatic action of nitric oxide synthase (NOS). NO is known to play important functional roles in a variety of physiological systems. It is a vasodilator produced by endothelial cells, signaling molecule generated by neurons, and bactericidal agent produced by macrophages and other inflammatory cells during infection. NO reacts with superoxide to produce the damaging oxidant peroxynitrite ($ONOO^{-}$). Peroxynitrite is able to induce cell necrosis and apoptosis and

acts as a terminal mediator of cellular injury in various pathophysiologic conditions [15–20].

4. REACTIVE OXYGEN SPECIES

There are two major free radical groups: (1) oxygen free radicals and (2) nitrogen free radicals. Reactive oxygen species (ROS) is a collective term used to denote oxygen-derived free radicals and related reactive species. ROS represent the most important class of free radicals and related reactive species generated in the body. The most important ROS include superoxide radical ($O_2^{\bullet -}$), hydroxyl radical ($OH^{\bullet}$), and hydrogen peroxide (H_2O_2). Hypochlorous acid (HOCl) is another important oxygen-derived reactive species (also see Section 11.2).

4.1. Superoxide

Superoxide is the main precursor of most ROS in biology. It is formed when only a single electron is added to the diatomic oxygen molecule. It is considered the primary ROS and can interact with other molecules to generate secondary ROS. Superoxide is produced mostly in mitochondria, caused by electron leak from the respiratory chain. It is also a product of oxidation reactions. Despite its 'super' name, superoxide seems relatively unreactive compared with many other free radical species. But, irrespective of its poor reactivity, superoxide is able to damage some important enzymes of energy metabolism (i.e., Krebs cycle) and amino acid biosynthesis. The oxidized enzymes have to be repaired to maintain their activity. Superoxide does not attack DNA directly. Superoxide generates other secondary radical species by participating in the so-called iron-mediated Haber-Weiss reaction ($H_2O_2 + O_2^{\bullet -} \rightarrow O_2 + OH^{\bullet} + OH^-$). In the Haber–Weiss reaction, superoxide reacts with hydrogen peroxide forming hydroxyl radical ($OH^{\bullet}$) and hydroxide anion (OH^-). Superoxide dismutates spontaneously to hydrogen peroxide ($2\ O_2^{\bullet -} + 2\ H^+ \rightarrow H_2O_2 + O_2$) [1, 21–23].

4.2. Hydrogen Peroxide

Hydrogen peroxide is a major biological ROS, excess of which can cause damage to cells and tissues. It is a by-product of respiration and an end-product of a number of metabolic reactions, particularly peroxisomal oxidation pathways. Dismutation of superoxide formed in the electron transport chain forms hydrogen peroxide. Peroxisomes are another organelle known to produce hydrogen peroxide. Hydrogen peroxide is produced continually in all tissues and can be detected in the exhaled breath as well as the urine [1].

Hydrogen peroxide is a relatively weak ROS per se, but gives rise to more damaging ROS. Production of hydrogen peroxide is tightly regulated in the cells, since a slight increase in its level may lead to cytotoxicity. Perhaps the most common reaction that hydrogen peroxide participates in is the Haber–Weiss reaction to produce hydroxyl radical (see Section 4.1). The O-O bond in hydrogen peroxide is relatively weak and is susceptible to dissociation when hydrogen peroxide is subjected to heating, ionizing radiation, ultraviolet radiation, or transition metals. This gives rise to the hydroxyl radical, which is responsible for many of the strong oxidizing (or disinfecting) actions of hydrogen peroxide. In contrast to superoxide and hydroxyl radical, the less reactive hydrogen peroxide is involved in a wide variety of biological processes, such as signal transduction, cell differentiation and proliferation, and immune responses. Hydrogen peroxide is a major factor implicated in the free radical theory of aging. According to the theory, free radicals provoke oxidative damage, and this is the cause of aging. There exists a close association between ROS generation, ROS-related damage, and aging. The classical physiological role attributed to hydrogen peroxide is its capability to induce bacterial killing. Hydrogen peroxide in conjunction with the amplification activity of myeloperoxidase is responsible for bacterial killing within the phagosome in the neutrophil cytoplasm [24–28] (also see Section 11.2).

4.3. Hydroxyl Radical

Hydroxyl radical is the most biologically active free radical. This species is much more reactive than superoxide, making it a very dangerous radical. Hydroxyl radical can damage virtually all types of macromolecules, including nucleic acids, lipids, and amino acids. Hydroxyl radical has a very short in vivo half-life of approximately 10^{-9} seconds and a high reactivity. This makes it a very dangerous species to the organism [1]. It should be noted that hydroxyl radical is distinct from hydroxyl or hydroxide ion

(OH^-), which is not a radical. Hydroxyl radical is formed from the reaction between ferrous iron and hydrogen peroxide (the Fenton reaction; see Section 3.5). Ultraviolet (UV) radiation of the skin generates ROS such as superoxide, hydrogen peroxide, and the hydroxyl radical. Hydroxyl radical is produced by UV-induced splitting of hydrogen peroxide (gamma-radiation + $H_2O_2 \rightarrow 2\ OH^{\bullet}$). Gamma-radiation, the most dangerous form of ionizing radiation, is high-energy radiation. It passes through the human body, like a microscopic bullet, until the radiation is stopped by the tissues due to absorption. The first consequence of ionizing radiation is ionization of water. Since water represents 70% of the chemical composition of the adult body, its chemical transformation by ionizing radiation merits serious consideration. Gamma-radiation splits water molecules producing hydroxyl radical and hydrogen atom (gamma-radiation + $H_2O \rightarrow OH^{\bullet} + H^{\cdot}$). Of the ROS, the highly reactive hydroxyl radical destroys biologically active molecules by either removing electrons or removing hydrogen atoms. Cellular DNA, proteins, and lipids can all be damaged by hydroxyl radical. This may lead to damage to cell membranes, mitochondria, nucleus, and chromosomes, that either inhibits cell division, results in cell death, or produces a malignant cell [29, 30].

4.4. Hypochlorous Acid

Hypochlorous acid (HOCl) in biological systems is produced by a heme protein, namely, myeloperoxidase, which converts hydrogen peroxide to hypochlorous acid in the presence of chloride ion ($H_2O_2 + Cl^- \rightarrow HOCl + OH^-$) [1]. Myeloperoxidase is stored in azurophilic granules of neutrophils. The powerful bactericidal properties of hypochlorous acid have been well documented. Hypochlorous acid is the active ingredient in household bleach and the species responsible for the antibacterial properties of chlorinated water supplies [31–33].

5. REACTIVE NITROGEN SPECIES

5.1. Nitric Oxide

Nitric oxide (also see Section 3.6) is a colorless gas. As noted earlier, it possesses an unpaired electron making it a reactive free radical. NO is generated in cells by the action of the enzyme NOS which catalyzes the production of NO from L-arginine [1]. NO is an abundant chemical that acts as an important biological signaling molecule in a variety of physiological processes, such as smooth muscle relaxation, neurotransmission in the enteric plexus, neurotransmission in the central nervous system, defense mechanisms, and immune regulation. NO relaxes smooth muscle cells in arterioles during systole, with resultant vasodilation and blood pressure regulation. NO serves as an inhibitory neurotransmitter in the gastrointestinal tract and mediates relaxation of both circular and longitudinal muscle layers. NO serves as a neurotransmitter in areas of the brain that are specialized in cognition. Last but not least, NO produced in macrophages during inflammation is central in killing of engulfed microorganisms within phagolysosomes. Many of the reported toxic effects of NO are more likely mediated by its oxidation products rather than NO itself. NO may reversibly inhibit some enzymes such as cytochrome P450 and ribonucleotide reductase, but it generally does not directly attack DNA. The cytotoxic action of NO results largely from its oxidation product—the highly reactive peroxynitrite (see Section 5.3).

5.2. Nitric Oxide Synthase

There are three types of NOS: endothelial (eNOS), neuronal (nNOS), and inducible (iNOS). Endothelial and neuronal NOS are constitutively expressed in endothelial cells and neurons, respectively. Inducible NOS is not expressed constitutively but is induced by pro-inflammatory cytokines and lipopolysaccharide (LPS) in macrophages in sites of inflammation. iNOS and nNOS are soluble and found predominantly in the cytosol, while eNOS is associated with cell membranes. Both eNOS and nNOS synthesize NO, a signaling molecule on demand, for short periods of time (seconds to minutes) following enzyme activation. In contrast, iNOS is expressed after cell activation only and then produces NO for comparatively long periods of time (hours to days). The long duration of NO production makes it a cytotoxic rather than physiological molecule. iNOS activity may play a detrimental role in experimental autoimmune or chronic inflammatory processes. For example, overproduction of the vasodilator NO contributes to hypotension and vascular hyporeactivity to the vasoconstrictor agents during septic shock.

5.3. Peroxynitrite

Exposure of cells to excess NO can cause toxic effects, which are due to production of secondary reactive nitrogen species (RNS). NO reacts with superoxide producing peroxynitrite (also see Section 3.6). Peroxynitrite is synthesized when cells produce large amounts of both NO and superoxide ($NO + O_2^{\bullet -} \rightarrow ONOO^-$). iNOS can make substantial concentrations of NO when expressed. Peroxynitrite is also a strong oxidant but reacts relatively slowly with most biological molecules, which makes it unusually selective as an oxidant. Peroxynitrite is able to cross biological membranes and diffuse one to two cell diameters. This allows it to interact with most critical biomolecules, such as DNA, proteins, and lipids. Peroxynitrite reacts with lipids causing lipid peroxidation and with DNA causing DNA fragmentation. Peroxynitrite reacts with the tyrosine residues in proteins converting them to 3-nitrotyrosine. Nitration of proteins leads to enzyme inactivation. Tissue levels of 3-nitrotyrosine are widely used as a biomarker of peroxynitrite generation. Elevated levels of 3-nitrotyrosine have been observed in a vast range of diseases [15, 34–37].

6. CONSEQUENCES OF OXIDATIVE STRESS

In healthy organisms, production of free radicals is approximately balanced by antioxidant defense system. This balance is not perfect. Cells cannot eliminate reactive oxygen and nitrogen species (ROS/RNS) completely since free radicals are useful in controlled amounts. Oxidative stress refers to an imbalance between oxidants and antioxidants due to either increased oxidants or decreased antioxidants, or both. Cells exposed to oxidative stress are relatively unable to detoxify the reactive intermediates or to repair the resulting damage. Oxidative stress can result in adaptation or oxidative damage. Mild oxidative stress can result in adaptation. In this case, cells are able to cope with elevated concentrations of free radicals because of increased synthesis of endogenous antioxidants.

Oxidative stress adaptation is an important mechanism by which cells are able to accommodate with shifts in the level of oxidative stress. Body cells are exposed to a degree of oxidative stress that is not static but shifts with changes in environment, metabolism, diet, and age. Cells adapt to temporary changes in stress levels, through changing enzyme activity and overexpressing of a large number of protective genes. The adapted cell is then significantly more resistant to oxidative stress. If the stress is reduced to a lower level, cells gradually lose their previous adaptation [38].

Experimental work has demonstrated that adapted cells respond differently to single acute stress exposures. Initially, cells are exposed to a single, mild, non-toxic, dose of an oxidant such as hydrogen peroxide, then allowed to adapt for a period of time. The cells are then re-exposed to a much higher dose of the oxidant, which would normally be toxic without the pretreatment and adaptive period. Pretreated cells become considerably more resilient to the toxic challenge level of oxidative stress compared to non-pretreated cells [38].

Oxidative stress leads to potential cell damage. Such damage is called oxidative damage. It is noteworthy that the terms ‘‘oxidative stress’’ and ‘‘oxidative damage’’ are not synonymous. Oxidative damage usually occurs when important biological molecules, such as DNA, proteins, or cell membrane lipids, are robbed of their electrons and suffer chemical modification that can lead to cellular dysfunction. Oxidative damage occurs continuously in every cell. Examples of oxidative damage include DNA strand breakage, lipid peroxidation, and protein oxidation. Oxidatively damaged molecules can be repaired or replaced. Cell death (apoptosis, necrosis) occurs when oxidative stress is severe [39–41].

7. OXIDATIVE DNA DAMAGE

Of the ROS, hydroxyl radical, the most potent one, reacts with DNA by addition to double bonds of DNA bases. Oxidation damage of DNA matters more than that of other macromolecules because it can lead to genetic mutations associated with cancers. The guanine base is highly susceptible to oxidative stress. The remaining nucleotides are much harder to oxidize [40]. The most extensively studied DNA lesion is the formation of 8-hydroxy-2'-deoxyguanosine (8-OH-dG). 8-OH-dG is formed after hydroxyl radical addition to C8 of guanosine. 8-OH-dG is the most extensively studied DNA lesion. The levels of this guanine analogue increase with oxidative stress. Once formed, 8-OH-dG is a major pre-

mutagenic lesion. Further oxidation of 8-OH-dG opens the imidazole ring of the guanine (guanine is a purine) to give rise to a pyrimidine such as thymine. During the cell division and DNA replication, thymine pairs with adenine. As a consequence of this miscoding, the pair GC (guanine-cytosine) is replaced by the pair TA (thymine-adenine). If it is not corrected via base excision repair, GC-TA transversion mutations would lead to potential deleterious consequences. Many oxidative base lesions are mutagenic. For this reason, they have been considered as intermediate markers of cancer. GC→TA transversions, potentially derived from 8-OH-dG, have been observed in vivo in the RAS oncogene and the p53 tumor suppressor gene in lung and liver cancer. Also, tissue concentration of 8-OH-dG has been found increased 10–20 fold in breast carcinoma. Augmented urinary levels of 8-OH-dG have been found in many cancers [41–43].

8. OXIDATIVE STRESS AND LIPID PEROXIDATION

Lipid peroxidation refers to a process under which free radicals attack lipids containing carbon-carbon double bond(s), especially polyunsaturated fatty acids (PUFAs). The greater the number of double bonds in a fatty acid, the more readily it undergoes peroxidation. Saturated fatty acids such as palmitic and stearic acid, and monounsaturated fatty acids such as oleic acid do not undergo peroxidation. The highly unsaturated fatty acids are very susceptible to peroxidation.

PUFAs are structural components of lipid bilayer cellular membranes. Many of the permeability characteristics of cellular membranes depend on lipid bilayer integrity. The permeability characteristics of the bilayer allow gradients of metabolite and electrolyte concentrations to exist between the intra– and extracellular spaces. Damage of PUFAs would lead to rapid loss in membrane integrity, dissipation of these gradients, and compromise of organelle or cellular function. The destruction of membrane lipids by a free radical attack and the end–products of lipid peroxidation reactions are especially dangerous for the viability of cells.

The two most prevalent reactive species that can affect profoundly the lipids are hydroxyl radical and peroxynitrite. The overall process of lipid peroxidation consists of three steps: initiation, propagation, and termination.

8.1. Lipid Peroxidation by ROS

The first step in lipid peroxidation by oxygen free radicals is abstraction of a hydrogen atom from the acyl chain to form a lipid radical. The next step is the addition of molecular oxygen to the lipid radical yielding lipid peroxyl radical. When lipid peroxyl radical abstracts a hydrogen atom from one of the adjacent PUFAs, a hydroxyperoxy fatty acid (also called lipid hydroperoxide) is formed. Transition metals reduce hydroxyperoxy fatty acids yielding alkoxyl radicals and hydroxy fatty acids. Another possible direction of peroxidation reactions is the generation of complex cyclic compounds via intramolecular reactions yielding endoperoxides. The most studied endoperoxide products of peroxidation are isoprostanes. These are prostaglandin–like compounds formed via peroxidation of arachidonic acid without the direct action of cyclooxygenase (COX) enzymes. Isoprostanes are biologically active; they are potent vasoconstrictors acting via the thromboxane receptor.

The ultimate direction of peroxidation reactions is the fragmentation of fatty acid carbon chain. Fragmentation products include some of the most extensively studied products of lipid peroxidation, such as malondialdehyde (MDA), acrolein, and 4–hydroxy–2–nonenal (HNE). MDA is a naturally occurring end–product of lipid peroxidation that is mutagenic and carcinogenic. It is able to induce large insertions and deletions in the DNA, but base pair substitutions have also been detected. MDA causes up to a 15–fold increase in mutation frequency compared to background levels [44]. This level of increase is comparable with the mutation frequencies induced by ultraviolet light. Acrolein formed in vivo during lipid peroxidation exhibits facile reactivity with various key macromolecules, including proteins and phospholipids, has the potential to inhibit many enzymes, quickly deplete cellular glutathione levels, and potentially induce cell death. HNE is a highly abundant fragmentation product that results from lipid peroxidation of arachidonic acid. Elevated HNE concentrations have been found in several pathological conditions, such as atherosclerosis and cardiovascular diseases. HNE depletes cellular glutathione levels, easily forms adducts to cellular proteins lead-

ing to cellular dysfunction, disrupts cellular cytoskeleton, and induces apoptotic cell death [45].

8.2. Lipid Peroxidation by RNS

PUFAs can undergo modification by RNS as well. Attack to fatty acid double bonds by RNS gives rise to lipid nitrosylation. The initial steps in lipid nitrosylation are the formation of nitrosyl lipid radical and dinitro-fatty acids. Further steps in the formation of nitrated lipids give rise to a multiplicity of compounds, such as nitroalkene and alkyl nitrate. Bioactivities of nitrated lipids have been well characterized in the literature [44, 46–51].

9. OXIDATIVE PROTEIN DAMAGE

Protein modifications that occur due to reactive species generated during oxidative stress include fragmentation of the polypeptide chain, oxidation of amino acid side chains, and generation of protein–protein cross-linkages.

9.1. Oxidative Cleavage of the Polypeptide Backbone

Oxidation of a protein molecule is initiated by abstraction of a hydrogen atom from the polypeptide chain to form water and a carbon-centered radical. The next step is formation of a peroxyl radical as a consequence of the addition of molecular oxygen. Peroxyl radical is readily converted to protein peroxide and alkoxyl radical by reactions with transition metals. The protein alkoxyl radical can undergo peptide bond cleavage and fragmentation of the polypeptide chain. It has been demonstrated that it is the oxidation of proline residues that is most susceptible to peptide bond cleavage [52, 53].

9.2. Oxidation of Amino Acid Residue Side Chains of Proteins

Amino acid residues of protein side chains that are most susceptible to oxidation by free radicals include cysteine and methionine. Cysteine and methionine residues of proteins are by far the most sensitive to oxidation by almost all kinds of free radicals. The primary oxidation products of cysteine residues are protein disulfides. Other resides such as tyrosine can also be modified by free radicals. Abstraction of a hydrogen atom from the tyrosine residue forms the tyrosine radical. A tyrosine radical can react with RNS to form 3-nitrotyrosine. This modification is biologically irreversible. Tyrosine oxidation products, especially 3-nitrotyrosine, are considered as biomarkers for oxidative stress [54].

Protein carbonylation refers to a process that forms reactive ketones or aldehydes under oxidative stress conditions. Lysine, arginine, proline, and threonine residues of proteins are particularly susceptible to this process. Protein carbonylation reactions are considered biologically irreversible. Protein carbonylation is a commonly used marker for oxidative stress. The carbonyl content of proteins has become the most generally used marker for estimation of oxidative stress-mediated protein oxidation [55].

9.3. Generation of Protein–Protein Cross-Linkages

Free radical-mediated oxidation of proteins can lead to the formation of protein–protein cross-linked derivatives. Examples of oxidative cross-linking include reaction of a carbonyl group in one protein with the amino group of a lysine residue of another protein. Cysteine or methionine oxidation leads to intramolecular disulfide formation with another cysteine or methionine within the oxidized protein or intermolecular disulfide formation with a cysteine/methionine from another protein. These oxidations lead to alterations in protein conformation and loss of biological activity of the oxidized proteins. Protein–protein cross-links can be formed also by the interaction of lysine residues of two different proteins with the aldehyde groups of MDA produced during lipid peroxidation of PUFAs. Cross-linkages are also produced by a combination of tyrosine radicals in two different proteins [53].

9.4. Accumulation of Oxidized Proteins

Proteins which have been damaged through oxidative imbalances are degraded by the proteolytic machinery to their constitutive amino acids. There is evidence that mildly oxidized proteins are removed in a proteasome-dependent fashion. Heavily oxidized proteins and protein–protein cross-linked derivatives are misfolded and do not undergo ubiquitin-dependent degradation. Heavily modified substrates

are incompletely degraded, and they tend to aggregate and accumulate within the lysosomal compartments resulting in the formation of lipofuscin-like aggregates. Accumulation of misfolded proteins eventually results in impaired cell function, cell death, and tissue injury. Aggregation of oxidized proteins has been implicated in the development of cardiomyopathies, Alzheimer's disease, and protein folding diseases such as amyloidosis [53, 56–60].

10. ANTIOXIDANT DEFENSES

One of the biological mechanisms to counteract oxidative stress is by producing antioxidants. An antioxidant is a substance capable of preventing, reducing, or repairing the oxidative damage of a targeted macromolecule [61]. The human body is equipped with a wide variety of antioxidants, which are either endogenous or exogenously supplied through foods and/or dietary supplements. A narrow definition of antioxidants would include molecules that are capable of removing, neutralizing, or scavenging ROS/RNS and their intermediates. But antioxidant defense mechanisms also include the inhibition of free radical generation, binding of redox-active metal ions needed for catalysis of oxidative species production, and up-regulation of antioxidant defense activity. In fact, the elimination of free radicals by cells is not accomplished by a single biochemical pathway but is carried out by several cascades of intricately related events or processes.

Endogenous antioxidants can be classified into two categories: enzymatic and non-enzymatic. The major enzymatic antioxidants directly involved in the neutralization of ROS include superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase, among many others [62–64].

10.1. Superoxide Dismutase

Superoxide dismutases (SODs) are metalloproteins that catalyze dismutation of superoxide into hydrogen peroxide and molecular oxygen ($2\ O_2^{\bullet -} + 2\ H^+ \rightarrow H_2O_2 + O_2$). Depending on the transition metal ion found at their active site, SODs can be categorized into three types: copper, zinc superoxide dismutase (Cu,ZnSOD or SOD1), manganese superoxide dismutase (MnSOD or SOD2), and extracellular superoxide dismutase (ECSOD or SOD3). SOD1 is primarily a cytoplasmic enzyme, SOD2 resides in mitochondria, and SOD3, which contains copper and zinc in its active sites, is the major extracellular antioxidant enzyme. The role of SODs in modulating the pathophysiology of diverse disease processes has been demonstrated in genetically engineered mice [65, 66].

10.2. Catalase

Catalase is an enzyme found predominantly in peroxisomes. The main function of catalase is decomposition of hydrogen peroxide to water and molecular oxygen ($2\ H_2O_2 \rightarrow 2\ H_2O + O_2$). Catalase thus limits the accumulation of hydrogen peroxide, which is generated by various oxidases in cells and serves as a substrate for the Fenton reaction to generate the highly reactive hydroxyl radical (see Section 3.5 for the Fenton reaction). Catalase plays an important role in removing intracellular H_2O_2 and is the key enzyme for disposal of hydrogen peroxide in peroxisomes. It is a heme-containing homotetrameric protein, ubiquitously present in all types of mammalian cells. Catalase is a significant defense against oxidative tissue injury in various disease conditions. For example, mice deficient in catalase undergoing nephrectomy are more susceptible to oxidant tissue injury and renal fibrosis [8, 67, 68].

10.3. Glutathione System

The glutathione system is a major cellular antioxidant defense and consists of the reduced form of glutathione (GSH) and the enzymes that are involved in either its synthesis/regeneration or using it as an electron donor to detoxify reactive species. GSH is a tripeptide; its synthesis from glutamate, cysteine, and glycine is catalyzed sequentially by two cytosolic enzymes, γ-glutamylcysteine synthetase (GCS), which is more formally known as γ-glutamylcysteine ligase (GCL), and GSH synthetase.

Most of the cellular GSH is present in the cytosol. GSH scavenges free radicals and ROS (e.g., hydrogen peroxide, hydroxyl radical, peroxynitrite, and lipid peroxyl radical) directly and indirectly through enzymatic reactions. In such reactions, GSH is oxidized to form glutathione disulfide (GSSG). GSH is then regenerated from GSSG by the enzyme glutathione reductase (GR). In addition, glutathione peroxidase (GPx) catalyzes the GSH-dependent

reduction of hydrogen peroxide to water. GSH/GSSG is the major redox couple that determines the antioxidative capacity of cells. The ratio of intracellular GSH to GSSG is often used as an indicator of the cellular redox state. This ratio is 10:1 to 100:1 under normal conditions.

GPx is a family of enzymes with peroxidase activity. There are eight isozymes encoded by different genes, which vary in cellular location and substrate specificity. In mammalian tissues, GPx1, 2, 3, and 4 are selenoproteins with a selenocysteine in the catalytic center. All GPx enzymes are able to catalyze the reduction of hydrogen peroxide to water using GSH as a reductant. During the reaction GSH is oxidized to GSSG which can then be reduced back to GSH by GR [69, 70].

10.4. Nonenzymatic Antioxidants

Nonenzymatic antioxidants include low-molecular-weight compounds, such as vitamins C and E. Vitamin C (also known as ascorbic acid) is a water soluble antioxidant. Vitamin C is a cofactor for at least eight enzymes. Vitamin C acts as a reducing agent, donating electrons to various enzymatic reactions. Most mammalian organisms actually synthesize vitamin C, and the synthesis involves a series of enzymes. Humans must ingest vitamin C because we lack the last enzyme in the series of steps needed to make this molecule. Deficiency of vitamin C causes scurvy. Sources of vitamin C are vegetables and fruits (e.g., lemons, lime). Vitamin C is regarded as the first line natural antioxidant defense in plasma and a powerful scavenger of superoxide, hydroxyl radical, and peroxynitrite.

Vitamin E (α-tocopherol) is a generic term for a group of fat-soluble organic compounds known as tocopherols and tocotrienols. Vitamin E exists in eight different forms: 4 tocopherols (alpha, beta, gamma, and delta) and 4 tocotrienols (alpha, beta, gamma, and delta). The most biologically active form of vitamin E is α-tocopherol.

α-Tocopherol is a potent peroxyl radical scavenger, which protects cell membranes from lipid peroxidation. This would prevent the oxidation reaction from continuing. The oxidized α-tocopherol radical produced in this process may be reduced by a hydrogen donor (such as vitamin C). Compared with tocopherols, tocotrienols occur at very low levels in cells and tissues [71, 72].

11. ROS AS USEFUL MOLECULES

Although historically viewed as associated with tissue injury, ROS also play a major physiological role in several aspects of intracellular signaling and defense mechanisms against infection. Summarized below are representative scenarios where ROS play a part in cell signal transduction and innate immunity.

11.1. ROS in Signaling Pathways

The epidermal growth factor receptor (EGF-R) is a cell surface receptor that has an intrinsic ligand-activated protein tyrosine kinase activity. Ligand binding to the EGF-R leads to receptor autophosphorylation on specific tyrosine residues, which mediates cell response through a signal transduction pathway which, in turn, activates mitogen-activated protein (MAP) kinase. Studies have demonstrated that binding of ligands to EGF-R cannot produce enough phosphorylation to allow a full response. Transient inhibition of phosphatases by ROS is also required (**Figure 3**). The prevention of ROS accumulation by antioxidants blocks MAP kinase activation after EGF-R ligand binding. Moreover, direct exposure of cells to exogenous hydrogen peroxide, to mimic oxidative stress, leads to activation of EGF-R and MAP kinase pathways. The mechanism of EGF-R activation by ROS is not known. A plausible hypothesis is that oxidative modification of critical amino acid residues of the proteins participating in this signal transduction pathway is necessary for full activation of MAP kinase pathways [9, 73, 74].

11.2. ROS in Phagocytosis

It has been well documented that neutrophils and macrophages are able to generate ROS and use them to kill phagocytized bacteria (**Figure 4**). After phagocytizing a microorganism, activation of the phagocyte NADPH oxidase results in a rapid increase in oxygen uptake which is known as the respiratory burst. The increase in oxygen uptake during the respiratory burst can be 50 times the resting O_2 consumption of neutrophils. A key enzyme in this process is the oxygen radical-generating NADPH oxidase. It consists of six subunits, which are separated under physiological conditions to prevent spontaneous generation of free radicals with consequent cell damage [75]. In order to become activated, the subu

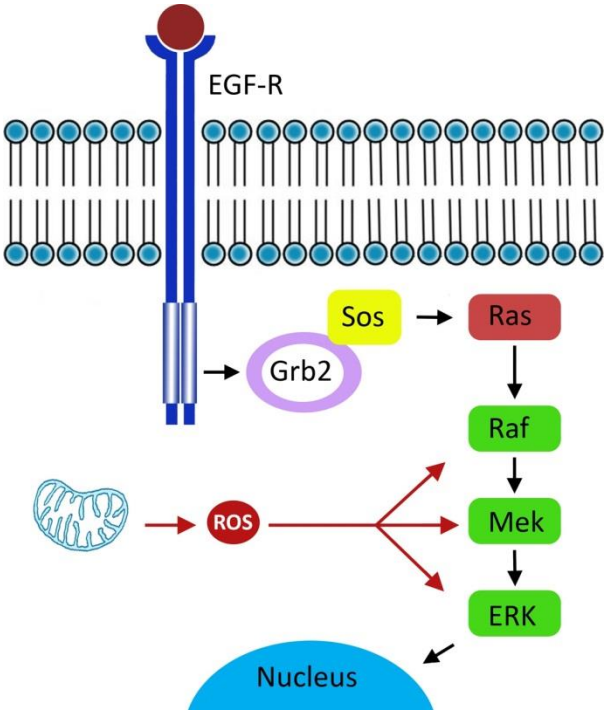

FIGURE 3. ROS in MAP kinase signaling pathway. See text (Section 11.1) for detailed description.

nits of NADPH oxidase have to assemble to form the multi-subunit enzyme. Two of these subunits, $gp91^{phox}$ and $p22^{phox}$ (phox refers to phagocyte oxidase according to the currently accepted nomenclature) are located within the phagolysosome membrane. These two subunits form cytochrome b which is the catalytic core of the enzyme, essential for the generation ROS in phagocytes. After neutrophil activation, three additional subunits ($p67^{phox}$, $p47^{phox}$, and $p40^{phox}$) along with GTPase binding protein Rac1 are dislocated from cytosol to phagolysosome membrane [9].

Activation of NADPH oxidase results in the generation of significant amounts of superoxide which accumulates in phagolysosomes. Subsequently, superoxide is converted into much more potent bactericidal oxidants species. Superoxide is rapidly converted to hydrogen peroxide by spontaneous dismutation. On the other hand, activated neutrophils undergo fusion of the cytoplasmic azurophilic granules with the phagolysosome with release of the granule content. Azurophilic granules are loaded with the enzyme myeloperoxidase (MPO). MPO is an abundant heme protein that accounts for 5% of the total neutrophil protein content. MPO produces hypochlorous acid from hydrogen peroxide and chloride anion (also see Section 4.4). Last but not least, hydroxyl radical is formed in phagolysosome by transition metal-catalyzed Haber–Weiss reaction or Fenton reaction (also see Sections 3.5 and 4.1). This reaction requires the presence of iron or copper, which is believed to be provided by the cell itself. Hydroxyl radical is a very powerful bactericidal agent [32, 76, 77].

12. MEASUREMENT OF OXIDATIVE TISSUE INJURY

Oxygen free radicals have been discovered more than fifty years ago. However, only within the last two decades, has there been an explosive research about their roles in the development of diseases. Free radicals are widely believed to contribute to the development of a large number of diseases and perhaps, even to the aging process itself. For example, diseases in which oxidative damage has been implicated include cancer, Alzheimer's disease, other neurodegenerative diseases, atherosclerosis, diabetes, hypertension, and among many others. But the causal role of oxidative damage in the onset and progression of many specific diseases remains to be elucidated. One way to study the participation of free radradicals in pathology is to be able to accurately measure chemical biomarkers of free radical damage in body fluids and tissues.

12.1. Biomarkers of Nucleic Acid Oxidation

The spectrum of oxidation products involving nucleic acids includes more than 20 different types of base damage. The most studied oxidation product is 8-OH-dG (also see Section 7). 8-OH-dG is used as a marker of DNA oxidation to evaluate carcinogenicity of ROS-forming chemicals. Cancer tissues contain abundant amounts of 8-OH-dG [78]. Tumor tissue from lung cancer patients contains 2–3 times higher amounts of 8-OH-dG than the apparently normal lung tissue. Smokers excrete 50% more 8-OH-dG in urine than nonsmokers. Also, there is a considerable amount of evidence supporting the involvement of DNA oxidation in the pathogenesis of neurodegener-

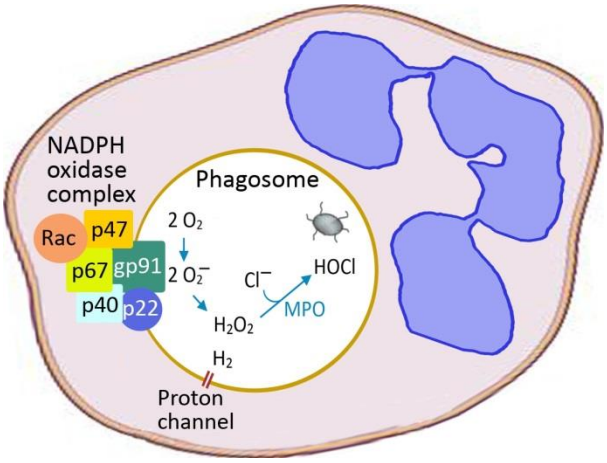

FIGURE 4. ROS in bacterial killing process inside the phagosomes of phagocytic cells. See text (Section 11.2) for detailed description.

ative diseases, especially Alzheimer's disease. Increased levels of 8-OH-dG have been found in the brains of patients with Alzheimer's disease [79].

12.2. Biomarkers of Lipid Peroxidation

There is evidence that lipid peroxidation of PUFAs is involved in the onset and progression of many specific disorders, such as diabetes, atherosclerosis, cancer, and neurodegenerative diseases (e.g., Parkinson's disease). Studies have demonstrated increased levels of products of lipid peroxidation, such as HNE, MDA, acrolein, and isoprostanes, in the brains of patients with Parkinson's disease. Immunohistochemistry studies have demonstrated increased HNE levels in dopaminergic cells in the substantia nigra and cerebrospinal fluid in patients with Parkinson's disease [80].

12.3. Biomarkers of Oxidative Protein Damage

Common markers of protein oxidation include protein carbonylation and protein nitration, which are used to study the possible involvement of oxidative stress in a variety of disease processes. For instance, increased amounts of carbonylated proteins have been found in the synovial fluid in patients with rheumatoid arthritis. Biomarkers of oxidative protein damage have been demonstrated in postmortem brain

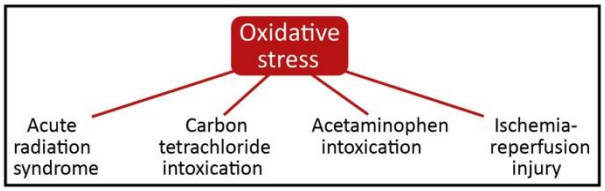

FIGURE 5. Diseases directly induced by oxidative stress. Illustrated in the scheme are some representative pathophysiological conditions in which oxidative stress may play a causal role.

tissue of patients with amyotrophic lateral sclerosis (ALS). Protein carbonyls and nitrotyrosine have been shown to be elevated in the spinal cord of patients with familial and sporadic ALS [81–84].

13. FREE RADICALS AND DISEASE

13.1. An Overview of the Relationship between Free Radicals and Disease

The significance of oxidative stress has become increasingly recognized to the point that it is now considered to be a component of almost every disease. Researchers in the field of oxidative stress can be classified into two large groups: enthusiasts and critics. Enthusiasts believe that increased free radical formation accompanies tissue injury in most if not all diseases, and oxidative stress may play an important casual role in many disease conditions. Critics, however, believe that in most disorders, oxidative stress is a mere consequence and not a cause of the primary disease process [83, 85].

In many diseases, oxidative stress may be just an unimportant consequence of and not a cause of the disease pathophysiology. Muscular dystrophy is a good example of this category: affected muscles demonstrate increased lipid peroxidation, which is a consequence of tissue damage, but makes no contribution to the damage. Attempts to slow down the course of muscular dystrophy with antioxidants have so far been unsuccessful [86]. On the other hand, some diseases are caused directly by oxidative stress (**Figure 5**). Examples include acute radiation syndrome, carbon tetrachloride intoxication, acetaminophen intoxication, and ischemia/reperfusion injury, among others [87–89]. In the third group of diseases,

ROS play a role in furthering tissue injury even when they are not the cause of the disease. Examples of this group include hereditary hemochromatosis and Wilson disease [90, 91]. A detailed discussion of the role of oxidative stress in specific diseases is beyond the scope of this article. Nevertheless, the section below uses cardiovascular disease as an example to illustrate the complexity of the oxidative stress mechanism of disease pathophysiology and antioxidant-based intervention.

13.2. Examples of Free Radical Disease: Cardiovascular Disorders

Cardiovascular disease (CVD) is the leading cause of death in the United States. Approximately 1.5 million cases of myocardial infarction are diagnosed annually and approximately 800,000 deaths related to the coronary artery disease and other CVDs occur each year [92]. In most cases, myocardial infarction occurs because of rupture or fissuring of a coronary artery atherosclerotic plaque containing a lipid-rich necrotic core with superimposed thrombosis causing reduced blood flow and consequent ischemic myocardial cell death [93].

Studies have shown that ischemic death of myocardial cells increases as a function of the duration of ischemia [94]. Fundamental experimental studies of Jennings et al. in the 1960s demonstrated that a 15-minute temporary occlusion of the left circumflex coronary artery in dogs caused no irreversible injury and was not associated with histopathologic changes of necrosis in the ischemic region. On the other hand, temporary ischemic episode lasting 20 minutes or longer was associated with ischemic necrosis of myocardial cells in the ischemic region [95]. Timely reperfusion of acute ischemic myocardium is essential for myocardial salvage. The case fatality rate of acute myocardial infarction has fallen dramatically in the past 3 decades, in part because of the widespread use of reperfusion therapy. Reperfusion is achieved through interventional procedures such as balloon angioplasty or the use of thrombolytic agents. Reperfusion of the ischemic tissue is beneficial because it supplies oxygen. However, there is evidence that reperfusion itself contributes to oxidative damage to myocardium. Hence, reperfusion is like a double-edged sword as ischemic tissues release myocardium-damaging ROS when reperfused with fresh oxygenated blood [93].

In classical experiments using open-chest, anesthetized dogs, a coronary occlusion lasting 15 minutes and followed by reperfusion caused no irreversible injury, but this episode of transient ischemia led to a period of hypokinesis of the previously ischemic wall after the restoration of flow. This mechanical dysfunction was not accounted for by myocardial necrosis as no histological signs of irreversible injury to cardiomyocytes existed. The contractile function usually restored several days or weeks later. This phenomenon of reversible reduction of function of heart contraction not accounted for by tissue damage or reduced blood flow after reperfusion has been referred to as myocardial stunning.

The degree of myocardial stunning and the level of the functional depression are proportional to duration, severity, and extent of the myocardial ischemia. The coronary occlusion-reperfusion cycles in open-chest dogs were shown to be associated with recurrent bursts of free radical formation. It was demonstrated that these bursts were markedly inhibited by mercaptopropionyl glycine, which is a hydroxyl radical scavenger [94]. Inhibition of free radical generation by mercaptopropionyl glycine resulted in attenuation of the ensuing postischemic contractile depression, indicating that free radical formation is necessary for the development of severe myocardial stunning in this setting. Taken together, the results of these experimental studies support an important pathogenic role of hydroxyl radical in myocardial stunning [94].

Recently, multiple antioxidant compounds with distinct structure have been shown to be protective against myocardial stunning in animal models [96]. However, it remains unknown if any of the antioxidant modalities would be effective in the clinical setting. In fact, for the entire spectrum of cardiovascular diseases, ranging from hypertension to ischemic heart disease and to heart failure, diverse antioxidant therapies have been shown to be effective in animal models, but none have been conclusively demonstrated to be effective in human patients [97–100]. This, on the one hand, highlights the complexity of the free radical nature of cardiovascular injury, and on the other hand, points to the need for further mechanistic studies of the oxidative stress mechanism of tissue injury and development of innovative mechanistically based strategies for the intervention of oxidative tissue injury in human patients with cardiovascular diseases as well as other disorders.

14. CONCLUSION AND PERSPECTIVES

The generation of oxygen and nitrogen free radicals is an unavoidable consequence of aerobic life. Sources of free radicals are numerous, and the major ones include electron leak from the mitochondrial electron transport chain, xenobiotics metabolism by the cytochrome P450 enzyme system in the endoplasmic reticulum, and activation of NADPH oxidase in inflammatory cells. The unpaired electron is responsible for the extremely high biological reactivity of free radicals. Oxygen and nitrogen free radicals represent a constant source of assaults upon cellular lipids, proteins, nucleic acids, and other biomolecules. Yet, it would be inaccurate to consider solely the damaging biological effects of ROS. At low or moderate concentrations, free radicals are vital to body health. They are important for host defense mechanisms, and play a key role in the regulation of intracellular signaling cascades in various types of nonphagocytic cells. Exposure of cells to elevated concentrations of reactive species through either endogenous or exogenous insults gives rise to oxidative stress. The harmful effect of oxidative stress is counteracted by the action of both antioxidant enzymes and non-enzymatic antioxidants. Oxidative stress has been recognized as a contributor to a number of pathophysiological conditions, especially cardiovascular injury. In this context, antioxidant compounds of diverse structures have been demonstrated to be protective in many disease processes in diverse animal models. However, for most, if not all, of the antioxidant-based modalities shown to be effective in animal models, their clinical efficacy in human patients remains to be established. As new knowledge on free radicals in health and disease increases, new opportunities would become available to help develop innovative mechanistically based modalities for the intervention of free radical disease without compromising the physiological function of these reactive species.

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