ROS

REVIEW ARTICLES

Superoxide in Biology and Medicine: An Overview

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ABSTRACT | Since 1933 when Linus Pauling, a twice-honored Nobel laureate, proposed its existence based on the theory of quantum mechanics, superoxide has gradually taken central stage in the research field of reactive oxygen species (ROS) in biology and medicine. Indeed, superoxide is considered the primary ROS that gives rise to secondary ROS. This oxygen free radical is generated in a wide variety of biological systems ranging from aerobic microorganisms to human cells, and also formed in the deep ocean and the soils of Earth and possibly the soils of Mars as well. Superoxide is now recognized as an important molecule that is formed via defined mechanisms and involved in diverse physiological and pathophysiological processes. This article provides an overview on the basic chemistry and biochemistry of this ubiquitous oxygen free radical and its significance in biology and medicine.

KEYWORDS | Cytochrome P450 system; Fenton reaction; Haber-Weiss reaction; Mitochondria; NADPH oxidase; Reactive oxygen species; Redox cycling; Redox signaling; Superoxide; Superoxide dismutase; Uncoupled endothelial nitric oxide synthase; Xanthine oxidoreductase

ABBREVIATIONS | Cu,ZnSOD, Cu,Zn superoxide dismutase; CYP, cytochrome P450; ECSOD, extracellular superoxide dismutase; EPR, electron paramagnetic resonance; METC, mitochondrial electron transport chain; MnSOD, manganese superoxide dismutase; NOX, NAD(P)H oxidase; ROS, reactive oxygen species; SOD, superoxide dismutase; XO, xanthine oxidase

CONTENTS

1. INTRODUCTION

Superoxide, also known as superoxide anion or superoxide anion radical, is designated as $O_2^{\bullet-}$, where the dot denotes the unpaired electron and the minus sign denotes that the species is negatively charged. The above three names for $O_2^{\bullet-}$ are used interchangeably in the literature, and for simplicity, superoxide is used throughout this article. Superoxide can exist in a protonated form, known as perhydroxyl or hydroperoxyl radical ($HO_2^{\bullet}$) with a pKa of 4.8. Because of this pKa, superoxide exists predominantly in the non-protonated form ($O_2^{\bullet-}$) under physiological pH.

Linus Pauling, a twice-honored Nobel laureate (Chemistry in 1954; Peace in 1962) proposed the existence of superoxide based on the theory of quantum mechanics in 1933 (see in ref. [1]). Two decades after Linus Pauling's proposal, in 1954, Rebecca Gershman and associates suggested that superoxide might be responsible for both oxygen toxicity and the deleterious effects of x-irradiation [2]. Subsequently, xanthine oxidase was proposed to produce superoxide in 1962 [3], and this was proved by electron paramagnetic resonance (EPR) studies in 1969 [4]. Meanwhile, methods involving electrolytic univalent reduction of molecular oxygen were used to produce superoxide. With superoxide generated via the above approaches, Joe McCord and Irvine Fridovich discovered Cu,Zn superoxide dismutase (Cu,ZnSOD) in 1969 [5]. Subsequently, Bernard Babior and coworkers reported superoxide generation by respiratory burst (activation of NADPH oxidase) of leukocytes as a potential mechanism of bactericidal activity [6]. Now superoxide is considered one of the most important reactive oxygen species (ROS) that plays a significant role in a variety of biological processes and disease conditions.

2. SOURCES OF SUPEROXIDE

Superoxide is produced from one electron reduction of molecular dioxygen (oxygen for simplicity): $O_2 + e^- \rightarrow O_2^{\bullet-}$ [7] (**Figure 1**). In biological systems, superoxide is generated from various metabolic processes. These include the mitochondrial electron transport chain (METC) complexes, NADPH oxidases (also known as NOXs), xanthine oxidoreductase, uncoupling of endothelial nitric oxide synthase (eNOS), and cytochrome P450 enzyme system, among others (e.g., Rac,) (**Figure 2**).

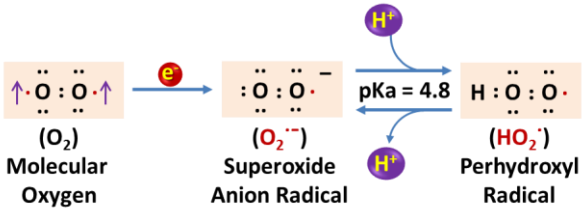

FIGURE 1. Electron configuration illustration of one electron reduction of molecular oxygen to superoxide. Molecular oxygen (O_2) is in fact a di-radical because it contains two unpaired electrons. The same spin direction of the two unpaired electrons in O_2 causes spin restriction, making O_2 less reactive. On the other hand, superoxide ($O_2^{\bullet-}$) contains one unpaired electron and has one more electron than O_2, and as such, superoxide is negatively charged. Superoxide can become protonated to form perhydroxyl radical ($HO_2^{\bullet}$.), which is believed to be more reactive than $O_2^{\bullet-}$.

Both NOXs and mitochondria are key machineries in cellular superoxide generation. Mitochondria are generally considered the chief cellular source of superoxide, whose formation is primarily derived from the univalent reduction of molecular oxygen by electrons leaked from the METC [8]. This is not surprising as mitochondrial respiration accounts for ~90% of total cellular oxygen consumption. Recently, NOX4 is also found to be present in mitochondria and acts as a potential source of mitochondria-derived superoxide [9, 10]. Hence, mitochondria-derived superoxide is not necessarily always due to electron leakage from the METC. In addition to its

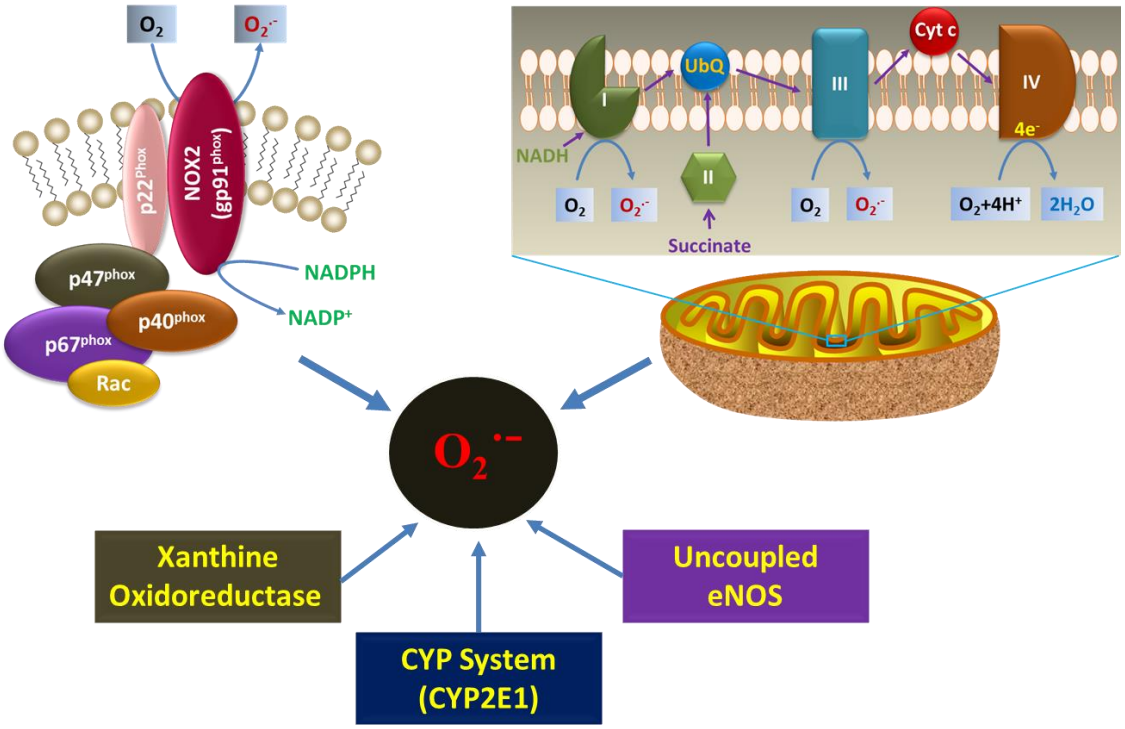

FIGURE 2. Major sources of cellular superoxide. NADPH oxidases (NOXs) and mitochondria are considered the major cellular sources of superoxide. Many other enzymes and cellular processes also contribute to cellular superoxide production, such as xanthine oxidoreductase, cytochrome P450 (CYP) enzyme system (especially CYP2E1), and uncoupled endothelial nitric oxide synthase (eNOS).

presence in mitochondria, NOX4 is also found to undergo translocation into the nucleus via a leukotriene C4-dependent mechanism, and its presence in the nucleus contributes to oxidative damage to the nuclear DNA under stress conditions, including inflammatory responses and treatment with cancer chemotherapeutic agents [11]

Notably, xanthine oxidoreductase has two interconvertible forms, namely, xanthine dehydrogenase and xanthine oxidase (XO). Both forms catalyze the conversion of hypoxanthine to xanthine, and xanthine to uric acid. Both forms also catalyze one- and two-electron reduction of molecular oxygen to form superoxide and hydrogen peroxide (H_2O_2), respectively, with XO being more active in generating the above ROS. The reversible conversion of xanthine dehydrogenase to XO occurs under certain conditions including hypoxia/tissue ischemia and oxidation of the cysteine thiol groups of the enzyme. Xanthine oxidoreductase-derived ROS have been implicated in a number of pathophysiological processes, such as atherosclerosis, inflammation, and tissue ischemia-reperfusion injury in the heart, liver, and kidneys [12].

Cytochrome P450 (CYP) enzyme system, especially CYP2E1, is also a significant source of superoxide formation in cells, particularly under conditions when these enzymes are induced by chemicals (e.g., ethanol). CYP2E1-derived superoxide/ROS upon ethanol consumption may also be responsible for the upregulation of CYP2A5/2A6 by alcohol [13]. Recently, a redox signaling heme-containing globin, namely, GLB-12, in *Caenorhabditis elegans* has been shown to generate superoxide, and the superoxide produced may play a critical signaling role in regulating apoptosis and controlling the reproduction of the organism [14].

In addition to the aforementioned endogenous sources, superoxide is also formed during biotransformation of certain xenobiotics, especially quinone

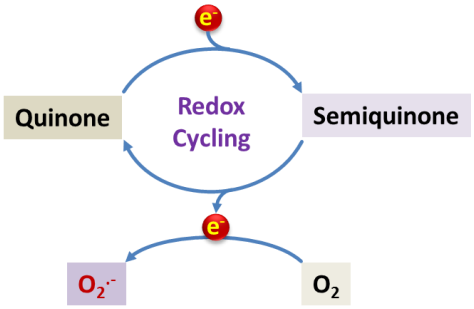

FIGURE 3. Schematic illustration of the concept of chemical redox cycling. Redox cycling of quinone compounds gives rise to an enormous amount of superoxide via one electron reduction of molecular oxygen by the semiquinone radical intermediate.

compounds, via a process known as redox cycling (**Figure 3**). Chemical redox cycling may be defined as the reduction and oxidation cycle between two forms of a compound, frequently a quinone molecule. Redox cycling is typically initiated by a univalent reduction of a quinone molecule to form a semiquinone radical species. The CYP enzyme system and METC are among the major machineries that carry out the one electron reduction. The semiquinone radical formed can then donate one electron to molecular oxygen, and during this reaction, molecular oxygen is reduced to a superoxide anion radical, and the semiquinone radical is oxidized back to the original quinone molecule. Hence, the entire process involving one electron reduction and oxidation forms a cycle, leading to the persistent production of superoxide and secondary ROS (e.g., hydrogen peroxide and hydroxyl radicals).

In the sea, superoxide is generated by photolysis of organic matter in the photic zone as well as bacteria in the deep ocean with the latter being a vast source of superoxide [15, 16]. Superoxide is formed in desert soils via photochemical reactions [17]. Superoxide may also exist in the soils of Mars. The Viking Landers were unable to detect evidence of life on Mars but, instead, found a chemically reactive soil capable of decomposing organic molecules. Using EPR spectrometry, Albert S. Yen and coworkers show that superoxide forms directly on Mars-analog mineral surfaces exposed to ultraviolet radiation under a simulated Martian atmosphere. The superoxide can explain the reactive nature of the soil and the apparent absence of organic material at the Martian surface [18].

3. CHEMISTRY AND BIOCHEMISTRY OF SUPEROXIDE

Although it is called "super-oxide", superoxide is in fact not a strong oxidizing species. Because of its lower reduction potential (–330 mV for the $O_2/O_2^{\bullet-}$ redox couple), superoxide often acts as a reducing agent rather than an oxidizing species. Indeed, the ferricytochrome c reduction assay, a method for detecting superoxide, is based on the ability of superoxide to reduce this small heme-containing protein [19]. The ability of superoxide to directly oxidize biomolecules, including lipids, proteins, and nucleic acids is much limited. However, superoxide is an important ROS that can result in cell and tissue injury [20]. As outlined below, the biologically damaging potential of superoxide is attributed to its several unique chemical and biochemical properties (**Figure 4**).

3.1. Oxidation of Iron-Sulfur Clusters

Although it generally acts as a reducing agent, superoxide does oxidize the iron-sulfur clusters in several enzymes, including aconitase, an enzyme of the tricarboxylic acid cycle [21, 22], and the mitochondrial electron transport chain enzyme complexes [23]. Oxidation of the iron-sulfur clusters by superoxide in these enzymes leads to the release of iron from the enzymes and the enzyme inactivation. The released iron ions may participate in the Fenton reaction, resulting in the formation of hydroxyl radicals (see Section 3.3 below). In contrast, reversible oxidation of the iron-sulfur cluster in prokaryotic transcription factors SoxR/SoxS leads to their activation (see Section 4.2 below).

3.2. Reaction with Nitric Oxide

Superoxide reacts with nitric oxide ($NO^{\bullet}$) at an almost diffusion-limited rate to form peroxynitrite anion ($ONOO^-$), which is frequently called peroxynitrite for simplicity. The reaction is as following: $O_2^{\bullet-} + NO^{\bullet} \rightarrow ONOO^-$. Peroxynitrite is a potent oxidant that causes damage to various biomolecules [24]. The fast reaction between superoxide and nitric oxide also contributes to the decreased bioavailability

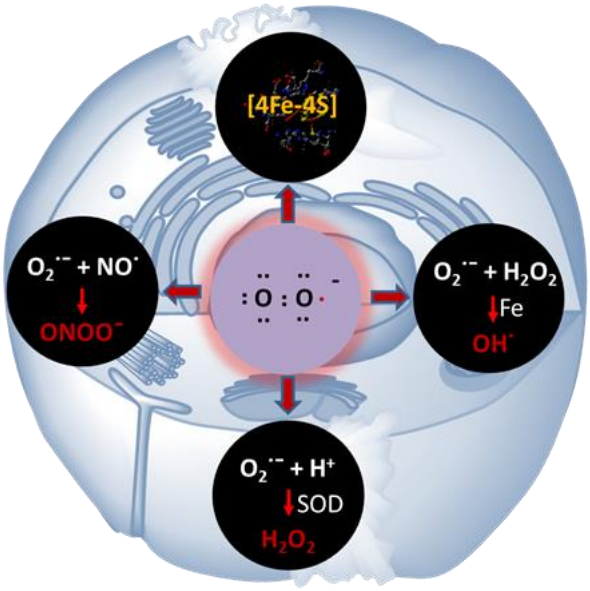

FIGURE 4. Chemical reactivity of superoxide. As illustrated, superoxide reacts with iron-sulfur clusters in enzymes, leading to enzyme inactivation. It reacts with nitric oxide (NO$^\bullet$) at a diffuse-limited rate to form peroxynitrite (ONOO$^-$), a potent oxidant. Superoxide is also a reactant of the iron-catalyzed Haber-Weiss reaction, giving rise to hydroxyl radical, the most potent ROS formed in biological systems. Spontaneous or superoxide dismutase (SOD)-catalyzed dismutation of superoxide produces hydrogen peroxide, another important ROS.

of nitric oxide under diverse pathophysiological conditions. Nitric oxide is generally regarded as a cardiovascular protective molecule, whose deficiency might contribute, at least partly, to such diseases as atherosclerosis, hypertension, coronary artery disease, and erectile dysfunction [25]

3.3. Haber-Weiss Reaction and Fenton Reaction

In the presence of transition metal ions, such as iron (Fe) ions, superoxide and hydrogen peroxide together can give rise to hydroxyl radical (OH$^\bullet$), the most potent ROS capable of damaging the entire spectrum of biomolecules. The reaction of superoxide and hydrogen peroxide in the presence of Fe ions to produce hydroxyl radical is known as iron-catalyzed Haber-Weiss reaction, which was first proposed by Fritz Haber and Joseph Weiss in 1934 [26] (**Figure 5**). In addition to his contributions to the field of free radical biology, Fritz Haber received the Nobel Prize in Chemistry in 1918 for the synthesis of ammonia from its elements.

In the absence of Fe ions, the above reaction proceeds slowly. The presence of Fe ions markedly accelerates the reaction to produce hydroxyl radical. The Fe ion-catalyzed Haber-Weiss reaction can be written in two sequential sub-reactions: (1) $O_2^{\bullet-}$ + Fe^{3+} → O_2 + Fe^{2+}, and (2) Fe^{2+} + H_2O_2 → Fe^{3+} + OH$^\bullet$ + OH$^-$. The second reaction is commonly referred to as the Fenton reaction, which is also frequently called the Fenton chemistry.

By definition, Fenton chemistry refers to the oxidation of organic substrates by Fe^{2+} and H_2O_2. In 1894, Henry J.H. Fenton first observed the oxidation of tartaric acid by H_2O_2 in the presence of Fe^{2+} [27], indicating that a potent oxidant(s) is formed by the reaction between H_2O_2 and Fe^{2+}, which are also known as the Fenton reagent. The Fenton reagent is commonly used to oxidize (destroy) organic chemicals, such as wastes in water. Later in the 1930s, Fritz Haber and Joseph Weiss proposed the formation of hydroxyl radical from the Fenton reagent, which is known today to be a major ultimate species responsible for oxidative damage of a wide range of biomolecules.

In addition to Fe^{2+}, other transition metal ions, such as cuprous ion (Cu^{1+}), also react with H_2O_2, forming hydroxyl radical or its equivalent: $M^{(n-1)+}$ + H_2O_2 → M^{n+} + OH$^\bullet$ + OH$^-$, where M denotes the transition metal ion. For example, Cu^{1+} + H_2O_2 → Cu^{2+} + OH$^\bullet$ + OH$^-$. Such reactions are commonly referred to as Fenton-type reactions.

3.4. Membrane Permeability and Protonation

Another factor influencing the biological activities of superoxide is its limited ability to cross biomembranes due to the negative charge and short half-life of this free radical species. However, superoxide may cross cell membranes through anion channels, such as the 4-diisothiocyano-2,2-disulfonic acid stilbene (DIDS)-sensitive chloride channel [28]. In contrast, perhydroxyl radical, the protonated form of superoxide, is highly membrane permeable, and is also a potent oxidizing species [29]. Perhydroxyl

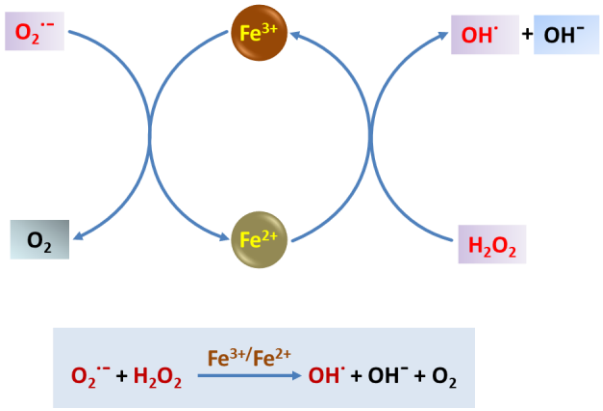

FIGURE 5. Iron-catalyzed Haber-Weiss reaction. The reaction between superoxide and H_2O_2 to form hydroxyl radical is commonly referred to as the Haber-Weiss reaction. This reaction proceeds slowly and can be dramatically accelerated by the presence of iron (Fe) ions. The reaction on the right side of the scheme is also called the Fenton reaction.

radical has a pKa of 4.8, and as such, may reach high levels in cellular compartments with high concentrations of protons, including the mitochondrial intermembrane space. A recent study by Michael A. Trush and coworkers provides direct evidence for the mitochondrial electron transport chain-derived superoxide to exit mitochondria and cross cell membranes to enter the extracellular space [8]. Exit of superoxide from mitochondria and its subsequent crossing of cell membranes to enter extracellular milieu provide a basis for superoxide to participate in diverse intracellular and intercellular processes under physiological and pathophysiological conditions.

4. SUPEROXIDE IN BIOLOGY AND MEDICINE

Superoxide is one of the most extensively investigated ROS in biology and medicine. A PubMed search on January 30, 2016, revealed 82,289 entries containing the term 'superoxide' in title/abstract. The number of entries for 'hydrogen peroxide' and 'hydroxyl radical' was 42,193 and 10,008, respectively. As outlined below, superoxide anion radical plays an important role in both physiological and pathophysiological processes.

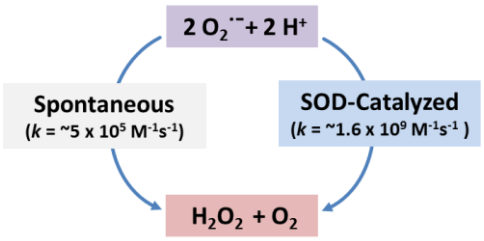

FIGURE 6. Spontaneous and SOD-catalyzed dismutation of superoxide. As illustrated, the dismutation reaction of superoxide is greatly accelerated by SOD with a reaction rate constant being over three orders of magnitude greater than that of spontaneous dismutation.

4.1. Phagocyte-Mediated Immunity

Superoxide produced from phagocytic NADPH oxidase during respiratory burst is an important precursor molecule leading to the formation of more potent ROS, especially hypochlorous acid (HOCl) and peroxynitrite that kill the invading microorganisms. Dismutation of superoxide generates hydrogen peroxide, which in turn reacts with chloride ion in the presence of the phagocyte-derived myeloperoxidase (MPO), forming hypochlorous acid. Likewise, superoxide released from the NADPH oxidase reacts with nitric oxide generated by inducible nitric oxide synthase (iNOS), resulting in the formation of peroxynitrite. Failure to generate phagocyte-derived superoxide and related ROS is the major defect in chronic granulomatous disease, causing recurrent infections and granulomatous complications [30].

4.2. Redox Modulation and Signaling

The role of superoxide in redox regulation in prokaryotes is well established. In bacteria, SoxR and SoxS are activated by superoxide via reversible one-electron oxidation of the [2Fe-2S] cluster and then enhance the production of various antioxidant proteins through the soxRS regulon [31, 32].

Superoxide stimulates cell proliferation. Overexpression of superoxide-producing NADPH oxidase induces cell transformation [33] though the responsible ROS seems to be hydrogen peroxide derived from the dismutation of superoxide [34]. Superoxide may also relay Ras protein's oncogenic message [35].

4.3. Autoregulation of Superoxide Production

Superoxide may regulate its own production. On the one hand, superoxide activates mitochondrial uncoupling proteins, leading to decreased formation of superoxide from the mitochondrial electron transport chain [36, 37]. On the other hand, superoxide flux across the endothelial cell plasma membrane occurs through chloride ion channels and induces intracellular calcium ion release, which in turn activates mitochondrial superoxide generation [38].

4.4. Cell and Tissue Defenses

Superoxide undergoes spontaneous dismutation to form hydrogen peroxide and molecular oxygen with a reaction rate constant of ~5 × 10^5 $M^{-1}s^{-1}$ at pH 7.0 (**Figure 6**) . The term dismutation reaction refers to a chemical reaction in which the same reactant is both oxidized and reduced. In superoxide dismutation reaction, one molecule of superoxide is oxidized to molecular oxygen, and another is reduced to hydrogen peroxide. The dismutation reaction of superoxide is catalyzed by superoxide dismutase (SOD) with a reaction rate constant of ~1.6 × 10^9 $M^{-1}s^{-1}$ which is over three orders of magnitude greater than that of spontaneous dismutation (**Figure 6**).

In mammalian species, including humans, there are three types of SOD: (1) Cu,ZnSOD, (2) manganese (MnSOD), and (3) extracellular SOD (ECSOD). All three forms of SOD catalyze dismutation of superoxide to hydrogen peroxide and molecular oxygen at a similar reaction rate constant.

Recently, NAD(P)H oxidoreductase 1 (NQO1), an enzyme that catalyzes 2-electrone reduction of quinone compounds, has been suggested to scavenge biological superoxide possibly by catalyzing its conversion to hydrogen peroxide [39, 40]. NQO1 appears to play a role in scavenging superoxide in tissues, such as vascular endothelium and myocardium, where SOD is relatively deficient and NQO1 is, on the other hand, highly expressed [40]. The fate of cellular superoxide is illustrated in **Figure 7**.

4.5. Pathophysiology

Although superoxide possesses physiological functions, dysregulated formation of this oxygen radical leads to tissue injury. In fact, formation of superoxide is an important mechanism of oxygen toxicity. The tissue damage may occur as a result of direct effects of superoxide on molecular targets but more likely due to the secondary formation of hydrogen peroxide from superoxide dismutation or the formation of peroxynitrite from the reaction of superoxide with nitric oxide (**Figure 4**).

The causal involvement of superoxide in disease pathogenesis is supported by numerous studies showing that genetic deletion of SOD causes diverse disease processes and transgenic overexpression of SOD renders resistance to disease pathogenesis in animal models [41–43]. The diseases or conditions whose pathogenesis is impacted by the alterations of SOD expression or content involve diverse organs and systems, and include aging [44, 45], cancer [46–48], cardiovascular diseases [49, 50], diabetes [42, 51], neurodegeneration [52, 53], immunological disorders [54, 55], pulmonary disorders [56, 57], hepatic disorders [58, 59], gastrointestinal diseases [60, 61], and kidney diseases [51, 62, 63].

Although substantial evidence supports a causal involvement of superoxide in diverse disease processes in animal models, the exact role of superoxide and SOD in disease pathogenesis in humans remains to be established. The only human disease that is, thus far, known to be affected by mutations in the SOD (Cu,ZnSOD) gene is familial amyotrophic lateral sclerosis [64]. Large-scale, well-designed, randomized controlled trials on using SOD in human disease intervention are currently lacking. Clinical research on the role of superoxide in disease process is hampered by the limited bioavailability of native SOD. In this context, recent development in selective SOD biomimetics with favorable pharmacokinetic properties [65] would facility research on the role of superoxide in human diseases.

5. PERSPECTIVES

The past several decades have witnessed substantial advances in understanding the chemistry, biology, and medicine of superoxide. Its ubiquitous presence in biological systems, its formation via defined cellular processes, and its unique biochemical properties make superoxide an important factor involved in diverse physiological and pathophysiological processes. Ongoing efforts on both basic and clinical studies of superoxide will further increase our understanding of this ROS with regard to its significance in biology

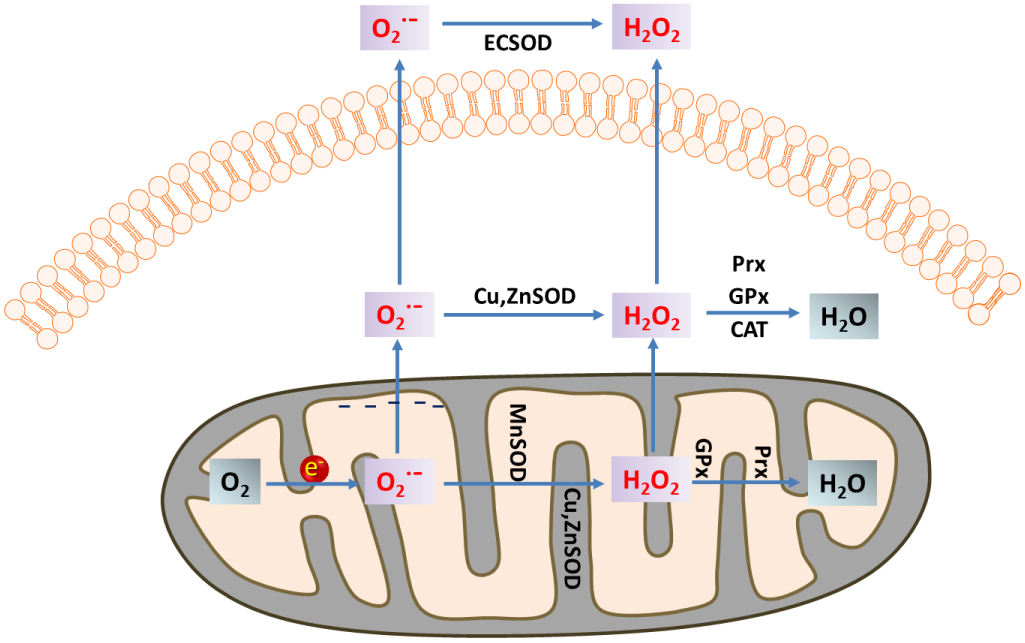

FIGURE 7. The cellular fate of mitochondria-derived superoxide. Mitochondrial electron transport chain (METC) is the major source of cellular superoxide formation. The METC-derived superoxide undergoes MnSOD-catalyzed dismutation to form H_2O_2, which is then converted to water by glutathione peroxidase (GPx) or peroxiredoxin (Prx) in mitochondrial matrix. Superoxide is able to cross the mitochondrial inner membrane and enter the cytosol, where Cu,ZnSOD catalyzes its dismutation to H_2O_2, and the H_2O_2 formed in the cytosol or escaped from mitochondria is converted to water by enzymes, including GPx, Prx, and catalase (CAT), in the cytoplasm. CuZnSOD present in the mitochondrial intermembrane space also catalyzes the dismutation of superoxide present in the intermembrane space. ECSOD acts on extracellular superoxide.

and medicine. The coming years will likely witness the translation of the new knowledge learned from studies with experimental models into the development of mechanistically-based strategies for modulating superoxide-mediated biological effects in humans so as to minimize its deleterious effects while maintaining the physiological function of this oxygen free radical.

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