ROS

RESEARCH HIGHLIGHTS

Nanomaterials for Selective Superoxide Dismutation

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ABSTRACT | Superoxide is a primary reactive oxygen species (ROS) formed from aerobic metabolism. Superoxide dismutase (SOD), by catalyzing the conversion of superoxide to form hydrogen peroxide and molecular oxygen, is a first-line defense against superoxide toxicity. Due to the protein nature of endogenous SOD, small molecule SOD mimetics are commonly used to protect against superoxide-mediated pathophysiological processes and identify the causal role of this ROS in diseases pathogenesis. Existing small molecule SOD mimetics, however, also are reactive toward other ROS and related species, including hydrogen peroxide, nitric oxide, and peroxynitrite, which makes it difficult to delineate the involvement of superoxide in the disease process. Nanomaterials, such as cerium oxide nanoparticles and fullerenes, also possess SOD activity, but again lack specificity for superoxide. A recent study by Samuel et al. reported in the Proceedings of the National Academy of Sciences of the United States of America (2015 Feb 24; 112(8):2343–8. doi: 10.1073/pnas.1417047112) demonstrates that poly(ethylene glycolated) hydrophilic carbon clusters (PEG-HCCs) efficiently catalyze the conversion of superoxide to form hydrogen peroxide and molecular oxygen, and more importantly, the carbon nanaoparticles are inert to nitric oxide and peroxynitrite. This work represents a major advancement in the development of SOD biomimetics that are highly specific for superoxide. Application of such specific SOD biomimetics would have significant impact on the field of superoxide biology and medicine.

KEYWORDS | Hydrophilic carbon clusters; Nanomaterials; Reactive oxygen species; Superoxide; Superoxide dismutase

ABBREVIATIONS | EPR, electron paramagnetic resonance; MPO, myeloperoxidase; PEG-HCCs, poly(ethylene glycolated) hydrophilic carbon clusters; RNS, reactive nitrogen species; ROS, reactive oxygen species; SOD, superoxide dismutase

CONTENTS

1. SUPEROXIDE AS A PRIMARY REACTIVE OXYGEN SPECIES

Utilization of molecular oxygen by aerobic organisms inevitably leads to the formation of reactive oxygen species (ROS). ROS simply refers to oxygen-containing reactive species. It is a collective term to include superoxide anion radical (or superoxide for short) ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$), singlet oxygen ($^1O_2$), peroxyl radical ($LOO^{\bullet}$), alkoxyl radical ($LO^{\bullet}$), lipid hydroperoxide (LOOH), peroxynitrite ($ONOO^-$), hypochlorous acid (HOCl), carbonate radical ($CO_3^{\bullet}$), nitrogen dioxide radical (commonly known as nitrogen dioxide) ($NO_2^{\bullet}$), and ozone (O_3), among others. As shown in **Figure 1**, superoxide is a primary ROS that leads to the formation of many secondary ROS or reactive nitrogen species (RNS). The term RNS refers to nitrogen-containing reactive species, such as nitric oxide ($NO^{\bullet}$), nitrogen dioxide, and peroxynitrite. Nitric oxide and nitrogen dioxide are free radicals, whereas peroxynitrite is a non-radical. Because biologically relevant RNS are almost exclusively also oxygen-containing species, the compound term ROS/RNS is frequently used in the literature. Also for this same reason, the oxygen containing RNS are sometime classified into the ROS category.

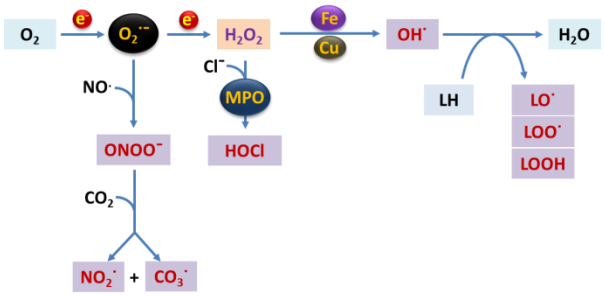

FIGURE 1. Superoxide as a primary ROS, leading to the formation of secondary ROS and RNS. As illustrated, the univalent reduction of molecular oxygen forms superoxide, which can then undergo one electron reduction to form hydrogen peroxide. Reaction of hydrogen peroxide with transition metal ions including copper (Cu) and iron (Fe) forms hydroxyl radicals which cause damage to all biomolecules, including lipids. Oxidation of lipid (LH) by hydroxyl radical gives rise to lipid peroxide (LOOH), alkoxyl radical ($LO^{\bullet}$), and peroxyl radical ($LOO^{\bullet}$). Superoxide reacts with nitric oxide forming peroxynitrite which can then react with CO_2 leading to the formation of carbonate radical ($CO_3^{\bullet}$) and nitrogen dioxide ($NO_2^{\bullet}$). In the presence of phagocyte-derived myeloperoxidase (MPO), hydrogen peroxide reacts with chloride ion to form hypochlorous acid (HOCl), a potent oxidant.

2. SUPEROXIDE DISMUTASE AS THE INTRINSIC CELLULAR DEFENSE AGAINST SUPEROXIDE TOXICITY

Although the harmful effects of oxygen were noticed in the 19th century, insights into the molecular mechanisms of oxygen toxicity only began to emerge during 1950's and 1960's. In 1954, Rebeca Gerschman et al. published an article in the Science magazine, hypothesizing that oxygen poisoning and radiation injury have at least one common basis of action, possibly through the formation of oxidizing free radicals [1]. Subsequently, in 1969 Joe M. McCord and Irwin Fridovich reported the discovery of an enzymatic function of a protein containing both copper and zinc, which then was known alternatively as erythrocuprein, hepatocuprein, or cerebrocuprein [2]. The function of this enzyme is the catalysis of dismutation of superoxide to produce hydrogen peroxide and molecular oxygen, and is known as Cu,Zn superoxide dismutase (Cu,ZnSOD). This discovery has triggered extensive investigations into the free radical mechanisms of oxygen toxicity. Substantial evidence accumulated over the past several decades suggests a critical involvement of superoxide and other ROS in the pathophysiology of a variety of human diseases. **Figure 2** illustrates the molecular mechanisms underlying superoxide toxicity.

It is now known that SOD exists in three forms in mammals: (i) Cu,ZnSOD, as mentioned above, (ii) manganese superoxide dismutase (MnSOD), and (iii) extracellular superoxide dismutase (ECSOD). Cu,ZnSOD is a homodimer with a molecular mass of 32 kDa. Both MnSOD and ECSOD are homotetramers with a molecular mass of 86–88 and 135 kDa, respectively. ECSOD also contains copper and zinc. Cu,ZnSOD is primarily present in cytosol. It may also be present in other subcellular compartments, including nucleus, mitochondrial intermembrane space, as well as peroxisome [3, 4]. The presence of

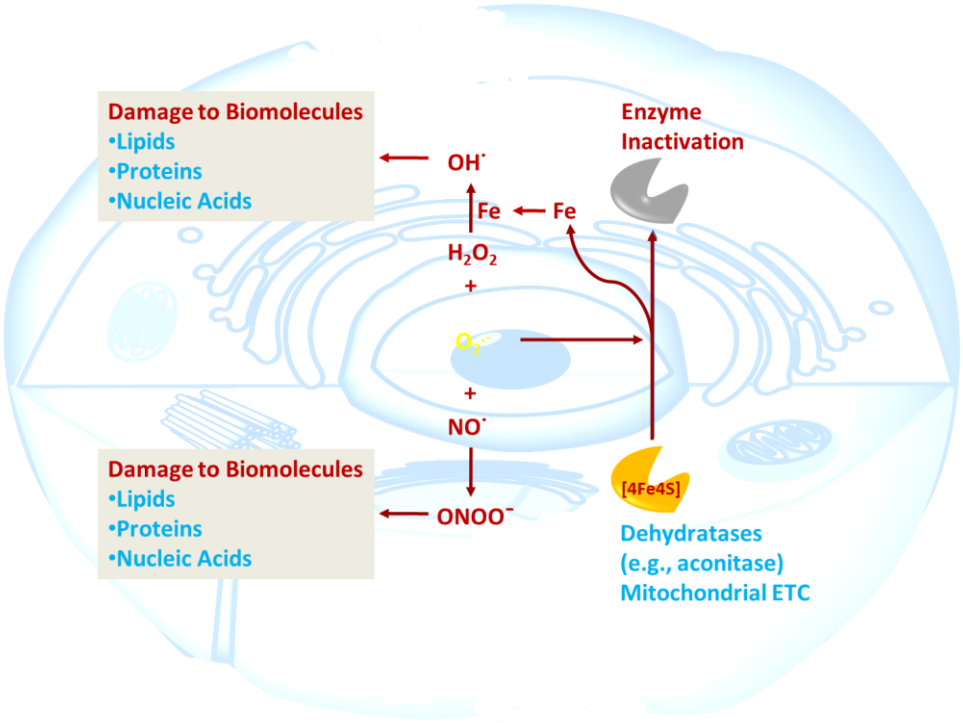

FIGURE 2. Molecular mechanisms of superoxide toxicity in biological systems. Superoxide is not a strong oxidizing species. Instead, due to its lower reduction potential (the standard reduction potential for the O_2/$O_2^{\bullet-}$ redox couple is -330 mV), superoxide often acts as a reducing agent rather than an oxidizing species. 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. As illustrated, the biologically damaging potential of superoxide is related to its chemical properties: (i) although in general it acts as a reducing agent, superoxide does oxidize the iron-sulfur clusters in several enzymes, including aconitase (an enzyme of the tricarboxylic acid cycle) and mitochondrial electron transport chain enzyme complexes. Oxidation of the iron-sulfur clusters by superoxide in these enzymes leads to the release of iron and enzyme inactivation; (ii) superoxide reacts with nitric oxide at an almost diffusion-limited rate to form peroxynitrite. Peroxynitrite is a potent oxidant that causes damage to various biomolecules; and (iii) in the presence of transition metal ions, such as iron ions, superoxide and hydrogen peroxide together can give rise to hydroxyl radical via a reaction known as iron-catalyzed Haber-Weiss reaction.

Cu,ZnSOD in nucleus is in line with a recent finding that Cu,ZnSOD may act as a transcription factor to regulate oxidative stress resistance and repair genes [5]. MnSOD exists in mitochondrial matrix. ECSOD is associated with plasma membrane or present in extracellular space.

All of the three isozymes of SOD catalyze dismutation of superoxide to form hydrogen peroxide and molecular oxygen with a similar reaction rate constant of ~1.6 × 10^9 $M^{-1}s^{-1}$ (Reaction 1). The reaction rate constant of SOD-catalyzed dismutation of superoxide is over three orders of magnitude greater than that of spontaneous dismutation, which is ~5 × 10^5 $M^{-1}s^{-1}$ at a pH of 7.0.

$$2\ O_2^{\bullet-} + 2\ H^+ \xrightarrow{SOD} H_2O_2 + O_2 \quad (1)$$

The overall mechanism by which SOD functions has been called "ping-pong" mechanism as it involves the sequential reduction and oxidation of the

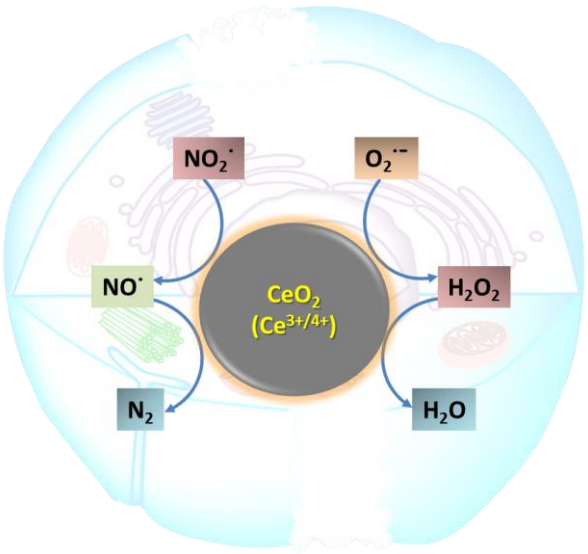

FIGURE 3. Cerium oxide as a non-selective SOD biomimetic. Cerium is a rare earth element of the lanthanide series. The oxide form (CeO_2) is used as a support material in automotive catalysis. Cerium oxide nanoparticles, also known as nanoceria, have been shown to scavenge superoxide possibly via the redox properties of Ce^{3+}/Ce^{4+} [9, 10]: (i) $O_2^{\cdot-} + Ce^{4+} \rightarrow O_2 + Ce^{3+}$ and (ii) $O_2^{\cdot-} + Ce^{3+} + 2\ H^+ \rightarrow H_2O_2 + Ce^{4+}$. Because of the above redox reactions, cerium oxide nanoparticles have been suggested to possess an SOD mimetic activity, i.e., the autocatalytic dismutation of superoxide. It is further reported that the reaction rate constant for cerium oxide nanoparticle-catalyzed dismutation of superoxide may exceed that determined for the enzyme SOD [9]. More recently, cerium oxide nanoparticles are found to exhibit a redox state-dependent catalase mimetic activity [11]. There is also evidence for the ability of cerium oxide to catalytically reduce nitrogen dioxide to nitric oxide, and nitric oxide to nitrogen gas (N_2) [12, 13]. In contrast to the ROS/RNS-scavenging activities, formation of hydroxyl radicals from a Fenton-type reaction between cerium and hydrogen peroxide has been observed in vitro via the use of electron paramagnetic resonance spectrometry in combination with spin trapping [14].

metal center, with the concomitant oxidation and reduction of superoxide (Reactions 2 and 3, where M stands for the redox metal ion of the SOD).

$$M^{n+}\text{-SOD} + O_2^{\cdot-} \rightarrow M^{(n-1)+}\text{-SOD} + O_2 \quad (2)$$

$$M^{(n-1)+}\text{-SOD} + O_2^{\cdot-} + 2\ H^+ \rightarrow M^{n+}\text{-SOD} + H_2O_2 \quad (3)$$

3. SMALL MOLECULE SOD MIMETICS

The potential involvement of superoxide in disease pathophysiology has led to the development of small molecule SOD mimetics that easily penetrate cell membrane to detoxify intracellular superoxide [6]. This would overcome limitations associated with use of the native SODs, including the size of the protein molecule, limited membrane permeability, short half-life, antigenicity, and high costs. Commonly used SOD mimetics are compounds that contain manganese (Mn) as the redox-active center, and include the following 3 classes: (i) Mn(III) metalloporphyrins (e.g., MnTBAP, MnTMPyP); (ii) Mn(III) salen complexes (e.g., EUK-8, EUK-134); and (iii) Mn(II) pentaazamacrocyclic ligand-based complexes (e.g., M40401, M40403). However, these SOD-mimetics lack specificity for superoxide, and may also scavenge other ROS/RNS and related species, including hydrogen peroxide, nitric oxide, and peroxynitrite, among others, which would make it impossible to delineate the causal involvement of superoxide in a pathophysiological process.

4. NANO-SOD BIOMIMETICS

Nanomaterials have received increasing attention in biology and medicine due to their unique interactions with biological systems and their potential applications as redox-active antioxidants [7, 8]. Several nanomaterials, including cerium oxide nanoparticles, platinum nanoparticles, and fullerene derivatives have been shown to possess antioxidative activities, including scavenging superoxide in biological systems. However, like small molecule SOD mimetics, these nanomaterials also show activities toward other ROS/RNS and related species [9–14] (**Figure 3**). In contrast to the above SOD biomimetics, the study by Samuel et al. shows that poly(ethylene glycolated) hydrophilic carbon clusters (PEG-HCCs) possess selective activity to superoxide and are inert to nitric oxide and peroxynitrite [15], thus offering a unique approach to studying superoxide.

Using 5-(diethoxyphosphoryl)-5-methylpyrrole-*N*-oxide (DEPMPO)-spin trapping technique in combination with electron paramagnetic resonance (EPR) spectrometry, the authors first demonstrated a scavenging activity of PEG-HCCs toward superoxide generated from potassium superoxide (KO_2) [15]. KO_2 decomposes to release superoxide in aqueous solutions and is widely used as a generator of superoxide. PEG-HCCs were also found to scavenge hydroxyl radicals generated from the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^-$) [15]. It is not surprising for an antioxidant compound to scavenge hydroxyl radicals, the most oxidizing ROS that can react with virtually all cellular constituents at a diffusion-limited rate.

To investigate the catalytic property of PEG-HCCs, the authors next applied a steady-state kinetic assay using low-temperature EPR and demonstrated that PEG-HCCs behaved as catalysts because the molar ratio of $O_2^{\bullet-}$ consumed to PEG-HCCs was far beyond the number of active sites on the PEG-HCCs. Using oxygen polarography and by measuring hydrogen peroxide formation, the authors showed the increased formation of oxygen and hydrogen peroxide (with a ratio of O_2 to H_2O_2 of 1) from PEG-HCC-catalyzed reaction of superoxide [15]. The authors further demonstrated that both self-dismutation and turnover of superoxide by PEG-HCCs followed the same mechanism leading to OH^- formation, and the stoichiometry between $O_2^{\bullet-}$ and OH^- was 1:1. Notably, the PEG-HCC nanoparticle was found to be a stable free radical species (PEG-HCC$^{\bullet}$), which might explain its high reactivity toward superoxide [15]. Collectively, the results led the authors to suggest that PEG-HCCs catalyzed $O_2^{\bullet-}$ conversion via a dismutation process involving 2 reactions (**Figure 4**): (i) PEG-HCC$^{\bullet}$ + $O_2^{\bullet-} \rightarrow$ PEG-HCC$^-$ + O_2 and (ii) PEG-HCC$^-$ + $O_2^{\bullet-}$ + 2 $H_2O \rightarrow$ PEG-HCC$^{\bullet}$ + H_2O_2 + 2 OH^- [15]. As indicated, the PEG-HCC nanoparticle acts as a catalyst to accelerate the dismutation of two molecules of superoxide to form one molecule of hydrogen peroxide and two molecules of hydroxide ions. To investigate the efficiency of the PEG-HCC nanoparticle as an SOD biomimetic, the authors compared the activity of PEG-HCCs with that of Cu,ZnSOD and observed that on a molar basis, PEG-HCCs were as efficient at turning over $O_2^{\bullet-}$ as Cu,ZnSOD [15]. Lastly, by using a standard hemoglobin assay, the authors showed that PEG-HCCs were not reactive toward nitric oxide (NO$^{\bullet}$) (15). Because nitric oxide reacts with superoxide at an almost diffusion-limited rate to form peroxynitrite (**Figure 1**), the reactivity toward peroxynitrite was also determined through examining peroxynitrite-induced quenching of the dye pyrogallol red. Again, PEG-HCCs were found to be inert to peroxynitrite [15].

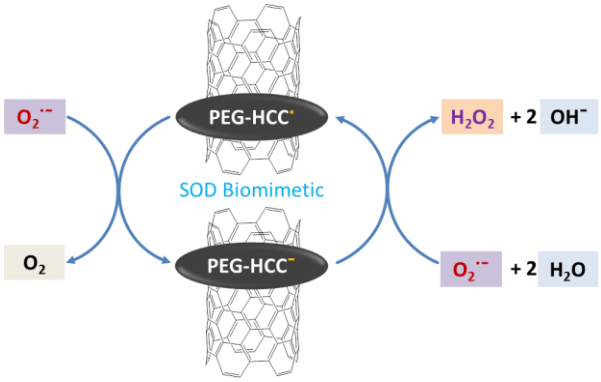

FIGURE 4. The proposed mechanism of action of the PEG-HCC nanotube as an SOD biomimetic to catalyze the dismutation of superoxide to form hydrogen peroxide and molecular oxygen. It is important to note that PEG-HCCs are inert to nitric oxide and peroxynitrite as well as hydrogen peroxide. Hence, the PEG-HCC nanoparticle may be considered as a highly selective SOD biomimetic, whose wide use may lead to a better understanding of the involvement of superoxide in biology and medicine. The scheme is based on Ref. [15].

5. IMPLICATIONS

The high selectivity of PEG-HCCs for superoxide makes it a valuable SOD biomimetic for investigation of superoxide in biological systems. Commercialization and subsequent wide use of PEG-HCCs in research would inevitably lead to a better understanding of the involvement of superoxide in both physiological and pathophysiological processes.

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