Proc. Indian natn Sci Acad. **B69** No.4 pp 453- 460 (2003)

Redox-Active Metal Ions and Oxidative Stress: Therapeutic Implications

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(Received on 2 August 2002; Accepted after revision on 5 October 2002)

Reactive oxygen and nitrogen species (RONS) such as superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($^{\bullet}OH$) and peroxynitrite ($ONOO^-$), have been ascribed an important role in oxidative stress contributing to the progression of inflammatory diseases such as rheumatoid arthritis (RA). RONS are generated by a number of pathways including enzymes, the inflammatory response and as side products of catabolism. A number of protective enzymes exist for the regulation of RONS. These include superoxide dismutase (SOD), catalase and glutathione peroxidase. In addition, vitamins play a secondary role in deactivating RONS. RONS can release redox-active metal ions such as Fe(II) and Cu(I) from the storage proteins ferritin and caeruloplasmin. Redox active metal ions further activate RONS and thus perpetuate their damaging effects. In addition to initiating and/or perpetuating the lipid peroxidation process these species are credited with degradation of DNA, destruction of endothelial cells and induction of increased vascular permeability. Therapies based on dual-function antioxidants that bind metal ions and scavenge RONS are currently being developed. The biological basis for this new therapeutic approach and recent advances will be presented in this review.

Key Words: Reactive oxygen & Nitrogen species, Metal ions, Oxidative stress, Inflammation, Rheumatoid arthritis

Introduction

Oxidative stress results from an imbalance between the generation of reactive oxygen and nitrogen species (RONS) and their suppression by antioxidant defence mechanisms. It contributes to the pathogenesis of a number of diseases including rheumatoid arthritis (RA), Alzheimer's disease, atherosclerosis and diabetes mellitus (Halliwell et al. 1999). During the past three decades oxidative stress has been the subject of an enormous amount of research. However, the mechanisms involved in oxidative stress have yet to be fully elucidated. The relatively recent discovery of new components such as nitric oxide and peroxynitrite and the mechanisms of their formation have added further complexities to the field (Halliwell et al. 1999).

Redox-active metal ions such as Fe(II) and Cu(I) are released from storage proteins by RONS and have been detected in a number of diseased tissues for example in the RA joint (Naughton et al. 1995). These metal ions have been shown to participate in several reactions that enhance oxidative stress (Liochey et al. 1999). By switching oxidation states these redox-active metal ions further activate species like hydrogen peroxide (H_2O_2) and superoxide ($O_2^{\bullet-}$)to the highly reactive hydroxyl radical ($^{\bullet}OH$). In addition, nitric oxide ($NO^{\bullet}$) reacts with $O_2^{\bullet-}$ to form the potent species peroxynitrite ($ONOO^-$). Damage to biological components by $ONOO^-$ is catalysed by Fe(III) ions. Our antioxidant defence consists of protective enzymes and natural antioxidants. As protective enzymes exist for H_2O_2 and $O_2^{\bullet-}$ but not for $ONOO^-$ and $^{\bullet}OH$, the latter two highly-reactive unregulated species pose a serious threat to biological systems (Halliwell et al. 1999).

In this review we will give an overview of oxidative stress. We will cover the generation and suppression of RONS with a particular focus on metal ions as they contribute significantly to the production of $^{\bullet}OH$ and the activation of $ONOO^-$.

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Oxidative stress within the inflamed rheumatoid joint has been particularly well studied (Halliwell et al. 1999). For this reason, in this review this clinical condition will be adopted as a representative systemic disease (Schett et al. 2001). Oxidative stress is a characteristic feature of a number of diseases that have poor therapeutic outcomes (including RA) and a requirement exists for the development of new therapies. In addition, oxidative stress has recently been ascribed an important role in neurological degenerative conditions such as Alzheimer's Disease (Aliev et al. 2002). A new therapeutic approach involving the development of metal binding compounds (chelators) that also scavenge RONS will be discussed.

Production and Regulation of RONS

Enzymes as Sources of RONS

Physiological production of NO• is a major source of free radicals *in vivo*. NO• is synthesised by a five-electron oxidation of arginine by the enzyme, nitric oxide synthase (NOS). The electrons required for this process are derived from NADPH. There are three isoforms of NOS. Neuronal NOS (nNOS) was first identified in the neurones, and similarly endothelial NOS (eNOS) was first identified in endothelial cells. Both these isoforms are constitutive NOS, which are activated by calmodulin (a Ca(II)-binding protein), via Ca(II) dependent mechanism. The third isoform, inducible NOS (iNOS), also binds to calmodulin, but it is bound so tightly that it is independent of Ca(II) (Lincoln et al. 1997).

NO• is a small-uncharged diatomic molecule comprised of nitrogen and oxygen, with an unpaired electron in a π bonding orbital. Following stimulation of eNOS, NO• is synthesised. Its lack of charge allows it to diffuse freely into the smooth muscle cell, activating soluble guanylate cyclase, increasing levels of cGMP, and resulting in vascular smooth muscle relaxation. In the peripheral nervous system (PNS) NO• acts as a neurotransmitter. In the central nervous system (CNS), NO• has a cyto-protective and neurotoxic role (Lincoln et al. 1997).

A detrimental role for NO• has been implicated in a number of diseases, which can be classified according to the regulating NOS isoforms. eNOS dysfunction gives rise to cardiovascular diseases such as hypertension, ischaemia and atherosclerosis (Dai et al. 2002). nNOS disorders such as stroke leads to neuronal damage within the peripheral and central nervous system. iNOS disorders are a result of immunological and inflammatory dysfunction, resulting in septic shock and autoimmune diseases such as RA.

Under normal conditions iNOS is not found in most cells. Activation of T_H-cells or macrophages results in the secretion of cytokines. These rapidly induce the expression of iNOS in many immune and non-immune cells. The ensuing production of NO• results in cell death of the pathogen by either apoptosis or necrosis. The increased levels of nitrite in RA synovial fluid have been linked to increased iNOS expression within the inflamed synovium (Farrell et al. 1992). Free nitrotyrosine has been detected in synovial fluid and sera of patients suffering from RA. In addition, using antibody labelling, proteins nitrated at tyrosine residues were detected in macrophages and vascular smooth muscle (Mapp et al. 2001). The prime candidate for tyrosine nitration *in vivo* is believed to be $ONOO^-$ the reaction product of NO• and $O_2^{\bullet -}$. Current research is investigating $ONOO^-$ as a principal mediator of cellular damage.

In the hypoxic synovial membrane of the RA joint, the enzyme system xanthine dehydrogenase/oxidase (XDH/XO) is converted to the oxidase form. XO reacts with molecular oxygen to generate $O_2^{\bullet -}$ and H_2O_2. Several studies have shown however that $O_2^{\bullet -}$ and H_2O_2 generated by xanthine oxidase, can react directly to give singlet oxygen ($^1O_2$), (eq. 1) (Mao et al. 1995, Kellog et al. 1975) by the Haber-Weiss reaction, resulting in lipid peroxidation. More recently, a new function of XDH in the reductive generation of NO• from nitrite has been demonstrated (Zhang et al.1997). This activity of the enzyme coupled to superoxide production led to the demonstration that XDH/XO can produce $ONOO^-$.

$$O_2^{\bullet -} + H_2O_2 \rightarrow OH^- + {}^{\bullet}OH + {}^1O_2 \quad \ldots(1)$$

The inflammatory response results in the recruitment and activation of inflammatory cells within the inflamed rheumatoid joint. These cells such as macrophages are a major source of RONS as a part of their protective role. $O_2^{\bullet -}$ is only generated by design in activated phagocytes by the enzyme NADPH oxidase (eq 2). Macrophages are known to secrete both NO• and $O_2^{\bullet -}$, which as described later

react to generate the highly reactive oxidising species $ONOO^-$.

$$2O_2 + NADPH \longrightarrow 2O_2^- + NADP^+ + H^+ \quad \ldots (2)$$

Superoxide ($O_2^{\bullet -}$) is generated as a result of leakage of electrons from the mitochondrial electron transport onto O_2, and by the oxidation of molecules such as glyceraldehydes and FADH_2 in the presence of O_2 (Lenaz et al. 2002). Superoxide is the one-electron reduction product of molecular oxygen, and subsequently has a negative charge. It has an unpaired electron in a π antibonding orbital. $O_2^{\bullet -}$ is not highly reactive in aqueous solution, but it will however rapidly react with nitric oxide to form peroxynitrite (as discussed later) (Munzel et al. 1997).

The greater part of $O_2^{\bullet -}$ generated in aqueous solution will undergo a dismutation reaction to form hydrogen peroxide. This generally advances by way of protonation of $O_2^{\bullet -}$ to give $HO_2^{\bullet}$, and its subsequent reaction with a second $O_2^{\bullet -}$ molecule. H_2O_2 is neither a strong oxidizing nor reducing agent, reflected by its reduction potential ($E^\circ = 0.32$ V) (Halliwell et al. 1999). It is freely diffusible, and moves easily within cells. It is generated in the body by the disproportionation of superoxide, generated by the mechanisms explained previously.

In addition, H_2O_2 is produced in peroxisomes, by flavoprotein dehydrogenases involved in β-oxidation of fatty acids. These enzymes catalyse the two-electron transfer to O_2 to form H_2O_2. The major site of catalase activity in the body is in peroxisomes (Dansen et al. 2001).

When $O_2^{\bullet -}$ is generated close to vascular endothelial cells it causes vasoconstriction (Zhilaiev et al. 2002); and will therefore oppose the effect of NO$^\bullet$. Any $O_2^{\bullet -}$ present on the NO$^\bullet$ endothelium-smooth muscle pathway will react with NO$^\bullet$ at diffusion-limited rates ($k_1 = 1.9 \times 10^{10}$ $M^{-1} s^{-1}$) (Richeson et al. 1998) to form $ONOO^-$. This would subsequently cause cellular damage, and would also prevent smooth muscle vasodilation. Once formed the $ONOO^-$ generally reacts with aqueous CO_2, due to its high biological concentration (~ 1mM), and their high reactivity which is independent of *p*H ($k_2 = 5.8 \times 10^4 M^{-1} s^{-1}$) (Squadrito et al. 1998). The reaction of $ONOO^-$ and CO_2 (eq 3) produces several short-lived reaction oxidizing and nitrating intermediates, which are thought to be the free radicals $^\bullet NO_2$ and $CO_3^{\bullet -}$. These intermediates are thought to nitrate tyrosine molecules, which can alter tyrosine phosphorylation, and consequently regulation of enzyme activity.

$$CO_2 + ONOO^- (Intermediates) \longrightarrow CO_2 + NO_3^- \quad \ldots (3)$$

As a consequence of the speed of the reaction between CO_2 and $ONOO^-$, most scavengers such as ascorbic acid and glutathione are ineffectual. Therefore the main biological defence against $ONOO^-$ is to restrict its formation by decreasing the concentrations of NO$^\bullet$ and $O_2^{\bullet -}$. NO$^\bullet$ cannot be limited due to the importance of its physiological functions. However the $O_2^{\bullet -}$ concentrations could be lowered by use of superoxide dismutase (SOD) and SOD mimics. $ONOO^-$ has been implicated as a contributory factor in various diseases, including rheumatoid arthritis, type I diabetes and Alzheimer's disease (Xie et al. 2002).

Antioxidant Enzymes that Regulate RONS Levels

To prevent oxidative damage by $O_2^{\bullet -}$ the body developed an antioxidant defence enzyme, specific for $O_2^{\bullet -}$, called superoxide dismutase (SOD). SOD is a metalloenzyme, of which there are three different isoforms in humans. The Mn SOD isoenzyme is found principally in mitochondria, and the extracellular (EC) SOD isoenzyme is found primarily in the extracellular matrix of tissue. A high concentration of EC SOD exists in the pulmonary and systemic arteries to regulate $O_2^{\bullet -}$ levels.

The third and most abundant isoenzyme is CuZn SOD. It is found predominantly in the cytosol and the nucleus of the cell. The Cu(II) is the catalytic part of the enzyme, whose structure is stabilised by the Zn(II) (Bergendi et al. 1999). The CuZn SOD catalyses the dismutation of $O_2^{\bullet -}$ to H_2O_2 and O_2 via an alternate oxidation and reduction reaction. (eq. 4,5)

$$SOD\text{-}Cu(II) + O_2^{\bullet -} \longrightarrow SOD\text{-}Cu^+ + O_2 \quad \ldots (4)$$

$$SOD\text{-}Cu^+ + O_2^{\bullet -} + 2H^+ \longrightarrow SOD\text{-}Cu(II) + H_2O_2 \quad \ldots (5)$$

SOD does not however just prevent damage from $O_2^{\bullet -}$. Of greater significance is the enzymes ability to prevent the reaction of $O_2^{\bullet -}$ with NO$^\bullet$ to form $ONOO^-$. Although H_2O_2 is not itself reactive, in the presence of ferrous iron it is reduced to the highly reactive hydroxyl radical ($^\bullet OH$). This is known as the Fenton reaction (discussed later). To counter this the body has an antioxidant enzyme

called catalase. There are two types of catalase, the Mn-catalase present in some prokaryotes, and the Fe-heme-catalase, which is present in mammals. Catalase is found in the peroxisomes, together with the enzymes that generate H_2O_2. The enzyme has two states the Fe(III) state and the 'compound I' state. The enzyme catalyses the two-electron transfer mechanism to disproportionate H_2O_2 into O_2 and H_2O (eqns 6,7) (Bravo et al. 1997).

$$\text{Catalase-Fe(III)} + H_2O_2 \rightarrow \quad \text{Compound I} + H_2O \quad \ldots(6)$$

$$\text{Compound I} + H_2O_2 \rightarrow \text{Catalase-Fe(III)} + H_2O + O_2 \quad (7)$$

A second antioxidant enzyme called peroxidase, catalyses the breakdown of H_2O_2 to H_2O, via the oxidation of a second molecule. $^{\bullet}OH$ is responsible for a large percentage of DNA damage, specifically double strand breaks that are hard to repair. Catalase and peroxidase therefore remove the H_2O_2 from the system.

Mechanisms of Redox-active Metal Ion Mediated RONS Formation

The Fenton reaction involves the transition metal catalysed reduction of H_2O_2 to generate a powerful oxidizing species. Transition metals have varying oxidation states, and therefore they are able to catalyse oxidation and reduction reactions. Iron is the most common transition metal in the body. Circulating iron is tightly bound to the protein transferrin, which reduces its reduction potential, and subsequently its reactivity with H_2O_2. In addition to circulating iron and other body stores, there is a pool of iron within cells referred to as the 'low molecular weight iron'. This iron is loosely bound to 'low molecular weight' compounds such as ATP, phosphate and citrate.

The standard reduction potential for Fe(III)-citrate/Fe(II)-citrate is 0.1 volts and is greater than the standard reduction potential for $O_2/ O_2^{\bullet -}$ at -0.33 volts (Halliwell et al. 1999). Thus it is thermodynamically feasible for low molecular weight iron to be redox-active. Fe(III) bound to the chelating agent citrate is reduced by $O_2^{\bullet -}$ (eq. 8). The rate constant for the Fe(II)-citrate in the presence of H_2O_2, where $\kappa_2 = 4.9 \times 10^3\ M^{-1}s^{-1}$ at 25°C (Halliwell et al. 1999) shows the viability of the Fenton reaction in the presence of H_2O_2

$$\text{Fe(III)-citrate} + O_2^{\bullet -} \rightarrow \quad \text{Fe(II)-citrate} + O_2 \quad \ldots(8)$$

$$\text{Fe(II)-citrate} + H_2O_2 \rightarrow {}^{\bullet}OH + OH^- + \text{Fe(III)-citrate} \ldots(9)$$

Therefore the oxidative damage caused as a consequence of this viable hydroxyl-radical production in cells, is dependent on the location of these pools of iron, and the localisation to ROS production.

A recent study has identified the distribution of these iron pools in rat hepatocytes and rat liver endothelial cells, with surprising results. The pool of iron was originally thought to be inherent in the cytosol, however this was disproved and redefined. Significant concentrations of chelatable iron were shown in the mitochondria and the cytosol but remarkably the largest concentration of chelatable iron was demonstrated in the nucleus (Petrat et al. 2001). This knowledge has important implications in understanding the potential for Fenton-mediated oxidative damage in cells. It is predominantly inferred that DNA site-specific damage is attributable to $^{\bullet}OH$ generation via redox-active iron associated with DNA. The presence of redox-active iron as a mediator of oxidative damage is reflected in a number of different disease processes, such as RA (Naughton 2001a). These pathological conditions lay emphasis on the importance of therapeutic use of chelators to selectively eliminate the iron, preventing further oxidative damage.

The catalytic effects of transition metals on the formation of reactive oxygen species are becoming evident as a major factor in many disease processes. This is demonstrated in Alzheimer's disease, a progressive neurodegenerative disorder characterised by amyloid plaques and neuronal cell death. These plaques are formed from the amyloid-β-protein (Aβ), which are proteolytically derived from the amyloid precursor protein (APP). Aβ is toxic to neuronal cells, and hence its production has an important role in neurodysfunction. APP binds to Cu(II) reducing it to Cu(I), leading to the formation of a disulfide bond in APP (Multhaup et al. 1997). The Cu(I) binding to APP may be involved in electron transfer reactions, although the normal function is unknown. In the presence of hydrogen peroxide the Cu(I) ion catalyses the reduction of H_2O_2 to generate $^{\bullet}OH$ by the Fenton reaction. $^{\bullet}OH$ generation results in lipid and protein damage, and oxidative stress in the neurones (White et al. 1999). The resultant amino acid oxidation and protein cross-linking induces the

aggregation of the Aβ to form amyloid plaques, a major pathogenic event in Alzheimer's disease.

The underlying chemical reaction of these diseases is the Fenton reaction, with consequent production of •OH. The hydroxyl radical is the most oxidising free radical species reflected by the reduction potentials. The reduction of H_2O_2 to H_2O has a large free energy decrease, where $E^\circ = 1.35$ V. However the half reaction of H_2O_2 to •OH only has a small decrease in free energy, where $E^\circ = 0.38$ V. Therefore the redox potential of •OH to H_2O is very high, where $E^\circ = 2.33$ V, and therefore the •OH oxidizing capabilities are very strong. The Fenton system can generate a series of reactions, which occur dependent on reaction conditions. (eq. 10 – 14) (Spiro 1980).

$$Fe(II) + H_2O_2 \rightarrow {}^{\bullet}OH + Fe(III) + OH^- \quad \ldots(10)$$

$$^{\bullet}OH + H_2O_2 \rightarrow O_2^{\bullet-} + H_2O + H^+ \quad \ldots(11)$$

$$Fe(III) + O_2^{\bullet-} \rightarrow Fe(II) + O_2 \quad \ldots(12)$$

$$Fe(III) + H_2O_2 \rightarrow Fe(II) + 2H^+ + O_2^{\bullet-} \quad \ldots(13)$$

$$2O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2 \quad \ldots(14)$$

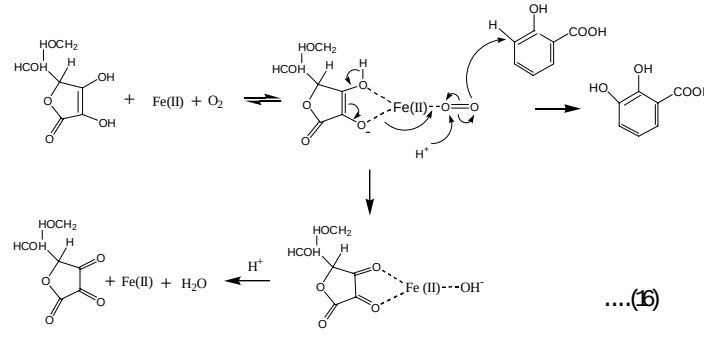
In addition to •OH generation and H_2O_2 decomposition, the recycling of Fe(III) to Fe(II) denotes that only small concentrations of Fe(II) are needed for •OH generation. Conversely there is still one point of debate on the Fenton reaction, and that is the generation of •OH. The Fenton reaction denotes that H_2O_2 is reduced at the iron centre to give •OH, in opposition some will argue that some of the •OH may remain bound to the iron centre to give the ferryl intermediate $(Fe{=}O)^{2+}$ (eq. 15) (Lloyd et al. 1997).

$$Fe(II) + H_2O_2 \rightarrow (Fe{=}O)^{2+} + OH^- \quad \ldots(15)$$

Several studies have tried to demonstrate the existence of this ferryl intermediate, and have concluded it exists in addition to the production of •OH. The ferryl intermediate appears to have oxidising properties comparable to •OH, however its reactivity patterns are consistent with an iron complex.

•OH generation is not limited to the Fenton reaction. The Udenfriend system has been proven to hydroxylate aromatic compounds, saturate hydrocarbons to alcohols and olefins to epoxides. Udenfriend's system involves ascorbic acid as a two-electron donor complexed to a transition metal such as Fe(II). It is speculated that in the presence of O_2, complexation between Fe(II) and ascorbic acid results in the formation of an active oxygen species speculated to be •OH (Kasai et al. 1984). The hypothesised mechanism (eq. 16) shows the oxidation of ascorbic acid to dehydroascorbic acid, by electron transfer through Fe(II), and subsequent hydroxylation of an aromatic compound (Martell et al. 1973).

This reaction has been shown to be enhanced when iron is coupled with a chelator for example in iron-citrate complexes, which is how a large



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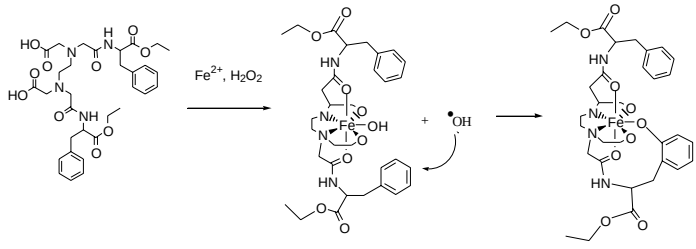
percentage of iron is found within biofluids. Considering the concentration of iron in and around the nucleus has been shown to be remarkably high, this system demonstrates another mechanism for DNA site-specific oxidative damage.

Therapeutic Suppression of RONS

Poor control of RONS levels either by enzymatic generation or suppression leads to oxidative stress. Natural anti-oxidants such as vitamins are restricted in their abilities to counter oxidative stress and in some cases can aggravate it. In the absence of metal ion chelating abilities, vitamins scavenge RONS in a random manner. In addition, vitamin C can reduce the metal ions to enable their participation in RONS activation. As the metal ion is key to several mechanisms of RONS activation, chelation therapies have been investigated. Chelators have been reported that have anti-oxidant activity based on stabilising the metal ion in the oxidised form to prevent RONS activation. An alternative approach comprises dual-functional chelating and scavenging molecules. Thus, the metal ion chelating and scavenging abilities are co-localised and any activated RONS can be readily scavenged.

One recently described system is centred on peroxide and hydroxyl radical scavenging. It is comprised of the chelator ethylenediaminetetraacetic acid (EDTA) and an aromatic moiety suitable for scavenging •OH radicals (Naughton et al. 2001b). In this report, metal ion binding was demonstrated coupled to Fenton generation of •OH. Subsequent directed scavenging of the •OH was shown by analysis of the isomeric tyrosines generated. Modelling and spectroscopic studies indicated that the hydroxylated aromatic ring stabilises the iron as Fe(III) and prevents the occurrence of potentially deleterious redox chemistry at the metal ion centre (see equation 17). Thus, in addition to stabilising the metal ion in a non-redox active form a molecule of H_2O_2 is removed.

More recently in an analogous system these results have been extended to investigate the



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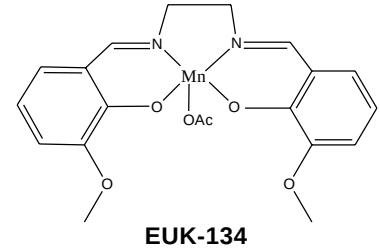
peroxynitrite scavenging abilities of both ferric and cupric complexes of the chelator. In this case nitrotyrosine formation was assessed as a measure of $ONOO^-$ scavenging ability. The rates of nitrotyrosine formation were greatly enhanced by metal ion complexation especially for the Fe(III) chelate. Thus, this system is particularly suited to scavenging the highly reactive $ONOO^-$ and •O H that are unregulated within the body. These results have interesting implications for the development of a unique assay to delineate the contribution of different RONS to oxidative stress within biological systems.

An analogous system is currently under development. Salen-manganese complexes are low-molecular weight compounds that exhibit both SOD and catalase activities and therefore remove superoxide and hydrogen peroxide. They are catalytic so are more potent than other low-molecular weight antioxidants. In comparison to SOD found in biological systems they contain a single Mn(III) ion. The metal is in an unstable oxidation state, but held in a stable environment, so upon oxidation or reduction it will revert back to its original state. The mechanism of its reaction is not completely understood (Baker et al. 1998). These compounds have been examined as potential therapeutics for various diseases including Alzheimer's disease, Parkinson's disease and stroke (Rong et al. 1999). These compounds are notable for their ability to dramatically extend the lifespan in *C. elegans*. These chelators exhibit scavenging abilities with ferric and cupric ions in place of the manganese ion and thus could be applied as dual-function chelator\scavengers to treat oxidative stress.



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Conclusions and Future Directions

Oxidative stress is characteristic of a number of diseases and results from an insufficient antioxidant defence. Thus, a requirement exists for the development of new antioxidant therapies. Here we have presented an overview of the mechanisms that contribute to oxidative stress and highlight the significant role of metal ions in these processes. Recently, a novel therapeutic approach to oxidative stress has been advanced by the development of dual function antioxidants comprising chelating and scavenging components.

Current therapies for oxidative stress are limited by poor therapeutic indices resulting from either poor efficacies and\or significant side effect profiles. The further development and testing of dual function chelating\scavenging antioxidants are warranted. This development will involve studying toxicity, pharmacokinetics and pharmacodynamics.

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