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Antioxidant Therapy: A New Pharmacological Approach in Shock, Inflammation, and Ischemia/Reperfusion Injury

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***Abstract*——A vast amount of circumstantial evidence implicates oxygen-derived free radicals (especially superoxide and hydroxyl radical) and high-energy oxidants (such as peroxynitrite) as mediators of inflammation, shock, and ischemia/reperfusion injury. The aim of this review is to describe recent developments in the field of oxidative stress research. The first part of the review focuses on the roles of reactive oxygen species (ROS) in shock, inflammation, and ischemia/reperfusion injury. The second part of the review deals with the novel findings using recently identified pharmacological tools (e.g., peroxynitrite decomposition catalysts and selective superoxide dismutase mimetics (SODm) in shock, ischemia/reperfusion, and inflammation. 1) The role of ROS consists of immunohistochemical and biochemical evidence that demonstrates the production of ROS in shock, inflammation, and ischemia/reperfusion injury. ROS can initiate a wide range of toxic oxidative reactions. These include initiation of lipid peroxidation, direct inhibition of mitochondrial respiratory chain enzymes, inactivation of glyceraldehyde-3-phosphate dehydrogenase, inhibition of membrane sodium/potassium ATPase activity, inactivation of membrane sodium**

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channels, and other oxidative modifications of proteins. All these toxicities are likely to play a role in the pathophysiology of shock, inflammation, and ischemia/reperfusion. 2) Treatment with either peroxynitrite decomposition catalysts, which selectively inhibit peroxynitrite, or with SODm, which selectively mimic the catalytic activity of the human superoxide dismutase enzymes, have been shown to prevent in vivo the delayed vascular decompensation and the cellular energetic failure associated with shock, inflammation, and ischemia/reperfusion injury. ROS (e.g., superoxide, peroxynitrite, hydroxyl radical, and hydrogen peroxide) are all potential reactants capable of initiating DNA single-strand breakage, with subsequent activation of the nuclear enzyme poly(ADP-ribose) synthetase, leading to eventual severe energy depletion of the cells and necrotic-type cell death. Antioxidant treatment inhibits the activation of poly(ADP-ribose) synthetase and prevents the organ injury associated with shock, inflammation, and ischemia/reperfusion.

I. Introduction

A. Oxygen Radical

A free radical is defined as any atom or molecule possessing unpaired electrons. Molecular oxygen, O_2, is a biradical with two such unpaired electrons. The biologically relevant free radicals derived from oxygen are the superoxide anion ($O_2^{\cdot-}$), the perhydroxyl radical (protonated superoxide, $HO_2^{\cdot}$), the hydroxyl radical ($HO^{\cdot}$), and free radical nitric oxide ($NO^{\cdot}$). The one electron reduction of oxygen (i.e., the addition of one electron to an oxygen molecule) results in formation of $O_2^{\cdot-}$ (also known as the superoxide radical), whereas the two-electron reduction product of oxygen, when fully protonated, forms hydrogen peroxide (H_2O_2). A third species of activated oxygen, known as singlet oxygen, is recognized as a possible contributor to oxidative stress in living systems. Singlet oxygen is a high-energy, electron-spin paired state of dioxygen that is approximately 1 eV higher in energy than ground-state triplet oxygen and is capable of oxidizing a number of biological molecules, including lipid- and olefinic-containing molecules. Finally, an additional reduction product of oxygen, $HO^{\cdot}$, is the most reactive and least selective of all the oxy radical oxidizing agents.

It was believed initially that the toxicity ascribed to the superoxide radical was caused by superoxide's direct interaction with biological targets. It is now clear that many tissue effects of $O_2^{\cdot-}$ result from the secondary formation of other oxygen radicals in addition to direct reactions of superoxide (or its conjugate acid) with biological targets, such as lipids (Aikens and Dix, 1991; Dix and Aikens, 1993), catecholamines (Misra and Fridovich, 1972; Heikkila and Cohen, 1973; Rao and Hayon, 1975; Macarthur et al., 2000), and DNA (Dix et al., 1996). Superoxide in aqueous media undergoes a spontaneous second-order reaction with itself, a dismutation reaction that yields one molecule each of H_2O_2 and oxygen (see reaction 1, Table 1) in a relatively slow reaction at pH 7.4 (the second-order rate constant is of the order of $10^{4.5}$) when compared with the rate at which superoxide or $HO_2^{\cdot}$ can abstract an H-atom from such key biological targets as catecholamines or the allylic CH in lipid where the second-order rate constant exceeds 10^7.

Although the dismutation would be spontaneous at physiological pH at high superoxide concentrations, the concentration of superoxide approaches 10 μM (physiological) as the self-reaction slows down considerably and its lifetime becomes extended to many seconds. Consequently, nature has evolved a class of superoxide dismutase (SOD[2]) enzymes to remove this deleterious free radical byproduct of oxygen metabolism. These enzymes can react rapidly with superoxide (rates approaching or exceeding 10^9) and dismutate the radical to the nonradical products, O_2 and H_2O_2, faster than superoxide can react with other potential biological targets. The short half-life should not be misinterpreted as mitigating the potential reactivity of $O_2^{\cdot-}$ because the half-life is actually quite long in relation to the phenomenal diffusion coefficient of the radical. Given that superoxide can interact with a variety of biological target molecules, the reaction with the enzyme literally can shunt the superoxide production into H_2O_2 and oxygen. Thus, it is conceivable that, in vivo, the presence of the highly active SOD enzymes will lead to an increase in the local concentration of H_2O_2.

The most reactive oxy radical is $HO_2^{\cdot}$. It was proposed many years ago that it could be produced from the interaction of $O_2^{\cdot-}$ and H_2O_2 by a chemical process known as the Haber-Weiss reaction (reaction 2, Table 1). However, detailed studies of the rate of this reaction have shown that it could not take place under physiological conditions. An alternative explanation, which is now widely accepted, is that trace amounts of metal ions, primarily ferrous ion, react with H_2O_2 in what is known as the iron-catalyzed Fenton reaction to produce the hydroxyl radical. Normally, ferrous ion is not present in vivo, but

TABLE 1
Free radical formation reactions

$O_2^{\cdot-} + O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2$	(1)
$O_2^{\cdot-} + H_2O_2 \rightarrow O_2 + OH^{\cdot} + OH^-$	(2)
$Fe^{3+} + O_2^{\cdot-} \rightarrow O_2 + Fe_2^+$	(3)
$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\cdot}$	(4)

[2] Abbreviations: SOD, superoxide dismutase; SODm, SOD mimetics; NOS, nitric-oxide synthase; AAR, area at risk; bNOS, brain NOS; BSO, L-buthionine-(*S*,*R*)-sulfoximine; ecNOS, endothelial cell NOS; ESR, electron spin resonance; ICAM, intercellular adhesion molecule; iNOS, inducible macrophage-type NOS; LPS, lipopolysaccharide; MnSOD, manganese superoxide dismutase; MnTBAP, *tetrakis*-(4-benzoic acid) porphyrin; NMDA, *N*-methyl-D-aspartic acid; PARP, poly(ADP-ribose) polymerase; PARS, poly(ADP-ribose) synthetase; RNS, reactive nitrogen species; ROS, reactive oxygen species.

it is produced by the action of superoxide on ferric ion (reactions 3 and 4, Table 1) present in iron storage proteins; thus, liberating soluble ferrous ion. There is considerable debate as to whether protein-bound metal ions (e.g., lactoferrin, hemoglobin, etc.) catalyze this reaction to any great degree. Sensitive measurements of the free (unbound) iron concentration in tissues, such as synovial fluid, have been reported to show a sufficient concentration to catalyze reactions (3) and (4) in an inflamed joint. Finally, recent attention has been drawn to what is called "site-specific" hydroxyl radical formation (Czapski et al., 1983), wherein an iron ion bound to a macromolecule catalyzes $HO^{\bullet}$ generation at the actual site on the substrate where cleavage eventually ensues.

In summary, tissue toxicity from extracellular superoxide generation seems to be based on its direct reactivity with numerous types of biological molecules (lipid, DNA, RNA, catecholamines, steroids, etc.) and from its dismutation to form H_2O_2 and the concomitant reduction of ferric ion to ferrous ion; reaction of these two products yields the highly toxic hydroxyl radical that may cleave covalent bonds in proteins and carbohydrates, cause lipid peroxidation, and destroy cell membranes. There are three strategies available to "detoxify" or prevent formation of locally produced oxygen radicals: 1) deliver SOD or an SODm to the area; 2) deliver catalase or a related peroxide scavenger, or 3) chelate (and thereby inactivate) the trace iron that catalyzes the reaction.

B. Nitrogen Species

$NO^{\bullet}$ is synthesized from the guanidino group of L-arginine by a family of enzymes termed $NO^{\bullet}$ synthases (NOSs). Three isoforms have been described and cloned: endothelial cell NOS (ecNOS or type 3), brain NOS (bNOS, nNOS, or type 1), and inducible macrophage-type NOS (iNOS or type 2). All of the NOS isoforms can be inhibited to varying degrees with *N*-substituted L-arginine analogs (e.g., *N*-methyl-L-arginine). The formation of $NO^{\bullet}$ is linked to the incorporation of O_2 into the molecule. All NOS isoforms are dependent on NADPH and calmodulin. In iNOS, calmodulin is present in a tightly bound form; thus iNOS produces $NO^{\bullet}$ in a sustained manner in the presence of adequate substrate (Geller and Billiar, 1998; Marletta, 1993; Stuehr, 1997). Many of the biological actions of $NO^{\bullet}$ are mediated through the guanylyl cyclase/cyclic GMP (cGMP) system. $NO^{\bullet}$, a lipophilic small molecule, diffuses to adjacent cells and readily enters the cytosol, where it activates soluble guanylyl cyclase by binding to the iron on its heme component, thereby moving the iron out of the plane of the porphyrin ring. Increased levels of cGMP trigger a reduction of calcium concentration by enhancing extrusion of calcium and its sequestration into intracellular stores. The decrease in intracellular calcium concentration is responsible for the $NO^{\bullet}$-mediated relaxation of vascular and nonvascular smooth muscle, inhibition of platelet adherence and aggregation, inhibition of neutrophil chemotaxis, and signal transduction in the central and peripheral nervous systems (Ignarro, 1991; Moncada et al., 1991; Dusting, 1995). It is now well established that $NO^{\bullet}$ also has several cGMP-independent actions. The cytotoxic effects of $NO^{\bullet}$ (in high local concentrations) involves the inhibition of key mitochondrial iron-sulfur enzymes, including NADH:ubiquinone oxidoreductase, NADH:succinate oxidoreductase, and aconitase (Nathan, 1992). cGMP-independent activation by NO of other enzymes, such as cyclooxygenase, has also been described. This action may be related to the reaction of $NO^{\bullet}$ with the iron-heme center at the active site of the enzyme (Salvemini and Masferrer, 1996). $NO^{\bullet}$ inhibits the activity of cytochrome P-450 enzymes (Khatsenko et al., 1993). $NO^{\bullet}$ may modulate gene transcription and translation: in endothelial cells, it activates c-fos (Felley-Bosco et al., 1994), whereas in neurons it potentiates the effect of calcium on gene expression linked to the c-fos promoter (Peunova and Enikolopov, 1993). Many inflammatory conditions are associated with production of comparatively large amounts of $NO^{\bullet}$, produced by iNOS, with consequent cytotoxic effects. iNOS, first identified in macrophages, can be expressed in essentially any cell type. Although constitutive expression of iNOS has been localized to the kidney, the intestine, and the bronchial epithelia, iNOS is expressed typically in response to immunological stimuli and produces nanomoles, rather than picomoles, of $NO^{\bullet}$. Once produced in high local concentrations, $NO^{\bullet}$ may act as cytostatic and cytotoxic molecules for fungal, bacterial, helminthic, and protozoal organisms, as well as tumor cells. Bacterial lipopolysaccharide and a variety of proinflammatory cytokines also induce the expression of iNOS in a number of nonhematopoietic cells, including fibroblasts, glial cells, and cardiac myocytes, as well as vascular and nonvascular smooth muscle cells (Nathan, 1992). iNOS produces large amounts of $NO^{\bullet}$ for prolonged periods. The expression of iNOS is regulated both at the level of transcription and at the level of iNOS mRNA stability. The mechanism of iNOS induction involves de novo transcription and the biosynthesis of new protein. Induction of iNOS can be inhibited by numerous agents, including glucocorticoids, thrombin, macrophage deactivation factor, tumor growth factor-β, platelet-derived growth factor, interleukin (IL)-4, IL-8, IL-10, and IL-13. Induction of iNOS may have either toxic or protective effects. Factors that seem to dictate the consequences of iNOS expression include the type of insult, the tissue type, the level and duration of iNOS expression, and probably the redox status of the tissue. Much attention has focused on the toxicity of iNOS. For example, induction of iNOS in endothelial cells produces endothelial injury (Palmer et al., 1992). Induction of iNOS has been shown to inhibit cellular respiration in macrophages and vascular smooth muscle cells; these processes can lead to cell dysfunction and cell death. Such

processes, when occurring within vascular smooth muscle cells, play a key role in the pathogenesis of the vascular hyporeactivity and progressive vascular decompensation associated with various forms of circulatory shock (Szabó, 1995). In clear contrast, expression of iNOS in liver cells suppressed endotoxin and tumor growth factor-α–induced toxicity (Kim et al., 1997; Ou et al., 1997). Overexpression of iNOS by gene transfer also limits lipopolysaccharide (LPS)-induced toxicity in endothelial cells (Tzeng et al., 1997).

Simultaneous generation of $NO^{\cdot}$ and $O_2^{\cdot-}$ favors the production of a toxic reaction product, peroxynitrite anion ($ONOO^-$) (Beckman et al., 1990), and this product may account for some of the deleterious effects associated with $NO^{\cdot}$ production. This peroxynitrite-forming reaction has since been shown to be diffusion controlled (k_{obs}, $= 6.7 \times 10^9\ M^{-1}\ s^{-1}$), indicating that competition of $NO^{\cdot}$ with SOD for superoxide is feasible (Huie and Padmaja, 1993).

Beckman noted that peroxynitrite production increases as the square of the fluxes of these precursors. Moreover, certain forms of SOD enzymes are inactivated by reaction with peroxynitrite, and this can create positive feedback for $ONOO^-$ formation (Ischiropoulos et al., 1992a; Beckman et al., 1994a). Hence, it is reasonable to conclude that peroxynitrite overproduction may occur readily in vivo. Once near or inside a cell, $ONOO^-$ can damage or deplete a number of vital components [e.g., DNA by strand scission (King et al., 1992; Groves and Marla, 1995; Groves et al., 1996), lipids by peroxidation (Radi et al., 1991a; Rubbo et al., 1994), aconitase (Castro et al., 1994; Hausladen and Fridovich, 1994), and antioxidant availability (Van der Vliet et al., 1994; Vasquez-Vivar et al., 1996)].

A considerable portion of the toxic effects previously attributed to $NO^{\cdot}$ or $O_2^{\cdot-}$ alone may in fact be modulated by peroxynitrite (Table 2). The resulting oxidative stress may cause cell death and tissue damage that characterize a number of human disease states, among them neurological disorders and stroke, inflammatory bowel disease, arthritis, toxic shock, and acute reperfusion injuries. In fact, recent studies suggest that peroxynitrite, and not $NO^{\cdot}$, may be the ultimate cytotoxic species in many conditions (Castro et al., 1994; Hausladen and Fridovich, 1994; Szabó et al., 1996a).

TABLE 2
Peroxynitrite biochemical impact

Mechanism of Action	Action	Tissue
Oxidation	Surfactant damage	Lung
Peroxidation	Lipid damage	Various
Oxidation	Glutathione depletion	Various
Alteration SH-groups	Mitochondrial respiration inhibition	Various
Nitrotyrosine formation	SOD inhibition	Neurons
Nitrotyrosine formation	DOPA synthesis inhibition	Neurons
Oxidation, deamination, nitrosilation	DNA damage	Various

DOPA, 3,4-dihydroxyphenylalanine.

In cells exposed to exogenous peroxynitrite or to compounds that simultaneously generate $NO^{\cdot}$ and superoxide, marked changes in the level of cellular energetics and DNA integrity occur. For instance, in pulmonary type II cells, inhibition by peroxynitrite of sodium uptake has been reported (Hu et al., 1994). Mitochondrial respiration is profoundly inhibited by peroxynitrite in neurons and glial cells (Bolanos et al., 1995), cultured monocytic macrophages (Szabó and Salzman, 1995; Szabó et al., 1996b), and cultured rat aortic smooth muscle cells (Szabó et al., 1996b). Although a decrease in the activity of succinate-cytochrome *c* reductase and cytochrome *c* oxidase was found in neurons exposed to peroxynitrite, only cytochrome *c* oxidase was affected in isolated mitochondria exposed to peroxynitrite. These findings suggest the contribution of secondary cellular pathways to the toxicity of peroxynitrite (Bolanos et al., 1995). Inactivation of mitochondrial enzymes increases the amounts of H_2O_2 generated by the mitochondria (Radi et al., 1994), which may further contribute to cellular injury, in an additive or synergistic fashion.

Similarly, in macrophages (Szabó and Salzman, 1995), smooth muscle cells (Szabó et al., 1996b), and neurons (Heales et al., 1994), immunostimulation leads to the inhibition of mitochondrial respiration. This inhibition is due to peroxynitrite, rather than "pure" $NO^{\cdot}$ formation, because the suppression of cell respiration can be restored by both NOS inhibitors and by superoxide or peroxynitrite scavengers.

Although exposure to high concentrations of peroxynitrite leads to rapid cell death associated with rapid energetic derangements, lower concentrations of peroxynitrite can, after several hours, lead to apoptotic cell death (Bonfoco et al., 1995; Estevez et al., 1995; Salgo et al., 1995). In isolated tissues and organs, peroxynitrite elicits a variety of alterations. Peroxynitrite infusion causes a reduction in myocardial contractility in isolated perfused hearts (Schulz et al., 1995) and induces an impairment of the endothelium-dependent relaxant ability (Villa et al., 1994). The finding that the development of this endothelial dysfunction can be prevented by $NO^{\cdot}$ donors (Villa et al., 1994) supports the notion that toxic acute effects are due to $OONO^-$ formation (Moro et al., 1994, 1995).

II. DNA Damage

Free radical-mediated reactions can cause structural alterations in DNA (e.g., nicking, base-pair mutations, rearrangements, deletions, insertions, and sequence amplification). The endogenous reactions that are likely to contribute to ongoing DNA damage are oxidation, methylation, depurination, and deamination (Totter, 1980; Ames, 1989). NO or, more likely, reactive products derived from it, such as NO_2^-, $ONOO^-$, N_2O_3 and HNO_2, are mutagenic agents, with the potential to produce nitration, nitrosation, and deamination reactions on DNA bases (Routledge et al., 1994). Methylation of cy-

tosines in DNA is important for the regulation of gene expression, and normal methylation patterns can be altered during carcinogenesis (Weitzman et al., 1994). Conversion of guanine to 8-hydroxyguanine, a frequent result of reactive oxygen species (ROS) attack (Halliwell and Aruoma, 1991; Dizdaroglu, 1993; Box et al., 1995), has been found to alter the enzyme-catalyzed methylation of adjacent cytosines (Weitzman et al., 1994)—thus providing a link between oxidative DNA damage and altered methylation patterns.

The chemistry of DNA damage by several ROS has been well characterized in vitro (Steenken, 1989; Dizdaroglu, 1993; Epe, 1993; Box et al., 1995), although specific information is needed about the changes produced by peroxyl ($RO_2^{\cdot}$), alkoxyl ($RO^{\cdot}$), ozone (O_3), and several of the reactive nitrogen species (RNS) (e.g., $ONOO^-$) is lacking. Different ROS affect DNA in different ways [e.g., H_2O_2 does not react with DNA bases at all (Halliwell and Aruoma, 1991; Dizdaroglu, 1993)], whereas $HO^{\cdot}$ generates a multiplicity of products from all four DNA bases, and this pattern seems to be a diagnostic "fingerprint" of $HO^{\cdot}$ attack (Halliwell and Aruoma, 1991). By contrast $O_2^{\cdot-}$ selectively attacks guanine (Epe, 1993; Van den Akker et al., 1994). The most commonly produced base lesion, and the one most often measured as an index of oxidative DNA damage, is 8-hydroxyguanine. It is sometimes measured as the nucleoside, 8-hydroxydeoxyguanosine (Floyd et al., 1986; Ames, 1989). These assay methods have been reviewed in detail (Floyd et al., 1986; Halliwell and Aruoma, 1991; Halliwell and Dizdaroglu, 1992; Dizdaroglu, 1993).

Damage to DNA by ROS/RNS seems to occur naturally, in that low steady-state levels of base damage products have been detected in nuclear DNA from human cells and tissues (Floyd et al., 1986; Ames, 1989; Halliwell and Dizdaroglu, 1992; Richter, 1992; Musarrat and Wani, 1994). The pattern of damage to the purine and pyrimidine bases suggests that at least some of the damage occurs by $HO^{\cdot}$ attack, suggesting that $HO^{\cdot}$ is formed in the nucleus in vitro (Halliwell and Dizdaroglu, 1992). ROS/RNS can also damage mitochondrial DNA, and such damage has been suggested to be important in several human diseases and in the aging process (Harman, 1992; Shigenaga et al., 1994). Mitochondria are often said to be the most important intracellular source of ROS, but it is difficult to unambiguously confirm this postulate (Halliwell and Gutteridge, 1985). However, it seems very likely that the mitochondrial electron transport chain generates ROS in vivo (Ambrosio et al., 1993; Guidot et al., 1993) and that mitochondrial DNA is damaged by them. The roles that ROS or RNS play in the DNA damage have not yet been completely elucidated. This apparent increased net oxidative damage in mitochondrial DNA compared with nuclear DNA could be because of the proximity of mitochondrial DNA to ROS generated during electron transport, the lack of histone proteins to protect the DNA against attack, or inefficient repair, so that base damage accumulates to higher levels.

DNA damage can be repaired by the action of a series of enzymes (Demple and Harrison, 1994). However, DNA from human cells and tissues contains low levels of DNA base damage products (Ames, 1989; Malins and Haimanot, 1991; Halliwell and Dizdaroglu, 1992; Bashir et al., 1993; Jaruga et al., 1994; Adachi et al., 1995), suggesting that these enzymes do not achieve complete removal of modified bases, perhaps because they operate at close to maximum capacity in vivo. DNA damage by ROS/RNS can cause multiple lesions, including single and double strand breaks, apurinic/apyrimidinic sites and modified pyrimidines and purines. Repair of these lesions occurs primarily by base excision repair, although nucleotide excision repair may also be involved. A repair system for the abasic apurinic/apyrimidinic sites produced by spontaneous depurination also exists. Areas of current interest include the role of poly(ADP-ribose) polymerase (PARP) in the rejoining of DNA strand breaks, including those induced by ROS (Satoh et al., 1993; Satoh and Lindahl, 1994).

III. Poly(ADP-Ribose) Synthetase

Poly(ADP) synthetase (PARS) [also known as PARP or poly(ADP-ribose) transferase] is a protein modifying and nucleotide-polymerizing enzyme that is present abundantly in the nucleus (Althaus and Richter, 1987; De Murcia and Menissier-De Murcia, 1994). The obligatory trigger of PARS activation is the nicks and breaks in the DNA strand, which can be induced by a variety of environmental stimuli and free radical (or oxidant) attacks; these include the oxidants $HO_2^{\cdot}$, $HO^{\cdot}$, and $ONOO^-$, ionizing radiation, and genotoxic agents, such as *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine. The physiological function of PARS and poly(ADP-ribosylation) is still under much debate. From studies using pharmacological inhibitors of PARS, poly(ADP-ribosylation) has been suggested to regulate gene expression and gene amplification, cellular differentiation and malignant transformation, cellular division, and DNA replication, as well as apoptotic cell death (Althaus and Richter, 1987; Lautier et al., 1993; De Murcia and Menissier-De Murcia, 1994; Lindahl et al., 1995; Wang et al., 1995; Simbulan-Rosenthal et al., 1996). However, recent studies using cells from PARS(–/–) mice have failed to demonstrate a role for PARS in the process of apoptosis induced by various apoptotic signals, such as the Fas ligand or dexamethasone (De Murcia et al., 1997; Morrison et al., 1997; Wang et al., 1995, 1997).

In the 1980s, Berger and Okamoto have observed rapid depletion of NAD+ due to PARS activation, leading to cellular ATP depletion, and functional alterations of the cell, with eventual necrotic-type cell death. The main cytotoxic triggers used in these studies in vitro were alkylating agents, radiation, and H_2O_2, whereas

the most frequently used PARS inhibitors were nicotinamide, 3-aminobenzamide, and benzamide.

Research into the "suicidal" role of PARS gained new momentum in the mid-1990s because of the observations in vitro that NO$^{\bullet}$ or peroxynitrite can trigger DNA single-strand breakage and PARS activation (Radons et al., 1994; Eliasson et al., 1997; Szabó et al., 1996b). NO$^{\bullet}$ and peroxynitrite can also inhibit mitochondrial respiration and exert other cytotoxic effects on their own. Thus, it is likely that a synergistic relationship exists between the PARS-mediated pathways and PARS-independent pathways of cellular metabolic suppression (Fig. 1). Furthermore, the observations that NO$^{\bullet}$ and peroxynitrite are important mediators of the cellular damage in various forms of inflammation and reperfusion injury suggest that the PARS-related suicide pathway might play a role in various pathophysiological conditions in vivo (Fig. 2).

IV. Relative Importance of Reactions of Glutathione with Nitric Oxide, Oxyradicals, and Peroxynitrite in Endotoxic Shock and Inflammation

Glutathione is a known oxyradical scavenger (Darley-Usmar and Halliwell, 1996). Moreover, glutathione can react with NO$^{\bullet}$ to form *S*-nitrosoglutathione, a vasodilator compound (Simon et al., 1993). Thus, theoretically, the mechanism of the observed vascular alterations in

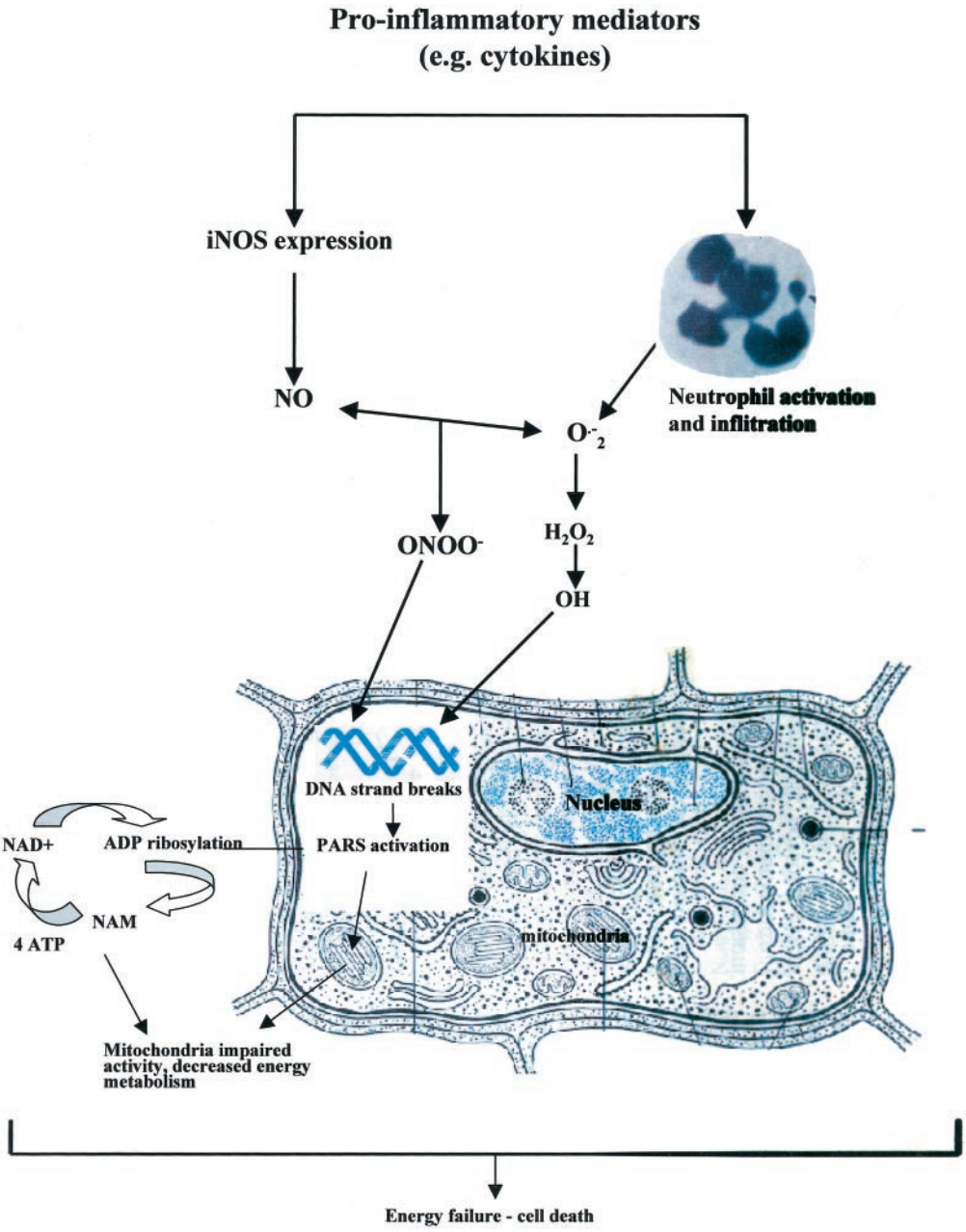

FIG. 1. Suggested mode of activation of PARS in inflammation and shock.

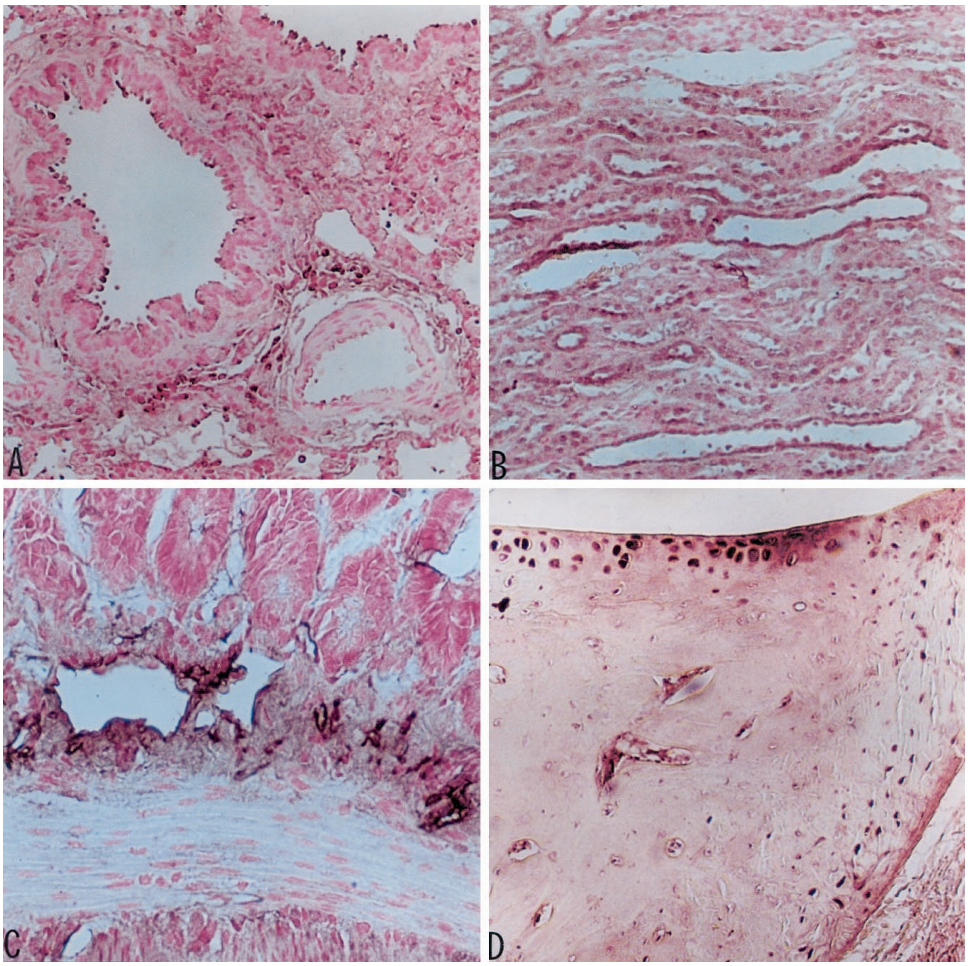

FIG. 2. PARS activity: Staining was observed at 4 h after carrageenan-induced pleurisy (A); at 5 days after gentamicin-induced renal injury (B); at 60 min after splanchnic artery occlusion shock (C); and at 35 days following collagen-induced arthritis (D). Original magnification, 125×. Figure is representative of at least three experiments performed on different experimental days.

the BSO-pretreated cells, and tissues may be related to peroxynitrite, oxyradicals, $NO^{\bullet}$, or the combination of these. From data in the literature, it seems unlikely that a glutathione-$NO^{\bullet}$ reaction plays a major role in the observed changes. This conclusion was based on the following considerations: 1) in accordance with previous studies (Jia and Furchgott, 1993; Stamler, 1995), we found no differences in the endothelium-independent relaxant effect of *S*-nitroso-*N*-acetyl-DL-penicillamine in control and BSO-treated animals, suggesting that the reduction in endogenous glutathione does not affect NO-induced relaxant responses; and 2) in vitro studies in macrophages and other cell types have established that endogenous glutathione only protects against very high (pharmacologically relevant) fluxes of $NO^{\bullet}$, but not against lower levels of $NO^{\bullet}$ production, such as the ones that are relevant to the in vitro or in vivo conditions in our experiments (Sakanashi et al., 1991; Walker et al., 1995). On the other hand, the possibility that the enhancement of cytotoxicity in BSO-treated cells or tissues after LPS treatment is related, in part, to increased oxyradical-induced cytotoxic effects is a good possibility. Data supports that depletion of endogenous glutathione enhances the cytotoxic effects of H_2O_2 and oxyradicals, and we have also observed an enhancement of H_2O_2 toxicity in endothelial cells and smooth muscle cells (Cuzzocrea et al., 1998c). In the experiments that involve LPS stimulation, it is conceivable that a more pronounced inhibition of mitochondrial respiration by oxygen-derived free radicals and oxidants can lead to a dysfunctional electron transfer, with more superoxide production from the mitochondria. This positive feedback cycle would also lead to an enhancement of peroxynitrite production, with subsequent increased cytotoxicity. It is noteworthy in this context that it is the production of superoxide, not the production of $NO^{\bullet}$, that represents the rate-limiting factor in the production of peroxynitrite during endotoxemia (Szabó and Salzman, 1995).

It must be kept in mind that, in immunostimulated cells, the production of various oxygen- and nitrogen-derived free radicals and oxidants occurs in a simultaneous fashion. Therefore, it is conceivable that important interactions exist between these various species in terms of oxidative potential and cytotoxicity. For instance, in respect to peroxynitrite-induced oxidative in-

jury, it is well established that the ratio of NO$^{\bullet}$ and superoxide determines the oxidant capacity, and excess NO$^{\bullet}$ reduces peroxynitrite-induced oxidative processes (Rubbo et al., 1994; Szabó and Salzman, 1995; Petit et al., 1996). H_2O_2, on the other hand, prolongs the half-life of peroxynitrite (Miles et al., 1996), and synergizes with peroxynitrite in terms of cytotoxicity. Thus, it is possible that the cytotoxic effects we observed in response to immunostimulation represent the sum of a complex interaction between various oxygen- and nitrogen-derived radicals and oxidants. Nevertheless, based on the similarities between the effects of exogenously added peroxynitrite and LPS treatment, and considering the simultaneous protective effects of *N*-methyl-L-arginine and *tetrakis*-(4-benzoic acid) porphyrin (MnTBAP) against the vascular failure in response to LPS challenge (see above), we propose that peroxynitrite, or a peroxynitrite-derived oxidant, contributes to protein oxidation in response to immunostimulation.

Recent studies demonstrate that endogenous glutathione plays an important role in reducing vascular hyporeactivity and endothelial dysfunction in response to peroxynitrite and endotoxic shock, as well as in acute inflammation. In fact we have shown that BSO-treated rats developed a significant inflammatory response, as compared with the animals that have a normal glutathione system. These findings are in agreement with previous suggestions that glutathione plays an important role in blocking the oxidant-induced injury and, specifically, in blocking the peroxynitrite-induced injury (Karoui et al., 1996; Cuzzocrea et al., 1998c). A variety of additive or synergistic cytotoxic processes triggered by peroxynitrite may contribute to acute and delayed cytotoxicity, and depletion of glutathione may also interfere with these pathways. This points out the importance of intact glutathione pools, as protective mechanisms against the vascular failure under conditions of oxidant stress, shock, and inflammation. There are several ways to improve glutathione status and/or replenish cellular glutathione stores. For instance, cell-permeable glutathione analogs have been described (Morris et al., 1995). These strategies may represent alternative or additional approaches to other approaches directed toward the prevention of the loss of vascular patency in shock and inflammation.

V. Superoxide Dismutase

Under normal circumstances, formation of $O_2^{\bullet-}$ (the one-electron reduction product of oxygen) is kept under *tight* control by SOD enzymes. These include the Mn enzyme in mitochondria (SOD2) and Cu/Zn enzyme present in the cytosol (SOD1) or extracellular surfaces (SOD3). The importance of SOD2 is highlighted by the findings that, in contrast to SOD1 (Reaume et al., 1996) and SOD3 (Carlsson et al., 1995), SOD2 knockout is lethal to mice (Lebovitz et al., 1996; Melov et al., 1999). In acute and chronic inflammation, the production of $O_2^{\bullet-}$ is increased at a rate that overwhelms the capacity of the endogenous SOD enzyme defense system to remove them. The result of such imbalance results in $O_2^{\bullet-}$-mediated damage. A proposal that $O_2^{\bullet-}$ was intimately involved with the inflammatory response was raised as early as the 1970s through the pioneering work of McCord and Fridovich (McCord and Fridovich, 1969). Some important proinflammatory roles for $O_2^{\bullet-}$ (Fig. 3) include endothelial cell damage and increased microvascular permeability (Droy-Lefaix et al., 1991; Haglind et al., 1994), formation of chemotactic factors such as leukotriene B_4 (Fantone and Ward, 1982; Deitch et al., 1990), recruitment of neutrophils at sites of inflammation (Boughton-Smith et al., 1993; Salvemini et al., 1996a, 1999), lipid peroxidation and oxidation, DNA single-strand damage (Dix et al., 1996), and formation of $ONOO^-$ (Beckman et al., 1990; Ischropoulos et al., 1992a; Crow and Beckman, 1995; Salvemini et al., 1998, 1999). Most of the knowledge gathered about the roles of superoxide in disease has been collected by the use of the native SOD enzyme and, more recently, by data generated in transgenic animals that

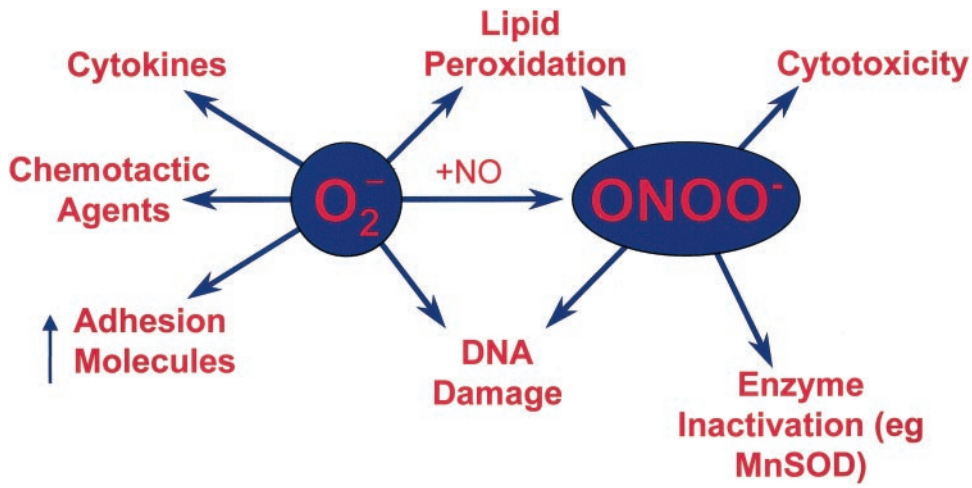

FIG. 3. Biochemical impact of superoxide generation.

overexpress the human enzyme (Huber et al., 1980; Uematsu et al., 1994; Fridovich, 1995).

VI. Radical Generation

A. In Ischemia/Reperfusion

Following ischemia, superoxide is produced during the reperfusion phase, and it rapidly reacts with NO$^{\bullet}$ and forms $ONOO^-$. This has been demonstrated in the heart (Matheis et al., 1992; Naseem et al., 1995; Schulz and Warnbolt, 1995), liver (Ma et al., 1995), kidney (Yu et al., 1994), intestine (Szabó et al., 1995a), brain (Cazevielle et al., 1993; Fagni et al., 1994; Gunasekar et al., 1995), and lung (Ischiropoulos et al., 1995; Kooy et al., 1995). Under these conditions, prevention of $ONOO^-$ generation by inhibition of NO$^{\bullet}$ biosynthesis markedly reduces reperfusion injury, as shown by reduced pulmonary lipid peroxidation (Ischiropoulos et al., 1995) or improved myocardial mechanical performance (Schulz and Warnbolt, 1995).

A growing body of evidence supports a role for $ONOO^-$ and other reactive species in neuronal injury associated with ischemia/reperfusion injury in the central nervous system. The original proposition (Beckman, 1991), that $ONOO^-$ (and not NO$^{\bullet}$ or $O_2^{\bullet-}$ independently) is a major cytotoxic mediator in the neuronal injury during stroke and *N*-methyl-D-aspartic acid (NMDA) receptor activation, was based on theoretical considerations and previous evidence showing that reperfusion injury in the central nervous system is associated with activation of NMDA receptors, which then triggers the production of $O_2^{\bullet-}$ and NO$^{\bullet}$. There is now indirect evidence to show that NMDA receptor activation is associated with a marked increase in HO$^{\bullet}$-like activity in the brain (blocked by inhibition of NOS), which is presumably due to $ONOO^-$ generation (Hammer et al., 1993). The involvement of $O_2^{\bullet-}$ and the protective effect of $O_2^{\bullet-}$ neutralizing strategies (Cazevielle et al., 1993; Dawson et al., 1993; Lafon-Cazal et al., 1993; Fagni et al., 1994; Beal et al., 1995; Crow and Beckman, 1995; Dawson, 1995; Gunasekar et al., 1995), as well as the involvement of NO$^{\bullet}$ and the protective effect of NOS inhibition (Huang et al., 1994; Smith et al., 1994; Schulz et al., 1995; Zielasek et al., 1995), has been well established in various forms of central nervous system injury.

Similar to inflammation and shock, the mechanism of $ONOO^-$-induced cellular damage in the ischemia/reperfusion remains a subject for future investigations, but presumably involves multiple mechanisms. Both in vivo and in vitro evidence clearly suggest the involvement of PARS in the neuronal damage associated with NO$^{\bullet}$ (or $ONOO^-$) production in response to NMDA receptor activation (Wallis et al., 1993; Cosi et al., 1994; Zhang et al., 1994, 1995).

Endothelial cells appear to be major regulators of neutrophil traffic, regulating the process of neutrophil chemoattraction, adhesion, and migration from the vasculature to the tissue. During the early phase of reperfusion, P-selectin is rapidly released to the cell surface from preformed storage pools after exposure to certain stimuli—such as H_2O_2, histamine, or complement—and allows the leukocytes to roll along the endothelium (Geng et al., 1990). ICAM-1, constitutively expressed on the surface of endothelial cells, is then involved in neutrophil adhesion (Geng et al., 1990; Farhood et al., 1995). Hypoxic endothelial cells synthesize proinflammatory cytokines, which can up-regulate endothelial expression of the constitutive adhesion molecule ICAM-1 in autocrine fashion (Shreeniwas et al., 1992). The expression of P-selectin and ICAM-1 corresponds with the induction of neutrophil recruitment, which is maximal within the first hour of reperfusion, and persists at a lower rate in the late phase of reperfusion (Clark et al., 1995). In accordance with these findings, it has been demonstrated that ischemia and reperfusion induced the appearance of P-selectin on the endothelial vascular wall and up-regulated the surface expression of ICAM-1 on endothelial cells. Thus, it has been hypothesized that oxidative and nitrosative damage in ischemia/reperfusion and shock requires the presence of ROS, which are mainly produced from the massive neutrophil infiltration (Cuzzocrea et al., 1998c).

B. In Shock and Inflammation

Important cardiovascular consequences of circulatory shock include reduced responsiveness of arteries and veins to exogenous or endogenous vasoconstrictor agents (vascular hyporeactivity), myocardial dysfunction, and disrupted intracellular energetic processes. Recent studies prompted these conclusions based mainly on results obtained with the use of NOS inhibitors, but they did not or could not distinguish between the effects of NO$^{\bullet}$ versus $ONOO^-$. Recent data demonstrate that authentic $ONOO^-$ is capable of mimicking many of the cardiovascular alterations associated with shock (endothelial dysfunction, vascular hyporeactivity, myocardial failure, and cellular energetic failure) (see above). In circulatory shock, proinflammatory cytokines invoke a pleiotropic cellular response, including the stimulation of oxygen-centered free radicals, such as $O_2^{\bullet-}$. The majority of NO$^{\bullet}$ produced by macrophages is converted to $ONOO^-$ (Ischiropoulos et al., 1992b). The production of $ONOO^-$ (evidenced by increased nitrotyrosine immunoreactivity or increased oxidation of the fluorescent probe dihydrorhodamine 123 to rhodamine 123) has recently been demonstrated in endotoxic shock and in hemorrhagic shock (Wizemann et al., 1994; Szabó et al., 1995b).

A large number of studies demonstrate the protective effect of SOD in various models of endotoxic and hemorrhagic shock and splanchnic artery occlusion/reperfusion injury (McKechnie et al., 1986; Wang et al., 1990; Rhee et al., 1991; Youn et al., 1991; McCord, 1993; Kapoor and Prasad, 1995; Salvemini et al., 1999). Fur-

thermore, there is a large amount of evidence to show that the production of reactive species such as $O_2^{\cdot-}$, H_2O_2, and $HO^{\cdot}$ occurs at the site of inflammation and contributes to tissue damage (Salvemini et al., 1996a; Cuzzocrea et al., 1997). Inhibitors of NOS activity reduce the severity of inflammation and support a role for $NO^{\cdot}$ in the pathophysiology associated with various models of inflammation (Tracey et al., 1995; Wei et al., 1995; Salvemini et al., 1996b; Cuzzocrea et al., 1997). In addition to $NO^{\cdot}$, $ONOO^-$ is also generated during inflammation damage (Salvemini et al., 1996b; Cuzzocrea et al., 1997). The involvement of $ONOO^-$ in these conditions is strongly supported by direct measurements. For example, in arthritis, nitrotyrosine levels increase in plasma and synovial fluid (Kaur and Halliwell, 1994). In ileitis (Miller et al., 1995) and in endotoxin-induced intestinal inflammation (Chamulitat et al., 1996), there is immunocytochemical documentation (increased nitrotyrosine immunoreactivity in the inflamed tissues) of augmented $ONOO^-$ production (Fig. 4).

The pathophysiological role of $NO^{\cdot}$ and $ONOO^-$ in the gastrointestinal damage elicited by endotoxin or chronic inflammation has been the subject of a variety of detailed investigations. The ability of authentic $ONOO^-$ to cause severe colonic inflammation has been documented (Rachmilewitz et al., 1993). The production of $ONOO^-$ in colitis may be even more pronounced because of the parallel down-regulation of SOD (Seo et al., 1995), which makes the $O_2^{\cdot-}$ available for coupling with $NO^{\cdot}$. Desferrioxamine, a putative peroxynitrite scavenger (Denicola et al., 1995), or SOD protects against the gastric damage elicited by $NO^{\cdot}$ donors, supporting the view that peroxynitrite (and not $NO^{\cdot}$ per se) is the cytotoxic species in these models (Lamarque and Whittle, 1995). Recent investigations have also concluded that inhibition of PARS exerts beneficial effects in shock (Szabó et al., 1997), reperfusion injury (Zhang et al., 1994; Zingarelli et al., 1996; Cuzzocrea et al., 1997; Thiemermann et al., 1997), and inflammation (Szabó et al., 1997, 1998; Cuzzocrea et al., 1998a,b).

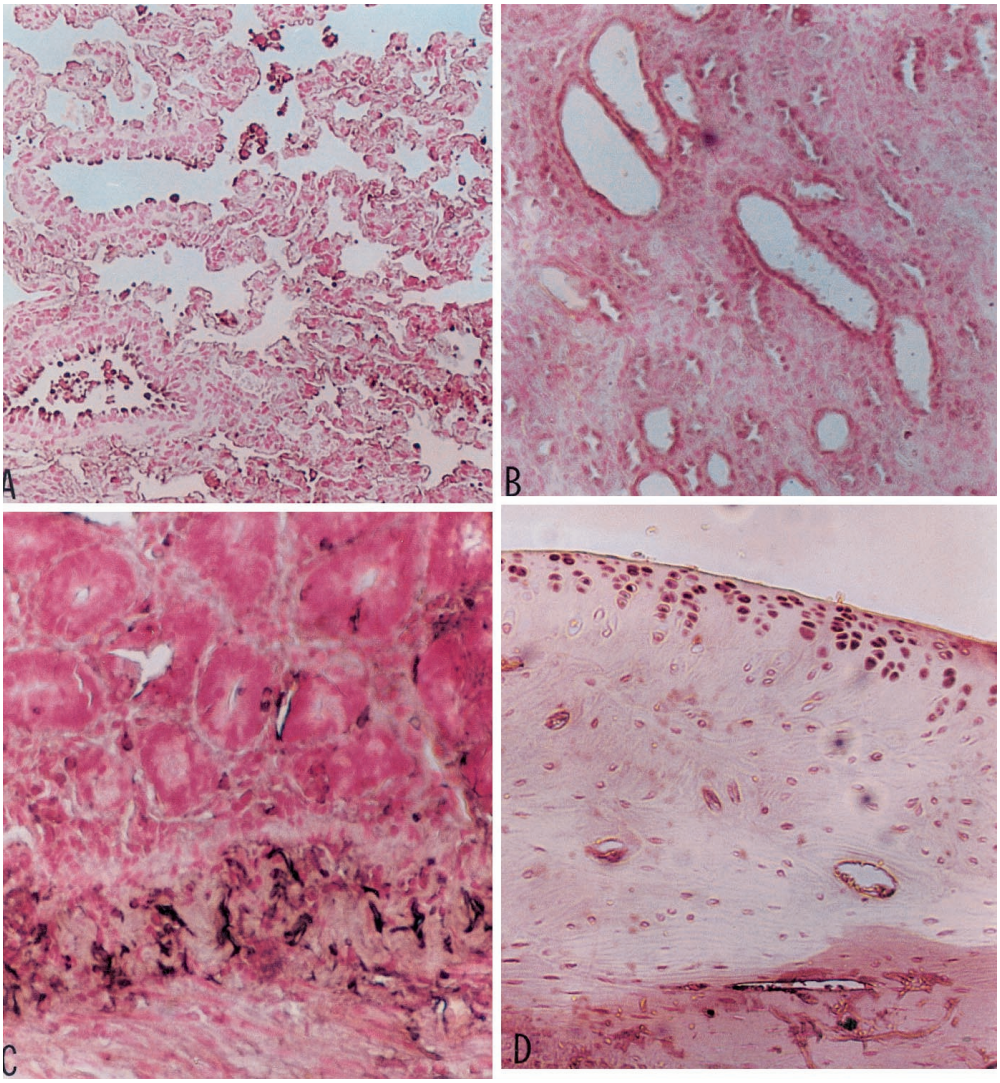

FIG. 4. Nitrotyrosine formation. Staining was present at 4 h after carrageenan-induced pleurisy (A); at 5 days after gentamicin-induced renal injury (B); at 60 min after splanchnic artery occlusion shock (C); and at 35 days following collagen-induced arthritis (D). Original magnification, 125×. Figure is representative of at least three experiments performed on different experimental days.

VII. Pharmacological Intervention to Reduce Reactive Oxygen Species Generation in Shock, Inflammation, and Ischemia/Reperfusion

Interventions, which reduce the generation or the effects of ROS exert beneficial effects in a variety of models of inflammation and shock. These therapeutic interventions include a vitamin E-like antioxidant (Cuzzocrea et al., 1999a), an SODm (Wang et al., 1990; Cuzzocrea et al., 1999b), and a $ONOO^-$ decomposition catalyst (Salvemini et al., 1998). The therapeutic efficacy of SOD itself in animals with systemic inflammation, hemorrhage, or shock is controversial. The following reasons may explain the lack of effect of SOD against the tissue injury associated with local or systemic inflammation: 1) SOD metabolized $O_2^{\cdot-}$ to H_2O_2. Without efficient removal of H_2O_2, however, H_2O_2 is converted to the highly toxic $HO^{\cdot}$ (Goode and Webster, 1993). Indeed, SOD may function as a pro-oxidant by catalyzing the conversion of H_2O_2 to $HO^{\cdot}$ (Yim et al., 1990), such as is believed to be the case in Down's syndrome. 2) Neither SOD nor $O_2^{\cdot-}$ easily cross biological membranes. Thus, an increase in the amounts of extracellular SOD does not attenuate the effects of the $O_2^{\cdot-}$ generated by intracellular sources (Meister, 1992). In contrast to SOD, spin-trapping nitrones, such as phenyl-*N*-*tert*-butyl nitrone, consistently improve outcome in rat models of endotoxic (McKechnie et al., 1986; Hamburger and McCay, 1989) and traumatic shock (Novelli et al., 1986; Novelli, 1992). The early phase of the inflammatory process is related to the production of histamine, leukotrienes, platelet-activating factor, and possibly cyclo-oxygenase products, whereas the delayed phase of the inflammatory response has been linked to neutrophil infiltration and the production of neutrophil-derived free radicals and oxidants, such as H_2O_2, $O_2^{\cdot-}$, and $HO^{\cdot}$, as well as the release of other neutrophil-derived mediators (Ohishi et al., 1989; Salvemini et al., 1996b).

A. Peroxynitrite Decomposition Catalysts as Anti-inflammatory Agents

Pathologies driven by the formation of peroxynitrite are amenable to pharmacological intervention at the level of the reactant ($NO^{\cdot}$ and $O_2^{\cdot-}$) or the product ($ONOO^-$). Indeed, SOD and/or inhibitors of NOS have been effective in attenuating both acute and chronic inflammatory responses in animal models of human diseases. We have recently identified a novel class of anti-inflammatory agents: peroxynitrite decomposition catalysts.

The peroxynitrite anion is formed and can be prepared via by a number of pathways (Fig. 5), particularly through various oxidations of nitrogen oxides and photolysis and radiolysis of solid nitrate salts. As previously noted, formation of peroxynitrite by the combination of $NO^{\cdot}$ and $O_2^{\cdot-}$ radicals is quite favorable (Huie and Padmaja, 1993), as is the combination of $NO_2^{\cdot}$ and $HO^{\cdot}$ ($k_{obs} = 4.5 \times 10^9\ M^{-1}\ s^{-1}$) (Logager and Sehested, 1993). Four practical laboratory syntheses of peroxynitrite are known, including: 1) the original route, in which intermediate NO^+ from dehydration of nitrous acid is trapped with peroxide (Beckman et al., 1994a); 2) nucleophilic attack of peroxide on alkyl nitrites (Moncada and Higgs, 1993); 3) ozonolysis of aqueous azide solutions (Gleu and Hubold, 1935; Pryor et al., 1995); and 4) photolysis and radiolysis of nitrate salts (King et al., 1992). Yields are readily observed and quantified spectrophotometrically by the characteristic yellow color of the anion ($\delta_{max} = 302$ nm, $\epsilon_{max} = 1670 \pm 50\ M^{-1}\ cm^{-1}$) (Beckman et al., 1994b).

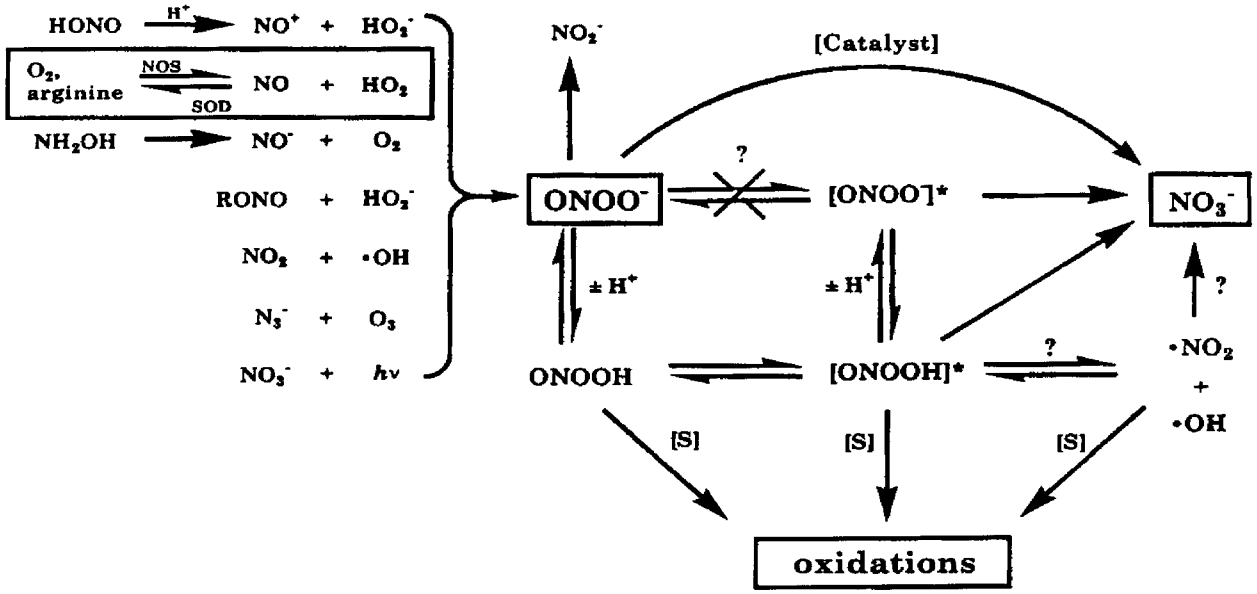

FIG. 5. Known and possible reactions of peroxynitrite.

Peroxynitrite is stable in alkaline solution, but the conjugate acid (pK_a = 6.80) (Logager and Sehested, 1993) is colorless and unstable, and isomerizes rapidly to nitrate, which is considerably more stable (ΔH° = 40 kcal/mol) (Squadrito et al., 1995). An additional acid-catalyzed pathway is observed at extreme acidity, which apparently corresponds to decomposition of $[H_2OONO]^+$ (Benton and Moore, 1970).

The rearrangement of peroxynitrite to nitrate is coupled intimately with the oxidation chemistry of this species, and both reactions have been the subject of recent investigations and intense debate. Peroxynitrite and its conjugate acid are strong oxidants, capable of effecting one- and two-electron reactions akin to those of $HO^\bullet$, nitrogen dioxide ($NO_2^\bullet$), and nitrosoniun cation. Oxidations of thiols (Radi et al., 1991b; Van der Vliet et al., 1994), sulfides (Pryor et al.; 1994; Padmaja et al., 1996), transition metal complexes (Goldstein and Czapski, 1995; Groves and Marla, 1995), deoxyribose (Beckman et al., 1990), halide ions (Hughes et al., 1971; Goldstein and Czapski, 1995), ascorbate (Barlett et al., 1995; Squadrito et al., 1995), olefins (Halfpenny and Robinson, 1992), benzenes (Halfpenny and Robinson, 1992), phenols (Halfpenny and Robinson, 1992, 1996; Beckman et al., 1992; Ischiropoulos et al., 1992a; Van der Vliet et al., 1994a,b; Groves and Marla, 1995; Ischiropoulos et al., 1996; Ramezanian et al., 1996), and other aromatics (Halfpenny and Robinson, 1996; Van der Vliet et al., 1994b; Alvarez et al., 1996) by peroxynitrite have been described.

Peroxynitrite is a particularly effective oxidant of aromatic molecules and organosulfur compounds that include free amino acids and peptide residues. Cysteine and glutathione, which are significant components of antioxidant reservoirs, are converted to disulfides (Radi et al., 1991b; Van der Vliet et al., 1994b). Methionine is converted to sulfoxide or is fragmented to ethylene and dimethyldisulfide (Pryor et al., 1994; Padmaja et al., 1996). Dimethyl sulfoxide is oxidized to formaldehyde (Beckman et al., 1990). Methyl acrylate is polymerized (Halfpenny and Robinson, 1992). Tyrosine and tryptophan undergo one-electron oxidations to radical cations, which are competitively hydroxylated, nitrated, and dimerized (Ischiropoulos et al., 1992b; Van der Vliet et al., 1994b; Alvarez et al., 1996; Ramezanian et al., 1996). The formation of nitrotyrosine is particularly favorable, and the appearance of this product in biological samples is taken as diagnostic of exposure to peroxynitrite (Salvemini et al., 1996b). Purine nucleotides are vulnerable to oxidation (Douki and Cadet, 1996; Uppu et al., 1996) and to adduct formation (Douki et al., 1996).

Peroxynitrite reacts with a number of metal-containing enzymes. It can inactivate aconitase as can superoxide (Keyer and Imlay, 1996) by oxidative cleavage of the labile iron site from the parent cluster (Hausladen and Fridovich., 1996), and certain SOD enzymes by nitration of critical residues (Ischiropoulos et al., 1992b). Peroxynitrite is proposed to down-regulate neuronal NOS by oxidation of a requisite cofactor (Huhmer et al., 1996). Heme prosthetic groups of cytochromes (Thomson et al., 1995) and peroxidases (Floris et al., 1993) are oxidized reversibly by peroxynitrite. Furthermore, peroxynitrite can release metal ions from the active sites of other enzymes (Swain et al., 1994; Crow et al., 1995).

The kinetics of substrate oxidation by peroxynitrite is extremely complex (Pryor and Squadrito, 1995). When the solution pH is fixed near physiologically relevant values, so that peroxynitrite and its conjugate acid are present in fixed ratios, observed rates of substrate oxidation and peroxynitrite loss are first-order in peroxynitrite. These rates typically increase from the peroxynitrite isomerization limit in proportion to increasing substrate concentrations; however, a significant zero-order limit is frequently encountered. Fairly high substrate concentrations, for example, several millimolar ascorbate (Barlett et al., 1995), are often required to attain first-order behavior. This behavior has been interpreted to involve competition between direct, first-order reaction of peroxynitrite with substrate and quenching by substrate of any activated form of peroxynitrite complex (Pryor and Squadrito, 1995).

The reaction of peroxynitrite with methionine at pH 7.4, 25°C, is the prototypical example (Pryor et al., 1994), although similar kinetics is observed with ascorbate (Squadrito et al., 1995). At high methionine concentrations, the sulfoxide is formed from a two-electron oxidation by peroxynitrite; observed peroxynitrite decay rates are first-order in methionine, but the extrapolated intercept with no added methionine is several times faster than the intrinsic isomerization rate of peroxynitrite. At lower methionine concentrations, the observed rates drop below the first-order limit, and an increasing mole fraction of the observed products is ethylene produced by decomposition of a sulfur-centered radical cation that results from a one-electron oxidation. The one-electron and two-electron oxidation products are proposed to arise from discrete reactions with activated and ground-state peroxynitrite, respectively.

The approach to a zero-order limit with increasing substrate indicates that the activated form is formed in a rapidly reversible equilibrium between peroxynitrite/peroxynitrous acid, which is a stronger oxidant. Furthermore, the rates of isomerization and zero-order oxidation are typically similar, which suggests that these processes follow coincident paths through the intermediate.

The nature of the activated state is the subject of considerable debate. Some researchers favor the formulation as a pair of radicals, $HO^\bullet$ and $NO_2^\bullet$, which results from reversible peroxide bond homolysis and can proceed either to oxidations or to recombination as nitrate (Halfpenny and Robinson, 1996; Beckman et al., 1990; King et al., 1992; Van der Vliet et al., 1994a; Alvarez et al., 1996). It has also been suggested that the interme-

diate is a *trans*-isomer that is formed via isomerization of the thermodynamically favored *cis* geometry by hindered rotation about the nitrogen peroxide bond (Hughes and Nicklin, 1968; Koppenol et al., 1992; Goldstein and Czapski, 1995; Pryor et al., 1994, 1996; Squadrito et al., 1995; Tsai et al., 1996a,b). The discrepancy in viewpoints is of more than academic interest, due to the extremely rapid and general oxidative reactivity of the product: $HO^{\bullet}$.

Interest in peroxynitrite chemistry recently has grown in significance, but experimental evidence on this point is limited and not completely definitive. Certain results seem to favor the radical hypothesis. For example, $HO^{\bullet}$ and $NO_2^{\bullet}$ radicals recombine to form peroxynitrite as a major product (Logager and Sehested, 1993), which provides reversibility. The bond dissociation enthalpies can be calculated with some assumptions and coincide closely with experimental activation enthalpies for isomerization (e.g., 17 versus 18 ± 1 kcal/mol) (Mahoney, 1970; Koppenol et al., 1992). However, other possible processes are calculated to have similar activation enthalpies, and it has been argued that the experimental entropy is too small to accommodate homolysis (Koppenol et al., 1992). Radical trapping products, particularly hydroxylated aromatics and oxidized spin traps, are reported to form (Halfpenny and Robinson, 1996; Beckman et al., 1990; Van der Vliet et al., 1994b), but yields relative to charged peroxynitrite are invariably very low (Shi et al., 1994; Richeson and Ingold, 1996). Finally, the rate of isomerization to nitrate shows none of the dependence on solution viscosity that is expected for diffusive radical recombination (Pryor et al., 1996). A detour around such objections is an efficient cage recombination process, but the distinction between tightly caged radicals and a geometric isomerization becomes an issue more of semantics than of physics.

Formulation of the activated state as the *trans*-isomer seems to be reasonable from a structural and theoretical standpoint. High-level calculations find that the *cis*-isomer is more stable (McGrath et al., 1988; Tsai et al., 1996a,b), and predictions for this isomer match experimental spectra (Tsai et al., 1996a,b). Isomerization of the *trans*-isomer to nitrate is predicted to be more facile than that of the *cis*-isomer, the terminal peroxide oxygen atom being more favorably disposed geometrically and electronically for intramolecular addition to nitrogen in the former (Tsai et al., 1996a). Furthermore, interconversion of the isomers by hindered rotation about the partial bond between nitrogen and the peroxide is predicted to be markedly more facile for the protonated acid than for the anion (McGrath and Rowland, 1994; Tsai et al., 1996a) that neatly explains the acid requirement of the nitrate isomerization reaction. However, reports of direct experimental observation of the *trans*-isomer are notably absent from the literature. In addition, yields of oxidized product versus peroxynitrite loading from some substrates that react only with the activated state (McGrath et al., 1988; Yang et al., 1992; Crow et al., 1994) and ferricyanide ($[Fe(CN)_6]^{4-}$) (Goldstein and Czapski, 1995) are reported to be substoichiometric and independent of substrate concentration. This result implies that substrate quenching of the intermediate is in competition with nitrate formation or some other decay reaction (e.g., to nitrite), directly from the ground state (Goldstein and Czapski, 1995).

Other activation mechanisms, such as heterolysis to $HO^{\bullet}$ and NO_2^{+}, are ruled out by the experimental observation that double labeling of peroxynitrite with ^{18}O across the peroxide bond produces double-labeled nitrate upon isomerization (Anbar and Taube, 1954). Wider use of isotope labels as tracers (^{18}O) or as magnetic probes (^{15}N) may assist future clarification of the nature of the activated state in the oxidation and isomerization reactions.

It should be noted that a few examples of alternative routes to peroxynitrite-like oxidative biochemistry have been suggested. One such possibility is a redox cycle that forms toxic hydroxyl radicals from adventitious ferrous ions and H_2O_2, which is closed by $NO^{\bullet}$ reduction of derived ferric ions (Farias-Eisner et al., 1996). Alternatively, the oxidation of nitrite to $ClNO_2$ by hypochlorite, which is formed in vivo from the action of peroxidase enzymes on chloride, provides another biologically derived radical precursor and nitration agent (Eiserich et al., 1996). Nitryl chloride has been shown to effect nitration of tyrosine in vitro, which is often taken as a biomarker for peroxynitrite exposure. Finally, peroxynitrite reacts rapidly with carbon dioxide, yielding an adduct that is a stronger oxidant than peroxynitrite alone (Lymar and Hurst, 1995). Because carbon dioxide is present in vivo in equilibrium with bicarbonate, the reactivity of peroxynitrite with various target molecules can be amplified (Lymar et al., 1996); however, bicarbonate actually seems to moderate the bacteriocidal properties of peroxynitrite (Zhu et al., 1992; Lymar and Hurst, 1996), perhaps by opening up benign decomposition pathways such as hydrolysis (Lymar and Hurst, 1996).

Known and possible reactions of peroxynitrite are summarized in Fig. 5. Two important notions emerge from these mechanistic investigations. First, formation of a highly oxidizing (possibly a radical) intermediate can be preempted by a competitive, direct reaction with ground-state peroxynitrite, especially as the unprotonated anion. Second, the kinetic barriers forming this oxidizing species, and nitrate-forming isomerization, are considerable; such reactivity may therefore be amenable to selective catalysis. These points lead the discussion to strategies for blocking the deleterious biochemistry of peroxynitrite.

Such strategies must aim to decrease either the flux or the intrinsic lifetime of the peroxynitrite. Three particular tactics would accomplish such purposes: 1) blockage of peroxynitrite formation by limiting the availability of

NO$^{\cdot}$ and $O_2^{\cdot-}$, either through inhibition of NOS or acceleration of superoxide dismutation; 2) competitive stoichiometric trapping of peroxynitrite; or 3) catalysis of peroxynitrite decomposition to benign products (e.g., isomerization to nitrate). All three approaches afford possibilities for pharmacological intervention, but the last one is particularly attractive. Identification of a highly active catalytic peroxynitrite isomerase would facilitate destruction of many equivalents of peroxynitrite formed over an extended time interval from a single, substoichiometric drug dose. As previously noted, the possibility of such catalysis is real and has now been demonstrated by highly stable molecules in vitro.

The initial experimental demonstration of peroxynitrite isomerization catalysis was reported recently (Stern et al., 1996). Addition of iron porphyrin complexes $(H_2O)Fe^{III}(L)$ (L = 5,10,15,20-*tetrakis*-(*N*-methyl-4′pyridyl) porphyrinato; 5,10,15,20-*tetrakis*-(2′,4′,6′-trimethyl-3′,5′-disulfonatophenyl) porphyrinato) to solutions measurably reduced the lifetime of peroxynitrite under physiologically relevant conditions (pH 7.4, 37°C). Furthermore, the product ion distribution shifted markedly toward innocuous nitrate at the expense of nitrite. The porphyrin complexes were extremely robust and attained high peroxynitrite isomerization rates at micromolar concentrations, even at peroxynitrite concentrations in excess of 100 μM. These qualities are quite remarkable, and the potential efficacy of these catalysts as drugs can be predicted from a purely chemical standpoint. For this reason, the search for other redox-active complexes that will accomplish catalysis of the peroxynitrite isomerization to nitrate continues.

The catalytic reactions are governed by Michaelis-Menten kinetics, typical of enzymes, and involve formation of an oxidized intermediate complex, $O = Fe^{IV}(L)$, which was observed by use of time-resolved stopped-flow UV-visible spectrophotometry. Therefore, irreversible turnover of peroxynitrite to nitrate results from a reversible formation of the catalytic intermediate, $O = Fe^{IV}(L)$, and $NO_2^{\cdot}$ radical. The intermediate was generated independently and was shown to quench rapidly upon addition of the $NO_2^{\cdot}$ gas or nitrite ion.

The proposed catalytic cycle is illustrated in Fig. 6 (Stern et al., 1996). In keeping with this scheme, yields of oxidized catalyst intermediate and observed peroxynitrite lifetimes were nonlinearly dependent on peroxynitrite concentration; higher loadings saturated the catalyst in the oxidized state and raised the lifetime of the peroxynitrite. Addition of antioxidants, such as ascorbate, competitively quenches the intermediate and accelerates catalysis (Jensen et al., submitted for publication). Peroxynitrite decomposition becomes more efficient simply by increasing the concentration of either the complex or antioxidant, and harmful oxidations become less competitive.

As previously noted, heme prosthetic groups are oxidized by peroxynitrite (Floris et al., 1993); for example,

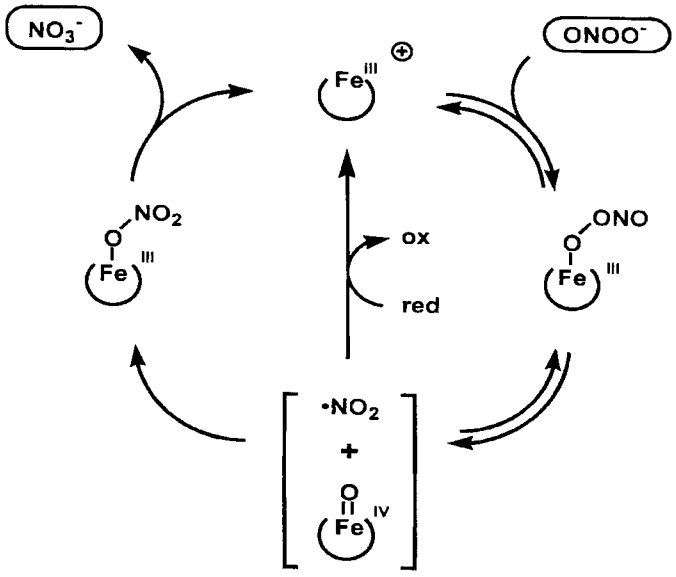

FIG. 6. Proposed catalytic cycle.

reaction of myeloperoxidase was reported to be as rapid as that observed for the synthetic catalysts. It is likely that peroxidase enzymes, in addition to their previously recognized biochemical role as peroxide scavengers, might function additionally as endogenous peroxynitrite isomerases (Lymar and Hurst, 1996). Myeloperoxidase is, for example, excreted by neutrophils, and may use $OONO^-$ in the inflammatory immune response. This hypothesis was supported by further experiments that demonstrated that mammalian heme haloperoxidases are as active as the synthetic porphyrins (Jensen et al., submitted for publication). Nature already practices such catalysis! Hence, an active drug will duplicate peroxidase reactivity at critical sites where these enzymes are not present in optimal quantities. The synthetic porphyrin complexes also have an advantage over the endogenous enzymes in higher reactivity with antioxidants, which permits reductase activity to compete with the isomerization reaction and further decreases peroxynitrite lifetimes (Jensen et al., submitted for publication).

Identification of the peroxynitrite decomposition catalysts offers the scientific community the exciting opportunity to elucidate and further our understanding of the roles of peroxynitrite in animal models of diseases. This may lead to breakthroughs in understanding the pathophysiological importance of this molecule. Therefore, direct pharmacological intervention with unique peroxynitrite decomposition catalysts specifically designed to decompose peroxynitrite to innocuous nitrate represents a novel strategy to tackle a wide range of disease states that are potentially governed and driven by the overt production of this cytotoxic molecule.

B. Catalytic Antioxidants

SOD and catalase are metalloproteins that use efficient dismutation reactions in their mechanisms to detoxify

ROS. A dismutation reaction involves a series of one- or two-electron transfers, where the electrons are accepted from one $O_2^{\cdot-}$ or H_2O_2 and then donated to another (Klug-Roth et al., 1973; Waldo and Penner-Hahn, 1995). These efficient reactions do not require reducing equivalents and thus do not require energy from the cell to operate. The overexpression of these enzymes in cell culture and in whole animals has provided protection against the deleterious effects of a wide range of oxidative stress paradigms (Krall et al., 1988; Sohal et al., 1995; Ho et al., 1998). The use of SOD and catalase as therapeutic agents to attenuate ROS-induced injury responses has had mixed success (Shaffer et al., 1987; Thibeault et al., 1991; Wispe et al., 1992; Lardot et al., 1996; Simonson et al., 1997). The main limitations of these natural products are their large size, which limits cell permeability, short circulating half-life, antigenicity, and expense. An increasing number of low-molecular weight SODm have been developed to overcome some of these limitations.

C. Metalloporphyrins

Manganese-based metalloporphyrin complexes have been shown to possess at least four distinct antioxidant properties (Lawrence and Sawyer, 1979; Faulkner et al., 1994; Day et al., 1995, 1997, 1999; Szabó et al., 1996a; Batinic-Haberle et al., 1998). These include scavenging $O_2^{\cdot-}$, H_2O_2, $ONOO^-$, and lipid peroxyl radicals. The manganese moiety of the SOD mimetics functions in the dismutation reaction with $O_2^{\cdot-}$ by alternate reduction and oxidation changing in its valence between Mn(III) and Mn(II), much like native SODs. The catalase activity of metalloporphyrins could be attributed to their extensive conjugated ring system that undergoes reversible one-electron oxidations, much like the heme prosthetic groups of endogenous catalases and peroxidases (Dolphin et al., 1971). In general, metalloporphyrins with higher SOD activity possessed greater catalase activity. It is noteworthy that the catalase activity of such complexes is less than 1% that of native catalases. However, despite this, manganese porphyrins are still able to protect cells from a toxic amount of H_2O_2 (Day et al., 1997). The mechanism by which metalloporphyrins scavenge $ONOO^-$ is thought to involve the formation of an oxo-Mn(IV) complex that can be readily reduced to the Mn(III) oxidation state by a wide variety of endogenous antioxidants (i.e., ascorbate and glutathione) and even by $O_2^{\cdot-}$. The exact mechanism by which metalloporphyrins inhibit lipid peroxidation is not known, but is thought to be similar to that described for $ONOO^-$ scavenging.

1. Effects of Metalloporphyrins in Inflammation. In vitro models of oxidative stress have been useful both in terms of confirming the antioxidant activities of metalloporphyrins obtained in cell-free systems and predicting their use as antioxidants in more complex in vivo models of human disease. Metalloporphyrins have been shown to be protective in a wide variety of in vitro oxidative stress models involving the generation of $O_2^{\cdot-}$, H_2O_2, and $ONOO^-$ alone or in concert. At micromolar levels, they seems to be nontoxic and protect cultured cells against the toxicity of $O_2^{\cdot-}$ generators [paraquat (Day et al., 1995) and pyocyanine (Gardner et al., 1996)], H_2O_2 generator [glucose oxidase (Day et al., 1997)], and $ONOO^-$ injury produced by endotoxin (Szabó et al., 1996a) or $ONOO^-$ itself (Misko et al., 1998). Metalloporphyrins are also potent inhibitors of lipid peroxidation (Day et al., 1999).

The inflammatory responses induced by injection of carrageenan into the footpad or the pleural space of the lung have been well studied and include production of histamine, leukotrienes and platelet-activating factor and a neutrophil influx (Tracey et al., 1995). Carrageenan-induced paw edema is a model of acute inflammation. Both iron^{III} *tetrakis*-(2,4,6-trimethyl-3,5-disulfonatophenyl) porphyrin and iron^{III} *tetrakis*-(*N*-methyl pyridinium-4-yl) porphyrin caused a dose-dependent reduction in swelling in paw tissue (Salvemini et al., 1998). In a rat model of lung pleurisy, the intraperitoneal treatment with MnTBAP before carrageenan administration was found to suppress inflammatory responses in a dose-dependent manner (Cuzzocrea et al., 1999b). The most profound effects of MnTBAP were on depressing the neutrophil influx and reducing nitrotyrosine formation, a marker of $ONOO^-$ formation in inflammation.

2. Effect of Metalloporphyrins in Endotoxic and Hemorrhagic Shock. A common complication of bacterial sepsis is the phenomenon referred to as endotoxic shock that results in oxidative tissue damage partially resulting from the formation of $ONOO^-$ (Kilbourn and Griffith, 1992). MnTBAP protected against some of the detrimental effects associated with endotoxic shock, including vessel contractility and cellular energy deficit (Zingarelli et al., 1997). MnTBAP also provided similar protective effects in a hemorrhagic shock model in the rat (Szabó, 1998). The efficacy of MnTBAP in these models probably relates to its $ONOO^-$-scavenging activity in addition to its $O_2^{\cdot-}$-scavenging activity.

3. Limitations of Metalloporphyrins. Although MnTBAP has proven to be a very effective compound in a wide range of oxidative stress paradigms, we have found that its potency and efficacy can be quite variable. One general limitation of metalloporphyrins is their poor blood-brain permeability that complicates their use in neurodegenerative diseases and will require development of new compounds to overcome this obstacle. A general problem for these SOD mimetics is that they can react with a wide variety ROS. This creates some confusion in the literature when used to demonstrate a specific role for $O_2^{\cdot-}$. Finally, because ROS/RNS have roles in cell signaling and, as a consequence, roles in controlling gene expression (Simon et al., 1998; Duranteau et al., 1998) and in host defense, antioxidant therapies may have an impact on these physiological processes.

D. New Rational Synthetic Enzymes: Manganese(II)-Based Superoxide Dismutase Mimics

Protective and beneficial roles of SOD have been demonstrated in a broad range of disease, both preclinically and clinically (Babior, 1982; Halliwell and Gutteridge, 1985; Maxwell, 1995). Orgotein (bovine Cu,ZnSOD) showed promising results as a human therapeutic in acute and chronic conditions, including rheumatoid arthritis and osteoarthritis, as well as side effects associated with chemotherapy and radiation therapy (Niwa et al., 1985). There are drawbacks and issues associated with the use of the native enzymes as therapeutic agents (e.g., solution instability, immunogenicity of nonhuman enzymes, bell-shaped dose response curves, high susceptibility to proteolytic digestion) and as pharmacological tools (e.g., they do not penetrate cells or cross the blood-brain barrier, thus limiting the dismutation of superoxide only to the extracellular space or compartments).

To overcome the limitations associated with native enzyme therapy the Salvemini group has developed a series of SOD mimetics that selectively catalyze the dismutation of $O_2^{\cdot-}$ (Fig. 7). An important and unique property of these SODm is that they are stable to dissociation and oxidation in the Mn(II) oxidation state and that the rate-determining step in the catalytic cycle for dismutation of superoxide is oxidation of the Mn(II) oxidation state not reduction of Mn(III) as occurs with the Mn^{III} porphyrin complexes. Those relevant biological oxidants (ROS) that have been demonstrated not to react with these Mn(II) complexes, **I**, include $NO^{\cdot}$, $OONO^-$, H_2O_2, O_2, and OCl^- (Riley et al., 1996). This property is not shared by other classes of SODm or scavengers, including several metalloporphyrins such as *tetrakis*-(*N*-ethyl-2-pyridyl) porphyrin and MnTBAP, since they have been shown to interact with a variety of oxidants, including $ONOO^-$ and H_2O_2 (Patel and Day, 1999). Although these agents are anti-inflammatory (Patel and Day, 1999), it is not clear at this stage how important the removal of superoxide is in the context of the inflammatory response because of their lack of selectivity for superoxide. Furthermore, this new class of SOD mimetics is not deactivated by $ONOO^-$ or H_2O_2 (Riley, unpublished results). This is an added advantage over the native manganese SOD (MnSOD) enzyme, since the native enzyme is nitrated and deactivated by $ONOO^-$ (Macmillan-Crow and Thompson, 1999).

1. Characterization of Superoxide Dismutase Activity. The discovery and activity of the SOD enzymes were first reported by Fridovich and McCord using a cytochrome *c* assay (McCord and Fridovich, 1969). In this assay, the ferricytochrome *c* is reduced by superoxide to give the reduced form of cytochrome *c*, which gives a spectrophotometric change. Inhibition of this reduction of cytochrome *c* was taken as a measure of SOD activity. Since then, the cytochrome *c* assay and other indirect assays have been used by investigators to assess the SOD activity of enzymes and putative SOD mimetics. Difficulty with these indirect assays can arise from several sources. For example, an agent with putative SOD activity can oxidize the reduced cytochrome (possibly resulting in a false-positive for SOD activity), reduce ferricytochrome *c* (potentially leading to a false-nega-

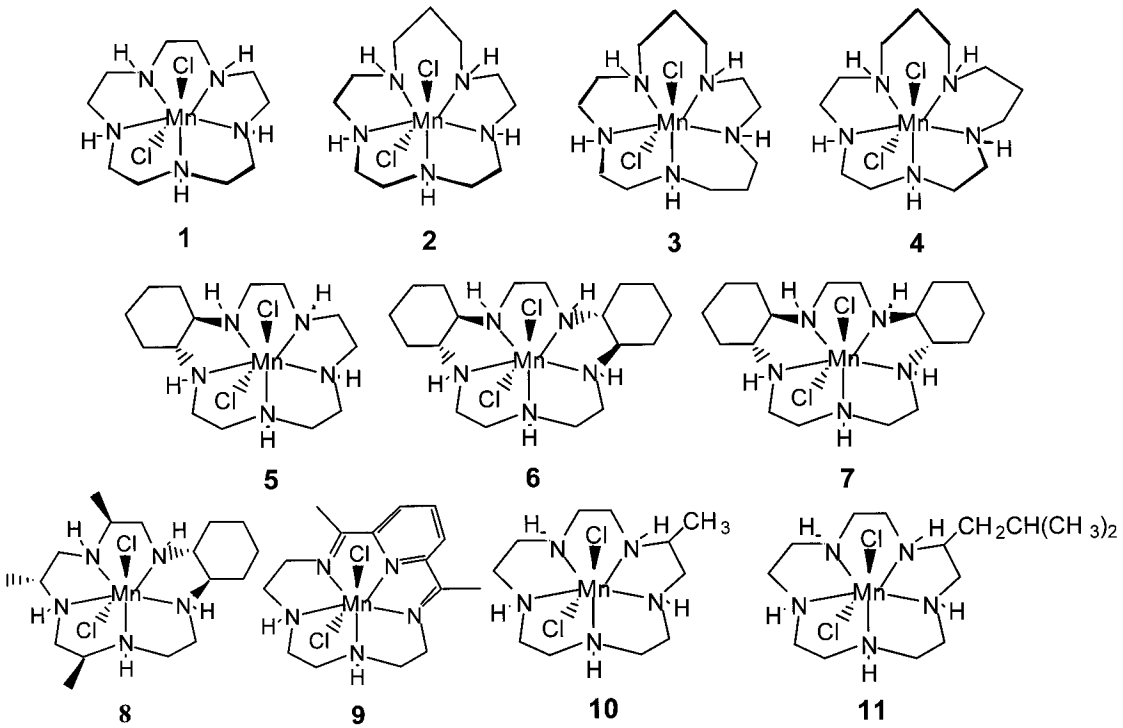

FIG. 7. Structures of the Mn(II) complex.

tive), or react stoichiometrically, not catalytically, with superoxide (i.e., a scavenger of superoxide) (Weiss et al., 1993). The indirect assays do not discriminate among these processes and additionally do not provide information regarding the mechanism of action of putative SODm.

To overcome the limitations of indirect assays, Riley et al. have utilized stopped-flow kinetic analysis as a direct technique for monitoring superoxide decays kinetics via the spectrophotometric signature of superoxide at 245 nm (Riley et al., 1991). From this type of analysis, an uncatalyzed decay of superoxide (second-order kinetics) can be distinguished from a catalyzed decay of superoxide (first-order kinetics) in the presence of a large excess of superoxide over the complex being screened. A second-order catalytic rate constant (k_{cat}) can be obtained for an agent with true catalytic SOD activity. This direct determination of a true k_{cat} can be used to directly compare and quantitate the SOD activities of enzymes and/or mimetics under a given set of conditions (e.g., defined pH and temperature). No direct comparisons can be made between the k_{cat} value and activity obtained from the cytochrome *c* assay or other indirect assays.

2. Catalyst / Drug Design. Initial efforts focused on the synthesis of Mn-based complexes as low molecular weight SOD mimics that could function as selective and active SOD catalysts. This decision to pursue Mn complexes was based largely on considerations of toxicity. Of the three metals known (Fe, Mn, and Cu) to catalyze $O_2^{\cdot-}$ to H_2O_2 and oxygen, manganese is the least toxic to mammalian systems as the free aquated metal ion and is also the least likely of the three M^{2+} ions to react with H_2O_2 to generate $HO^{\cdot}$ (Fenton chemistry). Although Mn-based complexes of desferal (Darr et al., 1987; Faulkner et al., 1994), quinolol (Howie and Sawyer, 1976), cyclam (Rush et al., 1991), and salen (Baudry et al., 1993) have been reported to have SOD activity based on indirect methodologies previously described, analysis of these complexes by the stopped-flow kinetic methodology (Riley et al., 1991) demonstrated that these complexes have no detectable *catalytic* SOD activity. More likely, these Mn complexes react stoichiometrically with superoxide (Weiss et al., 1993), resulting in their apparent activity in the indirect assays.

In early design efforts, the Riley group focused on the synthesis and screening of complexes of Mn(II), which could possess high chemical and thermodynamic stability. The reason for this is obvious, but the need in a pharmaceutical is for a stable complex that will not deposit a free redox-active metal ion in a biological compartment, where it may interact with the local biochemistry in unnatural ways. Such a situation is clearly capable of leading to toxicity problems. Consequently, synthetic efforts were focused on complexes of Mn(II) with macrocyclic ligands so that the needed stability could be achieved by incorporating the enhanced kinetic stability observed with such cyclic ligands.

By using the stopped-flow kinetic analysis as a primary screen for SOD activity, it was discovered that the Mn(II)-based complexes derived from **I**, containing the symmetrical [15]aneN$_5$ macrocyclic ligand framework (with r = H, complex **1**), efficiently catalyze the dismutation of superoxide ($k_{cat} = 4.13 \times 10^7\ M^{-1}\ s^{-1}$ at pH 7.4 and 21°C for **1**) (Riley et al., 1997). From structure-activity studies, we find that increasing the complex ring size to a 16-membered ring (complex **2**) results in a substantial loss of SOD activity with a k_{cat} of $1.0 \times 10^6\ M^{-1}\ s^{-1}$ (Riley et al., 1997). Further increasing the macrocycle size to a 17-membered ring (complex **3** or **4**) gives complexes with no detectable SOD activity ($k_{cat} < 5 \times 10^5\ M^{-1}\ s^{-1}$) as shown by stopped-flow analysis.

Importantly, it was found that, in general, increasing the substitution on the carbon backbone of the macrocyclic ring resulted in Mn(II) complexes with greatly enhanced stability toward dissociation while generally retaining SOD activity (Riley et al., 1996). Additionally, a large number of complexes were prepared with ligands possessing methyl substituents in various combinations of position and stereochemistry (Riley et al., 1996) to probe how position, stereochemistry, and number of substituents affect the catalytic SOD activity. Kinetic studies have also revealed that certain structural elements act synergistically giving complexes with substantially enhanced SOD activity and enhanced stability [e.g., Mn(II) complexes containing *trans*-cyclohexano groups (complexes **5** and **6**)]. Complex **5** with one *trans*-cyclohexano group has a k_{cat} value of $9.09 \times 10^7\ M^{-1}\ s^{-1}$ at pH −7.4 and complex **6** with two *trans*-cyclohexano (both *R,R*) groups has a k_{cat} value of $1.21 \times 10^8\ M^{-1}\ s^{-1}$ at pH 7.4 (k_{cat} for the all *S* mirror image isomer is identical to **6**, whereas the *R, R,S, S*-isomer, **7**, has no catalytic activity). By electron spin resonance (ESR) analysis, we have shown that complex **8** has high in vivo stability and is greater than 90% intact in the liver of rats 30 min after intravenous injection.

3. Anti-Inflammatory Activity of Superoxide Dismutase Mimics. The SOD mimetics **1** and **5** were tested for their ability to inhibit human neutrophil-mediated injury to human aortic endothelial cells in vitro (Hardy et al., 1994). Human neutrophils were activated to produce superoxide from exposure to tumor necrosis factor-α and the complement component C5a. The extent of injury to the endothelial cells, which was measured by the release of ^{51}Cr-labeled chromate from prelabeled cells, was linearly dependent on the amount of superoxide produced. Many of our SOD mimics have been shown to attenuate the neutrophil-mediated injury to the endothelial cells. Fluorescent studies of the neutrophil respiratory burst indicated that the SOD mimic does not prevent the generation of superoxide by the activated neutrophils. The SOD mimics also protected the endothelial cells against injury caused by xanthine/xanthine oxidase, a biochemical system that produces superoxide. The catalytic nature of the dismutation of superoxide by

the SOD mimics is evident from comparing the protective effects of the SOD mimics with the lack of protectiveness by the structurally related Mn(II)-based complex **9** that has no detectable SOD activity. In addition, the H_2O_2 scavenger catalase, the iron chelator desferrioxamine, and serine protease inhibitors did not protect against the neutrophil-mediated injury. These results were consistent with superoxide mediating the human neutrophil-induced injury to human aortic endothelial cells; thus, the Mn(II)-based SOD mimics may be viable agents to prevent oxidative injury due to neutrophils. Bovine erythrocyte Cu,ZnSOD protected the endothelial cells in a concentration-dependent manner from neutrophil-dependent injury, but the results were inconsistent (Hardy et al., 1994). On a SOD activity basis, as assessed by stopped-flow, the SOD enzyme was much less effective at protecting the endothelial cells than the SOD mimics. The efficacy of the SOD mimics in attenuating the neutrophil-mediated injury may be due to the compound's ability to gain access to the intracellular space. We have synthesized Mn(II)-based SOD mimics with a broad range of lipophilicities (log P values of -4 to $+2$) that may further allow control of the degree of intracellular penetration (Riley et al., 1996). At high doses, the SOD enzyme lost some of its efficiency to block the neutrophil-dependent injury to the endothelial cells (Hardy et al., 1994). This bell-shaped dose-response curve is a common characteristic of the SOD enzymes, and high doses of the SOD enzymes exhibit proinflammatory effects (Dowling et al., 1993). The proinflammatory effects of the SOD enzyme is not well understood, but it is speculated to be due to its reaction with the dismutation product H_2O_2 to generate $HO^{\cdot}$ radicals via Fenton chemistry (Mao et al., 1993). The lack of a bell-shaped dose-response curve with the SOD mimetics may be related to the selective reactivity of the SOD mimics with superoxide and the complexes' inability to react with H_2O_2.

Pretreatment of the mice with the SOD mimics **1**, **10**, or **11** resulted in the attenuation of the inflammatory injury induced by the dilute aqueous acetic acid as shown by the alleviation of epithelial destruction and no apparent bacterial colonization. Most interestingly, complex **1** inhibited the infiltration of the neutrophils in a dose-dependent manner, as assessed by enzymic myeloperoxidase activity (an enzyme marker for the presence of activated neutrophils), with a median effective dose (ED_{50}) of 10 mg/kg. The myeloperoxidase activity seemed to correlate with blinded scoring for severity of inflammation and with the histological appearance of the colonic tissue. The structurally related Mn(II)-based complexes **2** and **3** with no detectable SOD activity did not inhibit the inflammatory injury or the infiltration of neutrophils induced by the intracolonic treatment of the mice with dilute aqueous acetic acid. These results are consistent with a role for superoxide in the mediation of inflammation and neutrophil infiltration.

Bovine erythrocyte Cu,ZnSOD, but not *Escherichia coli* MnSOD, attenuated the inflammatory injury of the colons in dilute aqueous acetic acid-treated mice. Stopped-flow kinetic analysis demonstrated that the bovine erythrocyte Cu,ZnSOD effectively catalyzed the dismutation of superoxide ($k_{cat} = 2.3 \times 10^9\ M^{-1}\ s^{-1}$ at pH 8.1 and 21°C), whereas the *E. coli* MnSOD that we tested had no detectable SOD activity ($k_{cat} < 5 \times 10^5\ M^{-1}\ s^{-1}$) (Furchgott and Vanhoutte, 1989). Again, it is important to assay putative SOD mimics and the SOD enzymes by a direct method of analysis for SOD activity as the indirect methods (namely, the cytochrome *c* assay) indicated that both of these enzymes had comparable SOD activity.

When administered intravenously, the SOD mimic **1** attenuated the dilute aqueous acetic acid-induced inflammation of the colon in mice with an ED_{50} of 10 mg/kg, which is equivalent to the ED_{50} of the compound when it was administered intracolonically (Weiss et al., 1996). In contrast, the bovine erythrocyte Cu,ZnSOD failed to protect against the inflammatory injury when administered intravenously, presumably due to the enzyme's short in vivo half-life. The SOD mimic **1** was also effective at inhibiting the inflammation and neutrophil infiltration induced by the intradermal administration of the neutrophil chemoattractant leukotriene. These results are consistent with a role for superoxide in the mediation of oxidative tissue injury and the infiltration of neutrophils. These results indicate that the SOD mimetics may prove useful as novel anti-inflammatory agents for the treatment of conditions mediated, in part, by superoxide produced by activated polymorphonuclear leukocytes (neutrophils).

4. Attenuation of Myocardial Ischemia/Reperfusion Injury by Superoxide Dismutase Mimics. Reperfusion of the ischemic myocardium can result in a burst of superoxide production as shown by ESR spin-trapping studies (Zweier, 1988). The superoxide produced as a result of reperfusion of the ischemic tissue has been proposed to be a mediator of the reperfusion injury to the myocardium. We evaluated the cardioprotective effects of the Mn(II)-based SOD mimetics in isolated heart preparations and in the in vivo models of myocardial ischemia/reperfusion injury. When perfused at a concentration of 20 μM, the SODm **1** inhibited the release of creatine kinase and intracellular potassium in Langendorff-perfused rabbit isolated hearts subjected to 30 min of global ischemia followed by 45 min reperfusion (Kilgore et al., 1994). In the same model, complex **1** also decreased irreversible damage in the reperfused ischemic hearts as indicated by results obtained with a radiolabeled monoclonal antibody to the intracellular protein myosin. The SOD mimic **1** also protected against myocardial ischemia-reperfusion injury to the isolated primate heart.

The SOD mimetic **1** protected against myocardial injury resulting from a 90 min occlusion of the left circumflex coronary artery, followed by 18 h of reperfusion in

the anesthetized dog (Black et al., 1994). Myocardial infarct size was reduced from 44.2 ± 5.6% to 25.7 ± 4.3% by the SOD mimic when administered intravenously at a total dose of 16 mg/kg. Hemodynamic parameters were unaffected, except for a transient hypotensive effect that will be commented on in the following section on NO potentiation. At an identical dose, complex **9**, which had no detectable SOD activity failed to protect against the reperfusion injury of the ischemic myocardium in the dog, indicating that the injury was mediated by superoxide. Further myocardial ischemia/reperfusion studies were conducted in the cat to evaluate the mechanism of protection of the SOD mimetics (Venturini et al., 1994). The SOD mimic **5** protected the feline myocardium from necrosis as a result of a period of ischemia of 75 min followed by a 4.5-h period of reperfusion. The protection was dose-dependent and a statistically significant reduction in necrosis as a percentage of the area at risk (AAR) (9.9 ± 2.8% with **5** versus 29.0 ± 3.8% in controls) was observed at an intravenous dose of 5 μmol/kg. Creatine kinase release and the infiltration of neutrophils into the myocardium were also inhibited by complex **5**.

A dose-dependent, not bell-shaped, dose-response curve was observed with the SOD mimic **5** in the feline myocardial ischemia/reperfusion model (Venturini et al., 1994). With the SOD mimic **5**, the same amount of protection against necrosis was observed at 50 μmol/kg (9.3 ± 5.6% necrosis in AAR) as at 5 μmol/kg (9.9 ± 2.8% necrosis in AAR). However, as noted previously, the SOD enzyme had a bell-shaped dose-response curve in the feline model of the ischemia/reperfusion injury (Venturini et al., 1994). The SOD mimic **6**, which has a higher k_{cat} (more SOD activity) and is more lipophilic than complex **5**, significantly inhibited the myocardial necrosis at a dose of 2 μmol/kg (Venturini et al., 1994). Complex **6** also attenuated creatine kinase release and the infiltration of neutrophils in a concentration-dependent manner. ESR stability studies in the isolated rabbit heart indicated that the SOD mimic **6** has a high degree of stability in the myocardium under ischemic conditions. ESR studies also showed that complex **6** could partition into the myocardium and could dramatically attenuate the burst of superoxide-derived radicals at the time of reperfusion. As in the dog studies, the inactive complex **9** did not inhibit the necrosis arising from reperfusion of the feline ischemic myocardium, indicating that superoxide is a mediator of the injury (Venturini et al., 1994). Mn(II) chloride did not protect against the reperfusion injury, indicating that complex **1** was not attenuating reperfusion injury due to the release of Mn(II) (Venturini et al., 1994). Based on these in vitro and in vivo studies with the SOD mimics, we have established an important role for superoxide in reperfusion injury to the ischemic myocardium. By catalyzing the dismutation of superoxide, the SOD mimetics may prove useful as therapeutic agents to attenuate myocardial ischemia-reperfusion injury following myocardial infarction.

VIII. Conclusions and Future Directions

In initial studies, it was proposed that superoxide acts as an inactivator of NO$^{\bullet}$, since SOD prolongs the biological half-life of NO$^{\bullet}$. Along the lines of this concept, it has been suggested that NO$^{\bullet}$ can limit the cytotoxicity of superoxide. On the other hand the reaction of NO$^{\bullet}$ and superoxide has been shown to yield peroxynitrite, a reactive oxidant species, and an important mediator of cell damage under conditions of inflammation and oxidant stress. The evidence presented herein favors the view that the reaction of NO$^{\bullet}$ and superoxide yields peroxynitrite, which under many conditions enhances the cytotoxic potential of its "precursors". Clearly, the ratio of NO$^{\bullet}$ and superoxide is very important since NO$^{\bullet}$ can act as an inactivator of the biological activity of peroxynitrite. The finding that peroxynitrite can be formed by the combination of superoxide with NO produced by ecNOS in pathophysiological conditions, such as the early phases of shock and reperfusion injury, deserves further discussion. Previous studies, demonstrating protective effects of inhibition of the constitutive isoforms of NOS in the endothelium (ecNOS) and in the central nervous system (bNOS), have proposed that the toxicity is due to enhanced NO formation by these constitutive enzymes. Although this is certainly one possibility, another explanation should also be considered, namely that simultaneous generation of superoxide enhances the toxic potential of NO and inhibition of constitutive NOS activity prevents the formation of peroxynitrite. By recognizing that the formation of peroxynitrite can occur from superoxide and NO produced by ecNOS, the conventional wisdom of "small amounts of NO are beneficial, large amounts of NO are toxic" needs to be revised. In fact, as discussed previously, large amounts of NO may suppress the oxidant reactivity of peroxynitrite. Whether such an action plays an important role in the protective effect of NO donors in various pathophysiological conditions (such as ischemia/reperfusion injury and various forms of shock needs to be further investigated). In addition, it is clear that the cytotoxicity of peroxynitrite and ROS in various pathophysiological conditions will depend on the endogenous antioxidant status (glutathione levels, vitamin E, vitamin C, SOD, etc.). It is conceivable that small amounts of peroxynitrite are produced under basal, physiological conditions (since, in many cell types, NO from the constitutive NOS isoforms and superoxide from mitochondria and other cellular sources are always produced). It is also probable that the endogenous antioxidant systems are sufficient to neutralize such low-level peroxynitrite production. Selective pharmacological inhibition of iNOS in shock and inflammation is expected to be of significant therapeutic benefit, since it would maintain the physiological

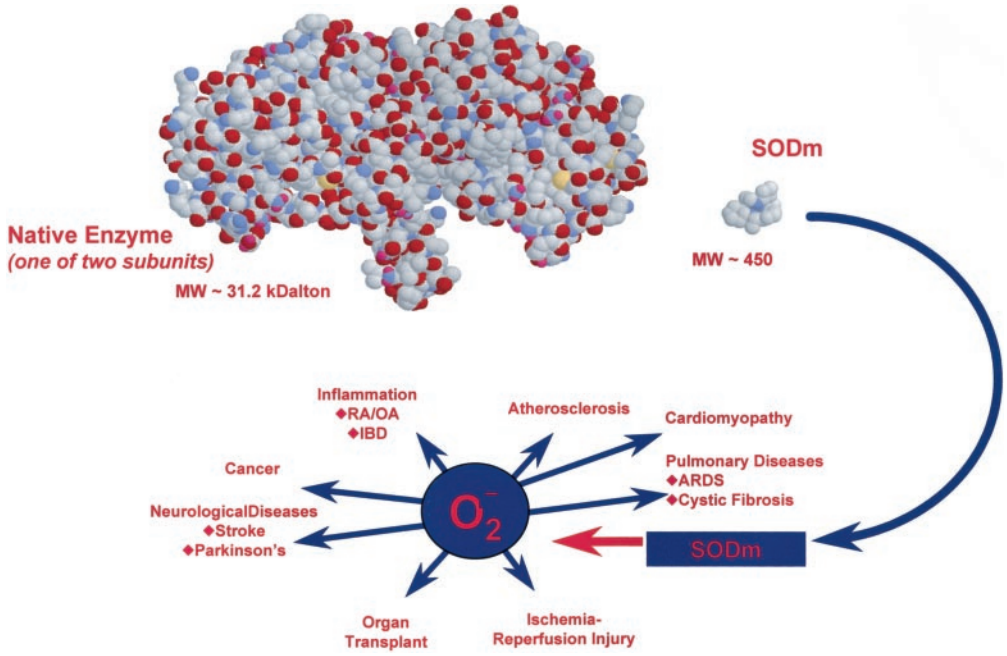

FIG. 8. Proposed clinical utility in diseases mediated, in part, by superoxide where SOD mimetics may be used. ARDS, adult respiratory distress syndrome; IBD, inflammatory bowel disease; MW, molecular weight.

functions of ecNOS (such as inhibition of platelet and white cell adhesion, maintenance of vasodilatory tone, etc.), while inhibiting the generation of cytotoxic concentrations of NO. In view of our current knowledge, however, such an approach would not completely eliminate the formation of peroxynitrite, especially during the phases of reperfusion or fluid resuscitation. On the other hand, nonisoform-selective inhibition of NOS, while having the potential of completely eliminating peroxynitrite generation, would have deleterious side effects on its own, due to the absence of ecNOS.

Understanding the signal transduction mechanisms used by free radicals to modify the course of disease will undoubtedly elucidate important molecular targets for future pharmacological intervention. It is clear that low molecular weight SOD "synzymes" can serve as powerful tools to pharmacologically dissect the mechanism(s) by which $O_2^{\cdot-}$ exerts its effects. The Mn(II)-based SOD mimetics **I** described herein provide an example of an unique approach for the development of artificial enzymes as future drugs. In certain cases where an enzyme of potential therapeutic benefit does not have the appropriate properties for a drug, a synthetic, small molecule enzyme mimetic can conceivably be designed with chemical and physical characteristics suitable for a therapeutic. The SOD mimics are *catalytic* drugs (i.e., the compounds do not involve a stoichiometric interaction with a biological target, such as a receptor, but instead enhance the rate of conversion of superoxide to O_2 and H_2O_2 without the complex itself being consumed). The SOD mimics have been rigorously characterized for SOD activity by stopped-flow kinetic analysis, for in vitro stability by kinetic and thermodynamic assays, and for in vivo stability by ESR and radiochemical studies. The ability of the SOD mimics to scavenge superoxide in vivo has also been demonstrated by ESR studies. In vitro and in vivo studies have demonstrated that the SOD mimetics have potent anti-inflammatory properties, attenuate myocardial ischemia/reperfusion injury, and prolong the half-life of $NO^{\cdot}$, an antithrombotic and vascular relaxant. Therefore, the SOD mimetics may find clinical utility in diseases mediated, in part, by superoxide (Fig. 8). Previous negative clinical studies with the SOD enzymes should not preclude clinical demonstration of utility by the SOD mimics because the SOD mimics have numerous advantages over the enzymes, including a normal dose-response curve, membrane permeability, stability, cost, and lack of immunogenicity, as well as potential oral activity. In light of the critical roles of superoxide in disease and cellular signaling, these new, highly potent "synzymes" have tremendous potential in the treatment of numerous diseases, ranging from acute and chronic inflammation to shock and ischemia/reperfusion injury.

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