

# Preface

Trauma and exposure to toxic and infectious agents invariably lead to organ damage followed by significant morbidity and mortality. Although these conditions have typically been associated with the battlefield, today they are more prevalent in urban areas. The events of September 11, 2001 have brought this problem to the forefront of national and international concern. The demand for solutions is justifiably high, and the research community needs to adjust its efforts appropriately.

*Combat Medicine* is meant to be a concise manual for the young clinical or basic investigator who is studying organ injury following trauma or toxic or infectious assaults either in an urban or battlefield setting, with an emphasis on current research issues in emergency and military medicine. The aim of *Combat Medicine* is to inspire surgical and medical residents and fellows, as well as biology and biochemistry students and fellows, to pursue research careers in the fields of military, trauma, and emergency medicine. *Combat Medicine* is not intended to be an exhaustive review; rather it is an introduction to key principles of this field.

The area of combat medicine research is enormously diverse, and in many ways it has not yet been defined. Mechanisms that lead to tissue damage include apoptosis, abnormalities in nitric oxide production, and disturbance of cell biochemistry. The affected organs are equally diverse and include the skin, the lungs, and the nervous system. Accordingly, we elected to divide the book into two parts. In the first, we asked experts to discuss the basic mechanisms that are invariably involved in the development of organ injury, such as apoptosis, nitric oxide production regulation, complement activation, and immune cell response to stressors. For the second part, we asked colleagues to discuss the current concepts that govern research aimed at understanding and reversing damage to major organs.

The authors explore mechanisms involved in the development of injury, review animal and, when available, human studies, and focus attention on future directions for research.

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## Nitric Oxide

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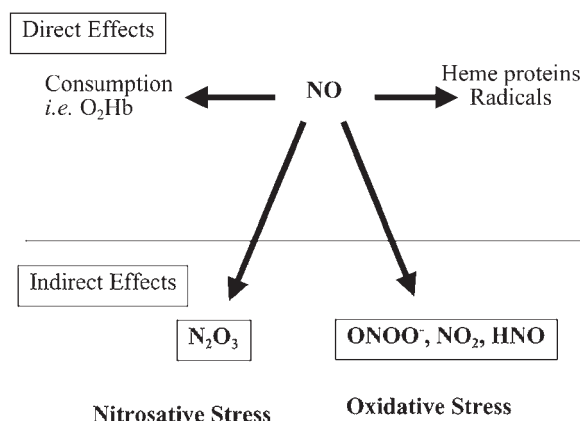
### INTRODUCTION

Nitric oxide (NO) is involved in numerous physiologic functions ranging from regulation of cardiovascular functions to participating in memory (1–4). In the immune system, this diatomic radical is involved in host defense and has tumoricidal functions (5,6). However, despite these properties, which are critical in maintaining homeostasis, NO has been implicated as a participant or causative agent in a variety of pathophysiologic conditions (7,8). Defining the exact role of NO under pathophysiologic conditions is further complicated by the fact that it has been shown to be both protective as well as deleterious even in the context of the same biologic setting. Therefore, the search for mechanistic explanations to account for these differing effects is ongoing.

Unlike other biologic mediators, the main determinants of the biologic effects of NO are its chemical and physical properties. In addition to the numerous potential reactions of NO in biologic systems, many of its effects can be attributed to additional reactive nitrogen oxide species (RNOS) that are formed and that have their own selective reactivity. Although a large variety of chemical reactions relate to NO in biologic systems, it is difficult to determine which ones are pertinent. In an attempt to sort out the diverse chemical reactions and their importance, we have developed the concept of the “chemical biology of nitric oxide” (9–11). The chemical biology of NO sepa-

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**Fig. 1.** The chemical biology of nitric oxide (NO).

rates the chemical reactions into two basic categories, direct and indirect effects (**Fig. 1**).

Direct chemical reactions are those in which NO directly interacts with biologic targets. The most common chemical reactions are those between NO and heme-containing proteins. These reactions are generally rapid, require low concentrations of NO, and are the genesis of most of the physiologic effects of NO. Conversely, indirect effects do not involve the direct reaction of NO with biologic targets but rather rely on the formation of other RNOS formed from the reactions of NO with oxygen or superoxide. These chemical species can then react with cellular targets, which may lead to the modification of critical macromolecules. It appears that indirect effects require much higher concentrations of NO than direct effects. This suggests that NO produced at low concentrations for short periods primarily mediates direct effects, whereas higher local NO concentrations sustained over prolonged periods mediate indirect reactions.

Indirect reactions can be further broken down into two subgroups: nitrosative and oxidative (9). A variety of chemical reactions result in nitrosative or oxidative chemistry depending on the species involved. The chemistry of nitrosation *in vivo* appears to be mediated primarily by  $N_2O_3$ , whereas oxidative chemistry is mediated by  $ONOO^-$  as well as  $HNO/NO^-$  (12). The chemistry of these reactive intermediates shows that they interact to produce either nitrosative or oxidative stress. Furthermore, several studies suggest that nitrosative stress is orthogonal to

oxidative stress with respect to the RNOS chemistry, implying that nitrosative and oxidative stress are produced by different mechanisms (9,13). It also appears that biologic effects such as cytotoxicity are different under nitrosative and oxidative stress (9). A balance exists between these two types of stresses, and therefore the functional outcome, such as cell death and signal transduction, may differ depending on which one predominates.

This separation of chemical reactions into direct and indirect effects is analogous to the function of the different isozymes of nitric oxide synthase (NOS) (14,15). NOS can be found constitutively in a variety of cells such as endothelial and neuronal cells. Constitutively expressed NOS (ecNOS) is thought to generate low amounts of NO at concentrations in the submicromolar range for short durations. On the other hand, inducible NOS (iNOS) generates NO for prolonged periods and in some cases at local concentrations as high as 1–5  $\mu M$ . Since, in general, the chemistry and the biologic outcome can be a function of NO concentration, it will be dictated ultimately by the type of isozyme present. An important consideration is the proximity of a biologic target to the NO source. Targets close to a source such as macrophages producing high levels of NO will be subjected to both direct and indirect reactions, whereas cells farther away will experience mostly direct effects as the primary mode of NO action. We explore here the different chemical reactions in the chemical biology of NO and discuss them in the context of oxidative and nitrosative stress.

## DIRECT EFFECTS

Most direct reactions of NO with biologically relevant substrates are not rapid enough to play a significant role *in vivo*. Hence at low concentrations of NO, there are a few reactions to be considered. Most of these reactions involve metals or other free radicals. The direct reaction of NO with thiols is far too slow to occur in biologic systems. The major metal-mediated reactions involve either the formation of metal nitrosyls, oxidation by dioxygen complexes, or metal-oxo complexes. Iron is the primary metal involved, in particular, reaction sites containing heme. Heme-containing proteins react with NO the fastest of the bio-organic complexes and should be the first to be implicated in any mechanism involving NO. In addition to metals, organic radicals can react with NO at diffusion-controlled rates. The production of lipid rad-

icals and carbon-centered radicals formed during exposure to ionizing radiation can be important under specific conditions.

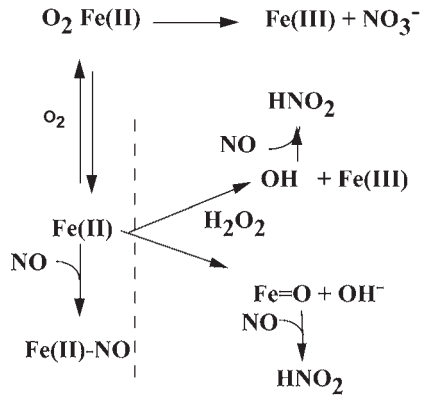
The basic regulatory NO reactions involve enzymes containing metal heme complexes such as guanylate cyclase, cytochrome P450, NOS, and hemoglobin. These reactions are facile enough that the NO source can be at greater distances from the target protein. On the other hand, some enzymes such as aconitase require higher NO concentrations and therefore must be in close proximity to the NO source. Under conditions of oxidative stress, NO can react rapidly with redox active metals and can catalyze the formation of oxidants such as hydroxyl radical. In the case of lipoxygenase and cyclo-oxygenase, NO can react with lipid radicals that are formed at low concentrations, whereas at higher concentrations they form metal nitrosyl complex, which inhibits the enzymes. Therefore the inhibitory effects of NO on arachadonic acid metabolism can occur at low or high concentrations of NO. As in the reactions of aconitase and those in the respiratory chain of the mitochondria, indirect as well as direct effects can be involved. We discuss below some of the relevant reactions and their relevance at different concentrations of NO.

### *Reactions Between NO and Metal Complexes*

The three major types of reactions between NO and biologic metals are the direct reaction of NO with metal centers (to form a metal nitrosyl complexes) and NO redox reactions with dioxygen complexes or high valent oxo-complexes (**Fig. 2**). These reactions are extremely rapid at near diffusion-limited rates, making them relevant under almost any physiologic or pathophysiologic condition.

NO may react with a variety of metal complexes to form metal nitrosyls. The vast majority of the reactions *in vivo* are with iron-containing proteins. Most copper complexes will react to form the nitrosyl but are too slow to be of major importance in the biology of NO. Cobalamin has been shown to react with NO, resulting in nitrosation catalysis (16). Other biologically important transition metals such as zinc do not react with NO under biologic conditions.

The most notable protein to form metal nitrosyl complexes *in vivo* is guanylate cyclase. The formation of heme nitrosyl adduct in guanylate cyclase causes the removal of the distal histidine, resulting in a 5-coordinate nitrosyl complex, which activates the enzyme (17,18). This alteration in protein configuration leads to the conversion of guanosinetriphosphate (GTP) to cyclic guanosine monophosphate (cGMP) in



**Fig. 2.** Important reactions of nitric oxide (NO) with iron complexes.

another domain of the enzyme. The formation of cGMP has many ramifications in a number of tissues, in particular vascular smooth muscle, in which NO mediates the vasodilation (19). The concentration of NO required to activate guanylate cyclase is relatively low [medium effective concentration ( $EC_{50}$ ) 100 nM] (20). The influence of NO on soluble guanylate cyclase has profound effects on vascular tone, platelet function, neurotransmission, and a variety of other intercellular interactions. Recent reports suggest that NO can prevent some apoptotic processes by activating guanylate cyclase.

In contrast to the activation of enzymes such as guanylate cyclase, NO can have an inhibitory effect as well; cytochrome P450 as well as other heme mono-oxygenases are inhibited (**Fig. 2**) (21–23). The inhibition of cytochrome P450 has important pathophysiologic sequelae. During chronic infection or septic shock, NO can be produced in copious amounts. Inhibition of liver cytochrome P450s (21,23) inhibits drug metabolism (22). Furthermore, the chronic exposure of NO to the heme domain of cytochrome P450 can result in release of free heme and the activation of heme oxygenase in hepatocytes (24). The activation of hemeoxygenase may serve as a protective mechanism against a variety of pathophysiologic conditions (25,26). The interaction of NO with cytochrome P450 can thus have a regulatory function as well as a positive or negative influence on pathophysiology.

Another important outcome of heme nitrosyl formation is in the regulation of NOS activity. NOS is a cytochrome P450-like enzyme that at

the heme domain catalyzes the oxidation of arginine to form citrulline and NO, similar to substrate oxidation by cytochrome P450 (14). However, it has been shown that NO will inhibit the oxidation of arginine, suggesting that the amount of NO produced from the enzyme is actually controlled by a negative feedback mechanism analogous to that of cytochrome P450 (**Fig. 2**) (27–30). Comparison of the different isozymes of NOS shows that ecNOS and neuronal (n)NOS are more susceptible to inhibition by NO than iNOS (30). This helps explain why significantly higher NO fluxes can be achieved with iNOS than with either ecNOS or nNOS. The difference in NO-mediated inhibition of NOS activity is apparently owing to the relative reactivity of NO and the stability of the resultant Fe-NO complex within NOS. The stable Fe-NO complex restricts the potential concentration of NO that can be produced by nNOS, and probably ecNOS. Even under conditions of hyper-intracellular calcium concentrations, this feedback mechanism will protect against the production of significant amounts of RNOS. Therefore, the predominant source of RNOS (indirect effects) *in vivo* may be from iNOS.

The competitive inhibition of NOS activity through a heme nitrosyl complex can serve to regulate tissue blood flow. Oxidation of arginine under catalytic conditions results in the formation of a Fe-NO complex in nNOS (28,29). This Fe-NO/nNOS complex competitively inhibits the binding of oxygen to the active site and prevents the oxidation of arginine. This binding of NO to nNOS increases the  $K_m$  for oxygen such that there exists a linear relationship in the range of physiologic oxygen concentrations. This suggests that NOS may serve as an oxygen sensor as well as attenuating oxygen supply to tissue (31). In recent studies, it was shown that NO produced from NOS in the lung alveoli responded to different concentrations of oxygen (32). This could regulate the blood flow in the lung depending on the oxygen tension. Since NO and oxygen compete for the heme binding site, the relative stability of the nitrosyl versus the dioxygen adduct determines the level of NO produced. It is thought that this mechanism may play a crucial role in regulation of blood flow through different tissues.

The iron metal nitrosyl complexes discussed above have involved heme-containing proteins. Other metals can also form nitrosyl complexes, such as the reaction of NO with cobaltamine, a cofactor in methionine synthase. The oxidized aquo form of cobaltamine reacts with NO to form

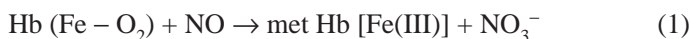


a nitrosyl, whereas the cyano and methyl adenosyl forms do not. The interaction of NO with cobaltamine reduces its ability to serve as a cofactor in methionine synthase. Studies show that the addition of cobaltamine prevents the loss of mean arterial blood pressure induced by lipopolysaccharide (LPS), suggesting that it could be an effective NO scavenger in vivo. In cell culture experiments, cobaltamine was shown to scavenge NO, thus preventing NO's inhibitor effects on cell proliferation. Nitrosyl cobaltamine can nitrosate thiols of protein and glutathione, providing a means for NO to form S-nitrosothiols. Taken together, this indicated that cobaltamine could modulate NO metabolism.

## BIOLOGICAL RELEVANCE

### *NO Interaction with Metal-Oxygen and Oxo Complexes*

The reactivity of NO with metals is not limited to covalent interactions with metal ions alone. Various metal-oxygen complexes and metallo-oxo complexes react rapidly with NO (**Fig. 2**). As with activation of guanylate cyclase, the reaction between NO and oxyhemoglobin is an equally important determinant of NO behavior in vivo. The reaction between NO and oxyhemoglobin results in met-hemoglobin and nitrate (33,34):

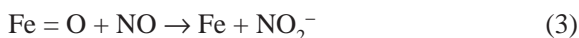


This reaction provides the primary endogenous mechanism to eliminate NO as well as control the movement and concentration of NO in vivo (35). In addition, it exerts important control on the mechanisms of indirect effects.

Another important reaction of NO with metal oxygen adducts is the rapid reaction between NO and metalloxo and peroxo species (discussed in ref. 9). Agents such as hydrogen peroxide form highly reactive metal complexes through their oxidation. In the absence of NO they can lead to cellular damage such as lipid peroxidation (36). When NO is present, it reacts rapidly with these complexes to abate oxidative chemistry mediated by metallo-oxo species (37-39):



NO results in the reduction of the hypervalent metal complex to a less oxidizing normal valent state:



These antioxidant properties of NO may be a primary mechanism by which this diatomic radical protects tissue from peroxide-mediated damage (39).

Another important reaction is the NO interaction with catalase. Kim et al. (40) demonstrated that cytokine-stimulated hepatocytes reduced catalase activity owing to the production of NO. Similar inhibition of hydrogen peroxide consumption by catalase was observed using NO donors (41). Farias-Eisner et al. (42) suggested that the NO inhibition of catalase could play a role in the tumoricidal activity of macrophages.

NO can inhibit catalase by two different mechanisms: (1) metal nitrosyl formation and (2) NO reacting with metalloxo species. Hoshino et al. (43) showed that NO could bind to the heme moiety, forming a ferric nitrosyl with a rate constant of  $3 \times 10^7 M^{-1}s^{-1}$  and a  $K_{diss}$  of  $1 \times 10^5 M^{-1}$ . This is analogous to the mechanism for P450 and NOS inhibition. The Fe-NO adduct prevents the binding of hydrogen peroxide to the metal ion by occupying the coordination site. It is estimated that between 10 and 15  $\mu M$  NO inhibits hydrogen peroxide consumption by 80%, by means of this mechanism (42). Cells that express iNOS have reduced catalase activity, suggesting that local NO concentrations near these cells may reach as high as 10  $\mu M$  for prolonged periods.

There is a second mechanism by which hydrogen peroxide may attenuate NO levels. During the enzymatic mechanism of catalase, hydrogen peroxide first reacts to form complex I and water (**Fig. 2**). Complex I will react with hydrogen peroxide to form  $O_2$ . However, NO can also rapidly react with compound I to form complex II, which reacts with an additional NO. This results in the conversion of 2 mole of NO and 1 mole of hydrogen peroxide to 2 mole of  $HNO_2$ . This results in NO consumption while hydrogen peroxide depletion is retarded. Brown (44) has shown that NO can partially inhibit hydrogen peroxide consumption while NO is consumed by catalase/peroxidase. The  $K_i$  for NO in this reaction was 0.18  $\mu M$ . This finding suggests that submicromolar NO levels, such as those produced by cNOS, may be partly controlled by this mechanism.

The reaction of NO with high valent heme derived from peroxide may be a mechanism to attenuate the NO levels in vivo. Reports have shown that an increase in glutathione peroxidase activity, which does not react with NO, increases the bioavailability of NO (from eNOS) (45). This implies that hydrogen peroxide, via a mechanism similar to that shown

in **Fig. 2**, may play a crucial role in regulation of the direct effects of NO *in vivo*. On the other hand, Fe-NO formation would be important under conditions in which NO concentrations are higher than H<sub>2</sub>O<sub>2</sub>. Both mechanisms may play a role during the sequences of NO and peroxide bursts under some physiologic and pathophysiologic conditions.

### *Reaction of NO with Radical Species*

Another direct effect of NO is its reaction with other radical species. The tyrosyl radical formed during the catalytic turnover of ribonucleotide reductase reacts with NO and inhibits the enzyme (46–49). Inhibition of this enzyme has been proposed to be a factor in the cytostatic properties of NO, owing to the suppression of DNA synthesis.

NO can react with oxyradicals formed during lipid peroxidation (50,51). Lipid peroxidation is an important component of cell death and in stages of inflammation (52). The process of lipid peroxidation results in the formation of variety of lipid oxy and peroxy adducts that perpetuate lipid oxidation. This can result in compromise of cell membranes. The reaction of NO with these peroxy-oxy radicals results in protection against ROS by termination of lipid peroxidation (53):



Reaction 4 has been proposed to play a role in the abatement of lipid peroxidation by NO that protects cells against peroxide-induced cytotoxicity (39,54,55). Lipid peroxidation induced by oxidants and formed as result of exposure to copper, xanthine oxidase, or azo-bis-amidinopropane is terminated by NO (51,56). The chain termination also prevents oxidation of low-density lipoprotein in both endothelial (57) and macrophage cells (56). It is thought that a reduction in oxidized cholesterol reduces initiation of arterioscleroses mediated by foam cells. Other processes in inflammation such as the production of leukotrienes are effected by NO. Lipoxygenase, which mediates a variety of lipid oxidations, is inhibited by NO.

## CHEMICAL TOXICOLOGY OF NO AND ROS

Although NO can act as an antioxidant in a number of reactions, there are other reactions affecting cellular processes that make tissue more susceptible to oxidative stress. Thus the formation of oxidants such as peroxynitrite, the proposed product of ROS and NO, is hypoth-

esized to lead to tissue injury. The cytotoxic effects of NO would be caused by formation of peroxynitrite and related species.

The complexity of the potential reactions within a cell has led to some differing opinions on the role of NO in oxidative stress (58). The clonogenic assay can be used to answer these questions at the cellular level. It is the gold standard for cytotoxicity mediated by different chemical substances and takes into account both necrotic and apoptotic death. To sort out these effects, the toxicity of hydrogen peroxide, alkyhydroperoxide, and superoxide was examined in the presence of NO.

Hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) mediates oxidation of biologic molecules, which can result in tissue damage. Although NO does not react chemically with  $\text{H}_2\text{O}_2$  (59), it can protect cells against toxicity mediated by  $\text{H}_2\text{O}_2$  (39,41,54,55,59). Recent studies on the biology and chemistry of NO have made use of a class of compounds known as NONOates, which release NO in a controlled manner over specific periods (60). In a recent study, lung fibroblasts exposed to increasing concentrations of  $\text{H}_2\text{O}_2$  exhibited marked increases in cytotoxicity (59). The presence of NONOates resulted in surprising protection against the cytotoxicity of  $\text{H}_2\text{O}_2$  (59). Treatment with these NO donor complexes before or after exposure to  $\text{H}_2\text{O}_2$  did not result in protection; in fact, the byproduct of the decomposition of NO, nitrite, increased the cytotoxicity of  $\text{H}_2\text{O}_2$ . Similar observations were made in neuronal (59), hepatoma (39), and endothelial cells (55,61). Other reports suggest that NO derived from endothelial cells is involved in the protection against damage to vascular smooth muscle mediated by  $\text{H}_2\text{O}_2$  (62).

These protective effects of NO were not restricted to NONOates. Compounds containing *S*-nitroso functional groups also protected against  $\text{H}_2\text{O}_2$ -mediated toxicity (41). However, clinically used nitrovasodilators such as 3-morpholinonydnimine (SIN-1) and sodium nitroprusside (SNP) increased the toxicity of  $\text{H}_2\text{O}_2$  (41,42). Angeli's salt ( $\text{Na}_2\text{N}_2\text{O}_3$ ;AS), a compound similar to the NONOates but one that donates nitroxyl ( $\text{NO}^-$ ) instead of NO, significantly potentiated the toxicity of  $\text{H}_2\text{O}_2$  (41). These results show that different putative NO donors can modulate the toxicity of  $\text{H}_2\text{O}_2$  differently.

The effects of the different NO donors on cellular antioxidant defenses as well as the amount and flux of NO produced during the experiment may explain the differences exhibited by the various NO donors. One of the major cellular defenses against  $\text{H}_2\text{O}_2$  is its consump-

tion by the enzymes glutathione (GSH) peroxidase and catalase (63). When the kinetics for the disappearance of  $\text{H}_2\text{O}_2$  were examined in the presence of the different NO donors, it was noted that several of the compounds inhibited the cellular consumption of  $\text{H}_2\text{O}_2$  to varying degrees. In these studies, SNP, DEA/NO, AS, and SNAP all increased the amount of time required to decompose 0.75 mM  $\text{H}_2\text{O}_2$  by as much as 30–200% (41). In the case of SIN-1 and GSNO, the consumption of  $\text{H}_2\text{O}_2$  was retarded by as much as 400% (41). Thus, the enhancement of  $\text{H}_2\text{O}_2$ -mediated toxicity by AS and SIN-1 might be explained partially by the inhibition of  $\text{H}_2\text{O}_2$  consumption. However, this cannot be the sole mechanism by which NO enhances or protects against  $\text{H}_2\text{O}_2$ , since GSNO, SNAP, and DEA/NO also decreased the rate of decomposition of  $\text{H}_2\text{O}_2$ .

Furthermore, different NO donors reduce intracellular levels of GSH to different degrees. Exposure of V79 cells to 1 mM nitrite, SNAP, SIN-1, GSNO, DEA/NO, or AS resulted in varying degrees of depletion of intracellular GSH (64). Exposure to SNAP, GSNO, or DEA/NO resulted in only a modest decrease (<30%), after which the levels of GSH recovered rapidly. However, SIN-1 and AS decreased intracellular GSH levels by as much as 85%. Nitrite (1 mM) decreased the levels GSH in these cells by 50% after a 1-h exposure (64). The increased reduction of GSH by SIN-1 and AS suggests a reason why these substances enhanced  $\text{H}_2\text{O}_2$  toxicity.

The other main explanation for the differences in protective effects among the various chemical NO donors may reflect the actual flux of NO produced by each compound. The temporal profiles of NO release by the different compounds demonstrate that different amounts of NO are released over time (41). Both the NONOates and the *S*-nitrosothiol complexes, which protect against  $\text{H}_2\text{O}_2$  toxicity, released NO over the time-course of exposure to  $\text{H}_2\text{O}_2$ . However, SIN-1, SNP, and AS did not produce measurable NO (<1  $\mu\text{M}$ ) under these experimental conditions, coincident with a lack of protection against  $\text{H}_2\text{O}_2$  (41).

SNP, however, appears to increase the toxicity of ROS by yet other mechanisms. Chemistry mediated by SNP can result in the formation of chemical species other than NO, such as cyanide ( $\text{CN}^-$ ) and iron. Desferrioxamine (DF) completely protected cells from  $\text{H}_2\text{O}_2$ , yet DF only partially protected against the toxicity mediated by SNP combined with  $\text{H}_2\text{O}_2$  (41). This discrepancy may be accounted for by the

enhanced  $\text{CN}^-$  released from SNP. Monocytes and polymorphonuclear leukocytes have been shown to facilitate the release of  $\text{CN}^-$  from SNP, a phenomenon believed to be mediated by  $\text{H}_2\text{O}_2$ . The authors suggested that a transition metal complex with a labile ligand could then further oxidize substrates via Fenton-type catalysis (65). Further evidence supporting this hypothesis comes from Imlay et al. (66), who showed that bacteria became more sensitive to  $\text{H}_2\text{O}_2$  in the presence of  $\text{CN}^-$ . The fact that DF completely protected against the toxicity of  $\text{CN}^-$  suggests that metal-peroxide reactions are required to initiate cytotoxicity. Thus, the DF-insensitive enhancement by SNP of  $\text{H}_2\text{O}_2$ -mediated toxicity could be attributed to an iron complex, which cannot be bound by DF; such a complex could catalyze the Fenton oxidation chemistry of cellular molecules.

Freeman and coworkers (51) have investigated the effect of NO on XO-mediated lipid peroxidation and found that NO acts as an antioxidant. We have also examined the effect of NO on organic hydroperoxide-mediated toxicity, thought to be mediated by oxidation of lipophilic membranes (54). Our studies further illustrate the importance of the presence of NO during the exposure to oxidants, showing that it is critical that NO be present during the exposure of the oxidant.

NO may be involved with several potential mechanisms in the protection against organic hydroperoxide-mediated toxicity. Intracellular metalloproteins such as those containing heme moieties react quickly with organic peroxides to form hypervalent complexes. These complexes can decompose and release intracellular iron, which in turn can catalyze damage to macromolecules such as DNA. Nitric oxide can react at near diffusion controlled rate constants with these hypervalent metalloproteins, which may restore these oxidized species to the ferric form (38,67). The reduction of these metallo-oxo proteins prevents both their oxidative chemistry and their decomposition, which releases intracellular iron (37–39), thus limiting intracellular damage mediated by oxidative stress.

Although NO can protect against the toxicity of  $\text{H}_2\text{O}_2$  to mammalian cells, the opposite effect is observed when the target is *E. coli*.  $\text{H}_2\text{O}_2$ , delivered either as a bolus or through the enzymatic activity of XO, exhibits only modest bactericidal activity (68). However, simultaneous exposure to both  $\text{H}_2\text{O}_2$  and NO, the latter delivered either as gas or by a NONOate complex, increases bactericidal activity by 4 orders of mag-

nitide. Addition of either catalase or superoxide dismutase demonstrated that NO/H<sub>2</sub>O<sub>2</sub> was the chemical species responsible for this bactericidal activity. Thus, the combination of NO and H<sub>2</sub>O<sub>2</sub> may be ideally suited for killing *E. coli* owing to the additional protective effect of NO on the host. This mechanism may hold true for other species of bacteria, albeit with different kinetics. Staphylococcal killing by O<sub>2</sub><sup>-</sup> was abrogated by NO at early time points, yet NO helped sustain killing at longer time intervals. Maximal killing depended on different timings of exposure to NO, H<sub>2</sub>O<sub>2</sub>, and O<sub>2</sub><sup>-</sup> (69). These findings may explain why NO and ROS are produced by immune effector cells at different times following exposure to different pathogens.

The diametrically opposite responses of mammalian cells and prokaryotes to the combination of NO/H<sub>2</sub>O<sub>2</sub> may reflect their different cellular structures and complements of metalloproteins. Bacteria utilize iron sulfur clusters to a greater extent than do mammalian cells, and these types of proteins are especially susceptible to degradation mediated by NO or RNOS (70,71). In *E. coli*, decomposition of iron complexes occurs in the periplasmic space, which is in close proximity to the cytoplasm. This relative lack of compartmentalization may allow iron to bind to and oxidize DNA. However, owing to the organellar structure of mammalian cells, metal labilization may be limited to the cytoplasm and mitochondria. In such a cellular arrangement, metals would be required to travel a large distance in order to reach the nucleus and bind to DNA.

### *Effect of NO/O<sub>2</sub><sup>-</sup> on Cytotoxicity*

Treatment of cells with peroxynitrite results in cell death in both the bacterial (72) and mammalian systems (see review in ref. (73)). However, lung fibroblast and neuronal cells treated with a superoxide source and NO donors showed no appreciable toxicity (59). Other studies have shown that ovarian carcinoma cells exposed to 5 mM SIN-1, a simultaneous NO/O<sub>2</sub><sup>-</sup> generator, resulted in no appreciable toxicity (42). In fact, cells treated simultaneously with XO- and NO-releasing compounds were protected against XO-mediated toxicity, and no appreciable toxicity owing to ONOO<sup>-</sup> formation was observed (41). These results suggest that there is a distinct difference between treating cells with bolus (mM) concentrations of peroxynitrite and generating similar amounts from NO and O<sub>2</sub><sup>-</sup> systems.



Part of the discrepancy between bolus peroxynitrite treatment and peroxynitrite derived from NO/O<sub>2</sub><sup>-</sup>-generating systems can be explained in terms of concentrations. Beckman and co-workers (72) noted that cells treated with bolus delivery of peroxynitrite required high concentrations of peroxynitrite for penetration. The cell membrane forms a formidable barrier for peroxynitrite penetration into intracellular targets. Generation of NO and superoxide over specific time intervals results in peroxynitrite; however, the short lifetime of this chemical species in solution does not allow high enough concentrations of peroxynitrite to accumulate in order to penetrate the cell. Therefore, the amount of peroxynitrite that could cross the cellular membrane under more biologically relevant conditions, despite product in stoichiometrically high amounts over a prolonged period, is dramatically reduced. Therefore, the cell membrane limits the toxicity of extracellular peroxynitrite.

Another factor to consider in respect to toxicity mediated directly by peroxynitrite is the reaction between NO and peroxynitrite to form NO<sub>2</sub>. As was discussed above, competition for superoxide by cellular components such as superoxide dismutase (SOD) and redox proteins increases the amount of NO required to form peroxynitrite. Since NO must outcompete these other reactions for O<sub>2</sub><sup>-</sup>, fluxes must become rather large. The peroxynitrite that is thus formed can potentially be converted to potent nitrosating agents since the extracellular chemistry of excess NO reacting with ONOO<sup>-</sup>, will convert it to nitrite. Thus, direct necrotic cell death mediated by oxidative chemistry of peroxynitrite from exposure of simultaneous NO/O<sub>2</sub><sup>-</sup> derived from reduced nicotinamide adenine dinucleotide phosphate (NADPH) is unlikely.

## INDIRECT EFFECTS

The indirect effects of NO are thought to result in the chemical species responsible for the etiology of numerous diseases related to NO. However, when determining the mechanisms under conditions that produce indirect effects, it is important to consider direct effects as well. Under conditions of high NO flux in the vicinity of a macrophage, the activity of guanylate cyclase, a direct effect of NO, still occurs.

The indirect effects can be divided into two basic types of chemistry, nitrosation and oxidation. Nitrosation chemistry results primarily in the formation of NO adducts on amines and thiols. Oxidation chemistry



results in the oxidation of different macromolecules ranging from mild reducing agents such as catecholamines and metal centers to those processes requiring higher oxidation potentials that damage DNA, proteins, and lipids. Oxidation and nitrosation chemistry can occur under nontoxic conditions or result in chemical intermediates that are cytotoxic. The terms nitrosative and oxidative stress are used to refer to chemical reactions that result in cytotoxicity. The chemical species responsible for NO-mediated nitrosative and oxidative stress involve RNOS. Under these conditions, molecules not normally associated with regulatory effects can be damaged. During chemical stress, proteins and DNA are often damaged, requiring the cell to repair itself. These chemical reactions are often invoked as the etiology of many diseases.

The ultimate chemical modifications resulting from RNOS species is a function of the intermediates formed under biologic conditions;  $N_2O_3$ ,  $ONOO^-$ ,  $NO^-$ , and  $NO_2$  are the most important (**Fig. 1**).  $N_2O_3$  is a relatively mild oxidant. It will only oxidize substrates with potentials less than +0.7 V and does not oxidize biomolecules such as DNA. However,  $N_2O_3$  readily nitrosates nucleophiles and may be the principle nitrosating species in vivo, at high local concentrations (11). On the other hand, peroxynitrite and nitroxyl, which do not nitrosate substrates, readily mediate oxidative chemistry of macromolecules in vivo. (13,74,75). A comparison of the resultant thiol products in the presence of RNOS can illustrate this point. An aerobic NO solution will autooxidize to produce  $N_2O_3$ . If GSH is present, it will form nearly 100% of the nitrosative product, GSNO (76). Conversely, if GSH is exposed to either peroxynitrite, nitrogen dioxide, or nitroxyl, the resultant product is oxidized thiols and not nitrosative products (75,77–79).

In addition to these bimolecular reactions, numerous other more complicated reactions of NO and RNOS/ROS can take place. For instance, NO can react with peroxynitrite, nitrogen dioxide, and nitroxyl, thereby inhibiting the oxidation of thiols (13,78). The reaction between NO and nitrogen dioxide or peroxynitrite can result in  $N_2O_3$ , which facilitates nitrosation of thiols. Since thiols are the primary reactive site for the indirect effects on cells, it can be seen that a balance of numerous reactions is what results in the eventual outcome. In addition to these interactions, NO can interact with ROS to abate oxidative chemistry. Therefore, it is not sufficient to consider just one reaction; they must be placed into perspective relative to each other. With this in

mind, we discuss the sources and conditions whereby nitrosative and oxidative stress might occur and the chemical reactions that are responsible as well as their likely biologic targets.

## NITROSATIVE STRESS

The study of nitrosation chemistry dates back to the turn of the century (80). Nitrosation of amines to form nitrosamines derived from nitrite in the gastrointestinal tract became a concern in the mid 1970s as a potential source of carcinogens (81). A decade later, the discovery of nitrosamines and nitrite production under conditions of infection or by activated leukocytes was a critical link in elucidating the formation NO in vivo (5,82–84). Later studies showed that under some types of chronic infection, nitrosamines are produced, thereby confirming that nitrosation does occur in vivo (85). Nitrosation of thiols and their ultimate biologic fate have been extensively studied in a number of diverse conditions from cardiovascular function to cancer (7,86).

The chemistry of nitrosation can occur by several different mechanisms. Nitrosation differs from nitrosylation in that there is the addition of a nitrosonium ion ( $\text{NO}^+$ ) equivalence to a nucleophile. Nitrosylation is defined as the formation of a nitrosyl adduct such as those formed between the reaction of NO and metals, as described for the direct effects. Simple nitrosation may occur from reactions mediated by metals as well as those from RNOS. Metal-mediated nitrosation can occur in the test tube and could have a role in vivo.

### *Metal-Mediated Nitrosation*

The formation of *S*-nitrosothiols and nitrosamines from metal complexes such as SNP rapidly catalyzes nitrosation reactions. This involves a simple transfer of an equivalence of nitrosonium ion to the nucleophile. Heme complexes have also been shown to form *S*-nitrosothiols and nitrosamines. However, nitrosonium ion involves the ferric state of the heme. Ferric nitrosyl complexes are much less stable than their corresponding ferrous state, but the ferrous nitrosyl heme complexes do not nitrosate thiols and amines. Ferric heme can nitrosate some thiols and amines; however, this process requires reactivity of iron (III) porphyrins and heme proteins with nitric oxide. Nitrosyls transfer to carbon, oxygen, nitrogen, and sulfur (86a).

One mechanism in particular that may be important in the physiologic transport of NO as well as the formation of *S*-nitrosothiols is the chemistry of iron sulfur nitrosyl complexes. The formation of nonheme nitrosyl complexes seems to occur under high fluxes of NO. Several studies have indicated that ferritin, aconitase, and even metallothionein are responsible for this (87,88). Nitrosation of thiols can occur under anaerobic conditions in the presence of ferrous iron. However, this chemistry is readily reversible. Therefore, this is not likely to account for inactivation of enzymes in cells exposed to high NO concentrations. However, an equilibrium exists among NO, dinitrosyl iron, and RSNO so that even a small fraction of GSNO may be all that is required for physiologic stimulation. These mechanisms remain to be elucidated.

An alternative possibility for the nitrosation of thiols could be catalyzed by proteins such as cobaltamine. As discussed above, a stable cobalt (III) nitrosyl can readily be formed even under physiologic conditions. In the presence of thiols, it was shown that *S*-nitrosothiols could be formed. This may play an important role in NO-mediated metabolism in the vascular system as well as in tumors.

Although some metal-mediated nitrosative chemistry could occur *in vivo*, the detection of nitrosamines under physiologic conditions is most likely a result of RNOS. Heme- and iron-mediated nitrosation of amines requires at least an atmosphere of NO and an exposure time of days. These are conditions not likely to be encountered *in vivo*. Hence, the formation of nitrosamines in stimulated cells is mediated by RNOS. The presence of these nitrosative products in cells and *in vivo* suggests that nitrosative stress is an important biologic effect of NO.

There are basically three potential sources of RNOS-mediated nitrosation: (1) NO autoxidation, (2) nitrite acidification, and (3) the NO/O<sub>2</sub><sup>-</sup> reaction under excess fluxes of NO (discussed just below). Except in gastric regions (pH < 1.5), the primary nitrosating intermediates are isomers of N<sub>2</sub>O<sub>3</sub>. The route of formation of these intermediates is important and determines where and when nitrosation occurs.

The chemical reaction most noted for the formation of N<sub>2</sub>O<sub>3</sub> is the reaction between NO and oxygen; this is referred to as autoxidation. This reaction has been studied for decades because of its importance in gas phase atmospheric nitrogen oxide chemistry (89). The autoxidation

of NO in the aqueous, hydrophobic, and gas phases has a third-order rate equation with second-order dependency on NO (89):

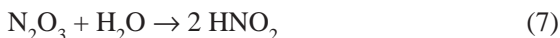
$$d[\text{NO}]/dt = k_{\text{NO}}[\text{NO}]^2[\text{O}_2] \quad (5)$$

This second-order dependence on NO helps to explain some of the confusion over how a toxic radical species like NO can participate in physiology. Because of the instability of NO in the presence of oxygen, as well as the formation of toxic chemical species like  $\text{N}_2\text{O}_3$  and  $\text{NO}_2$ , it was hard to envision why nature would choose NO as physiologic mediator. The second-order NO dependency of this autoxidation reaction dictates that the lifetime of NO is inversely proportional to its concentration (**Eq. 5**) (90,91). Therefore, when NO is formed and moves away from its cellular source, its concentration is diluted. As its concentration decreases, there is a concomitant increase in its lifetime. This allows NO to react with the other biologic targets such as guanylate cyclase without interference from the autoxidation reaction and production of the related RNOS. Conversely, when NO levels are high, the formation of RNOS increases dramatically. In local regions of high NO output, intermediates associated with the autoxidation can occur.

Where in the cell would the autoxidation be likely to occur? Comparisons between the rate constant for the autoxidation reaction in hydrophobic regions versus aqueous solution show a similar rate constant. This finding suggests that the surrounding medium does not influence the rate of autoxidation dramatically. The solubility of the reactants will, however, affect the rate of the reaction since NO and oxygen are more soluble in hydrophobic phases than in aqueous solutions. Reports have shown that NO and oxygen levels are 10–50 times higher in lipid membranes than in aqueous solutions owing to their increased solubility (92). For example, if a cell is exposed to a NO flux from a chemical donor or another cell, NO will partition such that the NO levels will be 10 times greater in the membrane than the surrounding aqueous solution. Therefore, since the rate of the autoxidation reaction is a function of reactant concentration, it should occur much faster in membranes than in aqueous solution based solely on the differences in the relative concentrations of NO and  $\text{O}_2$  in each region. A recent study has shown that in the presence of detergent micelles, the rate of autoxidation occurs 300 times faster in the hydrophobic region than in the surrounding aqueous solution (93). This work suggests that the nitrosation reac-

tions mediated by autoxidation would be mostly likely to occur in the membrane. Hence, membrane-bound proteins, which are functionally and structurally dependent on thiols, or amines would be most affected by nitrosative stress.

Another factor to consider in nitrosation chemistry is the mechanism of  $N_2O_3$  formation and whether it requires the intermediacy of  $NO_2$ . In gas-phase and hydrophobic solvents, the initial intermediate of autoxidation is the formation of  $NO_2$  (89). Nitrogen dioxide then reacts with another NO to form an equilibrium with  $N_2O_3$  (Eq. 6). In the presence of water,  $N_2O_3$  is converted rapidly to nitrite (Eq. 7):



An additional means for  $N_2O_3$  formation is the reaction of nitrite under acidic conditions. The protonation of  $NO_2^-$  to form  $H_2ONO$  will react with an additional  $NO_2^-$  to form  $N_2O_3$  (80).  $N_2O_3$  can then disproportionate into  $NO_2$  according to Eq. 6. This is the exact same species,  $N_2O_3$ , as is formed in the autoxidation reaction in aqueous solution; however, unlike the autoxidation reaction, there is formation of  $NO_2$ . Studies have also suggested that  $N_2O_3$  formed in aqueous solution is different from that in hydrophobic media (91,94). Several studies using competition reactions show that in aqueous solution  $NO_2$  cannot be trapped. This suggests that in aqueous solution the formation of  $NO_2$  from  $N_2O_3$  cannot escape the solvent cage. To illustrate best the differences between nitrosation versus nitration, reactions with phenol and tyrosine were examined (76,95). Nitrotyrosine was the exclusive product when tyrosine was exposed to acidic nitrite, or RNOS formed first in the gas phase. This is thought to occur through the reaction of  $NO_2$  (76). However, when RNOS are formed from the autoxidation in water, no nitrotyrosine is formed. This suggests that the autoxidation in aqueous solution will not produce nitrotyrosine but that in a hydrophobic environment such as that found in membranes it could occur.

The selectivity of the intermediates formed in the autoxidation reaction has been determined. Since  $N_2O_3$  is hydrolyzed to nitrite extremely rapidly (half-life of 1 ms), only substrates that are present in high concentration and have sufficient affinity will react (96). At neutral pH, thiol-containing peptides have an affinity for  $N_2O_3$  1000 times greater than any other amino acid (76,96). In addition, buffers

such as carbonate and phosphate have affinities less than 400 times that of thiol-containing peptides (96). These results suggest that the primary reaction of the NO/O<sub>2</sub> in aqueous solution will be to form *S*-nitrosothiols. *S*-nitrosothiols have been shown to have a variety of effects on biologic functions. This supports the possibility that this reaction can occur from NOS-generated NO (86).

### *Chemistry of RSNO*

The fate as well as the biologic action of *S*-nitrosothiols resulting from the nitrosative stress is important in understanding the biology of NO. In particular, small peptides such as GSNO or CysNO can play important roles in cellular metabolism and influence cardiovascular properties. GSNO has three major reactions that are important to the biologic outcome of nitrosative stress: reaction with other reduced thiols, reaction with metal complexes, and reaction with superoxide. It is important to understand that low molecular weight thiols are usually less stable than their corresponding protein adducts. For instance, CysNO in biologic solutions has a shorter half-life than GSNO, which in turn has a shorter lifetime than protein *S*-nitrosothiols. Therefore most of the *S*-nitrosothiol will be on proteins.

The reactions of RSNO with reduced thiols can result in two basic reactions, transnitrosation or reductive elimination of nitroxyl. Transnitrosation reactions have been proposed as a mechanism by which NO can be transported through biologic system. This is simply a transfer of a nitrosonium ion (NO<sup>+</sup>) from one thiol to another. Several reports have investigated the relative rates of these reactions. In general, CysNO will preferentially transfer NO<sup>+</sup> equivalence to higher molecular weight thiols, whereas the transfer from GSNO to proteins will be slower. These reactions are often in equilibrium with each other and hence favor the thermodynamically more stable product.

One of the most common and important reactions of RSNO in test tube experiments is the reaction with metal centers. Metals, especially copper in buffered solution, dictate the stability of RSNO. Kinetic analysis has revealed that Cu (I) and not Cu (II) is responsible for the decomposition of NO. The Cu (I) decomposition of GSNO may be important when CuZnSOD is present along with GSH. MnSOD, on the other hand, has no effect. GSNO with copper ions in SOD reacts with GSH to form GSSG and NO as products. The amount of GSH required

was 0.1–10 mM, which is in the range of physiologic GSH concentrations in the cytoplasm. This decomposition suggests that CuZnSOD may play a key role in the detoxification of nitrosative stress, either by direct scavenging of  $N_2O_3$  or via transnitrosative reactions.

Surprisingly, superoxide reacts with GSNO. Several papers have shown that the reaction of superoxide and GSNO will produce an oxidizing species resulting in GSSG. The kinetics for this reaction is the requirement of 2 GSNO for every superoxide in the rate-limiting step. GSSG and equal molar nitrite and nitrate are formed. Studies using millimolar GSNO suggest that the oxidant formed may be peroxyxynitrite. However, other kinetic analyses suggest that 2  $NO_2$  is the possible intermediate.

## OXIDATIVE STRESS

Oxidation is the removal of electrons from substrate and occurs under normal physiologic conditions. However, there is a significant difference between normal cellular redox chemistry and that associated with oxidative stress. Under conditions of oxidative stress, powerful oxidizing agents, resulting in products not normally found under normal physiologic condition. For example, oxidation of DNA results in strand breaks, and oxidation of nucleic acids can occur under oxidative stress (97). Oxidation of lipids results in lipid peroxidation, and oxidation of protein modifies their structure and impedes their function. These processes have been associated with the onset of different pathophysiological conditions, suggesting that chronic oxidative stress is the etiology of many pathophysiological states (97).

The chemistry of NO can result in different RNOS that are capable of causing conditions of oxidative stress. The three major RNOS that mediate oxidative stress are nitrogen dioxide, nitroxyl, and peroxyxynitrite. Nitrogen dioxide primarily originates in the same processes involved in nitrosative stress: autoxidation of NO, the  $NO/O_2^-$  reaction (as discussed in the  $NO/O_2^-$  Chemistry section below), and acidic nitrite.  $NO_2$  can directly nitrate substances such as tyrosine (76) and might be the source of nitrotyrosine observed in vivo.  $NO_2$  does not appear to alter DNA in such forms as strand breaks (98,99), but it can induce lipid peroxidation (100). Under conditions of excess NO, it can react rapidly with  $NO_2$  to form  $N_2O_3$ , which mediates nitrosative stress.



Oxidation mediated by  $\text{NO}_2$  would probably occur from acidic nitrite.  $\text{NO}_2$  produced in membranes from the autoxidation reaction or from  $\text{NO} + \text{HOONO}$  would most likely be converted to  $\text{N}_2\text{O}_3$  owing to the presence of excess  $\text{NO}$  (13). Therefore, the oxidative chemistry mediated by  $\text{NO}_2$  in vivo is probably limited.

Another nitrogen oxide species, nitroxyl ( $\text{NO}^-$ ), is a chemical intermediate of  $\text{NO}$ . It has been shown that formation of  $\text{NO}^-$  can result from different processes under a variety of biologic situations. One primary source of  $\text{NO}^-$  is the decomposition of *S*-nitrosothiols (101). The nucleophilic attack of thiols to  $\text{RSNO}$  can result in  $\text{NO}^-$  and disulfide. Decomposition of dithiothreitol (DTT)- $\text{SNO}$  results in  $\text{NO}^-$  and oxidized DTT (102). Other reports suggest that  $\text{NO}^-$  can be formed from the decomposition of iron dinitrosyl complexes (103). One intriguing possibility is that  $\text{NO}^-$  may be derived directly from  $\text{NOS}$  itself (104,105). Several reports have suggested that one initial product in the conversion of arginine to citrulline is  $\text{NO}^-$ . Other reports suggest that oxidation of the catalytic intermediate in  $\text{NOS}$  activity, hydroxyarginine, may result in  $\text{NO}^-$  (106). Taken together, these processes suggest that  $\text{NO}^-$  may play a role in the biology of  $\text{NO}$ .

Substances that release  $\text{NO}^-$  have provided a method of studying the effects of  $\text{NO}^-$  in biology (107). One of these is called Angelis's salt or sodium trioxodinitrate ( $\text{Na}_2\text{N}_2\text{O}_3$ ). At neutral pH, this complex releases  $\text{NO}^-$  and nitrite. Recent studies on AS have shown that  $\text{NO}^-$  is cytotoxic (108). Comparing survival in clonogenic assays with Chinese hamster V79 cells, AS at 2 and 4 mM was toxic. Comparisons between the toxicity of the  $\text{NO}/\text{O}_2^-$  donor SIN-1 and the  $\text{NO}$  donor DEA/ $\text{NO}$ , with AS, demonstrated that the toxicity of AS was more than 2 orders of magnitude greater. The toxicity of AS compared with hydrogen peroxide and alkyhydroperoxide was similar. Hypoxia abated the toxicity, suggesting that the RNOS chemistry responsible for cell death requires a reaction between nitroxyl and oxygen. The lack of an effect by metal chelators indicates that ROS via Fenton-type reactions are not involved. It appears that addition of AS results in a dramatic loss of GSH as well as DNA double-strand breaks. Since neither hydrogen peroxide nor peroxynitrite mediates double-strand breaks, they are probably not the chemical intermediate.

We have examined the oxidative, nitrosative, and nitrative properties of AS under aerobic and anaerobic conditions (109). A comparison of dihydrorhodamine (DHR) oxidation by peroxynitrite and  $\text{N}_2\text{O}_3$ , with



oxidation by AS demonstrated that AS had a selectivity similar to that of peroxynitrite. AS was also not quenched by azide, an  $N_2O_3$  scavenger. Despite these similarities, the yield of DHR oxidation was twice that of peroxynitrite. By examining the one-electron oxidation of hydroxyphenylacetic acid to its fluorescence dimer product in the presence of AS, it was shown that oxidation by AS was only minimal, whereas peroxynitrite was very effective. Hydroxylation of benzoic acid was more efficient with AS than with peroxynitrite. However, the nitration of HPA was not detected with AS, but peroxynitrite was readily nitrated under the same conditions. Therefore, it appears that  $NO^-$  in the presence of oxygen produces an intermediate other than peroxynitrite.

There appear to be two types of reactivates of nitroxyl in biologic systems, an oxygen-independent and an oxygen-dependent reaction. By using DHR oxidation, which requires oxygen, several substances were examined to test their reactivity with  $NO^-$ . It appears that amines such as hydroxylamine and thiols rapid react with  $NO^-$  rapidly and directly. In addition, NO, NADPH, and SOD all react with  $NO^-$  (108,110). Metalloproteins such as myoglobin and catalase have also been shown to react effectively with  $NO^-$ .  $NO^-$  oxidation and hydroxylation reactions appear to require oxygen, but  $NO^-$  in the presence of  $O_2$  mediates DNA damage or cellular toxicity.

## NO/ $O_2^-$ CHEMISTRY

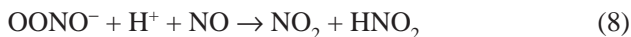
The reaction between superoxide and NO has been shown to be very important in the fundamental understanding of NO behavior in biology (73,111). Huie and Padmaja (112) showed that  $NO + O_2^-$  reacted at near diffusion controlled rate to form peroxynitrite. One of the first observations suggesting that this reaction could be important in biology was that SOD enhanced the effect of endothelium-derived relaxing factor (EDRF) (113). It was thought that NO concentrations might be partly controlled by superoxide. Because of the fast rate of peroxynitrite formation, it was thought that this reaction could play an important role in the contribution of NO to various pathophysiologic conditions (111). As we will discuss below, this reaction is much more complicated than the simple formation of peroxynitrite.

The reaction rate constant suggests that peroxynitrite could be formed in vivo. The question is when and where? One of the most important considerations in predicting whether a reaction will occur in

vivo is the relative pseudo-first-order rate constants and not just the rate constant itself. The concentrations of the reactants are as important as the rate constants in determining the participation of a given reaction (for a summary of RNOS evaluation, see ref. 96). The cellular concentrations of superoxide and NO under normal conditions are 1  $\mu M$  and 0.1–1  $\mu M$ , respectively. This suggests that superoxide production is the limiting determinant for the location as well as the amount of peroxynitrite formed between these radicals. The NO concentration determines whether the reaction occurs at any specific locality. The determination of whether this reaction occurs depends on competing reactions of superoxide with NO. Most important is the concentration of SOD. SOD reacts with superoxide at a similar rate constant as NO. Since the intracellular SOD concentration is thought to be between 4 and 10  $\mu M$  (50  $\mu M$  in the area of the mitochondria, where most of the cellular production of  $O_2^-$  would occur), the NO concentration would have to be 0.4–5  $\mu M$  for 10% of the superoxide to be converted to peroxynitrite. In addition, other reactants with superoxide such as aconitase ( $3 \times 10^7 M^{-1} s^{-1}$ ) and ferricytochrome c ( $5 \times 10^6 M^{-1} s^{-1}$ ) could play a role in the abatement.

Peroxynitrite at neutral pH has been shown to be a powerful oxidant. It can oxidize thiols, initiate lipid peroxidation, nitrate tyrosine, cleave DNA, nitrate and oxidize guanadine, and oxidize methionine. The oxidant responsible for this is an excited state of peroxynitrous acid (114). Peroxynitrite is in equilibrium with the protonated form, peroxynitrous acid. In the absence of an adequate substrate, this protonated form simply rearranges to nitrate. This can be thought of a detoxication pathway. However, at high enough concentrations, substrates such as tyrosine (1 mM to yield 50% yield) can react to give nitrotyrosine. It is thought that most of the nitration and oxidative chemistry proceeds through the HOONO species (114). Metals can react directly with  $ONOO^-$  at  $6 \times 10^3 M^{-1} s^{-1}$  (115). In the case of CuSOD and FeEDTA, the metal component enhances nitration reactions. It has also been shown that heme-containing enzymes such as myeloperoxidase ( $6 \times 10^6 M^{-1} s^{-1}$ ), lactoperoxidase ( $3.3 \times 10^5 M^{-1} s^{-1}$ ) and horseradish peroxidase ( $3.2 \times 10^6 M^{-1} s^{-1}$ ) react directly with  $OONO^-$  (116).

Another important consideration of the  $NO/O_2^-$  reaction is that NO and  $O_2^-$  can react with peroxynitrite to form nitrogen dioxide (74,114,117):

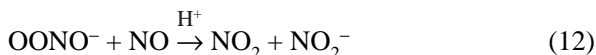


This places further restrictions on the chemistry mediated directly by peroxyxynitrite. To examine the fluxes of NO and superoxide in nitrosative and oxidative chemistry, several studies have examined NO donors with xanthine oxidase (XO) (74). For instance, NO does not alter the oxidation of xanthine by XO but does affect the production of superoxide (59,118–120). XO is considered a model of oxidative stress and generates the reactive oxygen species superoxide and hydrogen peroxide. In the presence of an NO<sup>-</sup> releasing agent, the amount of hydrogen peroxide produced by XO is unaffected (41). However, the amount of superoxide formed is dramatically reduced. It has been proposed that NO reacts with the produced superoxide formed from XO to form peroxyxynitrite, which then isomerizes to nitrate (59,118–120):



If the same conditions are used in the presence of DHR (74), an increase in oxidation is observed in the presence of NO/XO. Peroxyxynitrite oxidizes DHR (121), further supporting the hypothesis that peroxyxynitrite is an RNOS generated from NO/XO (**Eq. 10**).

What is intriguing about this study is that the fluxes of NO and superoxide were varied relative to each other. It was shown that maximal oxidation through peroxyxynitrite was only achieved when the reactants were present at a 1:1 ratio. It was shown that excess of either radical quenched the chemistry of peroxyxynitrite. In the presence of excess NO or superoxide, peroxyxynitrite is converted to nitrogen dioxide (74,114,122):



Nitrogen dioxide can rapidly react with NO to form the nitrosating species, N<sub>2</sub>O<sub>3</sub>:



Although nitrosation does not occur directly through peroxyntirite, there are several mechanisms by which they can occur through the  $\text{NO}/\text{O}_2^-$  reaction that may be important in the biology of NO.

## MIXED DIRECT AND INDIRECT

### *NO Inhibition of Mitochondrial Respiration*

One of the primary proposed cellular targets for the cytotoxic action of NO is the mitochondrion (1,87,123). Dinitrosyl adducts of aconitase are formed in cells after exposure to NO and may be an important factor in the inhibition of mitochondrial activity leading to cytoostasis and cytotoxicity (87). Further studies have shown that NO also de-energizes the mitochondria in a reversible manner (124,125), which, under normal physiologic conditions, involves regulation of intracellular calcium (126). So how does NO inhibit mitochondrial function as part of regulatory processes, yet also mediate cell death?

Inhibition of mitochondria mediated by NO appears to have a reversible and irreversible component. Knowles et al. (127) reported that NO derived from GSNO inhibited mitochondrial respiration by a distinctly different mechanism than  $\text{OONO}^-$ . They suggested that NO derived from GSNO reversibly inhibited respiration, whereas  $\text{OONO}^-$  resulted in irreversible inhibition of respiration. Several studies have shown that NO directly interacts with cytochrome c oxidase to inhibit respiration reversibly (128–133). The interaction with cytochrome c oxidase appears to require low concentrations of NO (submicromolar), characteristic of the NO concentrations resulting from cNOS production. However, under inflammatory conditions, complex I (NADH: ubiquinone oxidoreductase) and complex II (succinate: ubiquinone oxidoreductase) are irreversibly inhibited by NO (131). Under these conditions, RNOS may play a role in irreversible inhibition. Similar to the mechanism described for cytochrome P450, inhibition of mitochondrial respiration appears to have a reversible component mediated by direct effects and an irreversible component mediated by indirect effects.

Modulation of respiration by low amounts of NO at the cytochrome c oxidase will determine tissue oxygen gradients as well as cellular adenosine triphosphate (ATP) levels. Like other heme proteins,

cytochrome c oxidase can react with NO to form a nitrosyl adduct (134). Binding of NO to cytochrome c oxidase may influence the activity of mitochondrial enzymes depending on the oxygen concentration. Under both hypoxic and aerobic conditions, NO is consumed by the mitochondria through direct binding to the cytochrome c oxidase site. After the formation of Fe-NO, additional electrons from the respiratory chain reduce NO to nitrogenous products (135,136). However, under aerobic conditions, it appears that electrons in the respiratory chain are diverted from the reduction of NO at the cytochrome c oxidase site to form superoxide and (thus hydrogen peroxide), which can further react with NO (137). The partitioning between the reduction of NO and oxygen is dependent on oxygen tension versus the rate of electron reduction of the nitrosyl cytochrome c oxidase complex.

The inhibition of oxygen consumption at the mitochondria by low levels of NO may be important in regulating tissue oxygen. ecNOS (NOS-3) has been found in mitochondria (138), indicating that this source of NO may be important in various physiologic mechanisms. The presence of NOS in mitochondria suggests that the chemistry of NO is well regulated within this organelle. Some reports have proposed that NO plays a key role in the regulation of respiration. Other reports suggest that the influence of NO on the mitochondria may play a role in smooth muscle cell relaxation in both physiologic and pathophysiologic conditions (139,140).

As NO concentrations and time of exposure increase, there is an increase in RNOS formed in the mitochondria. The source of RNOS under aerobic conditions has been proposed to involve superoxide derived from decoupling of oxygen reduction at the cytochrome  $a_1a_3$  site, which reacts with NO to form peroxynitrite. However, MnSOD, which exists in the mitochondria at high concentrations, will compete with NO for superoxide, thus limiting the formation of peroxynitrite. The amount of NO required to form peroxynitrite results from NO fluxes that are higher than superoxide fluxes. As discussed above, this should create an imbalance in the  $\text{NO}/\text{O}_2^-$  ratio and would favor the conversion of peroxynitrite to  $\text{N}_2\text{O}_3$ . These conditions indicate that the oxidative chemistry mediated by peroxynitrite probably does not play a significant role in mitochondrial dysfunction but that other RNOS such as  $\text{NO}_2$  and  $\text{N}_2\text{O}_3$  may. Furthermore, according to **Eqs. 7–10**, it is more likely that nitrosative, not oxidative chemistry, would be the predominant indirect effect in mitochondria under high NO fluxes.

Most of the mitochondrial studies have been conducted in cell culture or with isolated mitochondria. However, a comparison of cellular and in vivo inhibition of mitochondria suggests that RNOS-mediated irreversible inhibition is less important in vivo. When cultured hepatocytes are stimulated with interferon- $\gamma$  and LPS to activate NOS and NO generation, inhibition of respiration results. In contrast, respiration in cells isolated from animals treated with LPS and interferon- $\gamma$  is not affected (141,142). This may suggest that oxyhemoglobin and diffusion of NO away from NOS-containing cells may play an important role in the extent of mitochondrial inhibition when RNOS formation is limited and reversible inhibition is only transient.

### *Metal Homeostatics*

An important role of NO under either physiologic or pathophysiologic conditions is the regulation of intracellular iron status (see reviews in refs. 70 and 71). There are different aspects of iron metabolism that NO can affect. NO can influence heme metabolism by activating heme oxygenase, which results in catabolism of heme complexes, as well as inhibiting ferrochelatase, an enzyme that places the iron in the porphyrinic complex (24). The inhibition reduces heme availability and decreases the amount of active NOS, which may serve as a negative self-regulation of NO formation.

In addition to influencing heme metabolism, NO affects the formation of the transferrin receptor and ferritin protein, which regulates the uptake and storage of cellular iron. The iron-responsive elements (IREs) are strands of RNA that are posttranscriptionally regulated (see reviews in refs. 70 and 71). The iron-responsive binding protein (IRB) that contains an  $\text{Fe}_{3-4}\text{S}_4$  cluster and possesses aconitase activity regulates the IRE synthesis of ferritin to transferrin receptor protein (143). The iron sulfur cluster within the IRB has two forms, apoprotein  $\text{Fe}_3\text{S}_4$  and holoprotein  $\text{Fe}_4\text{S}_4$ , in which the fourth iron is in the apical position. If the apical Fe is missing, then binding to the IRE results in the down-regulation of ferritin production and upregulation of transferrin receptor. These events result in increased cellular uptake of iron (143). However, if the apical iron is present, then ferritin protein increases and the protein for the transferrin receptors decreases, which results in reduction of iron uptake (143).

NO binds to the apical iron to form a nitrosyl complex, resulting in

inhibition of aconitase activity, but it does stimulate binding to IREs. It should be noted that peroxynitrite and superoxide inactivate both aconitase activity and the ability of the IRB to bind to the IRE (144,145), thus limiting the intake of iron. This inactivation of aconitase by either peroxynitrite or superoxide may be a *protective* mechanism against excessive iron uptake, limiting the iron available to catalyze oxidative chemistry. This may be another mechanism by which NO reduces intracellular oxidative stress. Inactivation of aconitase activity would also reduce the available electrons, thus reducing oxygen by the respiratory chain. Since NO may increase superoxide/hydrogen peroxide formation via inhibition of respiration (cytochrome c oxidase), the reduction of electron flow may also reduce ROS production. The inactivation of aconitase may be protective against intracellular hydrogen peroxide formation.

Iron metabolism and availability has a tremendous effect on oxidative stress as well as cell growth. NO may play a key role in inhibiting the availability of iron by inhibiting release of iron from ferritin. In mammalian cells, iron is released from ferritin by reduction from NADPH oxidase through the intermediacy of superoxide. The conversion of the ferric to ferrous state makes iron accessible to cells. NADPH oxidase assembly, not activity, is inhibited by NO (119). This would limit iron availability to the cell. In addition, NO scavenges superoxide, which inhibits the reduction of ferritin-bound iron. In addition to inhibition of ribonucleotide reductase, these two mechanisms may play an integral part in cytostatic mechanisms in a variety of disease states.

## CONCLUSIONS

Chemical biology can provide a road map for researchers who are investigating the molecular aspects of NO. The importance of concentration and timing with other reactive oxygen species cannot be overstated. Direct effects may predominate in the physiology of NO, but the indirect effects give NO some of its more pathophysiological characteristics. Temporal, stoichiometrical, concentration, and spatial considerations must be considered in order to place NO in the context of biologic systems.

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