

# Perspectives Series: Host/Pathogen Interactions

## Mechanisms of Nitric Oxide-related Antimicrobial Activity

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*As it takes two to make a quarrel, so it takes two to make a disease, the microbe and its host.*

Charles V. Chapin (1)

### *Roles of nitric oxide in infection*

With a molecular weight of 30 nitric oxide (NO)<sup>1</sup> is certainly one of the smallest biological molecular mediators. In mammalian cells, NO is produced along with L-citrulline by the enzymatic oxidation of L-arginine. Intensive investigation over the past two decades has demonstrated important roles of enzymatically produced NO in diverse physiological processes, many of which are relevant to understanding the pathogenesis of infection. NO may contribute to the morbidity of infection by acting as a vasodilator, myocardial depressant, and cytotoxic mediator. On the other hand, microvascular, cytoprotective, immunoregulatory, and antimicrobial properties of NO have a salutary and probably essential role in the infected host (2). This perspective article will provide a brief overview of current knowledge regarding the significance and mechanisms of NO-related antimicrobial activity, as well as preliminary investigations of microbial defenses against reactive nitrogen intermediates.

### *Evidence for NO-related antimicrobial activity*

The antimicrobial activity of NO has been demonstrated by a variety of approaches. First, there is circumstantial evidence implicating NO production as part of an effective host response to infection. NO production by the inducible NO synthase isoform (NOS2) is stimulated by proinflammatory cytokines such as IFN- $\gamma$ , TNF- $\alpha$ , IL-1, and IL-2, as well as by microbial products such as lipopolysaccharide and lipoteichoic acid. Infections in humans and experimental animals are often associated with significant increases in systemic NO production, when determined by measurement of NO end-oxidation products (nitrite and nitrate) in plasma and urine (3–5). Increased NOS2 expression and NO production can be

demonstrated at sites of infection in animal models such as toxoplasmosis and leishmaniasis (6), or in human infections such as tuberculosis (7).

Second, production of NO in many animal models can be directly correlated with the ability of the host to contain microbial proliferation, because abrogation of inducible NO synthase activity produces dramatic increases in microbial burden (6, 8). Although corresponding data in human infection cannot be obtained for ethical reasons, increased production of NO has been found to correlate with a better clinical outcome in children with falciparum malaria (3).

Third, in vitro studies of phagocytic cells and a variety of microbial targets have demonstrated cytokine-inducible microbistatic or microbicidal activity which is L-arginine-dependent and inhibitable by competitive NO synthase inhibitors such as N<sup>G</sup>-monomethyl-L-arginine (9).

Finally, NO-donor compounds have been shown to inhibit or kill microbes when directly administered in vitro. Although susceptibility is not universal, NO-related antimicrobial activity has been demonstrated against a remarkably broad range of pathogenic microorganisms including viruses, bacteria, fungi, and parasites (10).

NO has been most strongly implicated in host defenses against intracellular pathogens such as *Leishmania*, mycobacteria, and *Salmonella*. This may reflect the abundance of NO scavengers outside of the phagosomal compartment. NO scavenging by hemoglobin, for example, can antagonize antimicrobial actions of NO against trypanosomes and bacteria in experimental systems (11). Such antagonism is likely to be physiologically relevant in the setting of hemorrhage or bloodstream infection.

Evidence associating NO production and maintenance of microbial latency is particularly intriguing. Many pathogenic microorganisms, including *Leishmania* spp., *Mycobacterium tuberculosis*, Epstein-Barr virus, and *Toxoplasma gondii* are capable of causing prolonged asymptomatic latent infection. It is clear that latent infection requires active suppression by host cellular immune mechanisms, because subsequent impairment of cellular immune function by immunosuppressive drugs or illness results in reactivation of infection. The mechanism by which suppression of latent infection can be maintained without overt signs of inflammation and illness in the host has remained one of the least understood aspects of microbial pathogenesis. However, inhibition of NO production has been shown recently to induce prompt reactivation of *Leishmania major*, *M. tuberculosis*, Epstein-Barr virus, or *T. gondii* in experimental models (6, 8, 12), suggesting that NO may play a central role in persistent or latent infections.

The role of NO-related antimicrobial activity in human mononuclear phagocytes has been one of the most troublesome and controversial issues in NO biology. While it is clear

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1. Abbreviation used in this paper: NO, nitric oxide.

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that NO production is elevated in infected patients or in recipients of proinflammatory cytokines as adjunctive cancer chemotherapy (13), it is possible that this NO originates in cells other than macrophages. Many investigators have found that human macrophages from normal subjects produce little or no NO in response to in vitro stimuli that elicit copious quantities of NO from macrophages of murine origin. It remains difficult to detect NO production from human macrophages in vitro, although some investigators have demonstrated low levels of L-arginine-dependent NO production in response to such stimuli as CD23 or CD69 ligation, IL-4 administration, HIV infection, or cocultivation with tumor cells (14–16), and numerous independent studies have demonstrated induction of NOS2 mRNA or protein in human macrophages after proinflammatory stimuli. NO production by human neutrophils has also been observed (17, 18).

The most compelling demonstrations of inducible NO production by human macrophages have been obtained from patients with inflammatory conditions. Elevations in NOS2 protein expression by circulating mononuclear cells have been correlated with increased systemic NO production in Tanzanian children with malaria (3). Similarly, striking increases in NOS2 protein have been found in macrophages from patients with active pulmonary tuberculosis (7), acute respiratory distress syndrome, glomerulonephritis, and rheumatoid arthritis (19), and other evidence of macrophage-derived NO has been obtained from patients with alcoholic hepatitis or chronic granulomatous disease (20). One report even suggests that NO production by macrophages obtained from patients with active

tuberculosis may approach levels of production by murine cells (21), although this observation awaits confirmation. Taken together, the weight of evidence suggests that high-output NO synthesis is indeed part of the antimicrobial armamentarium of human macrophages, but the signals required for stimulation of this system remain incompletely understood.

#### Interactions between NO and reactive oxygen intermediates

Although inducible NO synthase and the phagocyte NADPH oxidase are differentially regulated, these systems may be co-stimulated by inflammatory stimuli (e.g., IFN- $\gamma$ ). Simultaneous production of reactive nitrogen and oxygen intermediates may lead to the formation of a variety of antimicrobial molecular species (Fig. 1), each with distinct stability, compartmentalization, and reactivity. In addition to NO radical (NO $\cdot$ ) itself, potentially important NO congeners include peroxyntirite (OONO $^-$ ), S-nitrosothiols (RSNO), nitrogen dioxide (NO $_2$  $\cdot$ ), dinitrogen trioxide (N $_2$ O $_3$ ), dinitrogen tetroxide (N $_2$ O $_4$ ), and dinitrosyl-iron complexes (DNIC). Peroxyntirite may be formed from the rapid interaction of NO $\cdot$  and superoxide (O $_2^{\cdot-}$ ), from the combination of hydrogen peroxide (H $_2$ O $_2$ ) and nitrous acid (HNO $_2$ ), which would exist in equilibrium with nitrite (NO $_2^-$ ) within an acidified phagolysosomal vacuole, or from the interaction of nitroxyl (NO $^-$ ) and oxygen. S-nitrosothiols such as S-nitrosoglutathione can be formed from NO $\cdot$  and reduced thiols in the presence of an electron acceptor (22). The potent oxidant NO $_2$  $\cdot$  can be formed by the autooxidation of NO $\cdot$ , or possibly by the oxidation of NO $_2^-$  by myeloperoxidase and H $_2$ O $_2$  (23). Additional potent nitrosating

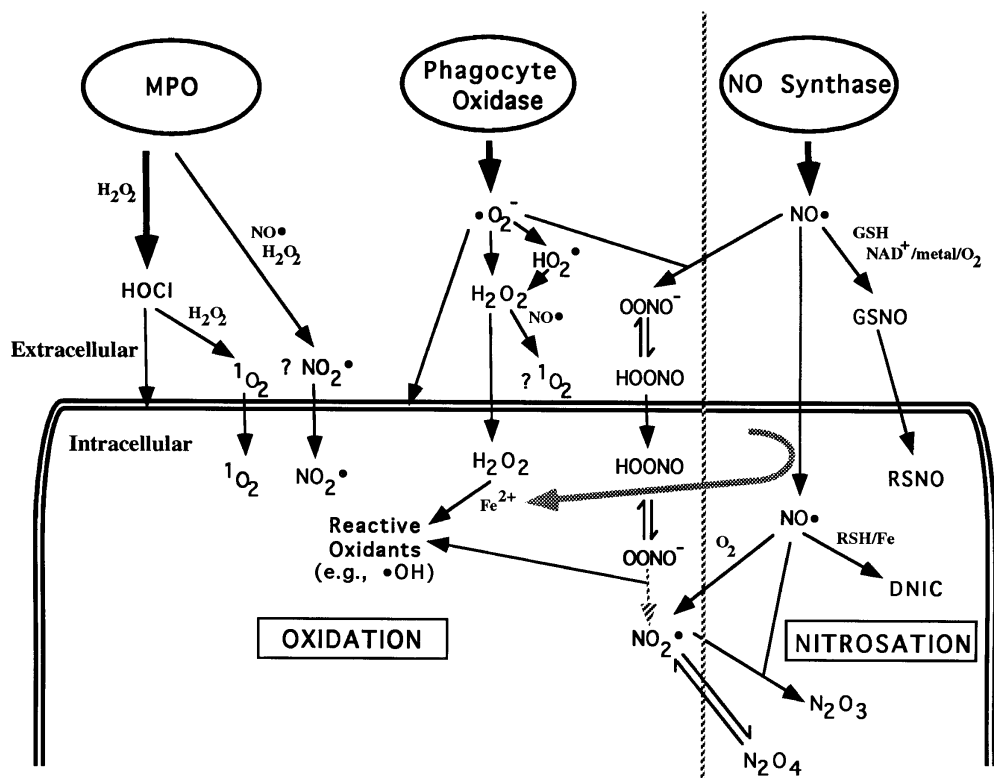


Figure 1. Potential interactions between phagocyte-derived reactive oxygen and nitrogen intermediates. Some possible reactions of products originating from NO synthase, phagocyte oxidase, and myeloperoxidase are shown in relation to a hypothetical microbe situated within a phagolysosome. "Extracellular" refers to the phagolysosomal compartment, and "intracellular" refers to the microbial cytosol. Chemical species are separated according to their predominant tendency toward oxidative or nitrosative reactivity. DNIC, dinitrosyl iron complexes; Fe, iron; GSH, glutathione; GSNO, S-nitrosoglutathione; H $_2$ O $_2$ , hydrogen peroxide; HOCl, hypochlorous acid; HOONO, peroxyntirous acid; MPO, myeloperoxidase; NAD, nicotinamide adenine dinucleotide; NO $\cdot$ , nitric oxide; NO $_2$  $\cdot$ , nitrogen dioxide; N $_2$ O $_3$ , dinitrogen trioxide; N $_2$ O $_4$ , dinitrogen tetroxide; O $_2$ , molecular oxygen;  $^1$ O $_2$ , singlet oxygen;  $\cdot$ OH, hydroxyl; OONO $^-$ , peroxyntirite; RSNO, S-nitrosothiol.

agents can arise from the autooxidation of  $\text{NO}^*$  ( $\text{N}_2\text{O}_3$ ,  $\text{N}_2\text{O}_4$ ), or from the interaction of  $\text{NO}^*$  with thiols and nonheme iron (DNICs).

Interactions between reactive oxygen and nitrogen intermediates provide a molecular basis for synergy between the respiratory burst and synthesis of NO. Reaction products such as peroxyxynitrite can have greater cytotoxic potential than  $\text{NO}^*$  or  $\text{O}_2^{\cdot-}$  alone. Recent observations indicate that peroxyxynitrite may be responsible for the candidacidal activity of murine macrophages (24). The combination of  $\text{H}_2\text{O}_2$  and  $\text{NO}^*$  appears to possess particularly potent antibacterial activity (25), possibly resulting from increased formation of the potent oxidant hydroxyl radical ( $\cdot\text{OH}$ ) in the presence of iron species (25) or from generation of singlet oxygen ( $^1\text{O}_2$ ) (26).  $\text{NO}^*$  has been shown to be capable of reducing  $\text{Fe}^{\text{III}}$  complexes (27), providing a mechanism for enhancement of the  $\text{Fe}^{\text{II}}$ -catalyzed Haber-Weiss reaction.  $\text{NO}^*$  can also inhibit antioxidant metalloenzymes such as catalase (28), thereby limiting  $\text{H}_2\text{O}_2$  disproportionation.

It should be noted that the combination of reactive oxygen and nitrogen intermediates may be antagonistic in some circumstances. Accordingly, it is interesting to observe that NO actually protects mammalian cells against oxidant injury, perhaps by forming iron-nitrosyl complexes (making iron less available for catalysis of prooxidant reactions), inhibiting the respiratory burst oxidase (29), directly scavenging radical species such as  $\cdot\text{OH}$  (30), or inducing the expression of protective stress regulons (31, 32).  $\text{NO}^*$  may also antagonize oxidant membrane injury by terminating lipid peroxidation reactions (27). These observations suggest that NO production may simultaneously enhance the antimicrobial function of the respiratory burst while protecting tissues from oxidant injury (33). It should also be noted that interactions with  $\text{O}_2^{\cdot-}$  reduce concentrations of  $\text{NO}^*$ , which may actually diminish antimicrobial activity toward microbes more sensitive to  $\text{NO}^*$  (or another NO congener) than to peroxyxynitrite; this is in fact suggested by studies in *Cryptococcus neoformans*, *L. major* (34), and *Giardia lamblia* (35).

#### Entry of NO into microbial cells

As a relatively nonpolar uncharged molecule with a small Stokes radius,  $\text{NO}^*$  would be predicted to cross membranes readily. Direct studies indicate that the diffusion of  $\text{NO}^*$  resembles that of oxygen, with the exception that oxygen is more lipophilic (36). Although superoxide ( $\text{O}_2^{\cdot-}$ ) does not appear to enter bacterial cells to a significant extent (37), its congener peroxyxynitrite can pass through membranes, probably as peroxyxynitrous acid ( $\text{HOONO}$ ); greater reactivity for lipids and proteins may nonetheless limit its effective diffusion into microbial target cells relative to  $\text{NO}^*$ . Peroxyxynitrite may also be formed in situ within target cells if both  $\text{NO}^*$  and  $\text{O}_2^{\cdot-}$  are present. Studies in the gram-negative bacterium *Salmonella typhimurium* indicate that *S*-nitrosoglutathione (GSNO) can be actively taken up and processed by microbial systems which ordinarily function to import glutathione and other short peptides (38). GSNO appears to be recognized as a substrate by the periplasmic enzyme  $\gamma$ -glutamyltranspeptidase, with subsequent conversion to *S*-nitrosocysteinyl-glycine. This nitrosated dipeptide in turn is imported into the bacterial cytoplasm across the inner membrane by a specialized dipeptide permease (Dpp). The presence of the dipeptide permease, a

member of the ABC (ATP-Binding Cassette) transporter family, is absolutely required for *S*-nitrosoglutathione-mediated inhibition of *S. typhimurium* growth in vitro (39). Transport mechanisms for other *S*-nitrosothiols have not been characterized.

#### Microbial cellular targets of NO

Although significant progress has been made in identifying interactions between specific cellular constituents and NO-related species, the critical targets responsible for microbial stasis or death remain for the most part uncertain. Multiple NO congeners and multiple cellular targets are almost certainly involved in NO-related antimicrobial activity. Moreover, it is also extremely likely that significant mechanistic differences will be established for various target microbial pathogens. For example,  $\text{NO}^*$  itself does not possess antimicrobial activity for *S. typhimurium* or *Escherichia coli* (25, 39), but *S*-nitrosothiols are bacteriostatic and peroxyxynitrite is bactericidal for these organisms. In contrast, *S*-nitrosothiols and  $\text{NO}^*$  are microbicidal for *Staphylococcus aureus* (40), *L. major* (34), and *G. lamblia* (35), under conditions in which peroxyxynitrite does not exert an apparent antimicrobial effect. Reactive nitrogen intermediates have been shown to modify DNA, proteins, and lipids (Fig. 2 A), as well as exert indirect effects on microbes by modulating immune responses or other host cell functions. The direct actions will be considered further in some detail.

Several lines of evidence indicate that DNA is an important target of reactive nitrogen intermediates. First, NO can deaminate DNA in vitro (41), evidently acting via an *N*-nitrosating intermediate (perhaps  $\text{N}_2\text{O}_3$ ) since deamination requires the presence of air.  $\text{NO}_2^*$  and peroxyxynitrite can also oxidatively damage DNA, resulting in abasic sites, strand breaks, and a variety of other DNA alterations (42). In vitro studies suggest that some of the effects of reactive nitrogen intermediates on DNA may involve interactions with DNA repair systems, as well as direct modification of deoxyribonucleotides. Second, NO-related DNA damage has been shown in intact bacteria. Exposure of DNA repair-deficient *S. typhimurium* TA1535 (*rfa uvrB hisG*) to NO donor compounds reveals mutagenicity consistent with a DNA deaminating mechanism (43). *S. typhimurium* deficient in recombinational DNA repair has been found to be hypersusceptible to inhibition or killing by *S*-nitrosothiols and 3-morpholinopyridone (a peroxyxynitrite generator) (39), as well as attenuated for virulence in mice. Lastly, preliminary observations in transgenic mice suggest that deficiencies in production of reactive oxygen or nitrogen intermediates can enhance the virulence of DNA repair-deficient mutant bacteria (Fang, F.C., unpublished observation), suggesting that dynamic interactions between NO-related species, DNA, and DNA repair systems are important during host-pathogen interactions in vivo.

NO interactions with proteins can involve reactive thiols, heme groups, iron-sulfur clusters, phenolic or aromatic amino acid residues, tyrosyl radicals, or amines. Peroxyxynitrite and  $\text{NO}_2^*$  can also nonspecifically oxidize proteins at a variety of sites (45). Initial studies of NO-related cytotoxicity demonstrated efflux of iron-nitrosyl complexes and inactivation of enzymes containing Fe-S clusters (e.g., aconitase, NADH dehydrogenase, succinate dehydrogenase), suggesting that  $\text{NO}^*$  might directly release iron from metalloenzymes and promote

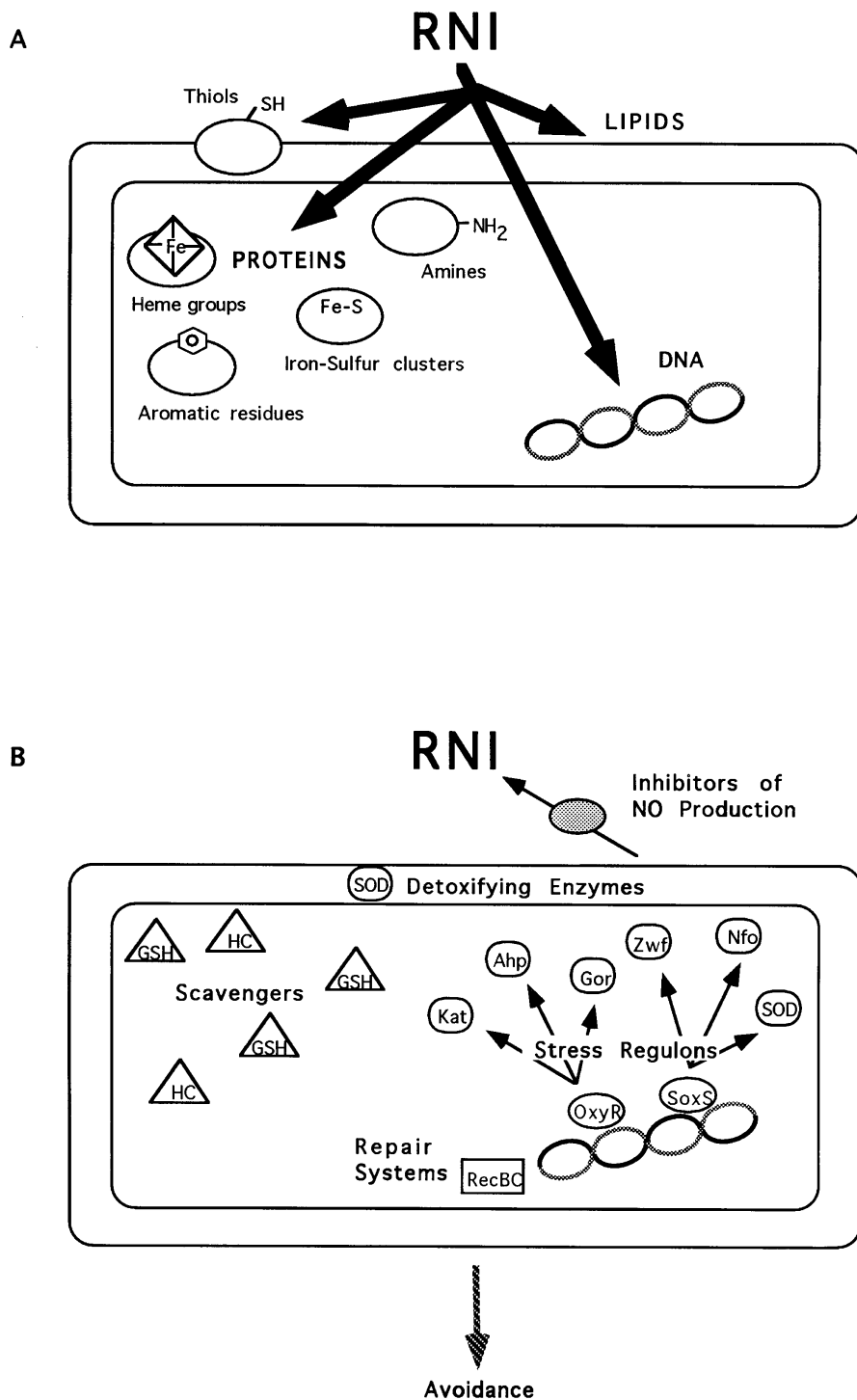


Figure 2. (A) Microbial cellular targets of reactive nitrogen intermediates. See text for details. (B) Microbial defenses against reactive nitrogen intermediates. *SOD*, superoxide dismutase; *GSH*, glutathione; *HC*, homocysteine. Other abbreviations are described in text.

iron depletion (46). However, more recent observations indicate that aconitase inactivation may be mediated by reactive species other than  $\text{NO}^*$  itself (47), and  $\text{NO}$ -related inactivation of Fe-S clusters may be an indirect result of reduced iron or sulfide availability (48). Nevertheless,  $\text{NO}$ -related inhibition of metabolic enzymes may constitute an important mechanism of  $\text{NO}$ -related cytostasis. Actions of reactive nitrogen intermediates on metabolic enzymes or membrane transporters may lead to dissipation of transmembrane electrochemical proton-

motive force, as has been demonstrated in mammalian cells. Both  $\text{NO}$  donors and metabolic inhibitors have been reported to exert similar cytotoxic effects on *Schistosoma mansoni*, suggesting common pathways of action (49).

$\text{NO}^*$  can interact with iron contained in heme proteins such as guanylyl cyclase, which accounts for many of its roles in physiological signal transduction (50). Although guanylyl cyclase is activated by  $\text{NO}$ ,  $\text{NO}$ -heme interactions can result in the inactivation of other heme proteins, such as catalase and

cytochrome systems (28). Ribonucleotide reductase, a non-heme metalloenzyme essential for DNA synthesis, has been implicated as a major target of NO in tumor cells (51). Nitrosylation of reduced thiol groups and quenching of an active site tyrosyl radical by a direct NO<sup>•</sup>-radical interaction, rather than a direct NO<sup>•</sup>-iron interaction, are believed to be involved. Ribonucleotide reductase inhibition has been implicated in the ability of NO to inhibit vaccinia virus, because supplementation with deoxyribonucleosides can partially relieve the antiviral effect (52). The ability of supplemental iron or tricarboxylic acid cycle intermediates to also partially restore viral replication (53) demonstrates the complexity of NO-related antimicrobial mechanisms. Prokaryotic cells also contain ribonucleotide reductase, but its importance as a target of NO in these organisms is unknown.

Thiols are among the most important protein targets of NO, and *S*-nitrosylation (attachment or transfer of NO<sup>+</sup> to sulfhydryl groups) is favored over *N*-nitrosylation under physiologic conditions. NO congeners including *S*-nitrosothiols, N<sub>2</sub>O<sub>3</sub>, and dinitrosyl-thiol-iron complexes are particularly potent nitrosating species in this context. Nitrosylation of thiols may modify protein function per se, facilitate subsequent modification such as ADP-ribosylation, or accelerate disulfide formation between vicinal thiols, as proposed by Stamler (54). Modification of surface thiols is believed to be responsible for *S*-nitrosothiol-mediated inhibition of *Bacillus cereus* spore outgrowth (55), but intracellular protein targets have been implicated in *S. typhimurium* (39). Numerous enzymes (e.g., glyceraldehyde-3-phosphate dehydrogenase,  $\gamma$ -glutamylcysteinyl synthetase) have been found to be inactivated by *S*-nitrosylation in vitro, but critical thiol target(s) involved in NO-related antimicrobial effects remain a matter of conjecture.

Nitration of tyrosine residues has received particular attention because this protein modification can be produced by peroxynitrite (56), although myeloperoxidase may also catalyze tyrosine nitration in the presence of NO<sub>2</sub><sup>-</sup> and H<sub>2</sub>O<sub>2</sub> (23). Tyrosine nitration may disrupt pathways involving tyrosine phosphorylation and modify protein function or turnover. NO-dependent tyrosine nitration of the surface of *S. aureus* was demonstrated recently after ingestion by human neutrophils (17), but NO did not appear to be necessary for antistaphylococcal activity in these experiments. More recently, the attenuated virulence of *S. typhimurium* lacking periplasmic superoxide dismutase has been found to be restored by either NO synthase inhibition or absence of the O<sub>2</sub><sup>-</sup>-generating phagocyte oxidase (57). These observations suggest that peroxynitrite production from NO<sup>•</sup> and O<sub>2</sub><sup>-</sup>, antagonized by the presence of bacterial superoxide dismutase, might constitute a significant component of the antimicrobial host defense in salmonellosis.

NO can be associated with membrane damage, and this action has principally been demonstrated with peroxynitrite. Peroxynitrite has been found to mediate lipid peroxidation of liposomal preparations, via a mechanism which does not require iron (58). NO<sub>2</sub><sup>•</sup> can also induce lipid peroxidation (59). The relationship between these actions and NO-related antimicrobial activity is unknown.

NO has been shown to exert immunomodulatory effects including effects on immune cell adherence and function, cellular proliferation, and cytokine production (60). While immunomodulatory roles of NO undoubtedly have important implications for microbial pathogens and NO-related anti-

microbial activity, their consideration is beyond the scope of this brief review.

#### *Microbial defenses against NO*

Mechanisms of microbial resistance to reactive nitrogen intermediates have been incompletely characterized, but appear to overlap considerably with antioxidant defenses (Fig. 2B). Low molecular weight thiols have an important scavenging role in microbes, as they do in mammalian cells. Mutant *Salmonella* strains unable to synthesize glutathione (GSH) are hypersusceptible to inhibition by *S*-nitrosothiols, peroxynitrite, and even NO<sup>•</sup> itself, suggesting that the high intracellular thiol concentrations of enteric bacteria may explain the lack of antimicrobial activity normally exerted by NO<sup>•</sup> against these pathogens. In contrast, *Staphylococci* contain low concentrations of GSH (61), and appear to be susceptible to NO<sup>•</sup> (40). Although mycobacteria and other actinomycetales do not synthesize GSH, they contain an alternative low molecular weight compound called mycothiol (61); trypanosomes and *Leishmania* sp. produce an analogous thiol called trypanothione (62). It remains to be determined whether these compounds can mediate analogous resistance to nitrosative and oxidative stress. The molecular interactions of *S*-nitrosoglutathione and glutathione have been characterized in detail recently (63), and similar transformations might occur within microbial cells. A complex set of reactions, dependent upon both relative glutathione concentration and the presence of oxygen, produces a mixture of products including oxidized thiol, ammonia, and nitrite (63).

Homocysteine is another low molecular weight thiol compound which has been implicated in resistance to *S*-nitrosothiols. *S. typhimurium* mutants with reduced synthesis of homocysteine are hypersusceptible to inhibition by *S*-nitrosoglutathione, and this inhibition can be reversed by the addition of exogenous homocysteine (64). Such mutants are also hypersusceptible to macrophages in vitro and attenuated for virulence in mice, but pathogenicity can be restored by NO synthase inhibition in vivo, suggesting that *S*-nitrosothiol-thiol interactions are biologically relevant in infection. It is particularly interesting to consider that antagonistic *S*-nitrosothiol-homocysteine interactions have been implicated in other pathophysiologic contexts, such as vascular disease. Inhibition of glutathione peroxidase by homocysteine with subsequent oxidative inactivation of NO equivalents has been proposed as a mechanistic explanation in mammalian cells (65). However, this may not be the case in microbes, since homocysteine antagonizes *S*-nitrosothiol-mediated *Salmonella* microbiostasis equally well under anaerobic conditions.

Microbial systems which repair oxidative injury appear to be similarly involved in repairing nitrosative injury. For example, the RecBCD exonuclease required for most homologous recombinational DNA repair processes helps to confer resistance to both hydrogen peroxide (66) and NO donor compounds (39). Glucose-6-phosphate dehydrogenase, which provides a major source of reducing equivalents (NADPH) used to regenerate thiols and other antioxidants, is similarly involved in defenses against both reactive oxygen and nitrogen intermediates (67). Cu,Zn-superoxide dismutase (SodC) may protect against both oxidative and nitrosative stress by removing periplasmic superoxide and limiting peroxynitrite formation (57).

Studies in *E. coli* have implicated specific antioxidant regulators in resistance to NO-related antimicrobial activity. The

*soxRS* regulon, originally identified as a genetic system responsive to elevated intracellular superoxide concentrations (68), can also be induced by NO (37). This regulon includes the *zwf* (glucose-6-phosphate dehydrogenase), *sodA* (manganese superoxide dismutase), and *nfo* (endonuclease IV) genes, loci which might contribute to antinitrosative defenses by generating reducing equivalents, reducing intracellular peroxynitrite formation, and repairing DNA damage, respectively. Expression of SoxRS-regulated genes is induced by dissolved NO<sup>•</sup> gas, and *E. coli* carrying a deletion of the *soxRS* locus is hypersusceptible to NO-dependent killing by murine macrophages (67). The *oxyR* regulon, originally described as a hydrogen peroxide-inducible genetic system (69), has been shown more recently to confer resistance to *S*-nitrosothiols in *E. coli* (70). Induction of OxyR-regulated genes (e.g. *katG* encoding catalase, *ahp* genes encoding alkyl hydroperoxide reductase, and *gorA* encoding glutathione reductase) by *S*-nitrosothiols is dramatically augmented in glutathione-deficient cells. One must be somewhat cautious in extrapolating from these interesting observations in laboratory strains of *E. coli* to pathogenic microbes which are more adapted to survival in phagocytic cells. *S. typhimurium* carrying mutations in *soxS* or *oxyR* retains virulence in mice (Fang, F.C., unpublished observation), suggesting that other important regulons conferring NO resistance are yet to be identified.

As described earlier, *Salmonella* can develop resistance to *S*-nitrosothiols by acquiring mutations which inactivate specific peptide transport systems (39). However, since these systems are required for chemotaxis and recycling of cell-wall components, such mutations are likely to confer a competitive disadvantage to bacteria under many conditions. It is also difficult to establish whether such systems are involved in resistance in vivo because the specific *S*-nitrosothiol(s) responsible for antimicrobial activity are unknown. Pyocyanin, a phenazine pigment produced by *Pseudomonas aeruginosa*, has been reported to inhibit NO in vitro (71), but the pathophysiological significance of this action is uncertain. Other mechanisms of NO resistance almost certainly remain to be discovered.

Finally, pathogenic microorganisms might resist NO-related antimicrobial activity by avoiding phagocytosis and avoiding or suppressing stimulation of NO synthase. Lipopolysaccharide phase-variants of *Francisella tularensis* have been described which fail to induce NO production by murine macrophages, thereby enhancing survival of the bacteria (72). Hemozoin, a heme-containing pigment produced by *Plasmodium* spp., has been reported to inhibit NO production (73). Pathogenic *Yersinia* spp. produce a tyrosine phosphatase (YopH, Yop-51) which subverts a variety of phagocytic functions, but specific effects on NO synthase have yet to be demonstrated.

### Summary

Accumulating evidence from multiple lines of inquiry suggests that reactive species derived from NO synthase possess biologically important antimicrobial activity: (a) increased NO production is associated with host defense both in experimental animal models and in human infections; (b) inhibition or genetic inactivation of inducible NO synthase enhances microbial replication in infected macrophages and experimental animals; (c) chemical NO donors possess antimicrobial activity in vitro; and (d) microbial resistance to NO donors can be correlated with virulence in experimental animals. NO appears to

be of particular importance in host defense against intracellular pathogens, and perhaps in the maintenance of microbial latency. NO may act in concert with reactive oxygen species to damage microbial DNA, proteins, and lipids. Microbial defenses against oxidative and nitrosative stresses share many elements in common, including specific stress regulons, scavengers, detoxifying enzymes, repair systems, and strategies to subvert or avoid host phagocytes. Further investigation of the complex interrelationships between NO-related species and microbes promises to provide important new insights into host-pathogen interactions.

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