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## Reactive Nitrogen Species in Host-Bacterial Interactions

Ferric C. Fang<sup>1,2</sup>, Andrés Vázquez-Torres<sup>3,4</sup>

<sup>1</sup>Department of Microbiology, University of Washington School of Medicine, Seattle, WA, 98195-7735 USA

<sup>2</sup>Department of Laboratory Medicine, University of Washington School of Medicine, Seattle, WA, 98195-7735 USA

<sup>3</sup>Department of Immunology and Microbiology, University of Colorado School of Medicine, Aurora, CO, 80045 USA

<sup>4</sup>Veterans Affairs Eastern Colorado Health Care System, Denver, CO 80220 USA

### Abstract

Reactive nitrogen species play diverse and essential roles in host-pathogen interactions. Here we review selected recent discoveries regarding nitric oxide (NO) in host defense and the pathogenesis of infection, mechanisms of bacterial NO resistance, production of NO by human macrophages, NO-based antimicrobial therapeutics and NO interactions with the gut microbiota.

### Introduction

Since the discovery that mammalian cells can produce large quantities of nitric oxide (NO) in response to inflammatory stimuli, the role of reactive nitrogen species in bacterial infections has been a focus of intensive investigation.

### NO in host defense

NO generated enzymatically by host NOS2 or abiotically in the gastric lumen by acidification of salivary nitrite exerts antimicrobial activity against diverse pathogens (figure 1), including *Clostridioides (Clostridium) difficile*, *Mycobacterium tuberculosis* and *Salmonella enterica* [\*1–3]. NO and congeners arising from its reaction with O<sub>2</sub><sup>-</sup>, O<sub>2</sub>, iron and low-molecular weight thiols have high affinity for Fe<sup>3+</sup>, Fe<sup>2+</sup> and Cu<sup>++</sup> in terminal cytochromes of the electron transport chain, [4Fe-4S] cluster-containing dehydratases, redox-active protein cysteine residues, and tyrosyl and glycyl radicals in ribonucleotide reductase [4]. Metabolism is a particularly salient target of this diatomic radical. Dihydroxyacid dehydratase, lipoamide dehydrogenase, methionine synthase, aconitase, and

Address correspondence to: Prof. Ferric C. Fang, Department of Microbiology, University of Washington School of Medicine, 1959 NE Pacific Street, Box 357735, Seattle, WA 98195-7735 USA, Phone: 1-206-221-6770, Fax: 1-206-616-1575, fcfang@uw.edu.

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fructose biphosphate aldolase are prominent targets of RNS [4–6]. Strict aerobes such as *M. tuberculosis*, *Burkholderia* spp, and *Pseudomonas aeruginosa*, which overwhelmingly rely on the electron transport chain to satisfy their energy needs, are particularly vulnerable to nitrosylation of terminal respiratory cytochromes [5, 7]. Conversely, *Staphylococcus aureus* and *S. enterica*, which can generate ATP via both oxidative and substrate-level phosphorylation, are relatively resistant to the antimicrobial actions of NO engendered by the innate immune response [8\*, 9]. Detrimental effects of NO on bacterial metabolism, DNA replication and repair, and protein quality control underlie the robust and broad-spectrum antimicrobial actions of RNS.

NO also targets regulatory proteins that coordinate bacterial virulence gene expression. For example, *S*-nitrosylation of AgrA Cys<sup>199</sup> interferes with the quorum sensing-dependent *S. aureus* virulence program [10\*] (figure 1). Similarly, *S*-nitrosylation or oxidation of SsrB Cys<sup>208</sup> prevents transcription of genes encoding the *Salmonella* pathogenicity island-2 (SPI-2) type III secretion system, which is of key importance for the pathogenesis of nontyphoidal *Salmonella* infections [11].

In addition to exerting direct antimicrobial activity, NO participates in host defense by modulating immunity [12]. It has recently been suggested that NO-mediated resistance to tuberculosis results in part from the negative effect of NO on neutrophil recruitment [13\*\*] (figure 1). Paradoxically, NOS2 may promote host defense against *S. aureus* by stimulating the accumulation of granulocytes in lungs [10\*]. It is presently unclear why NO exerts opposing effects on the granulocytic responses to *M. tuberculosis* and *S. aureus*.

## Bacterial NO resistance and evasion

Given the profound antimicrobial activity of NO, it is not surprising that pathogenic bacteria possess a variety of anti-nitrosative defenses [4, 5]. The intracellular pathogens *S. enterica* and *M. tuberculosis* downregulate NOS2 expression via RNA interference or PPE2-binding to the TATA box of the *Nos2* gene [14, 15], and *Francisella tularensis* inhibits NOS2 expression by suppressing IP-10 production and IFN $\gamma$ -induced STAT-1 signaling [16]. *Salmonella* also ameliorates RNS exposure by interfering with trafficking of NOS2-containing vacuoles in a SPI2-dependent manner [5]. NO crosses membranes and enters bacterial cells where low molecular-weight thiols such as glutathione and mycothiol, as well as cytochrome *bd*, provide a first line of defense against nitrosative stress [4, 17]. Expression of the *hmp*-encoded flavohemoglobin and cytochrome *bd* allows the adaptive detoxification of NO in *E. coli*, *M. tuberculosis*, *Salmonella*, *S. aureus* and *Yersinia pseudotuberculosis* [17–21]. Mechanisms of *hmp* regulation vary among pathogenic bacteria. The *hmp* gene is de-repressed in Gram-negative bacteria such as *Salmonella* and *E. coli* upon nitrosylation of the NsrR [2Fe-2S] cluster, whereas *hmp* transcription in *S. aureus* is under positive regulation by the SrrAB two-component regulatory system that monitors the redox status of the respiratory chain [18, 22]. Hmp is selectively expressed by bacteria in microenvironments where NO is present. Detoxification of NO by Hmp in marginal zones protects *Yersinia* within microcolonies, thereby orchestrating a spatially-dependent functional specialization of bacterial cells within infected foci [21].

Pathogenic bacteria reprogram their metabolism under nitrosative stress. A reduction in branched-chain amino acids that follows the nitrosylation of dihydroxyacid dehydratase is sensed by ribosomally-bound RelA monitoring the aminoacylation state of incoming tRNAs [23\*]. Guanosine tetraphosphate synthesized by RelA activates transcription of branched-chain amino acid biosynthetic genes, not only reestablishing amino acid homeostasis but also allowing the translation of NO-consuming Hmp [23\*]. Although terminal cytochromes of the electron transport chain are some of the most exquisitely-sensitive targets of NO [24], the electron transport chain is still important for the anti-nitrosative defenses of pathogenic bacteria. By working in reverse, the respiratory  $F_1F_0$  ATPase maintains an alkaline cytoplasm, thereby promoting skin and soft tissue *S. aureus* infections under conditions such as nitrosative stress and hypoxia that limit respiration [19\*]. The  $F_1F_0$  ATPase may serve a similar function in *Salmonella* pathogenesis [8\*]. In addition, the acquisition of manganese adds to the anti-nitrosative defenses of *S. aureus* and *S. enterica* [8\*, 19\*]. Analogous to its antioxidant role [25], manganese may substitute for iron, which is prone to NO toxicity.

*S*-nitrosylation of cysteine residues mobilizes  $Zn^{2+}$  from zinc metalloproteins involved in DNA replication and repair, protein synthesis, and metabolism [26\*].  $Zn^{2+}$  mobilized by NO can be detrimental to the cell. Thus, the ZntB and ZitB zinc efflux systems protect *Salmonella* from nitrosative stress [26\*]. Nevertheless, *Salmonella* must reacquire zinc to resume growth, and the high-affinity ZnuABC zinc uptake system promotes *Salmonella* pathogenesis during the nitrosative stress engendered by the innate response of macrophages and mice [8\*]. Given the widespread utilization of zinc metalloproteins, it is somewhat surprising that zinc-starved *Salmonella* tolerates nitrosative stress rather well as long as glycolytic fructose bisphosphate aldolase is functional. The metabolism of glucose satisfies cellular energy requirements of *S. aureus* and *S. enterica* by allowing the synthesis of ATP by substrate level phosphorylation in the payoff phase of glycolysis and acetate fermentation, while maintaining redox balance by the NADH-consuming fermentation of pyruvate to lactate [8\*, 9].

## NO in infection pathogenesis

Much effort has been focused on elucidating the multiple ways in which NO contributes to host defense. However, it is becoming increasingly clear that NO produced endogenously by bacteria or exogenously by host phagocytes may also promote bacterial pathogenesis in certain settings. NO produced endogenously by bacterial NOS plays a critical role in the pathogenesis of *S. aureus* and *Bacillus anthracis* infections [27\*\*, 28]. Endogenously-synthesized NO by bacterial NOS inhibits aerobic respiration while promoting the utilization of the oxidative branch of the tricarboxylic acid cycle [27\*\*, 29]. NADH generated by the tricarboxylic acid cycle powers reduction of the alternative electron acceptor nitrate, thus maintaining the membrane potential during microaerobiosis, which is essential for nasal colonization by *S. aureus* [27, 29]. As bacterial NOS is structurally similar to the oxygenase domain of mammalian NOS [30], the regulation of electron transport may represent the primordial role of NOS in biology. As with exogenous NO [31–33], NO produced endogenously prevents oxidative stress while tolerizing Gram-positive pathogens to antibiotics [29, 34, 35].

NO generated by the host can also paradoxically worsen bacterial infection. *E. coli* takes advantage of the energetic properties of nitrate derived from NOS2-expressing inflammatory cells to outcompete members of the resident gut microbiota [36\*\*] (figure 1), whereas migration of *Salmonella* toward nitrate generated constitutively by host cells in the lamina propria promotes invasion of Peyer's patches [37]. Alternatively, NO produced by the innate immune response can directly mediate immunopathology. For example, NO synthesized by infiltrating macrophages in response to *Mycobacterium leprae* phenolic glycolipids damages mitochondria of nerve cells, triggering demyelination that is pathognomonic of leprosy [38\*\*] (figure 1).

## NO production by human macrophages

After lipopolysaccharide and IFN $\gamma$  were shown to stimulate high-output NO production by murine macrophages [39, 40], it soon became apparent the human peripheral blood mononuclear cell (PBMC)-derived macrophages do not respond similarly. Although some studies have found varying levels of NOS2 mRNA, NOS2 protein, NO production or NO-dependent actions in human macrophages in vitro [41], marked quantitative differences compared to mice have suggested that human macrophages may not produce NO as an antimicrobial mediator [42].

However, analysis of NOS expression in macrophages from humans with active infections suggests that this is not the case [43]. For example, NOS2 protein is visualized within macrophages and epithelioid cells in most patients with leprosy [44, 45] where, as mentioned above, it may also play an important role in nerve damage [38\*\*]. NOS2 protein and mRNA are also observed within submucosal bladder macrophages, in association with increased NO formation, in patients undergoing BCG immunotherapy of bladder cancer [46]. NOS2 mRNA is found in PBMCs in children with moderately severe falciparum malaria but not in those with severe disease [47], suggesting that alternative macrophage polarization with loss of NOS expression may contribute to insufficient NO production in severe cases, resulting in poorer clinical outcomes [48].

Granulomas from patients with active tuberculosis exhibit a complex spatial organization of NO-producing cells, which is also seen in experimentally infected macaques. NOS2 is found within macrophages, epithelioid macrophages and neutrophils centrally situated within TB granulomas. Epithelioid macrophages, some containing bacteria, express high levels of NOS2 and low levels of arginase (Arg1), suggesting that NO is an important component of the antimicrobial response [49\*\*]. In contrast, higher arginase expression is evident in the surrounding lymphocyte cuff region, consistent with an immunoregulatory function of these cells. Even quiescent fibrocalcific granulomas contain NOS-positive cells, suggesting that NO may help to maintain microbial latency, as suggested in experimental models [50, 51].

Expression of NOS2 in human macrophages is not limited to infectious conditions. Macrophages containing NOS2 are seen in rheumatologic diseases and cancer [52–56]. NO production by tumor-associated macrophages can enhance or restrict tumor progression, depending upon the NO concentration and redox environment [57]. Whereas NO production

by tumor cells is associated with cancer progression and metastasis, NOS2 production by macrophages may be required for effective responses to chemotherapy [58, 59].

The recent discovery that the NOS2 gene promoter is highly methylated around the transcription start site in human macrophages [60\*] suggests that epigenetic regulatory mechanisms are of particular importance. This may account for the difficulty in eliciting high output NO production in human macrophages from healthy subjects. A better understanding of the mechanisms by which NOS2 promoter silencing is relieved during infection will be an important focus of future research.

## Therapeutic applications of NO in infections

High NO concentrations are inhibitory for a broad range of bacteria, viruses, fungi and parasites. Efforts to develop NO-based antimicrobial therapies have primarily focused on topical application or local delivery, to avoid unwanted physiological effects of systemic administration. Results are promising but still preliminary. A variety of NO-releasing scaffolds are in development [61]. Small clinical studies as well as animal and in vitro models have demonstrated efficacy of *S*-nitrosothiols, acidified nitrite or NO-releasing drugs in such diverse infections as cutaneous leishmaniasis, bacterial pneumonia and tinea pedis [62–65]. Acidified nitrite showed some efficacy in the treatment of viral warts but also caused local irritation [66].

The ability of NO to disperse bacterial biofilms by triggering specific sensor proteins [67] suggests that NO-based therapies may be useful for difficult-to-treat chronic infections involving biofilms, such as wound infections and cystic fibrosis-related lung infections [68] (figure 1). Inhaled NO was well tolerated in a phase I study of patients with cystic fibrosis and chronic resistant pulmonary infections, with a reduction in microbial burden and improved lung function after only 5 days of treatment [69]. Another trial of inhaled NO in cystic fibrosis patients observed a reduction in biofilm after 7 days' treatment [70\*\*]. As biofilm bacteria are more resistant to antibiotics [71], NO might be useful in combination with conventional antimicrobial agents. Synergy has also been shown against drug-resistant enteric bacteria treated with an NO donor, an antimicrobial peptide, and miconazole to inhibit the bacterial NO-detoxifying flavohemoglobin [72], although in other settings NO has been found to promote antibiotic tolerance [73].

Novel NO-charged materials have been developed as a strategy to create implantable catheters that are resistant to infection [74]. This approach could also reduce the incidence of thrombotic complications as a result of NO-mediated anti-platelet actions [75]. Yet another treatment approach is to stimulate the endogenous production of NO by host cells. Infergen, a synthetic interferon, enhances the ability of macrophages to restrict the growth of *M. tuberculosis*, in part via NO production [76]. L-arginine supplementation also augments NO production during infection [77] and might be a useful adjunctive therapy.

## NO and the gut microbiota

The development of new tools to analyze complex microbial communities is yielding exciting new insights into the role of the microbiome in metabolism and immunity.

Reduction of dietary nitrate by oral bacteria and reduction of nitrite by the intestinal microbiota play important roles in the enterosalivary circulation of nitrogen oxides and cardiovascular health [78]. Dysbiosis may contribute to the pathogenesis of hypertension, obesity and atherosclerosis. Dietary nitrate supplementation not only reduces systemic blood pressure but also alters the composition of the microbiome [79].

Complex interactions between enzymatic NO production and the microbiota have also been observed. NOS2 and reactive oxygen species maintain bacterial homeostasis in the gut and may limit overgrowth [80]. However, NOS2-driven carbohydrate oxidation [81\*] and NO<sub>x</sub> derived from NOS2 [36\*\*] provide a competitive advantage to enteric pathogens (figure 1). In turn, organic acids produced by gut bacteria modulate NOS expression to prevent the production of nitrate that can be exploited by pathogenic bacteria as a respiratory substrate [82\*\*]. Fecal microbiota transplantation is being utilized to restore gut homeostasis in an increasing number of conditions and may work in part by effects on NO production [83].

## Conclusions

Recent studies have provide new and interesting insights into the role of NO and other reactive nitrogen species in host defense and the pathogenesis of infection, as well as mechanisms of bacterial NO resistance, the production of NO by human macrophages, NO-based antimicrobial therapeutics and NO interactions with the gut microbiota.

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## Abbreviations

<b>NO</b>	nitric oxide
<b>NOS2</b>	nitric oxide synthase isoform 2, iNOS
<b>NO<sub>x</sub></b>	nitrate and nitrite
<b>RNS</b>	reactive nitrogen species
<b>SPI-2</b>	<i>Salmonella</i> Pathogenicity Island-2

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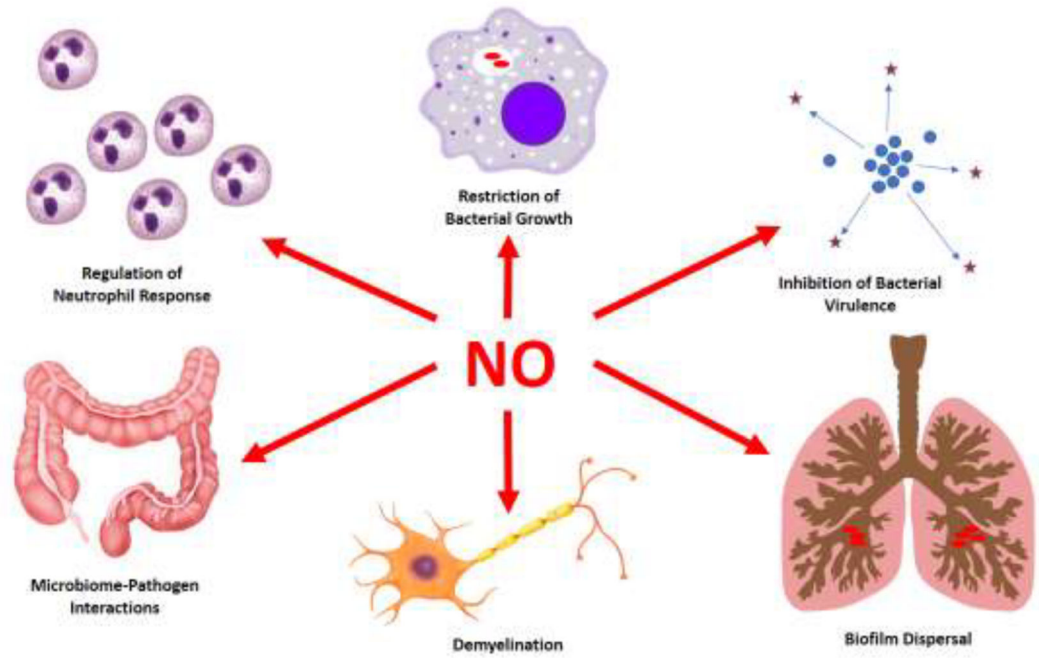
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**Figure 1. Diverse Actions of Nitric Oxide in Host-Bacterial Interactions.**  
Some important actions of NO are shown. Please refer to the text for details.