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Authors

Vázquez-Torres, Andrés
Bäumler, Andreas J

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Nitrate, nitrite and nitric oxide reductases: from the last universal common ancestor to modern bacterial pathogens

Andrés Vázquez-Torres[1,2] and **Andreas Bäumler**[3]

[1]Department of Immunology and Microbiology University of Colorado School of Medicine, Aurora, CO

[2]Veterans Affairs Eastern Colorado Health Care System, Denver, CO

[3]Department of Medical Microbiology and Immunology University of California Davis, School of Medicine, Davis, CA

Abstract

The electrochemical gradient that ensues from the enzymatic activity of cytochromes such as nitrate reductase, nitric oxide reductase, and quinol oxidase contributes to the bioenergetics of the bacterial cell. Reduction of nitrogen oxides by bacterial pathogens can, however, be uncoupled from proton translocation and biosynthesis of ATP or $NH4^+$, but still linked to quinol and NADH oxidation. Ancestral nitric oxide reductases, as well as cytochrome *c*oxidases and quinol *bo* oxidases evolved from the former, are capable of binding and detoxifying nitric oxide to nitrous oxide. The NO-metabolizing activity associated with these cytochromes can be a sizable source of antinitrosative defense in bacteria during their associations with host cells. Nitrosylation of terminal cytochromes arrests respiration, reprograms bacterial metabolism, stimulates antioxidant defenses and alters antibiotic cytotoxicity. Collectively, the bioenergetics and regulation of redox homeostasis that accompanies the utilization of nitrogen oxides and detoxification of nitric oxide by cytochromes of the electron transport chain increases fitness of many Gram-positive and –negative pathogens during their associations with invertebrate and vertebrate hosts.

Introduction

By reacting with metals, organic radicals, cysteine and tyrosineresidues, nitric oxide (NO) and its oxidative congeners elicit a panoply of biological responses. For example, NO modifies the redox active cysteine residue of lipoamide-dependent lipoamide dehydrogenase and the tyrosyl radical of ribonucleotide reductase, thus not only inhibiting amino acid and DNA biosynthesis but also exerting potent antimicrobial activity. If one considers the kinetics of the reaction of NO with molecular targets, it becomes readily apparent that this diatomic radical shows special selectivity for metal groups in hemoproteins. Thus, the heme group in mammalian guanylate cyclase was the first biological target of NO to be discovered

*Correspondence: ajbaumler@ucdavis.edu, and andres.vazquez-torres@ucdenver.edu.

and, with reactivities of up to 10^8 M^{-1} sec^{-1}, heme groups in cytochromes of the electron transport chain remain some of the preferred targets of NO. Inhibition of respiration through nitrosylation of Cu_B and Fe^{2+} of cytochrome *c* and quinol oxidases exerts strong antimicrobial activity. However, exciting recent publications have uncovered the importance that utilization of NO and its oxidative productsnitrate (NO_3^-) and nitrite (NO_2^-) by cytochromes of the electron transport chain has on bacterial pathogenesis and antibiotic resistance. This review discusses some of the most salient and contemporary findings of this vibrant and growing field.

Nitrate and nitrite reductases promote bacterial pathogenesis

A variety of pathogenic microorganisms utilize NO_2^- and NO_2^- during their associations with host cells. In this section, we discuss recent findings in *Enterobacteriaceae* and *Mycobacterium tuberculosis*.

Reduction of nitrate promotes growth of *Enterobacteriaceae* in the gut lumen

The enteric pathogen *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) actively triggers acute intestinal inflammation using its virulence factors [1]. Gamma interferon (IFN-γ generated during this process is a pro-inflammatory cytokine that induces antimicrobial responses, including the expression of the *Nos2* gene [2]. The enzyme encoded by *Nos2*, inducible nitric oxide synthase (iNOS), catalyzes the production of NO from L-arginine [3] (Fig. 1). NO can react with superoxide radicals generated by the host's NADPH oxidases to form peroxynitrite ($ONOO^-$), a reactive nitrogen species with potent bactericidal activity [4,5], or combine with O_2, iron or low-molecular weight thiols to generate a variety of nitrogen oxides and dinitrosyl-iron complexes [6]. The importance of these reactive nitrogen species in the host defense against *S.* Typhimurium is perhaps best illustrated by the finding that chemical inhibition or genetic ablation of iNOS activity reduces the ability of mice to control growth of the pathogen in tissue [7,8]. Given its established role in reducing the burden of *S.* Typhimurium in tissue, it seems paradoxical that in the intestinal lumen iNOS activity brings about the opposite effect, namely a pathogen expansion [9]. To understand this phenomenon it is important to know that while the production of $ONOO^-$ creates a hostile environment in close proximity to host cells, during its diffusion into the intestinal lumen this antimicrobial compound is quickly converted to harmless NO_3^- in a reaction catalyzed by carbon dioxide (CO_2)[10]. NO_3^- can independently form through either the denitrosylation of NO by the flavohemoprotein Hmp or oxidation by several hemoproteins [6]. As a result, NO_3^- accumulates in the gut lumen during conditions of intestinal inflammation [9,11,12]. Importantly, this conversion of an antimicrobial compound into a non-toxic by-product of inflammation brings about a significant change in the nutritional environment of the anaerobic gut lumen, because iNOS-derived NO_3^- serves as a substrate for bacterial nitrate reductases, thereby boosting NO_3^- respiration-dependent growth of *S.* Typhimurium [9]. Through this chain of events, the inflammatory host response fuels a luminal bloom of the pathogen that is required for transmission by the fecal oral route [13].

While *S.* Typhimurium is a cause of gastroenteritis, intestinal colonization with commensal members of the family *Enterobacteriaceae*, such as commensal *Escherichia coli*, is not associated with intestinal inflammation. However, while in healthy individuals commensal *Enterobacteriaceae* constitute only a small fraction of the gut associated microbial community, their relative abundance is markedly elevated during chronic intestinal inflammatory disorders, such as inflammatory bowel disease (IBD) [14–19]. Interestingly, NO concentrations are elevated in colonic luminal gas of individuals with inflammatory bowel disease [20,21] and a luminal expansion of commensal *E. coli* in animal models of IBD is driven by growth through NO_3^- respiration [12]. These observations suggest that intestinal inflammation fuels growth of commensal and pathogenic *Enterobacteriaceae* through an iNOS-dependent generation of NO_3^-. Furthermore, among the phylogenetic groupings that have been described within gut-associated microbial communities, the frequency at which a predicted nitrate reductase activity is present within genomesis highest for the *Enterobacteriaceae* [22]. These data suggest that intestinal inflammation specifically boosts growth of *Enterobacteriaceae* because within gut-associated microbial communities, members of this family are the ones most likely to encode the enzymes needed to take advantage of an important inflammation-derived nutrient, namely NO_3^-.

S. Typhimurium and *E. coli* encode three nitrate reductases encoded by the *narGHI*, *narZYV* and *napABC* genes. Nitrate reductase Z encoded by *narZYV* is synthesized constitutively and may function during the transition to anaerobic environments, in which expression of *narGHI*, encoding nitrate reductase A, and *napABC*, encoding periplasmic nitrate reductase, are induced by anaerobiosis [23]. While nitrate reductase A is synthesized optimally at NO_3^- concentrations of 10 mM or above [24], the periplasmic nitrate reductaseis synthesized optimally at NO_3^- concentrations below 1 mM [25]. These data suggest that the periplasmic nitrate reductase possesses a higher affinity for nitrate than nitrate reductase A. This high affinity comes at the cost of a lower energy yield, because the periplasmic nitrate reductase converts NO_3^- to NO_2^- at the periplasmic face of the cytoplasmic membrane, which does not resultin proton translocation [26,27] (Fig. 1). In contrast, the low-affinity nitrate reductases A reduces nitrate at the cytoplasmic face of the cytoplasmic membrane, a process coupled to proton translocation [23,28–30]. Nonetheless, the high-affinity periplasmic nitrate reductase is the major enzyme involved in NO_3^- respiration by *S.* Typhimurium during colitis, perhaps because NO_3^- concentrations in the inflamed cecum of mice are below 0.3 mM, a concentration too low for the low-affinity nitrate reductase A [31]. The limited availability of NO_3^- in the inflamed intestine necessitates *S.* Typhimurium to use motility and the chemotaxis receptor Tsr to seek out microenvironments in which concentrations of this electron acceptor are elevated [32].

Reduction of NO_3^- by the high-affinity periplasmic nitrate reductase generates NO_2^- in the periplasm. The periplasmic cytochrome c nitrite reductase encoded by the *S.* Typhimurium *nrfABCDEG* operon further reduces NO_2^- to ammonia (NH_4^+) at the periplasmic face of the cytoplasmic membrane [33] (Fig. 1). Although neither of these periplasmic reductases mediates proton translocation, either reaction can be coupled to energy-conserving respiratory electron transport performed by respiratory dehydrogenases. Thus, further reduction of periplasmic NO_2^- to NH_4^+ is predicted to bestow an energetic benefit upon *S.* Typhimurium during its growth in the inflamed gut. However, the contribution of the

nrfABCDEG operon to growth of *S.* Typhimurium during colitis remains to be investigated experimentally.

Reduction of nitrate / nitrite promotes intracellular growth of *Mycobacterium tuberculosis*

N_3^-/NO_2^- utilization also increases fitness of intracellular pathogens. For example, under hypoxic conditions, the alternative electron acceptor NO_3^- allows the strict aerobe *M. tuberculosis* to survive both inhibition of respiration and exposure to reactive nitrogen species [34,35], while fueling TCA cycle activity, and maintaining ATP levels and NADH/NAD^+ ratios [36]. NO_3^- produced by human macrophages can affect *M. tuberculosis* in two apparently opposing ways. First, the NO_2^- that arises from host-derived NO_3^- through the enzymatic activity of *M. tuberculosis* NarG inhibits bacterial growth, enhances ATP synthesis, and regulates the expression of 120 genes associated with adaptation to acid, hypoxia, oxidative and nitrosative stress, and iron deprivation [37]. This transcriptional response is believed to increase resistance of *M. tuberculosis* to host defenses. In this context, nitrite reductase can be important during *in vitro* dormancy [38], a genetic program activated by persistent *M. tuberculosis*. Second, NarG can promote growth of this intracellular pathogen in NO-producing human macrophages [39]. The different effects that NO_3^- utilization has on the outcome of the interaction of *M. tuberculosis* with human macrophages may reflect concentrations of NO_3^-, amounts and type of nitrogen oxides produced, oxygen tension and carbon and nitrogen sources used the population of human macrophages. Finally, the importance of NO_3^-/NO_2^- reduction in the pathogenesis of *M. tuberculosis* has been suggested by the fact that bis-molybdopter in guanine dinucleotide, a cofactor of nitrate reductases and other enzymes, is required for the persistence of this intracellular pathogen in guinea pigs [40].

Nitric oxide reductases in bacterial pathogenesis

Nitric oxide reductases, such as NorVW of *S.* Typhimurium, catalyze the $2e^-$ reduction of NO to nitrous oxide (N_2O) in the third step in the denitrification of NO_3^- to N_2 (Fig. 1). Nitric oxide reductases, members of the heme-copper oxidase superfamily that arose in ancestral bacteria for the utilization and detoxification of NO [41], are not only essential in the evolution of dissimilatory denitrification but also aerobic respiration. Nitric oxide reductases have been preserved throughout evolution and nowadays bacterial pathogens exploit these cytochromes in their efforts to parasitize metazoan hosts. Molecular epidemiology suggests a contribution of nitric oxide reductases in both hemolytic uremic syndrome caused by enterohemorrhagic *E. coli* and infections of cystic fibrosis patients with the opportunistic pathogen *Pseudomonas aeruginosa* [42,43]. It is likely that nitric oxide reductases help enterohemorragic *E. coli* and *P. aeruginosa* to cause these syndromes by antagonizing the negative effects that NO has on shiga toxin production, while allowing the production of NH_4^+, a cation that inhibits chloride transport. Epidemiological studies also indicate that *nor*$^+$ *S. aureus* belong to sequence types typically resistant to methicillin [44]. Experimental models of infection have also demonstrated roles for nitric oxide reductases in a variety of aspects of bacterial pathogenesis such as the dispersal of *P. aeruginosa* and *N. gonorrhoeae* biofilms; stimulation swimming and swarming motility in *P. aeruginosa*;

survival of *E. coli*, *Pseudomonas* and *Neisseria* in NO-producing macrophages or nasopharengial tissue; and virulence of *Pseudomonas* in a silkworm model of infection [45–50].

Nitric oxide reductases belong to either of two clades according to the substrates that feed reducing power to their enzymatic activity and their ability, or lack of, to translocate protons across cytoplasmic membranes. Nitric oxide reductases of *Neisseria*, *Moraxella* and *Staphylococcus* draw reducing power from membrane quinols and translocate protons across the membrane, thus combining NO detoxificiation with proton translocation that fuels ATP synthesis and energizes membranes [51]. Moreover, by promoting NADH dehydrogenase activity, quinol oxidation helps replenish NAD^+, there by maintaining redox balance that drives metabolism (Fig. 1). In this context, nitric oxide reductases of *M. catarrhalis* is thought to serve as a means to dispose of excess reductive power generated by oxidative metabolism of reduced carbon sources. In contrast to quinol nitric oxide reductases, the reduction of NO to N_2O by cytochrome c-type nitric oxide reductases such as those expressed by *P. aeruginosa* is not directly electrogenic. In other words, the exergonic energy released in the conversion of NO to N_2O by c-type nitric oxide reductasesis not conserved as an electrochemical gradient across the cytoplasmic membrane [52]. Recent structural analyses have revealed that hydrophobic isoleucine and phenolalanine residues, which areabsent in the water channel of cytochrome c oxidase and q-type nitric oxide reductases, prevent proton transfer in c-type nitric oxide reductases from the cytoplasm to the periplasm [53]. The c-type nitric oxide reductases indirectly contribute to the energetics of the cell by pulling electrons from the quinol pool through cytochrome c.

Regardless of their clade, nitric oxide reductases play a predominant role in the detoxification of NO. The enzymatic activity of nitric oxide reductases protects biological targets such as the [4Fe-4S] metalloproteins aconitase and 6-phosphogluconate dehydratase from the cytotoxic actions of NO [54]. Moreover, the NO-detoxifying activity of nitric oxide reductases limits the formation of S-nitrosating dinitrosyl iron complexes in *Pseudomonas* and reduces the amount of S-nitrosated proteins in *M. catarrhalis* [24,55]. NO detoxification on its own is a clear advantage afforded by nitric oxide reductases as suggested by the fact that *N. gonorrhoeae* and *Moraxella catarrhalis* express truncated denitrification pathways [24]. The lack of nitrous oxide reductase activity in these Gram-negative cocci could elevate NO concentrations to levels that limit inflammation [56,57]. Finally, in pathogens such as *N. meningitidis* and *P. aeruginosa* with complete assimilatory pathways, nitric oxide reductase enzymatic activity could play a nutritional role by participating in the formation of NH_4^+.

Quinol oxidases in the detoxification of NO

Phylogenetic, structural, functional and paleogeochemical lines of reasoning indicate that cytochrome *c* and quinol *bo* oxidases arose from nitric oxide reductases in the wake of aerobic metabolism [58–61]. Traditionally, quinol oxidases are known for the $4e^-/4H^+$ reduction of O_2 to H_2O, a process that catalyzes translocation of protons across the cytoplasmic membrane. The ensuing proton motif force energizes ATP synthesis, membrane transport, and flagellar rotation. More recently, quinol *bd* oxidase from *E. coli* has been recognized as a measurable source of NO consumption [62]. By virtue of its fast dissociation

rate and high affinity for both NO and O_2, quinol *bd* oxidase is believed to be at the forefront of NO detoxification under hypoxic conditions. Studies with a *fur* mutant *Salmonella* strain have hinted to the importance of NO detoxification by quinol *bd* oxidases in bacterial pathogenesis [63]. The hypersusceptibility of *fur* mutant *Salmonella* to NO generated by iNOS in a murine model of infection is partially dependent on diminished quinol *bd* oxidase content, perhaps due to a defect in aminolevulinic acid metabolism because the addition of this metabolite restored cytochrome content and resistance to NO. In addition to emerging as an important detoxification pathway, nitrosylation of quinol *bd* oxidases elicits antioxidant defenses in *Salmonella* by indirectly boosting NADH reducing power [63].

Nitrosylation of cytochromes of the electron transport chain modulates the cytotoxicity of antibiotics

The emergence of antibiotic resistant bacterial strains is a recalcitrant problem in clinical medicine. Efflux pumps, mutations in molecular targets, and detoxification systems are common strategies used by bacterial pathogens in their race to become antibiotic resistant. Recent studies have indicated that NO modulates the antimicrobial activity of commonly used antibiotics against several Gram-positive and –negative pathogens [64,65]. Although early studies suggested that the ability of NO to promote antioxidant defenses was at the heart of antibiotic tolerance [64], recent investigations indicate that in some instances NO modulates antibiotic tolerance through its interactions with cytochromes of the electron transport chain. It is also becoming apparent that NO can potentiate antibiotic activity under some circumstances. Degree of nitrosative stress along with respiratory and metabolic flexibility of the bacterial cells may explain the protean effects that nitrosylation of cytochromes of the electron transport chain has on antibiotic susceptibility.

NO congeners block antibiotic killing

By nitrosylating quinol oxidases of the electron transport chain and thus blocking the energy-dependent phases of drug uptake [65], NO protects *Salmonella*, *Pseudomonas*, *S. aureus* against aminoglycosides [65,66]. NO also promotes tolerance of *Burkholderia pseudomallei*, *S.* Typhimurium and *S. aureus* to β-lactams, vancomycin and daptomycin [67,68]. The mechanism of action by which NO induces tolerance to these drugs cannot be explained through the inhibition of drug uptake since the targets of β-lactams, vancomycin and daptomycin are located in the cell envelope. At least in the case of β-lactams, NO induces tolerance by inhibiting quinol oxidases of the electron transport chain [67]. Nitrogen oxides other than NO can also prevent antibiotic killing, as suggested by the fact thatnitrate reductase protects *M. tuberculosis* from isoniazid killing [69]. The mechanism of action underlying the protection afforded by nitrate reductase seems to rely on the inhibitory effects that nitrite has on catalase, an enzyme thatmakes possible the formation of anisoniazid radical intermediate needed for the inactivating reaction with NAD^+. Although it is not clear yet if NO and NO_2^- elicit antibiotic tolerance *in vivo*, NO produced by iNOS of macrophages protects intra cellular *Salmonella* and *Listeria* against the antimicrobial activity of aminoglycosides and β-lactams [65,67,70].

NO congeners synergize with antibiotics

NO can also potentiate antibiotic killing. NO enhances antimicrobial activity of tobramycin against established *P. aeruginosa* biofilms [45], and NO produced by bacterial NOS enhances susceptibility of *S. aureus* to aminoglycosides [68]. In this context, NO generated by macrophages enhances killing of *B. pseudomallei* by β-lactam antibiotics [67], which might explain why IFNγ and ceftazidime synergize in clearing *B. pseudomallei* in a murine model of infection [71]. Moreover, NO_2^- synergizes with polymyxins and colismathate in killing *Pseudomonas*, perhaps by blocking respiration [72].

Conclusions

A large body of evidence indicates that NO and its congeners exert broad antimicrobial activity. However, recent investigations have shed light into the contribution that usage of nitrogen oxides by cytochromes of the electron transport chain plays in colonization and infection of mammalian hosts by bacterial pathogens. Reduction of nitrogen oxides by cytochromes of the electron transport chain energizes cytoplasmic membranes, drives ATP synthesis, oxidizes quinol and NADH. By doing so, cytochromes promote antioxidant defenses, modulate resistance to several classes of antibiotics, and foster bacterial virulence.

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•• of outstanding interest

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Highlights

- Bacterial cytochromes are conserved throughout evolution for the utilization of nitrogen oxides and oxygen.
- These metalloproteins support cellular bioenergetics by aiding with ATP synthesis, and quinol and NADH redox balance.
- Cytochromes modulate antioxidant and antinitrosative defenses, and antibiotic susceptibility.
- Cytochromes contribute to bacterial pathogenesis.

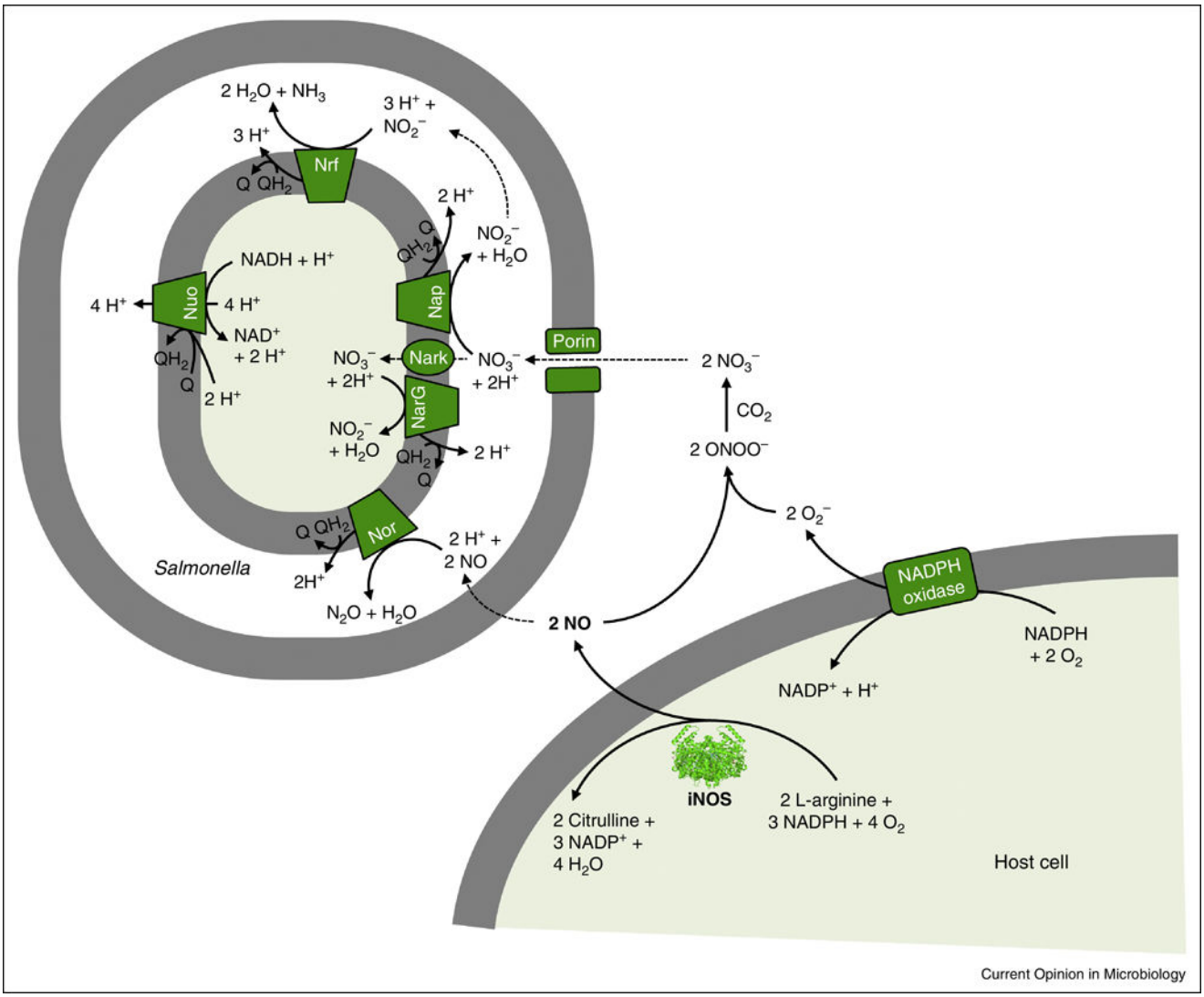

Figure 1.

Host-derived nitrate supports growth of *S.* Typhimurium in the intestinal lumen. The nitric oxide reductase (norVW) of *S.* Typhimurium (*Salmonella*) can transfer electrons from the respiratory quinone (Q) pool to nitric oxide (NO) produced by the hosts' inducible nitric oxide synthase (iNOS, image by courtesy of Protein Databank Europe) to generate nitrous oxide (N_2O). NO and superoxide (O_2^-) produced during inflammation react to form peroxinitrite ($ONOO^-$), which rapidly decomposes to nitrate (NO_3^-). Nitrate enters the perimplasm through porins where it is reduced to nitrite (NO_2^-) by the periplasmic nitrate reductase (Nap) or it is further transported into the bacterial cytosol by NarK and serves as an electron acceptor for nitrate reducatse A (NarG). Periplasmic nitrite is further converted to ammonia (NH_3) by the periplasmic nitrite reductase (Nrf). Electrons required for these reactions can be transferred to the quinone (Q) pool by NADH:ubiquinone oxidoreductase (Nuo) in a reaction coupled to energy-conserving proton translocation.

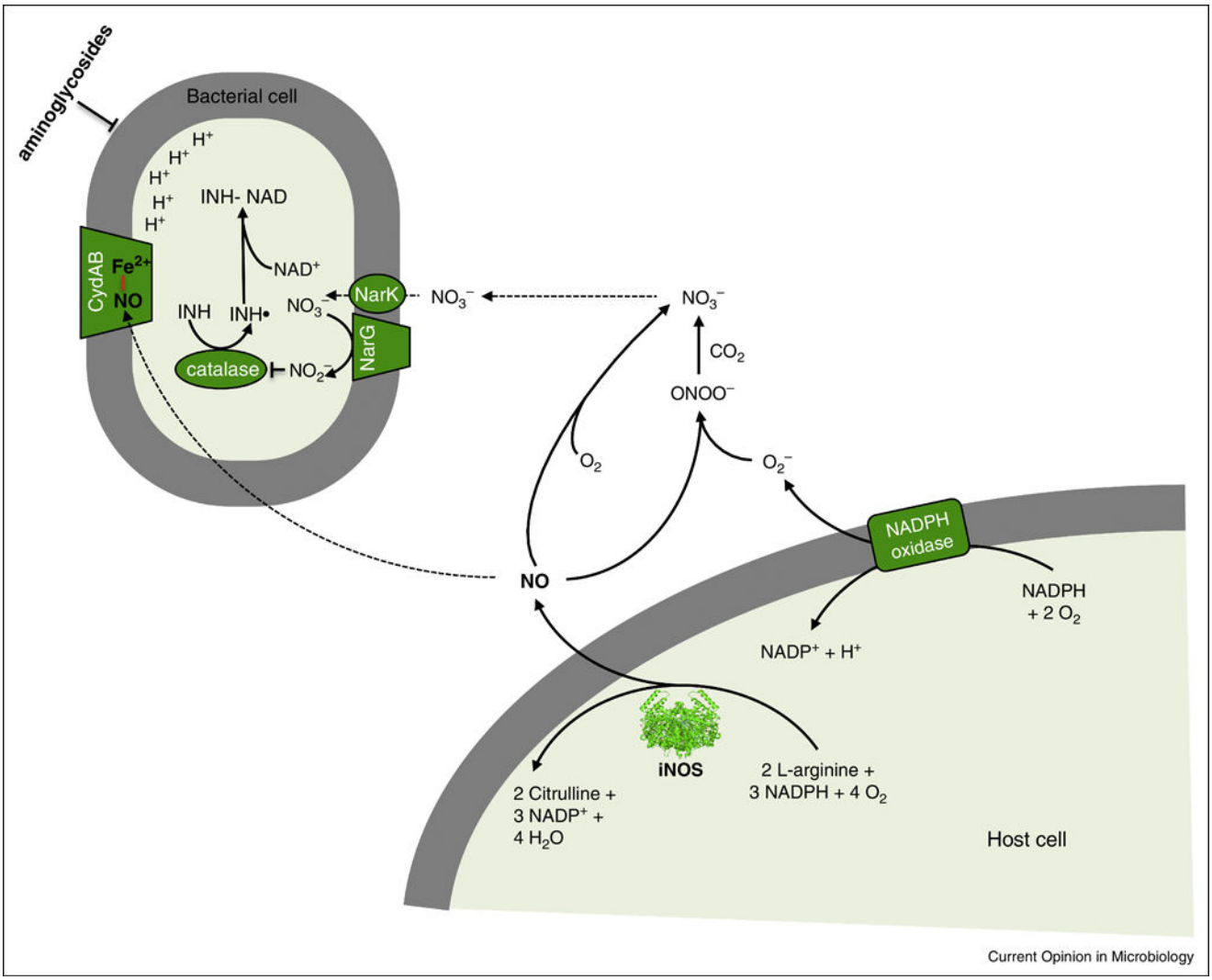

Figure 2.

Nitrogen oxides modulate antibiotic activity. Nitric oxide (NO) and its oxidative products nitrate (NO_3^-) and nitrite (NO_2^-) modulate the antimicrobial activity of different classes of antibiotics that target ribosomes (e.g., aminoglycosides) of several Gram-positive and –negative bacteria, and InhA-encoded fatty acid elongation system II (e.g., isoniazid, INH) of mycobacteria. Nitrosylation of heme *d* in quinol *bd* oxidase encoded by the *cydAB* gene inhibits the proton motive force needed for aminoglycoside uptake, thus preventing inhibition of ribosomes and accumulation of cytotoxic mistranslated proteins. NO_2^- produced by reduction of NO_3^- by nitrate reductase inhibits catalase activity required for the activation of INH. Consequently, INH cannot produced the INH-NAD adduct that mediates poisoning of InhA. The molecular mechanisms by which NO modulates the antimicrobial activity of several antibiotics remain unknown.