

Nitrite and Nitrite Reductases: From Molecular Mechanisms to Significance in Human Health and Disease

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Abstract

Nitrite, previously considered physiologically irrelevant and a simple end product of endogenous nitric oxide (NO) metabolism, is now envisaged as a reservoir of NO to be activated in response to oxygen (O₂) depletion. In the first part of this review, we summarize and compare the mechanisms of nitrite-dependent production of NO in selected bacteria and in eukaryotes. Bacterial nitrite reductases, which are copper or heme-containing enzymes, play an important role in the adaptation of pathogens to O₂ limitation and enable microorganisms to survive in the human body. In mammals, reduction of nitrite to NO under hypoxic conditions is carried out in tissues and blood by an array of metalloproteins, including heme-containing proteins and molybdenum enzymes. In humans, tissues play a more important role in nitrite reduction, not only because most tissues produce more NO than blood, but also because deoxyhemoglobin efficiently scavenges NO in blood. In the second part of the review, we outline the significance of nitrite in human health and disease and describe the recent advances and pitfalls of nitrite-based therapy, with special attention to its application in cardiovascular disorders, inflammation, and anti-bacterial defence. It can be concluded that nitrite (as well as nitrate-rich diet for long-term applications) may hold promise as therapeutic agent in vascular dysfunction and ischemic injury, as well as an effective compound able to promote angiogenesis. *Antioxid. Redox Signal.* 17, 684–716.

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Reviewing Editors: Markus Bachschmid, Srinivas Bharath, John Calvert, Sergey Dikalov, Martin Feelisch, Carlos Gutierrez-Merino, Christopher Kevil, João Laranjinha, Tienush Rassaf, and Jay L. Zweier

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I. Introduction

A. Nitrite is the Cinderella molecule in biological signaling

In bacteria, nitrite is a well-known source of nitric oxide (the NO• radical, hereinafter NO) under anaerobic conditions (380). In eukaryotes, on the other hand, for years nitrite has been considered physiologically irrelevant and a simple end product of endogenous NO metabolism (113, 185); only in the last decade, the relevance of nitrite as a source of NO has emerged.

In mammals, NO is mainly synthesized by the enzyme nitric oxide synthase (NOS) *via* oxidation of the amino acid L-arginine; this reaction requires oxygen (O₂) as an essential substrate (Fig. 1A), with a K_m in the 5–20 μM range (*in vitro*) (5, 199, 287). The exact O₂ threshold level at which NOS-dependent NO generation is compromised and fails to signal is unknown mainly due to uncertainties on the *in vivo* value of the K_m for O₂ of NOS enzymes (220). Organs and tissues are characterized by their own unique normoxic status (57, 352),

given that the local oxygen pressure (*p*O₂) is a key component of the physiological state of an organ and results from the balance between O₂ consumption and delivery (Fig. 1B). However, in the human body, NO is also produced under hypoxic conditions (352); under these conditions, NO formation is not blocked by NOS inhibitors (74, 131, 382) and nitrite reduction is found to be enhanced (44, 131). These lines of evidence suggest that, in eukaryotes, an NOS-independent system exists, able to ensure sufficient NO formation when O₂ supply is limited (Fig. 1A), and identify nitrite as a main source of NO under hypoxic conditions. As a consequence, nitrite has recently obtained novel relevance and continuously expanding popularity.

This review summarizes and compares the molecular mechanisms of nitrite-dependent NO production in selected bacteria and in eukaryotes. Moreover, the review analyses the involvement of nitrite in human physiology and the possible therapeutic applications of this molecule. As will be detailed in the review, the nitrite reductase (NiR) activity is carried out by enzymes and proteins with intrinsically different cellular

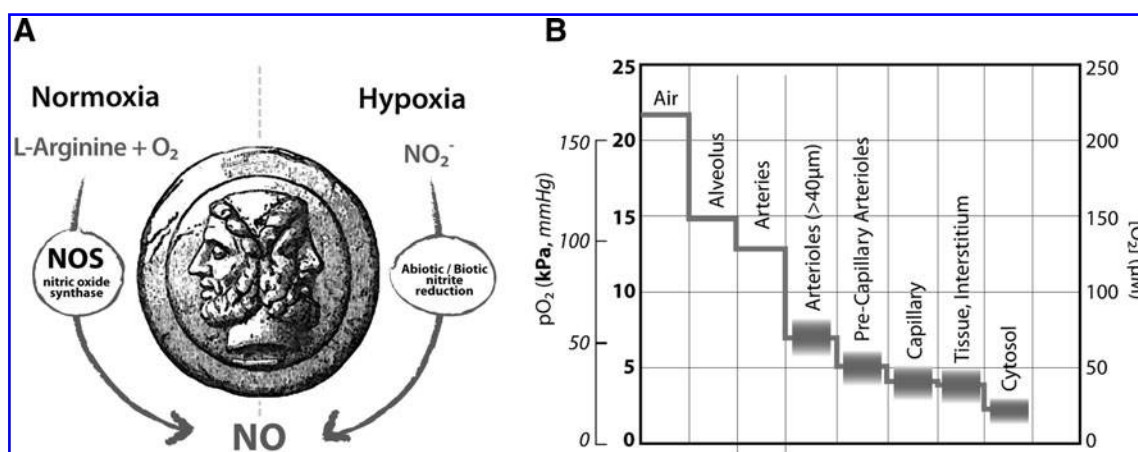


FIG. 1. Nitrite reduction: the two faces of nitric oxide (NO) production. (A) Under normoxic conditions, NO is synthesized by nitric oxide synthase (NOS) from L-arginine and oxygen (O₂) (305). On the other hand, under low O₂ tension (hypoxia) NO generation occurs in an NOS-independent way, *via* the reduction of nitrite carried out by abiotic or biotic systems. This double-faced system guarantees the formation of NO during the different physiological or pathological conditions. (B) Normoxia and hypoxia: values of the oxygen pressure (*p*O₂) measured from the airways to the cell cytosol in the human body. Each region of the body is characterized by its own unique normoxic status whose *p*O₂ value is indicated by a line; the thickness of the line represents variation between reports (352). [O₂] = concentration of free O₂; 1 mmHg = 0.13 kPa; 1 mmHg corresponds to [O₂] = 1.32 μM in H₂O at 37°C (345). Figure modified with permission from (352).

roles and biochemical features, spread across the two kingdoms; the wide distribution of the NiR activity highlights the importance of nitrite in cellular homeostasis.

II. Bacterial NiR and Their Significance

In this section we describe the reaction mechanism of two different classes of bacterial enzymes involved in the reduction of nitrite and the significance of this activity for human health, with particular attention to host–pathogen interactions. Three examples are reported: AniA from *Neisseria* (section A), NirBD from *Mycobacterium tuberculosis* (section B), and cytochrome cd₁ from *Pseudomonas aeruginosa* (section C).

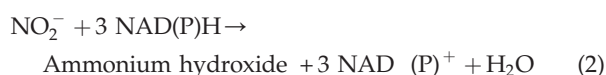
The human body is heavily colonized by bacteria. These bacteria are frequently exposed to anoxia and to NO (and, thus, also to nitrite) generated by the host. Dealing with these two environmental factors often forces the bacteria to metabolic changes to (i) maintain growth and survive in the absence of O₂, and (ii) detoxify the free radical NO and/or the toxic compound nitrite. While microbial colonization is part of the normal physiology of the human body, the body can also be exposed to pathogenic bacteria such as *P. aeruginosa*, *M. tuberculosis*, and the pathogenic *Neisseria* species *N. meningitidis* and *N. gonorrhoeae*. The ability to reduce nitrite can therefore confer to these species a selective advantage in the host–pathogen arms race to survive in an O₂-limited and nitrite-rich environment.

Two distinct classes of NiR are responsible for the reduction of nitrite in bacteria. The first group comprises the enzyme NiR (EC 1.7.2.1), which reduces nitrite to NO during denitrification, the anaerobic respiratory process widely found in both autotrophic and heterotrophic microorganisms, in which oxidized nitrogen compounds such as nitrate and nitrite are used as electron acceptors for energy production (380). Denitrification has been implicated in the virulence of several bacterial species, including *Brucella* (12), *Pseudomonas* (342), and *Neisseria* (17).

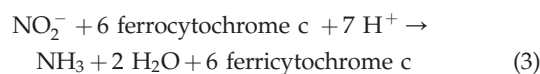
Depending on the bacterial species, the NiR enzyme might be a copper-containing protein or a hemoprotein (cytochrome cd₁ nitrite reductase [cd₁NiR]), encoded by the *nirK* and *nirS* genes, respectively. The reaction catalyzed by these enzymes is



The second group of NiR includes two quite distinct enzymes catalyzing the reduction of nitrite to ammonia. The first type is the (NAD(P)H)-dependent enzyme NirBD (EC 1.7.1.4), which reduces nitrite to ammonium coupled to the oxidation of either NADH or NADPH. It is a flavin-dependent enzyme that also contains a specialized heme cofactor named sirheme (119, 326); this enzyme participates in assimilatory nitrite reduction in bacteria but also in algae, fungi, and higher plants. The reaction catalyzed by NirBD NiR is



The other class of enzymes able to convert nitrite to ammonia is the multiheme NiR (cytochrome-ammonia forming) NrfA (EC 1.7.2.2). The enzyme also reduces NO and hydroxylamine to ammonia, and sulfite to sulfide. The reaction catalyzed is:



In the following paragraphs three relevant examples of bacterial NiR strategic for the survival of pathogens within the infected host are reported.

A. *Neisseria* and the copper NiR AniA

The three closely related bacterial species, *N. meningitidis*, *N. gonorrhoeae*, and *Neisseria lactamica*, colonize mucosal surfaces in humans. *N. gonorrhoeae* is the causative organism of the sexually transmitted disease, gonorrhoea, one of the most frequently reported communicable diseases; *N. meningitidis* does occasionally cause severe, life-threatening illness known as meningitis, whereas *N. lactamica* is a common, harmless commensal of children.

N. gonorrhoeae is an obligate human pathogen that colonizes O₂-limited environments of the genitourinary tract. As in *N. meningitidis*, it conserves energy during electron transfer from physiological substrates via a membrane-bound respiratory chain to a single terminal cytochrome oxidase, cytochrome cbb₃ (65, 266, 319). When the O₂ supply is growth-limiting, the bacterium produces a truncated denitrification pathway in which nitrite is reduced to NO by the anaerobically induced outer membrane protein AniA, a copper-containing NiR of the NirK family (34, 188, 231). NO is then reduced to N₂O by a single-subunit nitric oxide reductase (Nor) B subunit (NorB) that receives electrons directly from ubiquinol (153). Therefore, AniA and NorB cooperate together to facilitate the anaerobic growth of gonococci: the *nirK* and *norB* genes are differentially controlled by a group of transcriptional regulators that respond to changes in the levels of O₂, nitrite, and NO (146, 163, 164).

The crystal structure of the soluble domain of AniA shows that the protein adopts a fold typical of copper-containing bacterial NiR (34): a tightly packed trimer of identical subunits, containing a type I and a type II copper atoms (Fig. 2).

The catalytic mechanism of CuNiRs requires one electron and two protons to convert nitrite into NO and water (2, 7, 249) (Fig. 2C). The protons are donated by a conserved active-site aspartate, which hydrogen bonds directly to the nitrite molecule, and by a histidine residue; these aminoacids are linked through a solvent-bridged hydrogen bond. The aspartate and histidine residues are conserved in all known Cu-NiR sequences and correspond to Asp97 and His240 in AniA (Fig. 2B). The most likely electron donors to AniA are the CcoP subunit of cytochrome oxidase cbb₃ and, by sequence similarity to the CcoP subunit, also the cytochrome c5 (10, 152).

Interestingly, *aniA* is the most highly induced gene during anaerobic growth; expression of AniA during infection has been detected immunologically, supporting the induction of nitrite reduction *in vivo* (54, 67, 297). The ability to reduce nitrite during O₂-limited growth appears therefore to confer a selective advantage for the survival of pathogenic *Neisseria* in the human host (66). The analysis of *aniA* expression has been recently extended to the biofilm mode of growth of the bacterium; biofilms are organized bacterial communities in which the cells are embedded in a self-produced extracellular polymeric substance, attached to a surface. Biofilms formed by pathogens play an important role in the infection of living tissues and are responsible for the resistance to antibiotics and

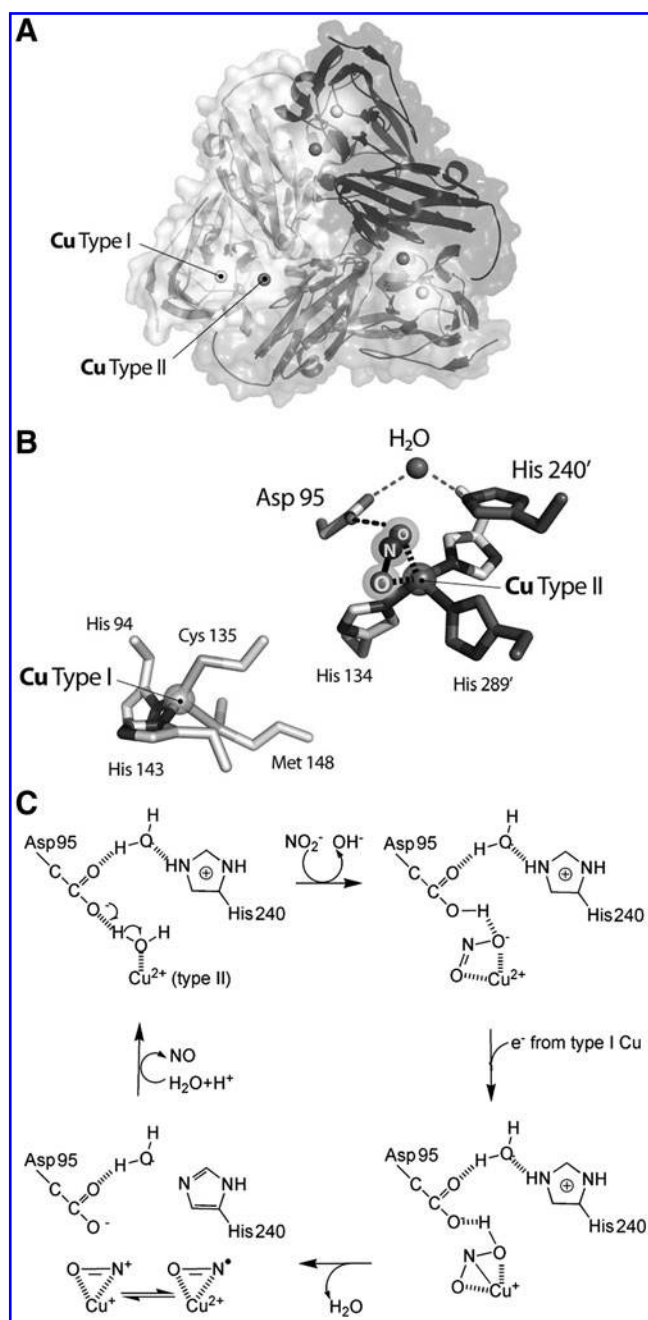


FIG. 2. Structural organization and catalytic mechanism of the copper nitrite reductase (NiR) AniA from *Neisseria gonorrhoeae*. (A) Overall organization of the enzyme [pdb id: 1kbv (34)]. AniA is a homotrimer, with each subunit binding two copper atoms. The type I Cu atom is bound in the internal part of each monomer, while type II Cu is positioned at the interface between two adjacent monomers. (B) Structure of the two copper sites, showing nitrite bound to type II Cu atom (pdb id: 1kbv). The metal coordinates residues coming from adjacent monomers (labeled Asp95, His134, and His289', His240', respectively). (C) Possible scheme of the catalytic cycle, adapted from that proposed for the copper NiR from *Alcaligenes faecalis* (7).

to the host immune system (45). *N. gonorrhoeae* readily forms biofilms over abiotic surfaces, over primary and transformed cervical epithelial cells and over cervical tissues *in vivo*. Expression of AniA in biofilms is induced over time; this evidence shows that a combination of anaerobic/aerobic respiration is used by *Neisseria* to support growth in the biofilm and that nitrite appears to be the preferred substrate for anaerobic respiration (110, 111).

Interestingly, Aspholm *et al.* (10) reported the identification of a single-nucleotide polymorphism (SNP) unique in the species *N. meningitidis* that leads to truncation of the c-type heme protein CcoP, an essential component of cytochrome oxidase. This SNP was found in all strains of *N. meningitidis* but not in strains of *N. gonorrhoeae* and *N. lactamica*. Although this mutation results in the truncation of an essential component of the cytochrome *cbb*₃ oxidase, it also conditionally affects nitrite consumption, providing evidence that an alteration in the circuitry of respiratory electron-transfer networks is associated with *N. meningitidis* speciation.

B. *M. tuberculosis* and NirBD NiR

M. tuberculosis, the etiologic agent of tuberculosis, is a facultative intracellular pathogen that can persist within the host; the bacterium may lie dormant in the human body for decades, only progressing to active disease in 5%–10% of individuals. One of the primary host defense mechanisms against mycobacterial diseases involves the formation of a granuloma-like structure. Immune containment by granuloma formation creates a microenvironment in which nutrient limitation, low pH, reactive nitrogen and O₂ species, and reduced O₂ tension are believed to be factors that coincide with the establishment of chronic infection. Under these conditions, *M. tuberculosis* changes its metabolism and, to survive, it can utilize various nutrients, including nitrate, as a source of nitrogen. Assimilation of nitrate requires the reduction of nitrate *via* nitrite to ammonium, which is then incorporated into metabolic pathways (89, 143); assimilation of nitrite is therefore essential for the survival of *M. tuberculosis* *in vitro* and *in vivo*.

The second step in nitrate assimilation is the reduction of nitrite to ammonium, catalyzed by the siroheme-dependent NADH-NiR, encoded by the *nirBD* operon; this enzyme is known to catalyze nitrate assimilation in various bacteria and fungi (208). The NirB protein is produced by *Mycobacterium* throughout infection, as recently shown in a proteomic study, together with the proteins required for nitrate/nitrite transport (such as NarX) and nitrate reduction (*narGHJI* and *narK2X* operons) (192).

In *M. tuberculosis*, NirBD has been proposed to be involved in the assimilatory reduction of nitrite (228). A recent study suggests, however, that the NirBD complex is not required for nitrate-dependent protection from acid-induced death under hypoxia (325); under these conditions the nitrite produced upon nitrate reduction through the NarGH complex is not further reduced into ammonium by the NirBD complex. Thus, in *Mycobacterium*, protection from nitrite toxicity at acidic pH, is most likely achieved by exporting nitrite outside the cell, likely through predicted nitrite extrusion proteins, including NarK3 and NarU (69). An alternative role for the *nirBD*-encoded NiR enzyme has been proposed for *Escherichia coli* and other enterobacteria; in these microorganisms, NirBD is induced under

anaerobic conditions and it is involved in detoxifying nitrite that accumulates from nitrate respiration (126).

C. *P. aeruginosa* and *cd₁NiR*

The ubiquitous gram-negative bacterium *P. aeruginosa* is an opportunistic pathogen responsible for both acute and chronic infections. *P. aeruginosa* is an etiologic agent common in several infections, including those affecting ears (94), burn wounds (244), and eyes (169). In addition, *P. aeruginosa* chronic lung infection is the major cause of death in cystic fibrosis (CF) patients, a genetic disease affecting 1/2500 newborn in Europe (99). *P. aeruginosa* is frequently resistant to conventional antibiotic therapy and to the host antimicrobial effector mechanisms. A major problem in the control of *P. aeruginosa* infection is given by the sessile, biofilm-mode of growth adopted by this bacterium in many infection sites, and typically in CF lung chronic infections (137, 139). *P. aeruginosa* survives in the low O₂ environment of the airway mucus of CF patients by using anaerobic metabolism and forming robust biofilms (246). The stagnant mucus overlaying the CF lung epithelium constitutes a nitrate-rich microaerobic/anaerobic environment (Fig. 3); nitrate in CF mucus is generated in part by the host inflammatory response to infection *via* NO. In this environment, *P. aeruginosa* produces energy from nitrate also using the metabolic pathway of denitrification (6, 139) (Fig. 3). Four reductases are involved in this process (380), namely, nitrate reductase (Nar), NiR, Nor, and nitrous oxide reductase (N₂OR), whose expression is tightly regulated, being the intermediate NO a cytotoxic compound. Genetic mutants lacking *nar* and *nir* genes show swarming defects and reduced virulence (342).

The molecular mechanisms controlling enhanced biofilm formation during anaerobic growth are not clearly defined. Low concentrations of NO have been shown to promote biofilm

dispersion (15); on the other hand, Yoon and coworkers (369) have shown that *P. aeruginosa* PAO1 grown anaerobically is more elongated than that grown aerobically and is defective in cell division. Elongated cells easily form highly cohesive clumps, thus yielding a robust biofilm. Cell elongation is dependent on the presence of NiR and is repressed in *P. aeruginosa* PAO1 in the presence of an NO antagonist (2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide [carboxy-PTIO]); these evidence suggests a link between cell elongation, NO, and anaerobic respiration (369). Importantly, the nonelongated NiR-deficient mutant failed to form biofilm, while the wild-type PAO1 is highly elongated and formed robust biofilm.

In addition to its role in anaerobic growth of *P. aeruginosa*, the NiR activity controls other important aspects of pathogenesis even under conditions where O₂ is apparently not limiting, including motility, initiation of biofilm formation, and virulence. As an example, a recent study has demonstrated that the NO produced by *P. aeruginosa* NiR regulates the activity of type III secretion system (343), an apparatus whereby cytotoxic effector proteins are directly secreted into the host cell cytoplasm after contact of the bacterium with a target cell.

Therefore, in *P. aeruginosa*, pathogenesis, biofilm formation, and denitrification, especially nitrite reduction, are closely related. The enzyme responsible for nitrite reduction to NO is *P. aeruginosa* cytochrome *cd₁* nitrite reductase (Pa-*cd₁NiR*), a homodimer containing one c-heme and one *d₁*-heme group in each subunit (Fig. 4A). Electrons are transferred from the soluble electron donor cytochrome *c₅₅₁* to the c-heme moiety of the enzyme (347) and thereby internally to the *d₁*-heme (Fig. 4B); in the active site (Fig. 4C), the substrate nitrite binds to the heme iron and is reduced to NO (380). The *d₁*-heme (3,8-dioxo-17-acrylate-porphyrindione) (Fig. 4B) is a partially saturated macrocycle with a set of oxo, methyl, and acrylate substituents, unique to the *cd₁NiR*s (4, 380) and synthesized by a specialized pathway present only in denitrifiers (strongly induced in *P.*

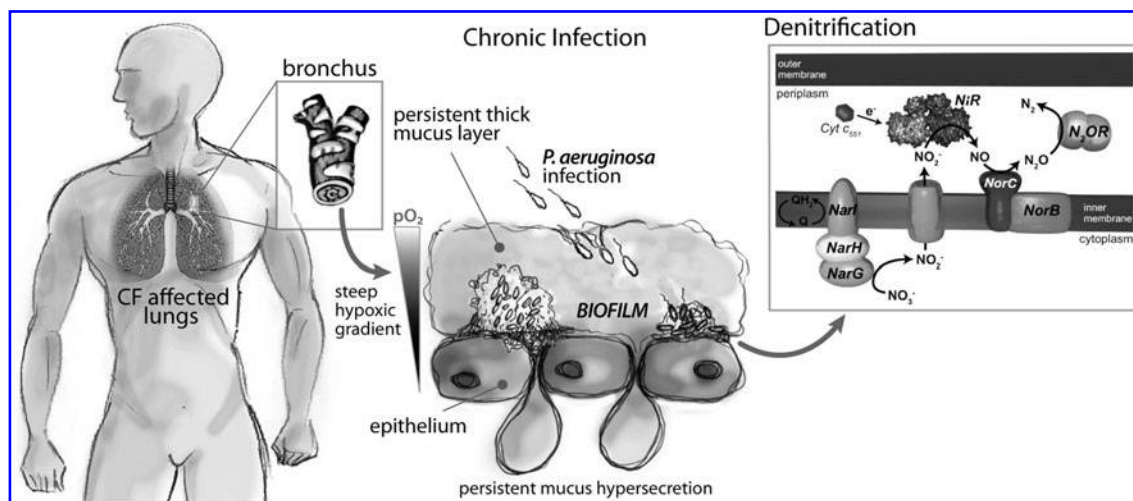


FIG. 3. Denitrification, pathogenesis, and biofilm formation in *Pseudomonas aeruginosa*. *P. aeruginosa* colonizes the lungs of cystic fibrosis (CF) patients (99). In the epithelium of the CF bronchus the formation of thick mucus is favored; in this environment, a steep gradient of pO₂ develops. This microaerobic/anaerobic environment is rich of nitrates (in part generated from the inflammatory response to infection) and favors the formation of *P. aeruginosa* biofilm (139). Under these conditions, *P. aeruginosa* survives thanks to the denitrification pathway involving the reduction of nitrates to N₂ and mainly occurring in the bacterial periplasm. The complete pathway is described in the box: four reductases are involved in this process, namely, nitrate reductase (Nar), NiR, nitric oxide reductase (Nor), and nitrous oxide reductase (N₂OR) (6, 139).

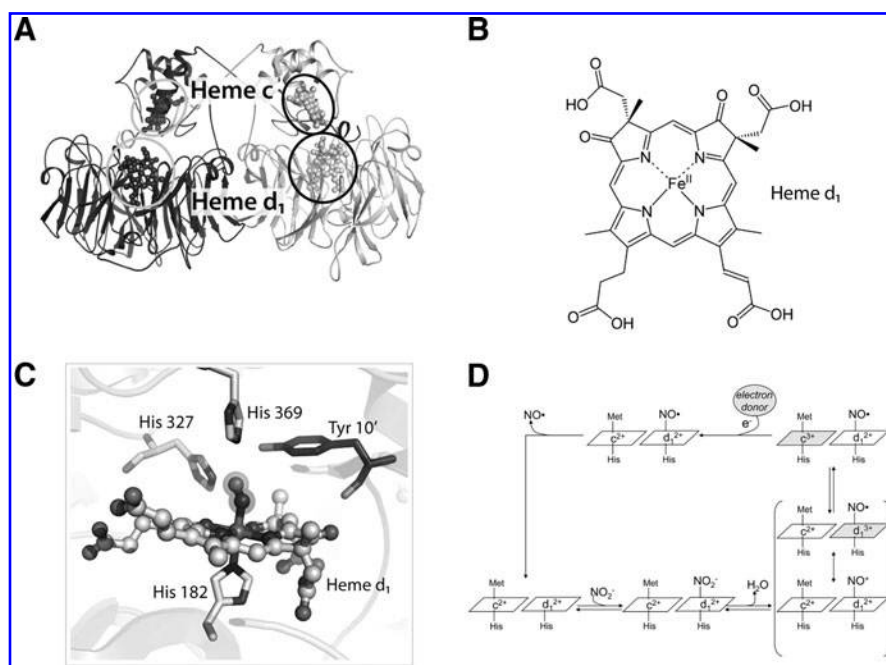


FIG. 4. Structural organization and catalytic mechanism of the cytochrome cd_1 nitrite reductase (cd_1NiR) from *P. aeruginosa*. (A) Overall organization of the enzyme [pdb id: 1nno (260)]. cd_1NiR is an homodimer, with each subunit binding one c-heme and one d1-heme. (B) Structure of d1-heme (3,8-dioxo-17-acrylateporphyrindione), a partially saturated macrocycle with a set of oxo, methyl, and acrylate substituents. The d1-heme is unique to the cd_1NiRs (4, 394) and is synthesized by a specialized pathway present only in denitrifiers. (C) Structure of the d1-heme pocket, showing NO bound to the heme iron. Residues belonging to the N-terminal region of each subunit (*i.e.*, Tyr-10') swap between domains and contribute to the formation of the d1-heme pocket of the adjacent subunit. (D) Possible scheme of the catalytic cycle: nitrite binds to ferrous (Fe^{2+}) d1-heme, and is then converted to NO (294); the oxidized (Fe^{3+}) hemes are shown in grey. Notice that dissociation of the product NO occurs from the ferrous iron (294).

aeruginosa upon nitrite treatment). Other hemes in which the porphyrin ring is partially saturated are the *d* heme in *E. coli* and siroheme of bacterial and plant sulfite and NiR (119, 326).

Nitrite reduction to NO is the physiologically relevant activity of cd_1NiR (289, 365); the expression of cd_1NiR is induced by low O_2 tension and presence of nitrogen oxides (380). NO is produced efficiently by Pa- cd_1NiR (turnover number = $6 s^{-1}$ at pH 7.0) (292) and the activity is pH dependent with an optimum between pH 5.8 and 6.5 (292, 365). The current knowledge on the individual steps in the catalytic cycle is summarized below and described in Figure 4D.

In cd_1NiR , nitrite binds to the ferrous (Fe^{2+}) d1-heme with high affinity ($K_m = 6 \mu M$) (81); binding of nitrite to the ferrous iron is expected in the reaction mechanism since this state of the iron has to supply the electron needed for the reduction process (Fig. 4D). In principle three different coordination modes of nitrite to monometallic centers are possible: the N-nitro, the O-nitrito, and the O,O-bidentate mode (Fig. 5) (364). The latter one is only observed in copper-containing

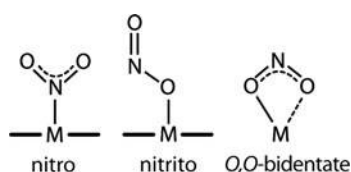


FIG. 5. Possible nitrite-binding modes. Experimentally determined nitrite-binding modes to metalloproteins.

bacterial NiR (16) and synthetic copper complexes (203). The N-nitro binding mode is observed in synthetic iron porphyrin nitrite complexes (64, 257, 363). Nitrite is thought to bind *via* the N-atom in Pa- cd_1NiR forming the so-called nitro-complex; this binding mode is inferred from the crystal structure of the nitrite-bound derivative of the homologous enzyme from *Paracoccus pantotrophus* (358). This observation agrees well with data on other heme-containing NiR (76, 105) and on synthetic iron porphyrin nitrite complexes in which, regardless of the iron oxidation state, the N-binding mode is observed (see Fig. 5) (64, 257, 363). The nitrite N-binding mode also agrees with the current mechanism of reduction of nitrite by cd_1NiRs , thought to occur *via* a double protonation of a terminal O atom of the nitrite molecule. Theoretical calculations have suggested that the O-binding mode is also possible for cd_1NiR (315) and for other hemoproteins such as hemoglobin (Hb) (272). Although nitrite can bind through the O-binding mode (the so-called nitrito mode) to the heme of myoglobin (Mb) and Hb (73, 367, 368), in the case of the d1-heme there is no experimental evidence that such O-binding mode may occur.

The high affinity for nitrite (and other anions such as cyanide) (166, 323) of the ferrous (Fe^{2+}) d1-heme is a peculiar and physiologically relevant feature of all cd_1NiRs . This behavior is remarkably different from that observed in the b-type heme-containing proteins in which the negatively charged molecules (nitrite and cyanide) usually bind much better to the ferric (Fe^{3+}) iron. The much higher affinity for nitrite of the d1-heme can be partially explained with the presence of two

electron-withdrawing carbonyl groups on the d_1 heme ring (Fig. 4B). Two conserved histidines (His327 and His369) in the active-site pocket (261) (Fig. 4C) also contribute to the stabilization of the nitrite anion (NO_2^-), as confirmed by site-directed mutagenesis (81).

In Pa- cd_1 NiR, nitrite can displace the NO bound to the ferrous enzyme (293), allowing the enzyme to enter a new catalytic cycle (Fig. 4D); therefore, the high affinity of the active-site ferrous d_1 -heme for nitrite (see above) actively contributes to NO dissociation during the catalytic cycle. In agreement with this observation, if the affinity of Pa- cd_1 NiR for nitrite is decreased (by mutation of a conserved active-site residue) the fully reduced-NO bound derivative accumulates (81, 293). The observation that NO and nitrite can compete for binding may suggest that the formation of dinitrogen trioxide (N_2O_3) could in principle occur, for example, in a reaction similar to that proposed for Hb (23). This event is, however, highly unlikely, mainly because during catalysis the d_1 -heme is maintained in the reduced state by internal electron transfer from the c -heme. Moreover, ferric d_1 -heme has low affinity for nitrite (329), a feature that likely limits further reaction with free NO to produce N_2O_3 .

In the catalytic cycle of Pa- cd_1 NiR, the formation of a complex between NO and the reduced d_1 -heme might slow down product release (11). Trapping of ferrous hemes by NO is highly likely, given the high affinity of this ligand for Fe^{2+} (182, 245). However, we have clearly shown that the rate constant of NO dissociation from the reduced d_1 -heme is fast (up to 70 s^{-1}) not only for Pa- cd_1 NiR (292) but also for the cd_1 NiR from *P. pantotrophus* (294). Consequently, the affinity of reduced Pa- cd_1 NiR for NO is relatively low ($\sim 10^{-7} \text{ M}$) and the ferrous enzyme is not firmly inhibited by NO (292, 293). Noteworthy, nitrite reduction can still be monitored after preincubation of reduced Pa- cd_1 NiR with NO (292).

The rapid dissociation of NO is largely controlled by the d_1 -heme cofactor itself, as recently shown using a complex of the d_1 -heme and apomyoglobin (294). This evidence underscores that the d_1 -heme has evolved to have low affinity for NO, as compared with other ferrous hemes. The results on the d_1 -heme suggest that the reactivity of porphyrins with nitrite and NO can be modulated very extensively by the functional groups present on the heme macrocycle—information of general significance in light of the emerging important biological functions of nitrite as a source of NO under hypoxic conditions.

III. Nitrite Reduction in Mammals

In this section we first describe the sources and the distribution of nitrite in mammalian tissues (section A) and then we discuss the chemical (abiotic) and biochemical (biotic) mechanisms of nitrite reduction in mammals (section B).

A. Sources, levels, and distribution of nitrite

There are several sources of nitrite in mammals, both endogenous and exogenous. The endogenous source of nitrite is mainly the oxidation of the NO produced by NOS (Fig. 6) (241). The oxidation of NOS-derived NO is slow *in vitro*, but is enhanced in plasma mainly by the copper protein ceruloplasmin (314) and in membranes by the accumulation of NO in the lipid bilayer by preferential partition (210).

The main exogenous source of nitrite is dietary nitrate, which is converted to nitrite by commensal bacteria in the

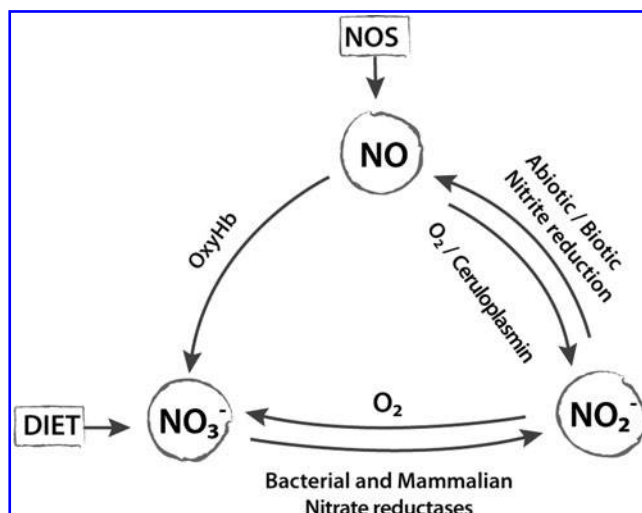


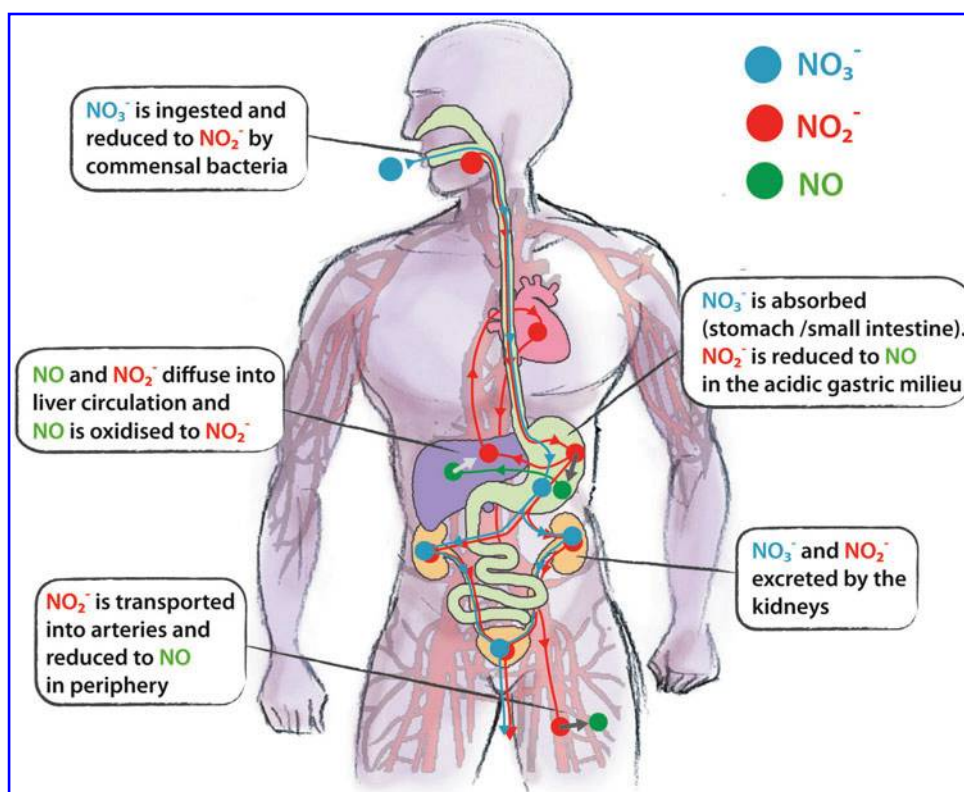
FIG. 6. Nitrogen-oxides metabolism. A percentage of the nitrate ingested with the food is reduced to nitrite by bacterial and mammalian Nar. Afterward, the nitrite produces NO *via* either biotic or abiotic reduction: the first one involves different putative NiR, such as deoxygenated hemoglobin (deoxyHb) or xanthine oxidase; the second one is enhanced by acidic conditions and reducing environments (*e.g.*, vitamin C). On the other hand, the production of NO in normoxia depends on the activity of the NOS enzyme. NO is rapidly oxidized to nitrite (by ceruloplasmin and O_2) or to nitrate (by oxygenated hemoglobin [oxyHb]) (215). Nitrite may also produce NO thanks to the oxidative denitrosilation activity of oxyHb (not shown in the figure).

mouth or intestines (Fig. 6). Dietary nitrate levels are particularly high in specific fruits and vegetables; as an example, one serving of spinach, lettuce, or beetroot contains more nitrate than that generated endogenously over a day by the oxidative metabolism of NO carried out by all isoforms of NOS (220). A considerable variability in the nitrate concentrations of plants is observed depending on different species, environmental conditions, nutrient availability, insect damage, and application of nitrogen-based fertilizers. On the other hand, only a small amount of nitrite is contained in dietary food (345); nitrite is used for meat preservation, to cure flavor and color and to enhance meat appearance (59, 219). In general, nitrite intake varies from 0 to 20 mg/day (270).

Nitrates and/or nitrites are also contained in drinking water ($< 10 \text{ mg/L}$ in the absence of bacterial contamination) (42). Other environmental sources of nitrate and nitrite include cigarette smoke (258) and car exhausts (219). These and other environmental pollutants contain volatile nitrogen oxides, some of which are converted to nitrate or nitrite in the body (219). The relative contribution from these different sources of nitrite during normal conditions is variable (218). Nitrite levels show considerable variation between individuals and are significantly affected by dietary habits and lifestyle (216): for example, plasma nitrite and nitrate may be lowered by about 50% by dietary restriction (288).

Dietary nitrate is rapidly absorbed from the gastrointestinal tract into the blood stream, and distributed throughout the body, mixing itself with the endogenous nitrate (219) (Fig. 7). Some nitrate is excreted and concentrated 10-fold in salivary glands (354): the amount of nitrate secreted in saliva is up to 25% of plasma nitrate level (320). The dorsal face of tongue

FIG. 7. Metabolic fate of nitrates and nitrites. Dietary nitrate (NO_3^-) is reduced to nitrite (NO_2^-) by bacterial Nar on the dorsal surface of the tongue and swallowed in the stomach. Nitrite is also reduced to NO under the acidic conditions of the gastric milieu and in the resistance vessels of the arterial circulation. The kidneys finally excrete excess nitrates and nitrites. Blue, red, and green lines depict the different physiological routes of nitrate, nitrite, and NO in the body, respectively.



harbors a specialized flora of symbiotic facultative anaerobic bacteria expressing enzymes that can reduce nitrate to nitrite (306) (Fig. 6). These bacteria use nitrate as an alternative terminal electron acceptor during respiration to produce ATP (356). The biologic effect of ingested nitrate, as well as the concomitant increase in plasma nitrite, is abolished after avoiding swallowing of saliva (216, 354) or by the use of an antibacterial mouthwash (135, 274). Therefore, if mammals were germ free, the endogenous or ingested nitrate would not be metabolized because of the lack of the enzymatic machinery for its reduction (42, 219).

The metabolic fate of dietary nitrite in humans is depicted in Figure 7. After ingestion, nitrite reaches the stomach, where it is reduced to NO. In the acidic gastric milieu, nitrite is protonated to form nitrous acid (HNO_2), which then decomposes to NO and other nitrogen oxides (27, 221). Low pH and reducing compounds, such as ascorbic acid and polyphenols, enhance this reaction (28, 122). The level of NO gas in the stomach can be considerable (more than 100 ppm) (356). Most of the salivary nitrite escapes from this conversion and diffuse in systemic circulation (216); nitrite is transported in the arterial circulation to resistance vessels, where the low O_2 tension favors the reduction of nitrite to NO, causing vasodilatation and lowering the blood pressure (354).

Plasma levels of nitrite are usually in the range 0.1–0.6 μM with a mean of $0.345 \pm 0.017 \mu\text{M}$ (128, 186, 216) (Table 1). The plasma concentration of nitrate is much higher (20–50 μM); as previously described, in the whole blood, nitrite is rapidly oxidized to nitrate (97, 241) (Fig. 6). Nitrate and nitrite increase greatly in saliva, plasma, and urina after a nitrate load (216).

Each tissue has a different concentration of nitrite (Table 2). As an example, inside the erythrocyte the levels are higher than in plasma (44, 86); given that the hematocrit represents between 40% (children) and 50% (adult males) of total blood

volume, the erythrocytes contribute the largest nitrite pool in whole blood.

The levels of nitrite in the body show significant variability due to differences in dietary habits, lifestyle (e.g., tobacco consumption), and physical exercise (37, 255, 265, 375). Circulating nitrite may be significantly enhanced in individuals suffering from an infection (332, 333) and markedly lowered during pregnancy. Plasma levels of nitrite also depend on NOS activity (186): in the presence of different NOS inhibitors, a change in vascular resistance occurs, paralleled by a reduction in plasma nitrite by $30\% \pm 8\%$ (186).

It is important to mention that the stationary levels of free nitrite or NO may also be influenced by the buffering effect of reduced glutathione (GSH), one of the main antioxidants present in cells and tissues. Under acidic conditions, nitrite reacts with GSH to form S-Nitrosoglutathione (GSNO), an endogenous S-nitrosothiol (SNO) (187, 337); on the other

TABLE 1. PLASMA NITRITE CONCENTRATION IN DIFFERENT MAMMALIAN SPECIES

Mammalian species	Nitrite (μM)
Human	0.305 ± 0.23
Monkey	0.367 ± 0.62
Minipig	0.319 ± 0.24
Dog	0.305 ± 0.50
Rabbit	0.502 ± 0.21
Guinea pig	0.412 ± 0.44
Mouse	0.457 ± 0.51

Data are taken from (186). Concentration of free nitrite (μM) in plasma in a variety of species has been measured using three different analytical approaches: flow injection analysis technique, reductive gas phase chemiluminescence, and sensitive high-pressure liquid chromatography techniques. Data are given as mean \pm standard error of the mean (SEM).

TABLE 2. NITRITE CONCENTRATIONS IN BLOOD AND TISSUES OF WISTAR RAT

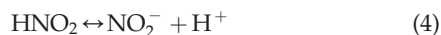
Localization	Nitrite (μM)
Blood	
Plasma	0.29 ± 0.05
Erythrocytes	0.68 ± 0.06
Tissues	
Brain	1.68 ± 0.31
Heart	0.77 ± 0.08
Liver	0.46 ± 0.06
Kidney	0.61 ± 0.09
Lung	0.45 ± 0.06
Aorta	22.5 ± 9.2

Data are taken from (44). Copyright (2004) National Academy of Sciences, USA. Nitrite levels were measured by high-performance liquid chromatography (HPLC) (284). Mean \pm SEM from 10–14 animals.

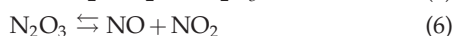
hand, GSNO may be formed by reaction of GSH with N_2O_3 , with production of nitrite and protons, or, to a lower extent, with peroxynitrite (ONOO^-) (25, 183, 344). GSNO, in turn, is one of the most relevant biological molecules to carry out nitrosation reactions under physiological conditions (9, 373).

B. Mechanisms of nitrite reduction

1. Abiotic nitrite reduction: solution chemistry of nitrite. Nitrite is the conjugate base of HNO_2 [$\text{pK} \sim 3.1\text{--}3.2$ (83)]. Therefore, at physiological pH, HNO_2 is essentially in the deprotonated form, the NO_2^- , according to the following equation:



At lower pH values, HNO_2 decomposes to various nitrogen oxides, including N_2O_3 , which can dissociate to NO and NO_2 ($K = 2 \times 10^{-5} \text{ M}$) (335).



Moreover, the reduction of nitrite in aqueous solution strictly depends upon pH, according to the following reaction:



Therefore, the so-called disproportionation of nitrite to NO, promoted by acidic pH, includes these reactions; this abiotic pathway is responsible for the production of NO in the gastric milieu of humans (27, 221, 230). The disproportionation of nitrite to NO is enhanced in the presence of reducing agents such as ascorbate (vitamin C) (213, 239) and other compounds such as polyphenols (122, 271, 295). Interestingly, disproportionation of nitrite to NO in biological tissues is enhanced under ischemic conditions when the pH drops from the normal value (7.4) to values as low as 5.5 (304, 382).

2. Biotic nitrite reduction. Several examples of NiR activities have been found in blood, tissues, and mitochondria. All of them can be ascribed to proteins, which, under aerobic conditions, play an O_2 -dependent biological role but turn into NiR under hypoxic conditions.

a. Hemoglobin. Hb is the heme-containing metalloprotein involved in O_2 transport in humans. It is located in the erythrocyte, where it binds and releases O_2 in response to the partial pressure of this gas. The heterotetrameric adult HbA ($\alpha\text{2}\beta\text{2}$) exists in two quaternary conformations, R-state and T-state, which display different affinity for the heme ligands, including O_2 and nitrite. Hb has been assigned a central role in the physiological reduction of nitrite: Hb binds and reacts with nitrite in both deoxygenated (deoxyhemoglobin [deoxyHb]) and oxygen-bound forms (oxygenated hemoglobin [oxyHb]), yielding different intermediate species and products, as detailed below.

(1) Reaction of deoxyHb with nitrite. The ability of deoxyHb to act as an NiR and produce NO was known since the pioneering work of Brooks (36). In blood, the reaction of nitrite with deoxyHb has been proposed to represent a source of NO bioactivity, according to the following reaction (129):



The Hb-dependent NiR activity is allosterically controlled by the quaternary structure of the protein (Fig. 8) (53, 129). The bimolecular rate constant of the reaction varies as the allosteric conformation of Hb changes: the R-state Hb has a bimolecular rate constant of $6 \text{ M}^{-1} \text{ s}^{-1}$, whereas the constant for T-state Hb is $0.12 \text{ M}^{-1} \text{ s}^{-1}$, giving the reaction an average bimolecular rate constant of $0.35 \text{ M}^{-1} \text{ s}^{-1}$ (pH 7.4, 25°C) (156). The R-state Hb is a better reductant due to a more negative redox potential and/or to a more accessible heme pocket relative to T-state Hb (30, 77, 302).

The maximal rate of nitrite reduction is reached at the $p\text{O}_2$ value of about $35 \mu\text{M}$, which corresponds to the P_{50} (i.e., the $p\text{O}_2$ at which Hb is half-saturated) (Fig. 8). The observed kinetics of the reaction is the combination of two processes, where the rate of nitrite reduction increases with increasing O_2 fractional saturation in parallel with the increased R-state character of Hb; at high fractional saturation, the concentration of deoxyHb (one of the substrates) decreases, thereby slowing down the rate. This peculiar chemistry has been described as an allosteric autocatalytic reaction (130, 156), where

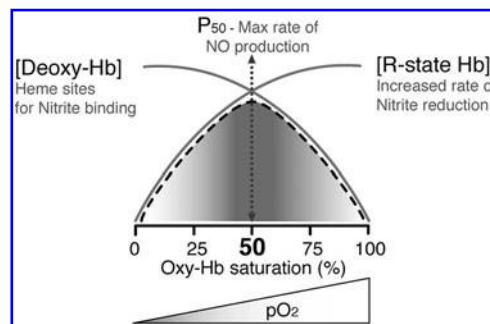
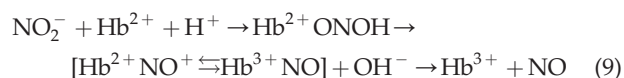


FIG. 8. Schematic representation of the NiR activity of hemoglobin (Hb). The NiR activity, depicted as a gray area, can be described as a bell-shaped curve in which the maximal rate of NO generation is reached at the P_{50} , when Hb is 50% saturated with O_2 . The NiR activity is allosterically controlled by the quaternary structure of Hb and depends on the oxygen tension ($p\text{O}_2$). The increase in $p\text{O}_2$ decreases the amount of deoxyHb available to bind nitrite and, at the same time, increases the fraction of R-state Hb, which reduces nitrite to NO more efficiently. Figure modified with permission from (220).

autocatalysis is controlled by the allosteric transition of the Hb tetramer from the T- to the R-state. Stabilization of Hb in either the T- or R-state (by chemical cross-linking) has recently confirmed this interpretation (53).

Accordingly, fetal Hb, in which the γ -subunits replace the β -subunits and the R-state is favored, shows an increased efficiency of nitrite reduction (30). Conversion of nitrite to NO in the fetus is thus favored (i) by the molecular properties of fetal Hb and (ii) by the fetal arteriovenous oxyHb concentrations ($\sim 75\%$ to 45%) that fall in the range where nitrite reduction is maximal. The reaction of Hb with nitrite is potentially of great importance for the fetus because it provides an O_2 -sensitive mechanism for NO production in the vasculature and may contribute to maintain the low resistance to blood flow, characteristic of the fetal circulation. However, taking into account that tissues are generally more effective than blood in reducing nitrite to NO (113, 204) (see also below), it remains to be demonstrated whether reduction in blood or in tissues is more important for the low vascular resistance in fetal circulation.

The exact molecular mechanism of nitrite reduction by deoxyHb is still a question of debate. A possible reaction pathway has been suggested (251, 252, 290, 291, 301) and is summarized in the following scheme:

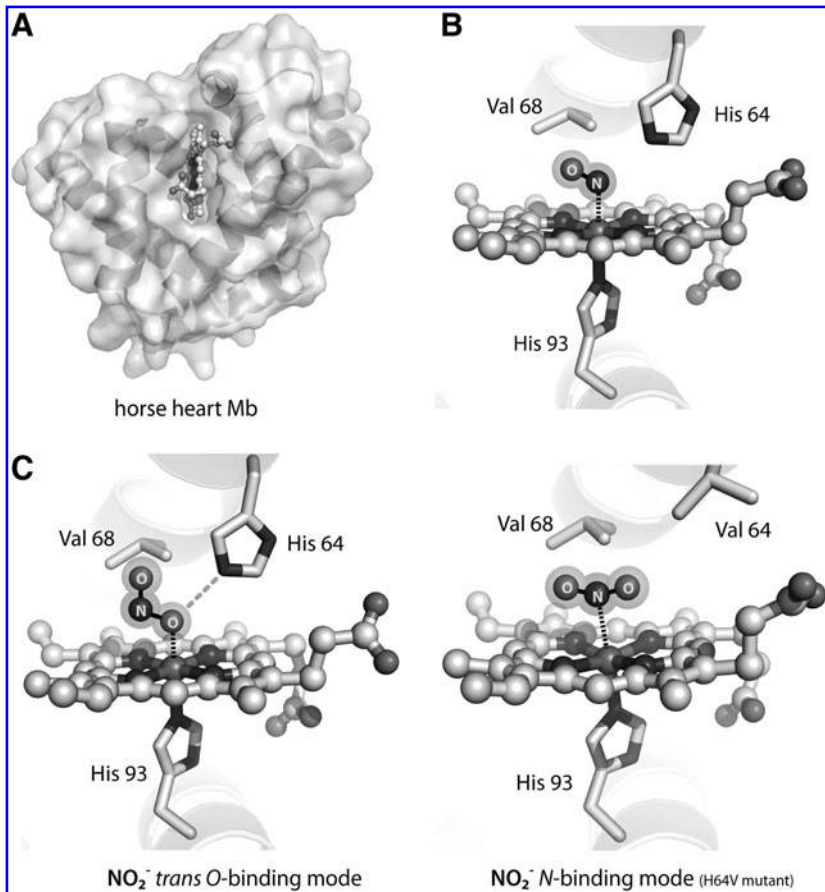


In this scheme, the initial intermediate is the nitrite-associated Hb, while the second one has an electron delocalized between the heme iron and the ligand ($\text{Hb}^{2+} \text{NO}^+ \rightleftharpoons \text{Hb}^{3+} \text{NO}$)

and is stable even in the presence of a large excess of oxyHb and deoxyHb, particularly at low nitrite/Hb ratios found *in vivo*. The release of NO in this reaction is facilitated by excess nitrite and is conformationally regulated: the R-state quaternary conformation favors the formation of the second intermediate, whereas the T-state quaternary conformation favors the release of NO from the intermediate. This observation has been explained in terms of a conformational effect on the distal heme pocket, involving hydrogen bonding of nitrite to the distal histidine (291).

Despite the relevance of the interaction between nitrite and the heme iron, no crystal structure of the ferrous (Fe^{2+}) iron-nitrite complex of heme globins (including Hb) is available. The only adducts for which crystal structures are available are the nitrite-bound ferric (Fe^{3+}) complex of Hb at 1.8 Å resolution (368) and of Mb at 1.2 Å resolution (73) (Fig. 9). Interestingly, in both cases, the nitrite molecule is bound in the O-nitrito mode (Fig. 5); the ligand is stabilized by a hydrogen bond to the distal histidine residue. The hydrogen bonding capability of the distal heme pocket is crucial to direct the binding mode of nitrite: substitution of His64 with Val in Mb leads to the reorientation of the nitrite molecule to the N-nitro binding mode (Fig. 9C) and decreases the rate of nitrite reduction (366). Altogether, the existence of the N- and O-binding to hemoproteins (see also section on bacterial NiR) may suggest that the efficiency of nitrite reduction could be a function of the binding mode of the nitrite molecule. Thus, in principle, the mechanism of the reaction with deoxyHb should involve the formation of a nitrite complex of ferrous heme (either N- or O-bound), followed by proton transfer by

FIG. 9. Nitrite and NO binding modes in horse heart myoglobin (Mb). (A) Surface representation of horse heart Mb (hhMB) [pdb id: 2frf (73)]; the heme is shown as balls and sticks. (B) NO bound to Mb [pdb id: 2frk (73)]. (C, Left panel) Nitrito derivative: nitrite is bound to the heme iron through one of the O_2 atoms: *trans* O-binding mode [pdb id: 2frk (73)]. His-64 forms a hydrogen bond with the same O_2 atom of the nitrite molecule. (C, Right panel) Nitro derivative: nitrite is bound to the heme iron through the nitrogen atom: N-binding mode [pdb id: 3hep (366)]. The nitro derivative was observed only after substitution of the distal His64 with Val (H64V mutant of hhMB).



a nearby histidine. In the case of the N-nitro complex, dehydration of nitrite then yields the Hb (Fe³⁺)-NO derivative; on the other hand, starting from the O-nitrito complex the final derivative is the Hb (Fe³⁺)-OH complex, and thus NO is released. Regardless of the mechanism employed, one of the products of the reaction is methemoglobin (MetHb or Hb³⁺), whose accumulation is associated with the pathological status named methemoglobinemia (see section on nitrite therapy for detailed discussion). However, metHb can be recycled to Hb, thus yielding an enzymatic conversion of nitrite into NO (179), by the NADH-cytochrome b5 reductase system. There are two forms of the NADH-cytochrome b5 reductase in humans: a soluble, erythrocyte-restricted form, which is active in metHb reduction, and a ubiquitous membrane-associated form involved in lipid metabolism. Genetic alterations of these genes are associated with congenital methemoglobinemia due to an enzyme defect in the reductase activity (179).

In summary, deoxyHb reacts with nitrite to produce NO. The main conundrum of the reaction of deoxy Hb with nitrite as a source of bioactive NO lies in the expectation that the NO produced is likely to be either immediately oxidized to nitrate by reaction with oxyHb or trapped by the excess of deoxyHb, yielding a stable ferrous-nitrosyl complex ($k_{\text{off}}\text{NO} = 10^{-3}/10^{-5} \text{ s}^{-1}$) (182, 245). Possible chemical tricks to overcome this problem include the oxidative denitrosilation carried out by nitrite itself and the nitrite anhydrase activity of Hb forming N₂O₃, which may diffuse out of the erythrocyte, later forming NO or acting by nitrosylating a thiol. Both possibilities are analyzed in more detail below.

(2) Reaction of nitrite with oxyHb and oxidative denitrosylation. Nitrite can react with oxyHb in a complex reaction to produce metHb and nitrate (184, 190). Thus, by reacting with oxyHb, the majority of NO and nitrite end up as nitrate, which may serve as another storage form of these N-oxides.

The reaction of nitrite with oxyHb does not produce NO directly; however, as it will become clearer below, it is relevant to analyze the mechanism of this reaction and the possible crosstalk with the reduction of nitrite with deoxyHb, given that, under oxygenated conditions, the two reactions (*i.e.*, with deoxyHb and oxyHb) compete with one another (184).

In the reaction of nitrite with oxyHb, the rate of metHb production is about $0.5\text{--}1 \text{ M}^{-1}\text{s}^{-1}$ (345). This reaction has an autocatalytic kinetics, as it is initially slow (in the lag or induction phase) but then enters a rapid autocatalytic phase involving radical-mediated chain reactions and branching steps (96, 98, 180, 209, 351). The reaction can be divided into at least two steps, that is, initiation and propagation: the initiation step yields metHb and hydrogen peroxide (H₂O₂), which then react to produce the FerrylHb (Fe^{IV}=O) radical. In the second step, oxidation of Ferryl-Hb by nitrite produces the nitrogen dioxide (NO₂•) radical, which reacts again with oxyHb to produce nitrate and ferrylHb, resulting in an autocatalytic loop (propagation step) (180).

The intermediate species of the nitrite/oxyHb reaction, most probably (NO₂•), can also oxidize (Fe²⁺)Hb-NO, thus releasing NO in the so-called oxidative denitrosylation (22, 136). However, given that the nitrite levels in the erythrocytes and plasma are in the sub-micromolar range, it is unlikely that the reaction can proceed to the autocatalytic step *in vivo*, and thus low levels of NO₂• are produced.

(3) The nitrite anhydrase reaction of Hb. An attractive hypothesis to explain NO bioactivity from nitrite is the formation of a carrier molecule, less reactive and more easily diffusible, which can reach the target tissue and be converted again to NO. A likely candidate is N₂O₃, which can produce NO (see below) and is also able to form nitrosothiols (357) adding an extra possibility of chemical signaling. Formation of N₂O₃ might be readily explained by the nitrite anhydrase reaction of MetHb:



Different possibilities for metHb-catalyzed formation of N₂O₃ have been proposed, involving initial formation of an Fe³⁺-nitrite (23) or Fe³⁺-NO (116) complex. A recent theoretical study of the various mechanistic alternatives (151) shows that both pathways of Fe³⁺-mediated N₂O₃ formation are energetically feasible. N₂O₃ back-conversion to NO has also been proposed to be mediated by Hb (23).

The formation of N₂O₃ is supported experimentally by the formation of S-nitrosothiols *in vivo* and *in vitro* (23, 212, 252, 311). It is thus likely that Hb may function *in vivo* as a nitrite anhydrase and that N₂O₃ may be an important player in nitrite-mediated NO and S-nitrosothiol signaling (see also section IV).

b. The other globins: Mb and neuroglobin. Mb is one of the most extensively studied hemoproteins; it is a monomeric globin bearing a single b-heme group. Mb concentration in human skeletal and cardiac muscle is as high as 200–500 μmol/kg wet tissue (362).

Mb has been implicated not only in the storage and facilitation of O₂ diffusion (236, 360, 361), but also in the scavenging of NO to protect mitochondrial respiration (39, 118). The major mechanism of attenuating intracellular NO bioactivity in cardiac muscle is the reaction of MbO₂ with NO to give metmyoglobin (metMb) and nitrate. Mb-deficient (myo^{-/-}) mice are more sensitive to endogenously formed and exogenously applied NO; regeneration of metMb by metMb reductase to Mb and subsequent association with O₂ leads to reformation of MbO₂ available for another NO degradation cycle.

Therefore, the reactivity of Mb with different ligands depends upon O₂ concentration: under normal O₂ levels, Mb mainly acts as an O₂/NO binding protein. Accordingly, Mb displays a functional relevance in O₂ supply and NO scavenging on the whole animal level: loss of Mb leads to impaired myocardial contractile function and exercise endurance (233).

When the O₂ concentration decreases to a value around the P₅₀ of Mb (3–4 μM), the protein becomes significantly deoxygenated; under these conditions, Mb is able to reduce nitrite to bioavailable NO in the red muscle and in the heart (312). Therefore, Mb comes into play as an NiR mainly under hypoxic or ischemic conditions (144). Mb has distinct properties from Hb as an NiR: first of all, it has a very low redox potential, and therefore it reduces nitrite ~50 times faster than T-state Hb (154). Moreover, since Mb is a monomer without allosteric behavior, the reaction of nitrite with deoxyMb is a second-order reaction with a bimolecular rate constant of 6–12 M⁻¹s⁻¹ (between 25°C and 37°C, pH 7.4); the products of the reaction are equimolar amounts of metMb (Fe³⁺) and iron-nitrosyl-Mb (312). Using an Mb-knock out mouse model, Hendgen-Cotta and coworkers (145, 285) provided

unequivocal evidence that deoxyMb reduces nitrite to form NO that regulates mitochondrial respiration and cardiac contractility during hypoxia and ischemia/reperfusion.

Interestingly, both neuroglobin (Ngb) (41) and cytoglobin (273) have low heme redox potential and high O₂ affinity, suggesting similar properties as Mb in terms of nitrite reduction. This hypothesis has been recently investigated for Ngb, a highly conserved hemoprotein that evolved from a common ancestor to Hb and Mb. Ngb possesses a six-coordinate heme with proximal and distal histidines ligands; coordination of the sixth ligand is reversible. Gladwin and coworkers have recently shown that deoxygenated human Ngb reacts with nitrite to form NO (330). This reaction is regulated by two redox-sensitive surface thiols (cysteine 55 and 46) controlling the fraction of five-coordinate heme together with nitrite binding and NO formation. Replacement of the distal histidine by leucine or glutamine leads to a stable five-coordinate geometry; these Ngb mutants reduce nitrite to NO ~2000 times faster than the wild type, whereas mutation of either Cys-55 or Cys-46 to alanine stabilizes the six-coordinate structure and slows down the reaction. The NiR activity of Ngb was found to inhibit cellular respiration *via* NO binding to cytochrome c oxidase (Cox), thus suggesting that Ngb may function as a physiological oxidative stress sensor and a post-translationally redox-regulated NiR. Therefore, NO generation by Ngb is controlled by the transition from six-to-five-coordinate heme state (138, 330). The authors also speculate that the six-coordinate heme globin superfamily may serve a function as primordial hypoxic and redox-regulated NO-signaling proteins. This hypothesis is in agreement with the observation that also mitochondrial cytochrome c can act as an NiR only in the five-coordinate state.

c. Xanthine oxidoreductase and aldehyde oxidase. In tissues, nitrite-derived NO production is due to the activity of both xanthine oxidoreductase (XOR) and aldehyde oxidase (AO) (aldehyde: O₂ oxidoreductase), belonging to the family of molybdenum-containing hydroxylases (232), classified under a single EC number (EC 1.2.3.1). These enzymes require, for their catalytic activity, flavin adenine dinucleotide (FAD) and a particular form of organic molybdenum, known as the molybdenum cofactor (MoCo). MoCo is a molybdopterin in eukaryotes, while it is a molybdopterin nucleotide in prokaryotes.

XOR enzymes have been isolated from a wide range of organisms, from bacteria to human, and catalyze the hydroxylation of a wide variety of purine, pyrimidine, pterin, and aldehyde substrates. The mammalian enzymes, which catalyze the hydroxylation of hypoxanthine and xanthine, the last two steps in the formation of urate, are synthesized as the dehydrogenase form (xanthine dehydrogenase [XDH]) and exist mostly as such in the cell but can be readily converted to the oxidase form (xanthine oxidase [XO]) by reversible oxidation of critical cysteine residues (535 and 992) or limited proteolysis (108, 149). Conversion of XDH to XO is enhanced by hypoxic conditions and ischemia (299). XDH shows a preference for NAD⁺ reduction at the FAD reaction site, whereas XO fails to react with NAD⁺ and exclusively uses dioxygen as its substrate, leading to the formation of superoxide anion and H₂O₂.

Mammalian XO is a complex homodimer (Fig. 10); in addition to MoCo, two different [2Fe–2S] centers and one FAD are present in the enzyme (35, 148). The ability of XO to

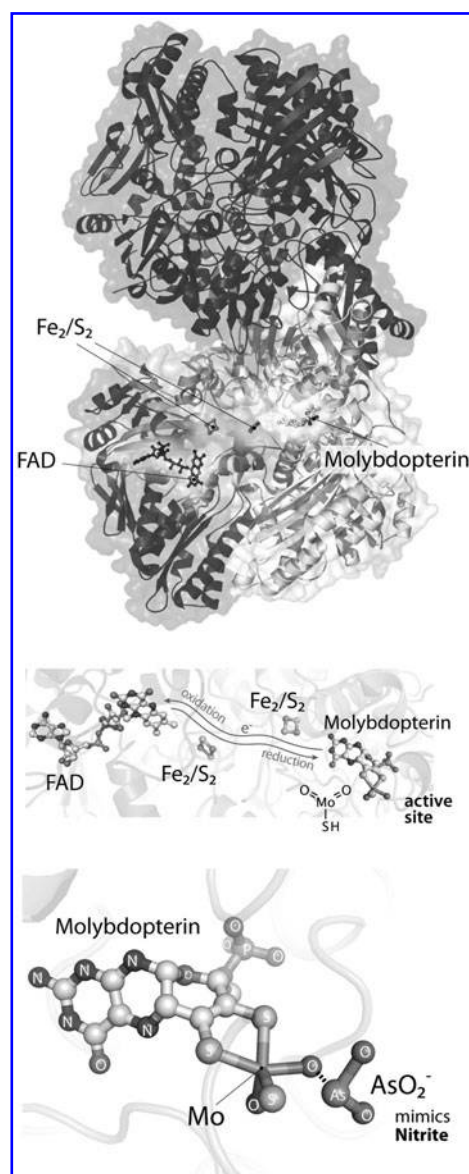


FIG. 10. Structural organization of bovine xanthine oxidase. This enzyme is a complex molecule formed by two symmetric trimers. Each trimer binds several specialized molecular tools for handling electrons. At one end of the enzyme, the purine substrate binds to an active site that includes a molybdenum atom. A hydroxyl group is added to the substrate and the electrons are funneled by a string of iron–sulfur clusters from the purine active site to the opposite side of the enzyme, which ultimately transfers the electrons to NAD or O₂; the electron flow will revert when the enzyme works as a reductase. The figure illustrates the relative position of the different cofactors. The structure of xanthine oxidase is obtained in the presence of sodium arsenite, which contains a polymeric linear anion [AsO₂[−]]_n (pdb id: 3nvv). The arsenic atom is bound in the form of AsO₂[−] anion, in which the AsIII atom is pyramidally coordinated with the two bound O₂ atoms and with the terminal O₂ of molybdopterin (200). It is highly likely that nitrite binds to the active site in the same position. FAD, flavin adenine dinucleotide.

catalyze, under normoxic conditions, the reduction of nitrate is well recognized (93, 120, 235); evidence for Nar activity in an endothelial NO synthase-deficient and germ-free mice highlights the contribution of XOR to the overall nitrite levels and nitrite homeostasis (Fig. 6) (167). Expression of XOR in the liver is increased in germ-free mice compared to conventional animals, which may explain the apparently greater tissue Nar activity observed in the germ-free animals (155), representing a compensatory response to uphold nitrite homeostasis in the absence of commensal bacteria.

The XO-catalyzed reduction of nitrite to NO has also been reported over the last decade (132, 235, 376). This activity has been proposed to be a major source of NO in tissues (204) and to exert a protective role during myocardial infarction and ischemia-reperfusion (I/R) damage (13, 353). In rat and mouse models of pulmonary hypertension, sodium nitrite is converted to biologically active NO *via* reduction in large part by XOR; in these model systems, NO production was attenuated by allopurinol (200 μ M), an inhibitor of XOR (379).

The reaction catalyzed by XO is



During the reduction of nitrite, one O₂ atom is abstracted from the nitrite molecule, resulting in the production of NO: under anaerobic conditions, NADH and xanthine are used as reducing agents. Involvement of the molybdenum site of XO in nitrite reduction was shown by the fact that alloxanthine inhibits xanthine oxidation competitively with nitrite (132). Moura and coworkers have recently shown that the molybdenum metal center is the direct electron donor to nitrite (225). The K_m for nitrite is in the mM range and the reduction is dependent on the concentration of O₂, which acts as a competitive inhibitor (207). Despite these lines of evidence, under hypoxia and anoxia, the acidosis and the increased concentrations of xanthine and NADH will probably be sufficient to support the XO-mediated nitrite reduction physiologically, as supported by *in vivo* studies (13, 102, 353).

Interestingly, also the mammalian AO was shown to catalyze the reduction of nitrite to NO. AO is a cytosolic enzyme that plays an important role in the biotransformation of drugs and xenobiotics (371). AO is present in highest levels in the liver but is also broadly distributed in other tissues, such as lung, blood vessels, heart, and kidney (26, 247). Similarly to XOR, also AO contains two iron-sulfur clusters, a flavin cofactor, and a molybdopterin cofactor. The K_m for nitrite of AO is 3 mM (205) but the affinity for NADH (K_m = 24 μ M) is much higher than that of XOR (K_m = 0.9 mM). Therefore, this pathway would be predicted to better retain its nitrite reduction ability in the presence of O₂: AO-mediated NO generation could exceed the NO generation from XOR in the lung and approach that from XOR in the heart and liver under anaerobic conditions.

d. Other mammalian proteins acting as NiR. In the last decade, multiple proteins, besides those discussed above, have been implicated in nitrite reduction to NO. As an example, nitrite reduction by mitochondria under low O₂ concentrations has been reported and ascribed by different authors to Cox (mitochondrial respiratory complex IV), to mitochondrial complex III (191) and to the soluble electron carrier cytochrome c.

Castello *et al.* (60) have reported that yeast and rat liver mitochondria produce NO at O₂ concentrations below 20 μ M.

This NO production is nitrite dependent, is carried out by Cox in a pH-dependent fashion, and is accompanied by an increase in protein tyrosine nitration. The ability of Cox to reduce nitrite in yeast can be modulated by O₂ by altering the subunit composition of the complex: the presence of the isoform COX5b of subunit V, preferentially expressed at low O₂ tensions, enhances NO production (61). The authors suggest a positive feedback mechanism in which mitochondrially produced NO induces expression of COX5b, whose protein product then functions to enhance the ability of Cox to produce NO in hypoxic/anoxic cells. The NO generated in the mitochondria by Cox might be released from cells, thereby reaching external targets (280). Interestingly, it has been reported that the nitrite-derived NO synthesis catalyzed by Cox is enhanced by low intensity light, offering a new explanation for the increase in NO bioavailability experienced by tissue exposed to light (14, 279).

In agreement to what has been observed for the six-coordinate hemoprotein Ngb previously discussed, also cytochrome c is able to reduce nitrite to NO when in the five-coordinate state (22, 62). These data ascribe a possible role for cytochrome c as an NiR, possibly relevant in the hypoxic, redox, and apoptotic signaling pathways within the cell.

Other possible NiR at the tissue level include the ubiquitous enzyme carbonic anhydrase (CA), a crucial player in CO₂ transport (1), and rat liver cytochrome P450s (206), a family of proteins involved in the metabolism of xenobiotics (including organic nitrates). In the latter case, mammalian cytochrome P450 reductase (CPR) and cytochrome P450 cooperate to function in a sequential manner to produce nitrite and then NO and nitrosothiols, serving as the link between organic nitrates and NO-mediated signaling.

Altogether, these lines of evidence reinforce the idea that multiple proteins may function as NiR under low O₂ tension; however, the precise regulatory pathways controlling these activities is still far from being understood.

IV. Significance of Nitrite in Human Health and Disease

In this section we first discuss the involvement of nitrite in vasodilation, focusing on its role as a source of NO (section A); then, we analyze the cytoprotective effects of NO in the I/R injury and the role of nitrite as endocrine reservoir of NO (section B); finally, we briefly describe the other NO-independent physiological activities of nitrite (section C).

A. Nitrite in vasodilation

The primary function of vasodilation is to increase blood flow in the body to tissues that need it most; if the supply of O₂ is not sufficient, for example, in a working muscle or under hypoxia, an increase of O₂ delivery must occur, to selectively distribute the blood according to variable needs (90, 303, 339).

Recent evidence suggests that nitrite is a putative physiological signaling molecule with a potential role in hypoxic vasodilation, signaling, and cytoprotection after I/R (130). Several studies have suggested and confirmed the involvement of nitrite in vasodilation in humans, also in the presence of NOS inhibitors (74); the effect of nitrite has been confirmed in mouse, rat, sheep, and primates (29, 88, 338). Interestingly, intra-arterial infusion of nitrite during normoxia and hypoxia has shown that arteries are modestly affected under normoxic

conditions, but are potentially dilated under hypoxic conditions (224).

Nitrite-dependent effects on vasodilation are mostly mediated by NO, which stimulates soluble guanylate cyclase (sGC), thereby increasing cGMP levels, activating cGMP-dependent protein kinase and producing smooth muscle relaxation (130, 241). A physiologic NO-dependent post-translational regulation of vascular sGC in mammals involving S-nitrosylation as a key mechanism has recently been suggested (264). NO can also relax smooth muscle by cyclic GMP-independent mechanisms, including the direct modifications of sarco/endoplasmic reticulum calcium ATPase (SERCA), the enzyme essential for the control of intracellular free Ca^{2+} levels. NO modification causes, in smooth muscle cells, reduction of intracellular Ca^{2+} levels and, consequently, vascular relaxation (331). It has been demonstrated that NO, *via* ONOO⁻ and N_2O_3 formation, can adduct GSH to SERCA cysteine thiol; this modification predominantly activates SERCA and refills Ca^{2+} stores in sarcoplasmic reticulum. This NO-dependent mechanism of SERCA modification is crucial for proper vascular relaxation; in altered redox-state background such as in atherosclerotic arteries, NO does not stimulate SERCA activity because of the irreversible oxidation of the target cysteine thiol by the high levels of oxidants accompanying the disease (331).

A key role in the control of hypoxic vasodilation by nitrite has been assigned to the erythrocyte (100, 107, 130), mainly due to the NiR activity of deoxyHb, previously discussed (74, 91, 170). An open question is how NO escapes the erythrocytes to exert its vasodilatory effect. Different potential mechanisms could be operative (130): one hypothesis suggests that the NO escape is inefficient, but sufficient to regulate vascular tone due to lipophilicity and potency of the molecule. Another hypothesis is that the erythrocyte membrane provides a potential NiR metabolon, a system that would concentrate chemical reactants, nitrite, protons, and deoxyheme with highly hydrophobic channels. A third solution is that nitrite reduction produces intermediate(s) that could facilitate the transport of NO, such as N_2O_3 . Finally, the formation of S-nitrosothiols can also occur (130, 170). The formation of Hb derivatives, such as the S-nitrosated Hb, as a possible reservoir of NO bioactivity has been proposed (172, 321), but was recently critically analyzed and disputed by *in vivo* experiments in rats (168).

Nitrite-dependent vasodilation at low pH may also occur in a protein-independent pathway. For more than a century, it was known that acidic conditions allow vascular smooth muscle relaxation; indeed, to date, it has been suggested that abiotic reduction of inorganic nitrite to NO, a phenomenon known as acidic metabolic vasodilation, regulates local blood flow under hypoxia or ischemia (213, 238).

B. Nitrite-based cytoprotection in I/R injury

I/R injury is the major cause of death and illness in the Western World (281). This injury consists of multifaceted cellular events that take place on the recovery of O_2 delivery after a period of hypoxia. The heart, the kidneys, and the brain are among the organs that are the most quickly damaged by loss of blood flow. Insufficient blood supply to the myocardium can lead to myocardial ischemia infarction; timely restoration of the blood flow to the acute ischemic myocardium is

essential to reduce morbidity and mortality of the patients. However, the process of reperfusion after an ischemic episode can paradoxically lead to a unique form of damage, termed myocardial reperfusion injury. As initially observed by Hearse *et al.* (141), the reoxygenation required during reperfusion of ischemic myocardium generates reactive oxygen species (ROS), which trigger cellular injury (340, 341).

One of the most effective strategies, when applicable, to preserve tissues from I/R damage is the ischemia preconditioning, consisting of short periods of ischemia followed by reperfusion before a long ischemic period (250, 281); this setting restricts its potential clinical utility to planned acute myocardial I/R injury such as coronary artery bypass graft surgery, cardiac transplantation, and percutaneous coronary intervention (355). The ischemia preconditioning guarantees the activation of the protective signaling pathway (ROS-induced, see below) at the time of the reperfusion, which is able to reduce tissue infarction significantly (32, 281); the same protective effect (reduction of infarct size and attenuation in inflammatory response) can be obtained by ischemic post-conditioning (378).

Under normoxic conditions, the mitochondrial respiratory chain produces a small amount of ROS and reactive nitrogen species, which are scavenged by different antioxidant enzymatic systems and compounds (24). On the other hand, after ischemia, reperfusion of ischemic tissue leads to an exceptional production of ROS: this oxidative burst depletes or damages the pool of antioxidants available, causing tissue injury (106, 281, 377). More in detail, during ischemia, the electron transport chain functions as a reservoir of electrons since O_2 availability is limited. This high reductive potential, particularly for complex I and III, promotes incomplete O_2 reduction, and thus radical formation, when a massive entrance of O_2 occurs during reperfusion (47). Incomplete O_2 reduction during reperfusion leads to the production of superoxide anion, H_2O_2 , and hydroxyl radical to levels that are 1 to 2 orders of magnitude higher than those detected in non-ischemic background (381).

It should be mentioned that other enzymes participate in ROS generation during I/R injury, such as NADPH oxidase, NOS (during substrates depletion), and XO; in the latter case, during ischemia, ATP is predominantly metabolized as hypoxanthine and xanthine, which reacts with XO (whose formation from XDH is favored in postischemic heart) upon reoxygenation, resulting in superoxide generation (381).

In the last years, many studies demonstrated that NO production is associated with cytoprotection against I/R injury in many organs, such as the heart, liver, lungs, and kidneys (174, 275). One of the first evidence associating NO with cardioprotective effect was obtained 20 years ago by using acidified nitrite, which was shown to exert a significant protective action during ischemia and reperfusion injury (173). A relative decrease in NO bioavailability appears to be central in the pathogenesis of this injury, and therapeutic strategies aimed at replacing NO by inhalation, nitrite/nitrate anion supplementation, or *via* donor drugs represent a novel means for ameliorating I/R injury (275, 318).

The mechanism activated during ischemic postconditioning to minimize ROS-dependent tissue damages during I/R injury has been extensively studied in last two decades (91); even though the molecular details controlling this phenomenon remain to be deciphered, Bolli and coworkers demonstrated that

NO plays a prominent role in mediating the cardioprotective response (33). The authors demonstrated that a brief ischemic stress causes a burst of NO production as well as $\cdot\text{O}_2^-$ production, which in turn could then react to form ONOO^- ; this reaction triggers a complex signal cascade involving the protein kinase C (PKC ϵ) and the transcription factor NF- κB (33). ONOO^- formation prevents further ROS production occurring *via* the Fenton reaction and, despite the cytotoxic effects observed in the presence of excess of ONOO^- , its decomposition produces intermediates, which are scavenged by NO itself (359). This reaction ultimately results in production of the nitrosating species N_2O_3 , which in turn contributes to the overall NO-dependent cellular response (359). As demonstrated both *in vivo* and *in vitro* by using chemical models, this nitrosative stress provide an optimal antioxidant environment rather than toxic species, indicating that NO acts to counterbalance oxidative stress, beside its role in controlling signaling events *via* ONOO^- formation (85, 328).

NO modulates a plethora of processes during I/R injury, including inflammation, ROS formation, and apoptosis (Fig. 11). NO can also exert its cytoprotective role by inhibiting caspase through S-nitrosation and *via* cGMP to avoid the release of cytochrome c from mitochondria, thus preventing apoptosis (91). NO inhibits calcium overloading, responsible for the opening of a nonselective mitochondrial permeability transition pore, which uncouples oxidative phosphorylation and worsens ions/energetic homeostasis, leading to cell death by necrosis or apoptosis (277). At the same time, it is known that NO reversibly inhibits complex IV (40, 72) and, more importantly, complex I (38). Inactivation of complex I involves mainly S-nitrosation of critical thiols (68); since this modification is reversible, the inhibition of complex I is slowly reduced over time, thus allowing the respiratory chain to gradually recover the normal electron transfer between complexes, without the deleterious instigation of oxidative

damage (248) (see below). Inhibition of complex I activity seems to be crucial for cytoprotection during I/R injury being the inhibited form of this enzyme unable to generate ROS (47) and involved in limiting calcium overload (277). Moreover, as mentioned above, the respiratory chain is directly involved in the induction of some hypoxic nuclear genes (hypoxic signaling); as proposed recently (60), mitochondrially produced NO functions in a signaling pathway to the nucleus *via* ONOO^- , which may directly or indirectly modify specific proteins and activate hypoxic signaling.

Thus, the concept of reversible ROS inhibition in mitochondria during the early phases of reperfusion may represent an intriguing therapeutic chance to minimize tissue damages caused by I/R injury. In hypoxia, it is plausible that nitrite functions as an alternative NO source under those conditions. As previously reported, Zweier and coworkers in 1995 demonstrated for the first time the pivotal role of nitrite as source of NO (382); the finding that nitrite may act as endocrine reservoir of NO has prompted many studies demonstrating the capability of nitrite to mediate potent cytoprotection (*via* NO) in a number of organs and animal models of I/R injury [see (102)], also by modulating mitochondrial electron transfer (313).

The pathway by which nitrite forms NO in hypoxic tissue remains to be determined; several mechanisms have been proposed/identified over the years (281).

Two groups suggested the involvement of XO on the basis of reduced efficacy after treatment with allopurinol, an XO inhibitor (336, 353). Other groups have shown the involvement of Mb in nitrite conversion to NO and thus in cytoprotection. Addition of nitrite to rat heart homogenates containing both Mb and mitochondria resulted in NO generation and inhibition of respiration; these effects were blocked by Mb oxidation with ferricyanide but not by the addition of XO inhibitor allopurinol (312). Moreover,

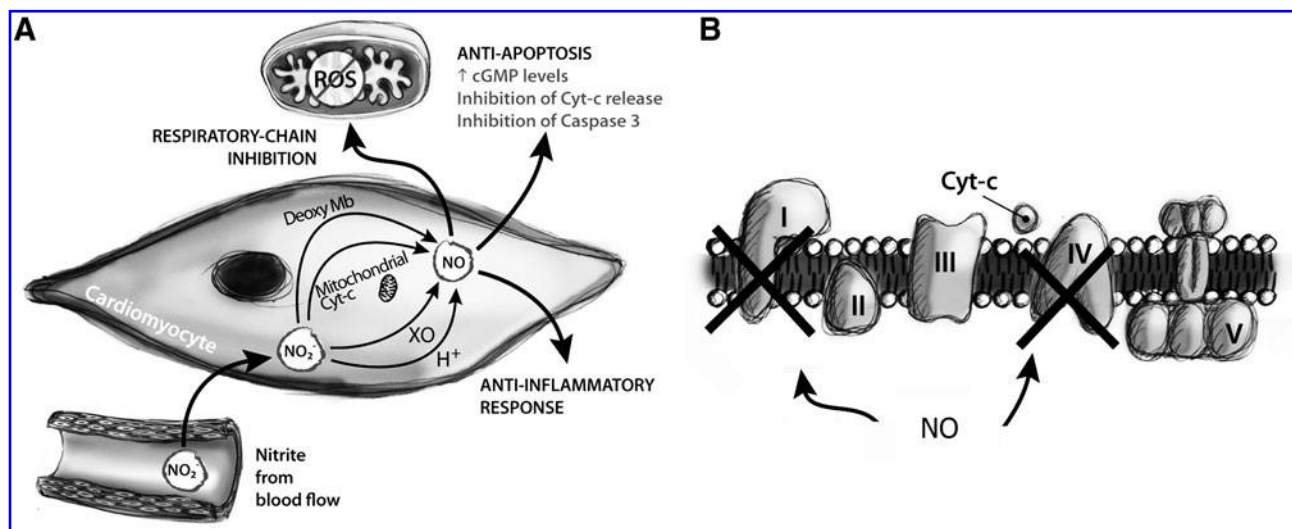


FIG. 11. Nitrite-mediated cytoprotection. (A) Nitrite enters in cardiomyocytes from blood and is reduced to NO by either biochemical mechanisms involving deoxyMb, xanthine oxidoreductase (XO), and mitochondrial cytochrome c (Cyt-c), or *via* pH-dependent chemical reduction. NO can be cytoprotective, *via* different pathways such as activation of the anti-inflammatory response, prevention of apoptosis (by inhibition of caspase 3 through S-nitrosation or *via* cGMP to avoid Cyt-c release), and inhibition of the respiratory chain (91) (B). Mitochondria exposed to hypoxia and reperfused with O_2 generate abundant and potentially damaging reactive oxygen species (ROS). (B) NO can prevent ROS formation by inhibiting the respiratory chain, mainly at the level of complexes I and IV (38, 40).

intracoronary application of nitrite in wild-type and myo^(-/-) mice (285) clearly showed that the NO generated by reaction of deoxyMb with nitrite is functionally relevant and leads to a downregulation of cardiac energy status, not observed in mice lacking Mb. The involvement of other hemoproteins and mitochondrial heme-containing complexes (such as cytochrome P450 and complex IV) in nitrite reduction has also been demonstrated (281).

However, even though the strategy of NO production is not unequivocally identified, enough NO seems to be produced from nitrite either directly within the mitochondria (22, 60, 61) or in the cytoplasm, as it occurs in myocytes where deoxyMb acts as an NiR (75, 145).

The discovery that nitrite may act as an endocrine reservoir of NO has suggested the idea that in the future this anion may represent an alternative strategy for an effective NO-based therapy by acting as an NO prodrug (181, 324). An overview of the main results focused on nitrite-mediated cytoprotection and more in general on the state-of-the-art of nitrite therapy is reported in chapter V.

C. Other activities of nitrite

To date, the majority of studies have described the nitrite effect as NO-dependent, but a novel NO-independent role of nitrite has been recently suggested, which may involve nitrite-mediated nitrosation without passing through a free NO intermediate (113). Nitrite-mediated nitrosation may protect tissues against inflammation (349).

Finally, a global role of nitrite in hypoxic signaling has been described: it affects cyclic-GMP production, cytochrome P450 activity, heat shock protein 70, and heme oxygenase-1 expression in a variety of tissues (43). These cellular activities of nitrite, in addition to its stability and abundance *in vivo*, suggest that this anion has a distinct and important role in mammalian biology, perhaps by serving as an endocrine messenger and synchronizing agent.

All these unexpected roles of nitrite in human physiology have opened the research to the possibility of therapeutic application associated with hypoxia and vasoconstriction.

V. Nitrite Therapy

In this section we discuss the advantages and the applications of nitrite-based therapy. We first analyze its feasibility and the pharmacokinetics of nitrite (section A); then, we discuss the tolerance and the toxicity of the compound (section B); finally, we described the most recent results concerning the use of nitrite in therapy (section C).

In 2006, David Lefer, a leader in I/R injury research, stated in an Editorial Focus that “the field of nitrite chemistry and biology is a truly exciting area of research that is certain to expand in the near future and lead to a dramatically improved understanding of the physiology of NO synthases and NO in terms of cytoprotection” (202).

It is now clear that reduction of nitrite to form NO under both physiological and pathological conditions is a challenging topic in medicinal chemistry (50). The potential of nitrite-based therapy is indeed clearly demonstrated by the rapidly growing number of studies on animal models of injury, the review articles (58, 91, 177, 248, 259, 316), and special issues (such as volume 89, issue 3, of *Cardiovascular Research*) on this topic, by the clinical trials aimed at investigating the action of

nitrite in protection [ClinicalTrials.gov and (181)] and by the number of patents (*e.g.*, uspto.gov).

The state of the art on the activity of nitrite under physiological and pathological states and on the translation of nitrite and nitrate research for clinical applications has been presented, from 2005 and every 2 years, during a dedicated international meeting held in Atlanta (for info on past meetings, see www.strategicresults.com/nitrite2011/). The outcome of the nitrite meeting 2011 will be published as a special abstract supplement of *The NO: Biology and Chemistry Journal*.

The advantage to use nitrite therapy instead of NO therapy is closely related to the high stability of nitrite, which can be transported in the circulation and stored in tissues. Moreover, since nitrite reduction occurs under conditions close to those of injured tissues (ischemia, hypoxia, and low pH), nitrite-dependent NO release targets injured tissues preferentially, thereby reducing the risk of systemic side effects (51). A summary of the possible effects of nitrite-based therapy is given in Figure 12.

Currently, NO therapy has been exploited *via* inhaled NO (iNO) gas from pressurized tanks; this approach is, to date, the preferred and the only approved NO treatment for acute pulmonary hypertension (78, 92, 372) even though it is inconvenient and onerous (92, 160). Alternatively, other strategies imply compounds containing either NO or an NO precursor in a stable form (178, 195), which may represent a clinical promise once it will be possible to control carefully the sustained delivery (49).

However, contrary to NO, nitrite allows multiple administration strategies, including oral, topical, intravenous, intraperitoneal, and aerosolized.

A. Pharmacokinetics and feasibility

It is known that nitrite intake occurring *via* nitrate as dietary source leads to its rapid absorption in the duodenum and jejunum and distribution in the whole body (18, 181, 296) (Fig. 7). Studies focused on nitrite administration/infusion have been obtained recently on both nonhuman primates (87) and human volunteers (87, 158). These experiments demonstrated that infused nitrite is a rapid vasodilator at physiological concentration and that nitrite-induced effects in terms of forearm blood flow are immediate (ranging from 15 to 60 sec, depending on the dose) (87). Upon infusion, nitrite levels increased to micromolar levels and then decayed with an apparent biological half-life of 42 min, while nitrite-induced hypotension lasted for 3 h (87).

A detailed study focused on the pharmacokinetics of ingested nitrite by human volunteers has shown that nitrite is able to reach the systemic circulation (158).

Very recently, the outcomes of a completed clinical trial, developed to determine the safety and feasibility of prolonged sodium nitrite infusion, have been published (278); this study provided pharmacokinetic and toxicity parameters needed for using nitrite as a therapeutic agent, such as maximal tolerated dose and dose-limited toxicity for long-term intravenous infusion of sodium nitrite (266.9 and 445.7 mg/kg/h, respectively) and the mean half-life of nitrite in plasma and whole blood (45.3 and 51.4 min, respectively). More interestingly, the small increase of metHb seen in this study was asymptomatic and the decrease of blood pressure was transient (278). This study suggests that prolonged intravenous

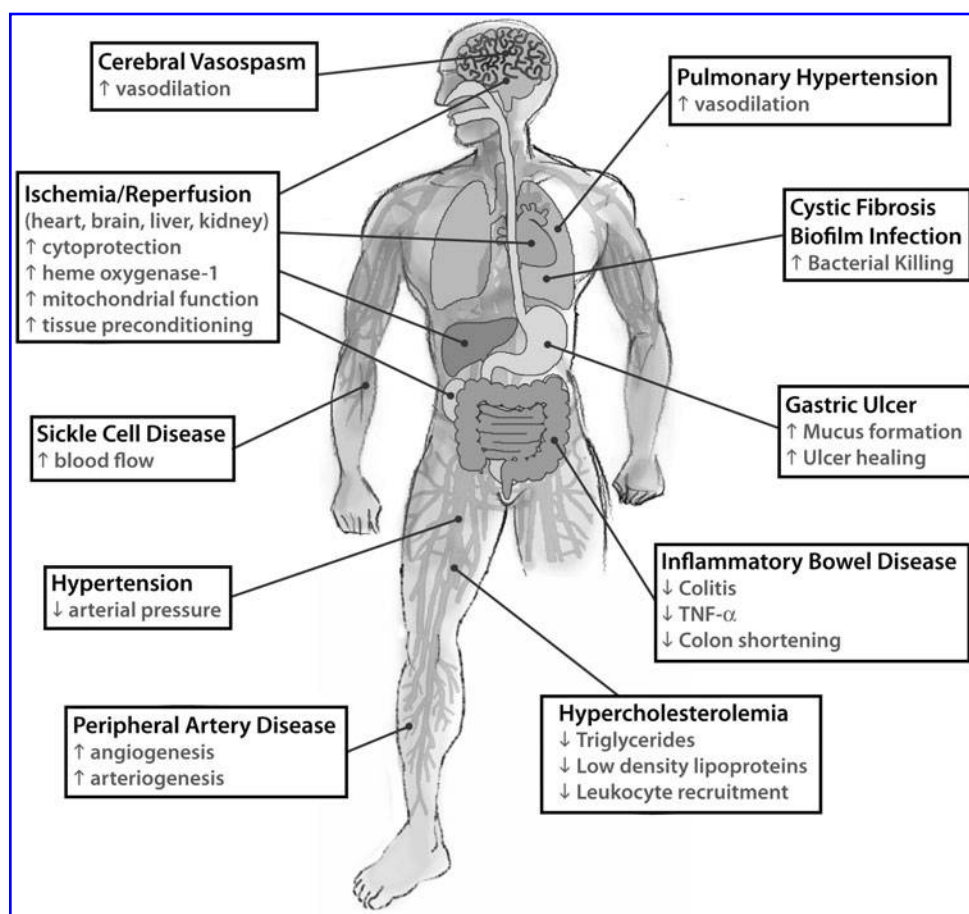


FIG. 12. Effects of nitrite-based therapy. Summary of the main effects exerted by nitrite as a therapeutic agent in different pathological conditions: these properties are supported by experimental and/or clinical results. Figure modified with permission from (181).

infusion of sodium nitrite (at doses within the maximal tolerated dose) is safe and should provide the proper amount of nitrite required to exert its therapeutic role (278).

B. Tolerance and toxicity

It is worth to notice that, contrary to organic nitrate class of drugs such as nitroglycerin (327), continuous sodium nitrite administration in nonhuman primates does not induce tolerance (87), that is, decrease of sensitivity of vascular smooth muscle to further vasorelaxation. In 2002 Ignarro underlined how nitroglycerine tolerance limits the chronic use of such compounds to treat angina pectoris and how “the discovery either of ways to avoid tolerance or of new NO-generating drugs that do not cause tolerance” is of great interest (161). Nitroglycerin tolerance arises likely from the inhibition of the enzymes involved in nitroglycerin bioactivation, such as the mitochondrial aldehyde dehydrogenase, cytochrome P450, and glutathione-S-transferase, which function also as nitroglycerin reductases (63, 121, 237). The capability of nitrite to bypass the enzymatic tolerance may represent a convenient alternative to organic nitrate therapy, as wished by Ignarro about 10 years ago.

Since more than 95% of orally ingested nitrite is absorbed, its toxicity during extended exposure to nitrite was evaluated. The intake of nitrite has been historically associated with poisoning due to methemoglobinemia (52, 229); this condition can interfere with the ability of blood cells to carry O₂ when metHb concentrations reach 20%–30% of total Hb concentrations. In

healthy individuals without anemia, acquired methemoglobinemia causes few symptoms at 15% metHb, while levels of 20%–30% metHb cause headache, fatigue, and syncope; at levels of ~50% dys-rhythmias, coma and death occur (165). Infantile methemoglobinaemia has been associated with feeding reconstituted with well waters rich in nitrate (70, 95).

However, after intravenous administration of nitrite, the maximum percent metHb in blood was between 3% and 12% and ~4% after oral administration (87, 158); in both cases the maximum metHb level was reached about 0.8–1.2 h after intravenous or oral administration (87, 158). These results indicate that nitrite levels able to induce vasodilation do not cause clinically significant methemoglobinemia, probably thanks to the coupled methemoglobin reductase activity of the NADH-cytochrome b5 reductase, previously discussed.

Oral administration of nitrite has been causally associated with gastrointestinal cancer (229). It has been postulated that, under strong acidic conditions, swallowed salivary nitrite may nitrosate *via* HNO₂ secondary amines ingested in food to form nitrosamines (309); some of these nitrosamine have been found to be carcinogenic in animal and in epidemiological studies (20, 189, 211). However, the direct involvement of swallowed salivary nitrite [*via* nitrate reduction, (101)] in promoting gastrointestinal cancer has not been demonstrated (189); all available data indicate that there is not a direct causative role of nitrate in gastric cancer (229), possibly because food ingestion promotes an increase in pH to a value that hampers secondary amines nitrosation, a reaction preferentially occurring at pH 2.2–3.5 (19).

In conclusion, all data available to date on pharmacokinetics and toxicity of nitrite strongly support the use of nitrite therapy as a strategy to bypass the limitations of the NO therapy (51).

C. Effects of nitrate/nitrite administration and the therapeutic potential

Nitrite-based therapy has been used to control vasodilation and cytoprotection after hypoxic damage, but also inflammatory disease and host defense processes (181, 229). Accordingly, experimental data can be clustered into three main groups:

- Nitrite as a cardiovascular drug
- Nitrite use for inflammatory diseases
- Acidified nitrite as a tool against bacterial pathogens

A summary on these topics is reported below, with special attention to the use of nitrite therapy in cardiovascular diseases.

1. Nitrite as a cardiovascular drug. A growing number of studies reported the effects of nitrite administration on protection against I/R injury (176, 201, 317, 353), platelet function inhibition (222, 354), vasodilation (74, 131), reduction of blood pressure (127, 197), and increase of cGMP levels (43). All these results are in agreement with the evidence that most of the cardiovascular risk factors are associated with reduced NO availability in the vasculature (150, 256). Most of the studies indeed demonstrated that the protective effect of nitrite or dietary nitrate is significantly lowered by the presence of NO scavenger such as carboxy-PTIO and Hb (176, 194, 222).

Webb and coworkers (353) assembled 7 years ago a specially adapted NO-collecting chamber placed around isolated perfused rat heart to measure NO concentration, using the ozone chemiluminescence detection method previously developed by other groups (115, 186); this chamber was also employed to measure NO production in homogenized myocardium (rat and human). In both experimental settings, a significant NO production was observed upon addition of sodium nitrite (10–100 μ M) at pH 5–6, a condition typical of myocardial ischemia (353). Nitrite significantly reduced infarct size (~50%); the cytoprotective effect is abolished in the presence of NO scavengers (353).

a. *Ischemia/reperfusion injury.* The efficacy of nitrite administration in limiting I/R damages has been investigated extensively in myocardial and brain ischemia, as much as in liver and kidney; the main outcome of these studies is that the amount and the way of administration of nitrite are crucial to exert its protective role as therapeutics upon ischemic damage.

Duranski and colleagues performed studies in mice to evaluate the cardioprotective effects of acute nitrite therapy in the setting of coronary artery occlusion and reperfusion, exploring different nitrite dosages (2.4–1920 nmol) (102). The administration of nitrite into the left ventricular cavity 5 min before reperfusion significantly limited myocardial infarct size, in a dose-dependent manner with a maximal protective effect at 48 nmol of nitrite (102). Low doses of intravenous nitrite improved microvascular perfusion, reduced apoptosis, and improved contractile function (133). Nitrite can also exert potent cytoprotective effects after I/R injury on liver in a dose-

dependent fashion by limiting serum increase of the liver transaminases, hepatocellular injury, and apoptosis (102).

The effect of dose and duration of nitrite/NO exposure is critical, resulting in a precise setting and timing for NO-mediated cytoprotection in I/R pathophysiology. Different experimental protocols may give contradictory results, as shown in studies focused on the treatment of ischemic brain in rats by two different research groups. The group of Jung and coworkers (176) observed a significant reduction in infarct size, while Schatlo and colleagues did not observe the dose-protective effect of nitrite in ischemic brain (307). These contradictory results may arise from differences in the experimental setting employed by the two research groups, in terms of both duration of nitrite administration and age of the animals.

The route of administration may also explain discrepancies in studies on the efficacy of nitrite therapy. A clear example is represented by the results obtained on a rat model of I/R-induced renal injury by two different groups, both using the same amount and timing of nitrite application. Basireddy and colleagues observed that the intravenous or intraperitoneal administration of nitrite did not provide protection upon I/R induced renal injury (21), in contrast to its beneficial effects in cardiac and hepatic I/R injury (102, 133, 353). Tripatara *et al.* confirmed this result but were also able to attenuate the degree of renal dysfunction, reperfusion injury, glomerular dysfunction, and tubular injury upon topical administration of nitrite (336).

Oral nitrite administration in the treatment of I/R injury after transplantation was shown to improve cardiac allograft outcomes in rats by lowering allograft inflammation, apoptosis, and alloimmune responses (374). Nitrite administration resulted in prolonged allograft survival, while reduced oral intake of NO_x promoted rejection (374). Accordingly, administration of iNO (80 ppm) to patients undergoing orthotopic liver transplantation inhibits hepatic I/R injury (196). The concentration of NO metabolites (including nitrate and nitrite) was significantly increased in these patients, in agreement with other studies on iNO administration (253); Lang and coworkers (196) suggested that, in their study, the extrapulmonary effects of iNO were mainly due to nitrite.

b. *Cardiovascular disorders.* The therapeutic potential of nitrite in the field of cardiovascular diseases has been also evaluated for disorders associated with reduced NO availability, such as chronic tissue ischemia (31), sickle cell disease (SCD) (227), and pulmonary arterial hypertension (PAH) (79). Nitrite therapy has been studied also as a strategy to control vasodilation and hypertension (127, 181, 354).

Chronic tissue ischemia is mainly caused by peripheral arterial disease (PAD) (181, 267), responsible for hypertension, atherosclerosis, and diabetes (181). Currently, the treatment of such pathological status is based on the employment of anti-platelet agents and statins (267). The capability of nitrite to promote angiogenesis as a therapeutic strategy to treat PAD and more in general chronic tissue ischemia is a challenging issue; since NO plays an important role in stimulating angiogenesis (71), the effect of nitrite therapy in controlling ischemia-induced angiogenesis has been analyzed (194). Nitrite was found to improve angiogenesis in terms of increase in the quantity of endothelial cells and in the vascular density (194). A dose-dependent effect of nitrite in the increase of

ischemic tissue blood flow in mice was observed (310). The capability of nitrite to restore ischemic blood flow was significantly impaired by the presence of NO scavenger such as carboxy-PTIO (194). Interestingly, the capability of nitrite to augment angiogenesis was also demonstrated in the neural regeneration stimulated upon prolonged nitrite therapy in the ischemic brains (177).

The possibility to employ nitrite as source of NO in managing PAD has been supported also indirectly; pharmacological treatment of permanent tissue ischemia with the antiplatelet drug dipyridamole augments ischemic tissue reperfusion, promotes endothelial nitric oxide synthase (eNOS) activity, and, as a consequence, increases nitrite levels in ischemic muscle tissue (269). Nitrite reduction may enhance the vascular growth observed upon dipyridamole treatment (346), confirming the presence of a nitrite/NO endocrine system able to produce benefits not only locally but also in distal tissues (267).

As mentioned above, the reduced NO bioavailability also represents one of the pathological trait of SCD, a genetic disease due to a point mutation of the beta-chain of Hb (227); these patients accumulate huge amounts of free Hb, which reduces NO bioavailability (286, 348). The possibility to develop therapeutic strategies aimed at optimizing NO delivery has been considered at least 10 years ago (242); a recent phase I/II study demonstrated that sodium nitrite infusion into the brachial artery augmented in a dose-dependent manner the mean venous plasma nitrite concentration and stimulated forearm blood flow (223). Since this treatment was well tolerated (lack of hypotension and significative methemoglobinemia), nitrite could represent a plausible NO donor for future clinical trials in SCD (223).

The very efficient NO scavenging property of oxyHb (175) represents a limitation in using blood substitutes, where a secondary hypertension is associated with scavenging of NO by Hb-based oxygen carriers (HBOCs). A novel therapeutic strategy to attenuate this side effect has been proposed based on the use of nitrite as an adjunct therapy to attenuate HBOC-dependent hypertension (298).

Impaired NO bioavailability plays an important role also in the development of PAH, a chronic and progressive disease characterized by a persistent elevation of pulmonary artery pressure that progressively leads to right heart failure and ultimately death (79). Currently, iNO therapy is only approved for use in infants who have respiratory distress syndrome (140) even though the short-term effects and the elevated cost of such therapy made researchers to explore novel strategies (79). The administration of nebulized nitrite to treat hypoxic pulmonary hypertension in newborn lambs decreases the pulmonary artery pressure without measurable effect on mean arterial blood (159). More recently, the beneficial effects of nebulized nitrite under hypoxia or monocrotaline-induced PAH were confirmed in mice and rats (379). This therapeutic approach completely prevented or reversed PAH and pathological right ventricular hypertrophy and failure (379).

The evidence that nitrite may really target pulmonary circulation preferentially under hypoxic conditions has been verified on healthy human volunteers (162). The study was done in a hypoxic chamber (inspired O_2 , 12%; room air as control) where each volunteer received an infusion of sodium nitrite (1 μ mol/min). During hypoxia, nitrite increased fore-

arm blood flow (+36%) and reduced pulmonary arterial pressure (12%–17%). Interestingly, pulmonary, but not systemic, arterial effects persisted 1 h after stopping the infusion, in conjunction with plasma nitrite levels lowered to the baseline values (162).

It should be pointed out that, even though rapid nitrate administration is largely ineffective in the pathological settings discussed above (as most of these studies used nitrate as negative control), recent data report that sustained delivery of nitrate (over days/weeks) may represent an alternative strategy to increase plasma nitrite and achieve protection. Dietary nitrate can enhance exercise performance in PAD patients (179), representing a chance for these patients, where plasma nitrite levels could be indicative of vascular health and function (3).

The effects of dietary nitrate have been also analyzed in different pathological backgrounds. Dietary supplementation of nitrate was used in eNOS-deficient mice, which presents several features of the metabolic syndrome (55), a cardiovascular disorder associated with reduced NO bioavailability from eNOS, with increased cardiovascular risk and type 2 diabetes (117, 243). Dietary nitrate treatment (7–10 weeks) reduced both fat accumulation and circulating triglycerides; moreover, it reversed the prediabetic phenotype by improving glucose tolerance and reducing fasting blood glucose (55).

Nitrate/nitrite-derived NO may also control blood pressure (197). Dietary supplementation with sodium nitrate (0.1 mmol/Kg per day for 3 days) in healthy volunteers decreases the mean arterial pressure (by 3.2 mmHg) and increases plasma levels of both nitrate and nitrite (197). The effect of dietary nitrate has been recently analyzed in a model of renal and cardiovascular disease (56); chronic dietary nitrate supplementation attenuates hypertension and prevents the development of cardiac and renal damage (56).

Dietary nitrite therapy may play an important role in preventing cardiovascular disease associated with aging, such as vascular endothelial dysfunction and large elastic artery stiffening (316). Nitrite supplementation in old and young mice showed that the treatment ameliorated carotid artery endothelial dysfunction and reduced large elastic artery stiffness in old mice to a level similar to that of young mice, confirming the translational potential of nitrite administration to prevent cardiovascular disorders associated with aging (316).

2. Nitrite in inflammatory diseases. There is growing experimental evidence that inorganic nitrite therapy confers substantial benefit to numerous pathophysiological conditions includes experimental colitis, hypercholesterolemia, and gastric ulcer.

Colitis is a chronic inflammatory disorder of the intestinal tract characterized by mucosal inflammation of the large bowel (217) with excessive production of cytokines, adhesion molecules, and ROS (334). In a colitis model in mice (263), colonic nitrite levels decrease with concomitant increase in colonic TNF- α expression followed by increased iNOS and heme oxygenase-1 expression (262). Oral sodium nitrite administration (6.4–7.2 mg/mouse/day) attenuates acute inflammatory flares of colitis by preserving tissue NO bioavailability, as suggested by the increase in nitrite colonic levels and decrease in TNF- α expression (262).

Nitrite has shown a novel role as a therapeutic agent also to contrast microvascular inflammation and endothelial dysfunction associated with hypercholesterolemia (322). The hypercholesterolemia proinflammatory phenotype is characterized by a decline in NO bioavailability and increase of ROS production. Nitrite administration is able to restore NO biochemical homeostasis and significantly attenuates hypercholesterolemia-mediated leukocyte recruitment, which plays a key role in controlling vascular inflammation (262).

A recent whole genome array analysis (268), combined with previous data of nitrite-dependent induction of angiogenesis (194), suggests that nitrite therapy may also facilitate vascular remodeling during chronic tissue ischemia by modulating inflammatory gene expression. Nitrite-treated animals (330 $\mu\text{g/kg/day}$) present a significant decrease in inflammatory gene expression concomitant with an increase in genes that function to protect and preserve skeletal muscle tissue (268). Moreover, nitrite administration upregulates several genes involved in innate immune responses, such as the macrophage migration inhibitor factor and the heat shock protein 90 class B member (Hsp90ab1) (269). Since Hsp90ab1 promotes eNOS activation, the final outcome is NO production in ischemic tissue (123). On the other hand, nitrite also significantly downregulates other genes associated with innate immunity, for example, IL-10 (269). Several studies (84, 124, 254, 300) suggest that IL-10 expression acts in an anti-angiogenic manner in several pathological events, including chronic tissue ischemia; therefore, the nitrite therapy likely augments reperfusion in chronic tissue ischemia caused by this regulation of innate inflammation response.

3. Acidified nitrite as a tool against bacterial pathogens. As mentioned before, under acidic conditions, sodium nitrite forms HNO_2 that, in the presence of O_2 , generates reactive nitrogen intermediates, including ONOO^- , N_2O_3 , NO_2^\bullet , and NO (276). These small molecules readily cross cell membranes, where they can react with reduced thiols to form nitrosothiols, which are thought to be important in microbial killing (112).

A common perception of gastrointestinal host defence to pathogens ingested with food and water is that it depends entirely on immune factors secreted in the saliva and gastric acidity itself. However, for many gastrointestinal pathogens such as *Campylobacter*, *Shigella*, and *Salmonella*, even prolonged immersion in strong acid at pH 2, to emulate the normal fasting stomach, exerts only a bacteriostatic effect (104). Failure to kill these organisms means that viable pathogens would pass to the small intestine if the defence depended on acid alone. Salivary and gastric nitrites, converted to reactive nitrogen compounds under acidic conditions, may enhance the killing of pathogens in the stomach (103, 104, 283). An *in vitro* study (283) demonstrated that normal stomach acidity in combination with physiological concentrations of nitrite are required to kill *Clostridium difficile* spores that were not killed in acidic buffers or gastric content; moreover, nitrite enhances the killing of several nosocomial pathogens, including vancomycin-resistant *Enterococcus* spp., methicillin-resistant *Staphylococcus aureus*, and *Candida glabrata* (283). These data suggest that interventions to limit the overuse of acid-suppressive medications could potentially have an impact on multiple pathogens. For example, supplementing the diets of hospitalized patients with nitrates

could bolster gastric defenses by increasing levels of acidified nitrite (103, 104).

Recent data (226) suggest that the nitrite could be used in the treatment of chronic bacterial infections caused by opportunistic pathogens such as *P. aeruginosa*, *S. aureus*, and *Burkholderia cepacia* in the airways of CF patients. The most dangerous form of *P. aeruginosa* is the mucoid *P. aeruginosa* characterized by a mutation within the *mucA* gene and consequently by the overproduction of alginate, an exopolysaccharide that inhibits the diffusion of O_2 and antibiotics. As previously mentioned, mucoid *P. aeruginosa* biofilms are inherently resistant to antibiotics (134) and phagocytic neutrophils (48). Acidified nitrite ($\sim 15\text{ mM}$) kills the mucoid form of *P. aeruginosa* (370), inhibits nonmucoid planktonic *P. aeruginosa* PAO1, and kills *S. aureus* and, unexpectedly, planktonic and biofilm communities of *Burkholderia cepacia* (226). Although the concentration of acidified nitrite necessary for the antimicrobial effect is quite high, it is worth mentioning that 10 to 40 mg/kg of acidified nitrite are typically used in the cure of meat products (308), suggesting that humans have a high tolerance for nitrite, as discussed above. Therefore, acidified nitrite could be used for the clinical treatment of CF patients with lung infections.

The acidified nitrite antimicrobial activity is also effective on nosocomial mycoses (8) that represent an increasing problem among critical care patients (193). The susceptibility of *Candida albicans*, *Candida glabrata*, *Candida tropicalis*, *Cryptococcus neoformans*, *Aspergillus fumigatus*, and a *Rhodotorula* species; the lack of induction of resistance; and the possibility of a direct aerosol administration of acidified nitrite (8) provide a novel potential method for treating fungal infections.

Effective antimicrobial strategies are also important in industrial settings, including food processing environment, water treatment, and contact lens industry. One of the most used antimicrobial agents in disinfection processes is the oxidizing agent H_2O_2 , which causes microbial death by protein denaturation (125). However, the antimicrobial efficacy of H_2O_2 can be significantly enhanced by addition of acidified nitrite (142). This strategy could find an application in disinfection of contact lenses that are a main predisposing factor of blinding keratitis caused by the highly resistant *Acanthamoeba* spp. (147, 282). H_2O_2 (3%) used as a contact lens disinfectant is effective against *Acanthamoeba* cysts, but an exposure time of at least 4–6 h (157) and/or extensive neutralization are required before use of the contact lenses. On the other hand, if sodium nitrite is added to a 3% (v/v) H_2O_2 solution in one-step systems, significant killing of *Acanthamoeba* cysts is rapidly obtained (157).

VI. Conclusions and Open Questions

It is widely accepted nowadays that nitrite is a biologically relevant molecule: in bacteria, it may function as a terminal acceptor in electron transport chains and it may act as a key signal in many host–pathogen interactions, by producing NO. The role and biological fate of nitrite in humans is also closely interwoven with the homeostasis of NO (Fig. 13). Nitrite is not only an endocrine reservoir of NO under hypoxic conditions, but also an O_2 -dependent treasure house of NO [see also (80)].

In mammals, a wide variety of proteins are able to act as NiR both *in vitro* and *in vivo*. It is possible that more proteins will be identified in the near future, which may accomplish the moonlighting function of NiR under O_2 shortage conditions. We propose that this redundancy is not fortuitous for several

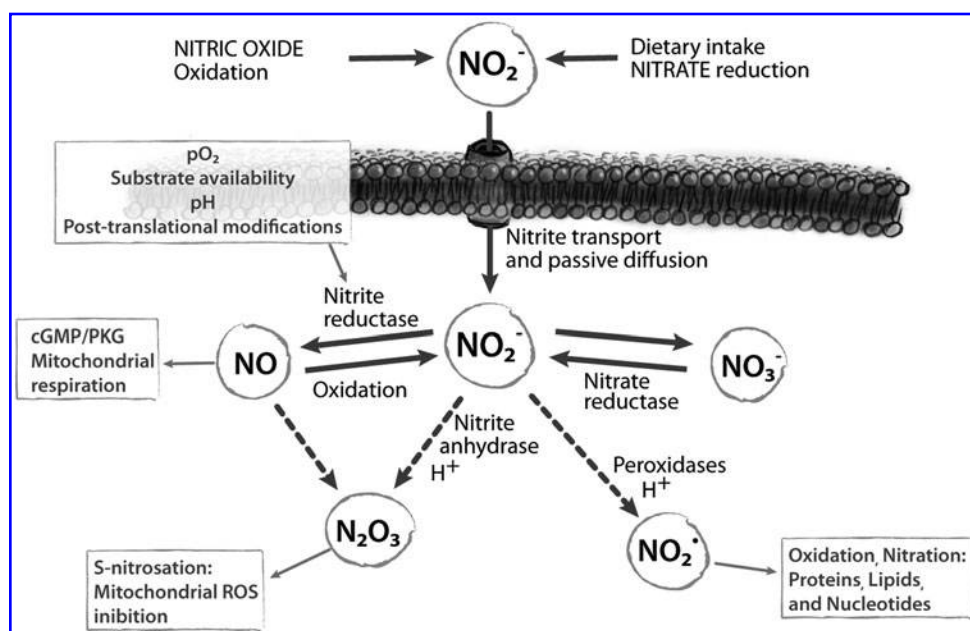


FIG. 13. Summary of the physiological functions of nitrite. The figure describes the fate of nitrite (produced from dietary nitrate or from NO oxidation) after its intake in the cells. Nitrite enters the cell following an electrochemical/concentration gradient, through either a channel and/or as nitrous acid (350), depending on the physiological request. Several enzymes are involved in nitrite reduction and the different species produced (NO or other reactive nitrogen species) could mediate acute and/or chronic effects of endogenous and therapeutic nitrite. Figure modified with permission from (349).

reasons. First of all, it may be considered an inheritance of a preaerobic past where N-oxides were available as terminal electron acceptors to produce energy (82, 114); second, it may represent an inherent property of the eukaryotic cell, which has several rescue pathways to escape harmful conditions such as O_2 depletion and redox unbalance.

It is very intriguing to notice that, in the bacterial kingdom, the heme-containing NiR mostly use specialized porphyrins, such as heme d_1 , to avoid trapping of the enzyme in an irreversibly inhibited NO complex, thus escaping the kinetic trap faced by mammalian hemoproteins such as Hb and Mb. Indeed, a major conundrum on to the relevance of these hemoproteins, especially Hb, in the nitrite/NO homeostasis is still the question of how the NO molecule can escape the trap of ferrous hemes. A recent mass spectrometry study has confirmed that, after addition of millimolar levels of nitrite to suspensions of deoxygenated red cells, no significant accumulation of extracellular NO can be monitored; these data support the hypothesis that the autocapture of NO by deoxyHb precludes efflux of NO from the erythrocyte (234). As recently suggested by other authors (349), it would be helpful to find an experimental setup able to uncouple the effect on the Hb/nitrite system from the effect on the intrinsic NO homeostasis within the erythrocyte.

The capability of Mb to reduce nitrite and its role in cytoprotection under hypoxic conditions is well established. Many experiments can still be planned on six-coordinate globins or on cytochromes: these hemoproteins may well serve the intriguing function of redox-controlled NiR, as recently suggested (22, 330), after switching their heme coordination from six- to five-coordinate.

Another important issue to be investigated in detail is the mechanism of nitrite transport across the cell membrane. Whereas this topic is well studied in bacteria [see (240)], the literature data on eukaryotic cells are mainly restricted to the erythrocyte, where the transport mechanism seems to involve both NO_2^- diffusion (possibly employing the anion exchanger AE1) and HNO_2 diffusion (109, 171, 350). To assess the uptake

rate of nitrite, more data on the mechanism of transport across the membrane of other cell types would therefore be highly desirable, in particular to identify O_2 -regulated processes.

In conclusion, evaluation of the contribution of blood-based and tissue-based nitrite reduction as a source of NO bioactivity seems to indicate that the tissue sources may play a more important role. A recent theoretical analysis of NO and O_2 transport around an arteriole, including superoxide generation and NiR activity in blood and tissue, confirms that NiR activity in blood should not be a very effective mechanism for conserving NO due to the strong scavenging activity of deoxyHb (46). These authors suggest that the NiR activity in tissue is much more effective in increasing NO bioavailability in the vascular wall and contributes progressively more NO as tissue hypoxia becomes more severe.

Finally, nitrite-based therapy has received a growing attention in the last years: several clinical trials are ongoing or planned in the next future [see *e.g.*, (278)]. Nitrite is a molecule that naturally occurs in the human body in blood and tissues (in nanomolar or micromolar concentrations, respectively); this confers a great advantage to nitrite-based therapy, which is safer than other strategies employing organic nitrates or NO donors. However, the interaction of therapeutic nitrite with other drugs is still poorly understood and this aspect will require further studies to assess possible toxic effects.

The variability of nitrite administration protocols, nitrite uptake rates, and final biological effects suggests that an effective nitrite therapy will require personalized protocols on the basis of the pathology and individual patient, considering also gender and race. Interestingly, even though further studies are needed, it was recently suggested that dietary nitrate may be as effective as nitrite administration for long-term therapy (214) and that it is also able to influence basal cell functions [such as mitochondrial activity (198)].

Acknowledgments

Funds from the Ministero della Università e Ricerca of Italy [RBRN07BMCT, 20094BJ9R7], from the Sapienza University

of Rome, and from Fondazione Italiana Fibrosi Cistica to F.C. are gratefully acknowledged.

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Date of first submission to ARS Central, July 28, 2011; date of final revised submission, January 24, 2012; date of acceptance, January 24, 2012.

Abbreviations Used

AO = aldehyde oxidase
 carboxy-PTIO = 2-(4-carboxyphenyl)-4,4,5,5-tetra-methylimidazoline-1-oxyl-3-oxide
 cd_1NiR = cytochrome cd_1 nitrite reductase
 CF = cystic fibrosis
 Cox = cytochrome c oxidase
 deoxyHb = deoxygenated hemoglobin
 eNOS = endothelial nitric oxide synthase
 FAD = flavin adenine dinucleotide
 Fe^{2+} = iron in the ferrous form
 Fe^{3+} = iron in the ferric form
 GSH = glutathione
 GSNO = S-nitrosoglutathione
 H_2O_2 = hydrogen peroxide
 Hb = hemoglobin
 HBOC = Hb-based oxygen carrier
 hhMB = horse heart Mb
 HNO_2 = nitrous acid
 iNO = inhaled NO
 I/R = ischemia-reperfusion
 Mb = myoglobin
 MetHb = methemoglobin
 metMb = metmyoglobin
 MoCo = molybdenum cofactor
 Nar = nitrate reductase
 Ngb = neuroglobin
 NiR = nitrite reductase
 N_2O_3 = dinitrogen trioxide
 NO = nitric oxide
 NO_2^- = nitrite anion
 $NO_2\bullet$ = nitrogen dioxide
 N_2OR = nitrous oxide reductase
 Nor = nitric oxide reductase
 NOS = nitric oxide synthase
 O_2 = oxygen
 $ONOO^{--}$ = peroxynitrite
 oxyHb = oxygenated hemoglobin
 $Pa-cd_1NiR$ = *Pseudomonas aeruginosa* cytochrome cd_1 nitrite reductase
 PAD = peripheral arterial disease
 PAH = pulmonary arterial hypertension
 pO_2 = oxygen pressure
 ROS = reactive oxygen species
 SCD = Sickle cell disease
 SEM = standard error of the mean
 SERCA = sarco/endoplasmic reticulum calcium ATPase
 sGC = soluble guanylate cyclase
 SNP = single-nucleotide polymorphism
 XDH = xanthine dehydrogenase
 XO = xanthine oxidase
 XOR = xanthine oxidoreductase

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