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Endogenous Hemoprotein-Dependent Signaling Pathways of Nitric Oxide and Nitrite

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Abstract

Interdisciplinary research at the interface of chemistry, physiology, and biomedicine have uncovered pivotal roles of nitric oxide (NO) as a signaling molecule that regulates vascular tone, platelet aggregation, and other pathways relevant to human health and disease. Heme is central to physiological NO signaling, serving as the active site for canonical NO biosynthesis in nitric oxide synthase (NOS) enzymes and as the highly selective NO binding site in the soluble guanylyl cyclase receptor. Outside of the primary NOS-dependent biosynthetic pathway, other hemoproteins, including hemoglobin and myoglobin, generate NO via the reduction of nitrite. This auxiliary hemoprotein reaction unlocks a “second axis” of NO signaling in which nitrite serves as a stable NO reservoir. In this Forum Article, we highlight these NO-dependent physiological pathways and examine complex chemical and biochemical reactions that govern NO and nitrite signaling in vivo. We focus on hemoprotein-dependent reaction pathways that generate

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M.R.D. prepared the initial drafts and final manuscript, as well as Figure 1. A.W.D. made all of the other figures and contributed to the writing of certain sections. A.W.D., J.T., and M.T.G. critically read, edited, and contributed to the preparation of the full manuscript.

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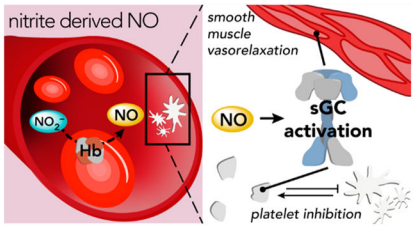
and consume NO in the presence of nitrite and consider intermediate nitrogen oxides, including NO_2, N_2O_3, and *S*-nitrosothiols, that may facilitate nitrite-based signaling in blood vessels and tissues. We also discuss emergent therapeutic strategies that leverage our understanding of these key reaction pathways to target NO signaling and treat a wide range of diseases.

Graphical Abstract



INTRODUCTION

The connection between nitric oxide (NO), a neutral diatomic molecule bearing a single unpaired electron, and human physiology was first realized in the context of vasoregulation when NO was identified as the endothelium-derived relaxing factor that activates the enzyme soluble guanylyl cyclase (sGC). Robert Furchgott, Ferid Murad, and Louis Ignarro received the Nobel Prize in Physiology or Medicine in 1998 for seminal contributions to this discovery of “nitric oxide as a signaling molecule in the cardiovascular system”. Central to this connection was the discovery that NO binding to heme directly enhances the activity of sGC, which catalyzes the formation of cyclic guanosine monophosphate (cGMP). As a second messenger, cGMP goes on to stimulate smooth muscle cells, primarily through the activation of protein kinase G (PKG). In the more than 30 years since this NO-dependent regulatory paradigm was established, rich collaborative efforts among investigators in inorganic chemistry, physiology, and biomedicine have elucidated much of the complex chemical reactivity and nuanced physiological regulatory functions of NO. Researchers have uncovered physiological sources of NO through enzymatic biosynthesis [by nitric oxide synthase (NOS)] as well as through redox bioactivation of the stable NO precursor nitrite (NO_2^-). Additional downstream effects of the NO–sGC–cGMP pathway have been determined, such as the regulation of platelet activation and inflammation. New physiological targets of NO and its derivatives have also been identified, including reactive thiols, tyrosine residues, and unsaturated fatty acids. These new discoveries have expanded the scope of NO-dependent regulation beyond cardiovascular homeostasis, and NO signaling is now implicated in many physiological and pathophysiological processes, such as neurotransmission, host defense, carcinogenesis, and response to oxidative stress. The primary objective of this Forum Article is to review critical oxidative and reductive reactions that govern NO and NO_2^- signaling, with a particular focus on reactions with hemoproteins. We will also highlight key NO-dependent signaling pathways and review emergent therapeutic strategies that target these pathways. Given our laboratory’s experience in nitrite bioactivation, we specifically emphasize the complex chemical and biochemical pathways that enable nitrite to modulate NO-dependent pathways and the therapeutic approaches that utilize this stable NO precursor.

SGC: THE CENTRAL NO RECEPTOR

NO regulates a number of critical biological processes via the NO–sGC–cGMP signaling pathway. In humans, picomolar-to-nanomolar concentrations of NO in vascular smooth muscle tissue enhance the guanylyl cyclase activity of sGC by several hundredfold, resulting in an increase of the second messenger molecule cGMP.[1–3] This activation of sGC occurs within milliseconds of NO exposure in cells.[4] cGMP subsequently interacts with downstream targets, including protein kinases and ion channels that regulate physiological processes such as vasodilation, platelet aggregation, and neurotransmission.[5] Disruption of the NO–sGC–cGMP pathway is implicated in a large number of cardiovascular diseases, including pulmonary hypertension, peripheral vascular disease, and atherosclerosis.[6–8] Myriad studies have sought to elucidate the detailed molecular mechanisms of NO-dependent regulation, largely because of the connection between the NO–sGC–cGMP pathway and cardiovascular diseases, which are responsible for 1 in 3 deaths worldwide. In addition to NO itself, sGC has garnered significant therapeutic interest as pharmacological stimulators, and activators of sGC can enhance the enzymatic activity (and subsequent production of cGMP) independent of or in conjunction with NO.[9]

sGC Structure and Function.

sGC is a 150 kDa heterodimeric protein that contains two polypeptide chains (α, 690 a.a. residues; β, 620 a.a. residues) with modest sequence similarity and identical domain architectures. While multiple sGC polypeptide isoforms exist in humans (α1, α2, β1, and β2), the α1β1 heterodimer is ubiquitously expressed and most commonly studied.[10,11] Both α and β subunits contain three domains: an N-terminal heme–nitric oxide binding (HNOB) domain (also referred to as a heme–nitric oxide–oxygen, or HNOX, domain), a HNOB-associated (HNOBA) domain comprised of a Per-Arnt-Sim (PAS)-like domain linked to a long α-helix that forms a parallel dimeric coiled-coil (CC) with the other monomer, and a C-terminal catalytic domain.[12,13] A b-type heme binds to the β-subunit HNOB domain via a histidine side-chain ligand (His105), and NO binding to this five-coordinate heme site modulates guanylyl cyclase activity in the catalytic cyclase domain.[14–16] Interactions between HNOBA domains facilitate α/β dimerization.[17–19] The sGC catalytic domain is a member of the class III cyclase family and contains a single active site at the interface of the α/β heterodimer.[17,20,21] In the active site, two Mg^{2+} cations and several hydrogen (H)-bond-donating amino acid residues stabilize GTP binding and facilitate intramolecular cyclization.[22,23]

NO-dependent activation of sGC occurs upon formation of a nitrosyl-heme species and ensuing scission of the axial His105 bond. Ignarro and colleagues first proposed this NO-dependent activation model based on several key observations: (1) heme is required to observe a NO-dependent increase in sGC enzymatic function, (2) direct addition of nitrosyl-heme complexes enhances the activity of apo-sGC, and (3) incubation of apo-sGC with iron-free protoporphyrin IX enhances enzymatic activity in a manner similar to that observed upon NO binding to heme-bound enzyme.[1,24] A slew of spectroscopic investigations subsequently confirmed this activation model through direct observation of five-coordinate heme-nitrosyl species, which correlated with increased enzymatic activity.[15,16,25–29]

Long-range structural reorganization accounts for NO-induced allosteric signal transduction in sGC. After years of structural studies using sGC truncates, Kang et al. recently reported moderate-resolution (3.8–3.9 Å) cryo-electron microscopy (cryo-EM) structures of full-length sGC in inactive (ferric and unliganded ferrous) and active (ferrous-NO-bound) forms (Figure 1).[30] NO binding to the β-HNOB heme triggers a structural reorganization of the N-terminal sensor module (encompassing HNOB and HNOBA domains from α and β subunits). This conformational change is accompanied by extension of the α and β CC helices, as well as a 70° rotation of the relative orientation of the two helices. Rotation of the CC helices drives reorganization of the catalytic module: the active site volume increases to accommodate Mg^{2+} cations and the GTP substrate, thereby lowering K_M and increasing guanylyl cyclase activity. A second cryo-EM study comparing active and inactive structures of sGC from the moth species *Manuca sexta*, published shortly after the *Homo sapiens* sGC study, largely corroborated the proposed structural model.[31] These authors also observed reorientation of the sensor module, accompanied by elongation and rotation of the CC helices and ultimately opening of the GTP-binding active site in the catalytic module.

Ligand Selectivity at the sGC Heme.

A critical detail of the NO–sGC–cGMP pathway is that NO binds sGC via the iron-containing cofactor heme. Metal cofactor binding and subsequent physiological signal transduction occur commonly for NO and other related “small molecule bioregulators”, such as dioxygen (O_2), carbon monoxide (CO), and hydrogen sulfide (H_2S). These Lewis base-type signaling molecules are historically described as “gasotransmitters”; however, relevant signaling interactions occur between molecules dissolved in solution, not in the gaseous phase, and we therefore refrain from using this term. Because of the ostensible similarity of these small-molecule bioregulators and their respective target metal centers, selectivity by small-molecule-sensing proteins is paramount.

Unlike most hemoproteins, sGC does not bind O_2 with a high affinity but exhibits exquisite selectivity for NO. The factors that dictate small-molecule heme-binding affinities have recently been reviewed in terms of a “sliding scale rule” for hemoproteins.[32–35] This sliding scale rule posits that five-coordinate iron(II) hemoproteins bearing an axial, neutral His ligand exhibit an intrinsic ligand selectivity for the diatomic small molecules NO, CO, and O_2: $K_D(CO)/K_D(NO) \approx K_D(O_2)/K_D(CO) \approx 10^3$–$10^4$. Four protein-derived structural factors in the heme pocket may alter the absolute and/or relative affinities of these ligands: (1) proximal Fe–N(His) bond strength (also described as “proximal ligand strain”), (2) distal pocket steric hindrance, (3) distal pocket electrostatics, and (4) the presence of a distal pocket H-bond donor. Differences in ligand access channels and heme distortions may also dictate hemoprotein selectivity, but detailed systematic investigations are required to fully understand these factors.[34]

In sGC, ligand selectivity against O_2 is primarily dictated by two factors: a weak proximal Fe–N(His) bond and lack of a distal pocket H-bond donor. The Fe–N(His) bond in ferrous sGC is very weak, as evidenced by the remarkably low Fe–N stretching frequency (204 cm^{-1})[29] compared to those of other His-ligated hemoproteins such as deoxyhemoglobin (215 cm^{-1})[36] and deoxymyoglobin (218 cm^{-1}).[37] Changes in the proximal ligand bond

strength modulate the ligand affinities for NO, CO, and O_2 in a similar manner: the weaker the Fe–N(His) bond, the lower the ligand binding affinity across the board. Distal heme pocket H-bond donation selectively stabilizes O_2 binding in five-coordinate hemoproteins, as observed for hemoglobin and myoglobin.[38] This structural factor likely contributes to poor O_2 binding in sGC, which lacks a H-bond donor in the distal pocket. Supporting this hypothesis, a bacterial H-NOX protein (with sequence and structural homology to sGC) bears a H-bonding Tyr residue in the distal heme pocket and forms a stable oxyferrous species.[39,40] While substitution of the analogous distal pocket residue in human sGC (Ile145) with Tyr does not give rise to a stable oxyferrous species,[41] steric clashes likely impart geometric constraints that preclude proper H-bond donation in this mutagenic model. Taken together, these factors lead to an estimated binding dissociation constant for O_2 of around 1.4 M, far exceeding the solubility of O_2 in aqueous solution (~260 μM).[32,34]

At equimolar concentrations, NO reversibly binds to the five-coordinate sGC heme to form a relatively stable six-coordinate species with a NO dissociation constant of 54 nM (K_D = $k_{off,6\text{-}c}/k_{on,5\text{-}c}$ = 27 s^{-1}/1.4 × 10^8 M^{-1} s^{-1} at 24 °C).[3,33] This NO affinity is consistent with the sliding scale rule: the dissociation constant for CO binding to sGC is 97 μM,[42] roughly 3 orders of magnitude lower than that of NO.[34] Taken in the context of physiological signaling in the vascular wall, which occurs at low nanomolar concentrations of NO,[43] it is likely that the above K_D value is an overestimation and/or that only a small fraction of sGC is required to trigger the activation of downstream PKG. Unfortunately, it has been difficult to rectify the kinetic properties ascribed to sGC through in vitro experimentation, with the complex NO signaling dynamics observed in vivo. Understanding the exact nature of NO binding and subsequent sGC signaling presents an extreme challenge for the field, and creative cross-disciplinary approaches are needed to fully understand these complexities.

Adding to the complexity of sGC-dependent signaling is the controversial notion that CO activates sGC under physiological conditions. Several studies have invoked CO-induced activation of sGC under physiological conditions in the context of neurotransmission,[44] regulation of vascular tone,[45] and inhibition of vascular smooth muscle cell proliferation.[46,47] However, as noted above, the CO binding affinity of the sGC heme is ~100 μM, while the basal physiological levels of CO vary between 2 and 5 nM.[42,48,49] Even though it is possible that localized CO concentrations may rise above basal levels under pathophysiological conditions (e.g., inhaled CO poisoning), CO-bound sGC remains six-coordinate and exhibits 80-fold lower enzymatic activity compared to that of five-coordinate, NO-bound sGC.[26] Likely the only relevant context of CO-induced activation of sGC is in the presence of pharmacological sGC stimulators, which enhance sensitivity to small-molecule bioregulators (vide infra).

While CO is unlikely to activate sGC, mounting evidence suggests that CO itself still possesses properties of a signaling molecule under physiological conditions, indicating other (metalloprotein) targets. CO, which is generated as a byproduct of heme degradation by O_2- and NADPH-dependent heme-oxygenase (HO) enzymes, has been implicated in maintaining homeostatic function through transcriptional regulation of circadian rhythm and regulation of large-conductance Ca^{2+} channels.[50–52] The precise molecular targets of CO that confer these effects remain elusive, although spectroscopic investigations have led

to the identification of several hemoprotein targets of CO, including NPAS2/CLOCK and Rev-Erbβ as well as Ca^{2+}- and voltage-gated K^+ channels.[53–59] These hemoproteins exhibit high (nM) CO binding affinities; however, the relative affinities of NO and O_2 have not been determined. Additionally, a large conceptual gap still exists between in vitro CO-binding properties of these putative mammalian CO sensors and in vivo conditions that could result in CO-dependent signaling. One step toward closing this gap is to better understand the spatiotemporal distribution of CO and NO in cellular and animal models. Understanding the interplay between and NO and CO signaling is a crucial area of future study because NO and CO likely compete for heme sites under certain physiological and pathophysiological conditions.

sGC as a Therapeutic Target: Pharmacological Stimulators and Activators.

Given the central role of sGC in the NO–sGC–cGMP signaling axis and the relevance of this pathway to cardiovascular disease, many therapeutic strategies that directly target sGC have been developed. Strategies aimed at targeting sGC via NO biosynthesis will be discussed in detail below. In this section, we briefly highlight pharmacological approaches to modulate sGC activity in the context of stimulators and activators. For a comprehensive overview of sGC therapeutic strategies, we direct the reader to recent reviews.[9,60]

sGC stimulators have emerged as a promising class of therapeutics currently undergoing preclinical and clinical studies to treat pathophysiological conditions including cardiovascular, fibrotic, hematologic, and metabolic diseases. sGC stimulators are organic small molecules that interact directly with the sGC β1 HNOB subunit bearing a ferrous heme that is primed for NO sensing. Pharmacologic enhancement of the sGC enzymatic activity was initially characterized as direct stimulation in an NO-independent manner,[61–64] but subsequent studies have demonstrated that sGC stimulators also sensitize the enzyme to NO (and CO), allowing for NO-dependent activation at lower concentrations of NO.[65] The detailed molecular interactions that confer stimulatory activity on this class of drugs are not completely understood but likely involve contacts between a stimulator molecule and the heme-containing β1 HNOB domain. Presumably, these interactions stabilize active sGC protein conformation[30,31] and/or enhance NO geminate recombination by blocking a heme pocket exit channel.[66,67] Preclinical animal models have suggested roles for sGC stimulators in the treatment of a wide variety of diseases, including pulmonary hypertension,[68,69] heart failure,[70] chronic kidney disease,[71,72] fibrosis,[73–75] metabolic disease,[76] and sickle cell disease.[77] Building on these preclinical findings, researchers have conducted (or are currently conducting) clinical trials to extend therapeutic applications of sGC stimulators to human patients. One stimulator compound, riociguat (BAY 63–2521), has been approved to treat pulmonary arterial hypertension (PAH) and chronic thromboembolic pulmonary hypertension.[78,79] An ongoing phase 2 clinical trial in our laboratory is assessing the use of riociguat to mediate severe adverse cardiovascular events associated with sickle cell disease (NCT02633397). Two recently completed phase 3 trials assessed the efficacy of vericiguat (BAY 1021189), another sGC stimulator,[80] as a treatment for heart failure with reduced ejection fraction (HFrEF) and heart failure with preserved ejection fraction (HFpEF). These studies found that vericiguat treatment improved outcomes (death and incidence of

hospitalization) for patients with HFrEF, but outcomes (physical limitation score and 6 min walking distance) for patients with HFpEF did not improve upon treatment.[81,82]

sGC activators comprise a second class of drugs that enhance the enzymatic activity of sGC with inactive (ferric) heme or no heme present.[83] These activators serve as heme analogues that allosterically enhance sGC activity by mimicking the structure of a ferrous nitrosyl-heme adduct, either through direct binding to apo-sGC or replacement of ferric heme.[84,85] Oxidation and loss of heme contribute to sGC inactivation and signal degradation. Such sGC loss is exacerbated under conditions of oxidative stress, and sGC activators may serve as a complementary pharmacologic intervention under pathophysiological conditions to enhance sGC activity and prevent enzymatic degradation.[86–89] In a preclinical study, mice that express heme-free sGC develop hypertension and exhibit blunted NO-dependent vasodilation; however, treatment with the sGC activator cinaciguat (BAY 58–2667) significantly decreased blood pressure, consistent with heme-independent activation.[90] While preclinical studies demonstrating the pharmacological benefits of sGC activators were promising, several clinical trials aimed at treating acute heart failure and peripheral arterial occlusive disease using cinaciguat were stopped after patients exhibited hypotension without clear benefits.[70] One open phase 1 clinical trial (NCT04609943) seeks to assess dose limitations of activator compound BAY 1211163 in patients with acute respiratory distress syndrome. Further research is required to better understand the limitations and ideal clinical applications of sGC activators.

L-ARGININE OXIDATION: NO BIOSYNTHESIS BY NOS

The canonical NO biosynthetic pathway features NOS enzymes that catalyze the oxidation of L-arginine to L-citrulline and NO. NOS enzymes are widely distributed among different tissues and are critical in the maintenance of vascular homeostasis as well as regulation of inflammation, immune response, and neurotransmission.[91] NOS-dependent generation of NO directly modulates the NO–sGC–cGMP signaling axis and therefore represents a valuable therapeutic target in NO signaling.

The NOS active site consists of a five-coordinate, thiolate-ligated heme center where two sequential O_2- and NADPH-dependent oxidation reactions occur via generation of a highly oxidizing iron–oxo species. First, one of the arginine guanidino N atoms is hydroxylated to form N^{ω}-hydroxy-L-arginine (NOHA), which is subsequently oxidized in a second reaction to form L-citrulline and a ferric nitrosyl species.[92] In order to become bioavailable, NO must dissociate from the ferric heme site. Untimely reduction of the ferric nitrosyl-heme to a nonlabile ferrous nitrosyl species initiates a “futile cycle” in which the enzyme returns to the ferric resting state upon reaction with O_2 and generation of nitrate. Thus, for optimal NO synthesis, reduction of ferric nitrosyl-heme occurs at a *slow* rate relative to NO dissociation from the ferric heme; however, reduction of the ferric–superoxo heme species and subsequent disproportionation occurs at a *fast* rate relative to superoxide dissociation.

To accommodate these kinetic constraints, the NOS enzymatic complex employs several cofactors and intricate quaternary structural interactions. The active site in the N-terminal oxygenase domain binds heme and the electron-transport mediator tetrahydrobiopterin

(BH_4). NADPH, which binds to a C-terminal reductase domain, reduces flavin adenine dinucleotide and flavin mononucleotide cofactors in the reductase domain before ultimately reducing the heme or BH_4 cofactors in the oxidase domain. An intervening calmodulin binding domain regulates electron shuttling from the reductase domain to the oxygenase domain: calmodulin binding to a NOS homodimer enables domain swapping in which electrons from one reductase domain monomer reduce the opposite oxygenase domain, triggering NO production in the presence of O_2 and L-arginine substrates.[93–96]

Three NOS isoforms exist in humans: neuronal NOS (nNOS, NOS I), inducible NOS (iNOS, NOS II), and endothelial NOS (eNOS, NOS III). These three isoforms are structurally homologous and share 50–60% overall sequence homology,[97,98] although significant differences exist between nNOS, iNOS, and eNOS. These NOS isozymes exhibit differential expression patterns at the tissue and subcellular levels.[60] Both nNOS and eNOS are constitutively expressed, and protein activity is primarily dictated by calmodulin/Ca^{2+} binding and post-translational modifications.[99–101] For example, half-maximal activity for nNOS and eNOS occurs at Ca^{2+} concentrations of 150 and 300 nM, respectively, while iNOS activity exhibits no Ca^{2+} dependence.[98,102] Unlike nNOS and eNOS, iNOS is primarily regulated at the transcriptional level.[102] Differences in kinetic parameters, including the heme reduction rate, O_2 binding rate, k_{cat}, and NO release rate, enable NOS isoforms to produce NO at different fluxes under different cellular conditions.[92,103] We direct the reader to several excellent reviews for more details regarding physiological regulation of isoform-specific NOS activity.[60,91,98,100,104,105]

Uncoupling of NOS enzymes, particularly eNOS, lowers NO production and often generates highly oxidizing superoxide and peroxynitrite species. Formally, NOS uncoupling occurs when NADPH consumption does not match stoichiometric NO production.[60,106–108] Such uncoupling may be caused by a deficiency of the L-arginine substrate, either through trafficking/compartmentalization or metabolism by arginases.[109–113] Alternatively, NOS uncoupling may arise when electron shuttling between reductase and oxygenase domains is disrupted. Changes in the quaternary structure, including calmodulin and/or dimer dissociation, give rise to NOS uncoupling, often with concomitant generation of superoxide.[114–119] Equivalents of superoxide may rapidly react with nearby NO, resulting in the formation of peroxynitrite, a highly reactive oxidant.[120] In the presence of strong oxidants, such as superoxide- or peroxynitrite-derived species, BH_4 can be oxidized to dihydrobiopterin (BH_2), which competitively binds to eNOS and is unable to facilitate heme reduction and subsequent L-arginine hydroxylation.[121–123] Post-translational modifications, including glutathionylation of eNOS cysteine residues and phosphorylation of a critical threonine residue, have been shown to disrupt electron flow and uncouple NOS activity as well.[101,124–126] Importantly, eNOS is responsible for the production of basal NO levels that maintain optimum cardiovascular function,[127] and eNOS uncoupling is implicated in myriad cardiovascular diseases.[128–133] Therapeutic strategies to combat eNOS uncoupling focus on increasing bioavailable L-arginine or BH_4 by direct supplementation, stimulation of production pathways, or inhibition of metabolic/decomposition pathways.[134–138]

NITRITE REDUCTION: THE NITRATE–NITRITE–NO PATHWAY

While NOS enzymes represent the canonical physiological source of NO, additional routes for NO generation have been established in recent years via the nitrate–nitrite–NO pathway. This pathway is critical for NO regulation and metabolism; nitrite and nitrate serve as long-lasting NO storage pools and have become valuable therapeutic targets in the past 20 years. Importantly, many of the chemical and biochemical reactions that convert nitrate and nitrite to NO occur under O_2-limited conditions, allowing the nitrate–nitrite–NO pathway to complement O_2-dependent NO production in NOS enzymes.

Inorganic nitrate (NO_3^-) and nitrite (NO_2^-) act as stable NO precursors under physiological conditions. Facultative anaerobes inhabiting the human salivary glands reduce dietary nitrate —abundant in leafy green vegetables, beets, and cured meats—to nitrite using reductase enzymes analogous to those found in soil-denitrifying bacteria.[139–141] Once swallowed, ingested nitrite and nitrate that exceed capacity for oral bacterial reduction can be absorbed in the gastrointestinal tract. Interestingly, as much as 25% of nitrate circulating in the plasma is reconcentrated in saliva via the sialin transporter, driving an enterosalivary recirculation of nitrate and additional nitrate reduction.[142,143] Nitrite and nitrate are also generated endogenously through oxidation of NO primarily derived from eNOS.[144] In plasma, oxidation of NO to form nitrite is catalyzed by the multicopper oxidase enzyme ceruloplasmin (eq 1).[145]

$$NO + Cu^{2+} + H_2O \rightarrow Cu^{+} + NO_2^{-} + 2H^{+} \quad (1)$$

Reoxidation of copper occurs in an O_2-dependent fashion. Under basal conditions, dietary nitrate reduction accounts for approximately half of the nitrite found in the plasma, while NO oxidation by ceruloplasmin accounts for the other half.[146–148] While exogenous sources likely contribute to the majority of nitrate found in the body,[149] endogenous oxidation of NO to form nitrate, a process known as NO dioxygenation, is facilitated by oxyferrous heme sites (eq 2).[150,151]

$$Fe^{II} - O_2 + NO \rightarrow Fe^{III} + NO_3^- \quad (2)$$

NO dioxygenation, a reaction common to all oxyferrous hemoproteins, has been specifically characterized in globin proteins found in the vasculature in vivo, including α-hemoglobin and cytoglobin,[152–155] and likely contributes to regulation of the NO levels in the vascular endothelium.

Noncatalytic nitrite reduction to NO can occur in vivo as the physiological pH decreases. Nitrite is a weak base with a pK_a value of 3.11 at 37 °C,[156] and the standard reduction potential for nitrite changes significantly from highly favorable under acidic conditions to unfavorable under basic conditions (eqs 3 and 4).[157,158]

Reductive half-cell reaction for nitrite under acidic conditions:

$$HNO_2 + H^+ + e^- \rightarrow NO + H_2O \quad E^\circ = 0.99\,V \quad (3)$$

Reductive half-cell reaction for nitrite under basic conditions:

$$NO_2^- + H_2O + e^- \rightarrow NO + 2OH^- \quad E^\circ = -0.46\,V \tag{4}$$

Further, a well-accepted mechanism of nitrite reduction in acidic, aqueous solution involves dehydration of nitrous acid to generate dinitrogen trioxide (N_2O_3; eq 5), followed by disproportionation of N_2O_3 to generate 1 equiv of NO and 1 equiv of nitrogen dioxide (NO_2; eq 6).

$$2HNO_2 \rightleftharpoons N_2O_3 + H_2O \tag{5}$$

$$N_2O_3 \rightleftharpoons NO + NO_2 \tag{6}$$

This acid-promoted nitrite reduction is viable under conditions of low pH and high nitrite concentrations.[159,160] During ischemia, reduced blood flow results in tissue acidosis that may support acid-promoted nitrite reduction.[161] Additionally, the above conditions are met in the stomach after a nitrate-rich meal, where NO has been shown to regulate gastric blood flow, mucous production, and host defense.[162–164] In addition to liberating NO, N_2O_3 is a powerful nitrosating agent that can react with primary and secondary amines to form *N*-nitrosoamines (vide infra). Subsequent metabolism of large quantities of *N*-nitrosoamines can lead to the formation of carcinogenic methylating agents,[165] and some associative studies suggest that this adverse reactivity may contribute to an increased risk of malignancy for those who consume large quantities of processed meats that utilize nitrite in the curing process.[166] On the other hand, ingestion of nitrate-rich leafy green vegetables leads to very high nitrite levels via nitrate bioconversion by oral bacteria, yet diets high in leafy green vegetables have not been consistently associated with significant risk of malignancy in large epidemiological studies.[143,149,167]

BIOACTIVATION OF NITRITE IN RED BLOOD CELLS (RBCS)

Because acid-promoted nitrite reduction occurs under limited conditions in vivo, nitrite would appear to serve little role in generating bioavailable NO. Very early work exploring the vasodilatory effects of nitrite in vitro seemed to support this theory: supraphysiological concentrations of nitrite were required to induce vasodilation in aortic rings, and these vasodilatory effects could be enhanced at lower pH values.[168–171] However, a series of investigations characterizing nitrite levels (which vary from 150 to 1000 nM in plasma and up to 10 μM in tissue)[172,173] and nitrite-dependent vasodilation in humans definitively identified a role for nitrite in the regulation of vascular tone through bioactivation. These studies demonstrated that plasma nitrite levels (1) exhibit an arterial–venous gradient, suggesting that nitrite is consumed across the physiological O_2 gradient, (2) correlate well with eNOS activity, (3) increase with NO inhalation, and (4) decrease under conditions of hypoxia or exercise-induced stress.[147,172,174,175] Additionally, infusion of nitrite (at near-physiological nanomolar concentrations) in the brachial forearm artery decreased systemic blood pressure and increased blood flow.[176,177] Subsequent investigations in animals and humans corroborated these findings: infusion of nitrite decreased blood pressure in a

dose-dependent manner, and blood pressure returned to basal levels several hours after infusion.[178–181] Furthermore, venous nitrosyl-heme levels increased significantly during nitrite infusion, consistent with nitrite bioactivation to generate NO and subsequent up-regulation of vasodilation via the NO–sGC–cGMP pathway.[176]

Originally, researchers suggested that molybdenum-containing oxidoreductase enzymes, which exhibit nitrite reductase activity under anaerobic conditions,[182,183] could facilitate nitrite bioactivation. In preclinical rodent models and in vitro studies at low O_2 tensions and very acidic pH values, specific inhibitors of the molybdopterin protein xanthine oxidoreductase (XOR) attenuated nitrite reductase activity and subsequent nitrite-dependent vasodilation;[179,184] however, specific inhibition of XOR activity did not inhibit nitrite-dependent vasodilatory response in humans given an infusion of nitrite.[176,178] While it is likely that molybdopterin enzymes contribute to nitrite bioactivation in tissue under specific physiological O_2 and pH conditions, another highly abundant metalloprotein, hemoglobin (Hb), primarily facilitates nitrite bioactivation in circulation.

Consistent with in vivo data indicative of nitrite-dependent vasodilation, in vitro experiments indicate that hemoglobin facilitates nitrite reduction in RBCs. For example, aortic rings exhibit vasodilatory activity in the presence of deoxyhemoglobin (deoxyHb) and nitrite but not in the presence of nitrite alone.[176,185] This Hb-facilitated bioactivation of nitrite modulates signaling along the NO–sGC–cGMP pathway because incubation of RBCs with nitrite induces cGMP production and subsequently inhibits platelet activation (vide infra).[186–189] Further, NO gas was indirectly observed by a chemiluminescent reporter in reactions of deoxygenated RBCs and rat aortas with nitrite.[171,185,188,190]

These seminal discoveries prompted exploration of the molecular mechanisms that drive nitrite-mediated signaling, and in this section, we review the proposed chemical and biochemical reaction pathways that occur between hemoglobin, NO, nitrite, and other nitrogen oxides in RBCs. While multifaceted and complex in nature, together these reactions may explain the observed pharmacological and in vivo effects of nitrite acting as a regulator of vascular tone under a variety of physiological and pathophysiological conditions. The elucidation of these pathways has been crucial for the development of nitrite therapeutics targeting cardiovascular diseases.

Hb-Facilitated Nitrite Reduction.

When O_2 tensions are low at physiologically relevant pH values, ferrous hemoproteins, specifically globins, facilitate nitrite reduction to generate NO (Figure 2, solid red pathway). Reduction of nitrite occurs via an inner-sphere electron-transfer mechanism in which an equivalent of nitrite binds to deoxyHb, is protonated, and then is reduced, resulting in 1 equiv of NO, ferric hemoglobin (metHb), and water (eq 7).[191–194]

$$NO_2^- + H^+ + Fe^{II} \rightarrow NO + Fe^{III} + HO^- \quad (7)$$

Importantly, NO binds to ferric heme with an affinity several orders of magnitude lower than that of ferrous heme[195] and therefore undergoes facile diffusion away from the site of nitrite reduction to participate in downstream signaling pathways.

Evidence from in vitro chemical and biochemical experiments, as well as in vivo preclinical and clinical studies, has coalesced to support a paradigm in which RBC-derived hemoglobin mediates nitrite reduction to produce bioavailable NO; however, three critical biochemical reactions deplete NO and thereby complicate this model. First, as aforementioned, ceruloplasmin (present at concentrations of 1–5 μM in plasma)[196,197] readily oxidizes NO and generates about half of the nitrite found in blood plasma (eq 1).[145,146] Second, at lower O_2 tensions, NO generated by Hb-mediated nitrite reduction in RBCs can rapidly bind to nearby deoxyHb sites (eq 8).

$$NO + Fe^{II} \leftrightharpoons Fe^{II} - NO \tag{8}$$

This nitrosylation or "autocapture" reaction occurs with a rate constant of 9×10^7 M^{-1} s^{-1} for deoxyHb[198] and effectively sequesters NO as a highly stable ferrous nitrosyl-heme species. Third, at higher O_2 tensions, NO reacts with oxyHb to generate nitrate through NO dioxygenation (eq 2; Figure 2, pink pathway), which occurs with a near-diffusion-limited rate constant of (6–8) $\times 10^7$ M^{-1} s^{-1} for oxyHb at 20 °C.[150,199,200] Taking into account these mechanisms of NO depletion, one model suggests that NO exhibits a half-life of 1 μs in RBCs.[201] Thus, a key mechanistic question surrounds nitrite-mediated signaling: how do RBCs facilitate nitrite bioactivation in a manner that produces *bioavailable* NO? Some evidence suggests that nitrite reduction occurs preferentially at the RBC cell membrane surface,[202] which would allow for more facile NO escape, especially considering that NO readily partitions to nonpolar media over aqueous media.[160] However, compartmentalization alone likely does not fully rectify the rapid rate of NO depletion in RBCs, and additional chemical and other Hb-mediated reactions are worth considering.

Role of N_2O_3 in Nitrite-Mediated Signaling.

Nitrite-mediated signaling in RBCs may be facilitated by N_2O_3,[203] which is generated in a very fast radical reaction between NO and NO_2 (reverse reaction of eq 6). The requisite equivalent of NO_2 may form as a product of NO autoxidation (eq 9).

$$2NO + O_2 \rightarrow 2NO_2 \tag{9}$$

While this third-order reaction does not typically proceed under physiological conditions in the aqueous cellular environment, more favorable reaction conditions may occur in the nonpolar environment of the RBC cell membrane, where it is estimated that autoxidation may occur up to 30 times faster than that in an aqueous environment due to enhanced solubility of NO and O_2.[204] Additional nitrite- and Hb-dependent pathways that generate NO_2 exist under physiological conditions (vide infra).

Another pathway to form N_2O_3 occurs via certain Hb-mediated reactions. Because deoxyHb facilitates nitrite reduction, a transient ferric-nitrosyl intermediate forms (Figure 2, orange pathway). This electrophilic intermediate may subsequently react with 1 equiv of nitrite, liberating N_2O_3 and ferrous hemoglobin (eq 10).[159,205,206]

$$\left[Fe^{III} - NO \Leftrightarrow Fe^{II} - NO^+\right] + NO_2^- \rightarrow Fe^{II} + N_2O_3 \tag{10}$$

Such reductive nitrosylation may also occur when water attacks the ferric-nitrosyl species, liberating 1 equiv of nitrite and ferrous heme (eq 11).

$$\left[Fe^{III} - NO \Leftrightarrow Fe^{II} - NO^{+}\right] + H_2O \rightarrow Fe^{II} + HNO_2 + H^{+} \quad (11)$$

Nitrite accelerates the rate of reductive nitrosylation, presumably because of the enhanced nucleophilicity of nitrite compared to water or hydroxide.[206] Alternatively, nitrite may first bind to a ferric heme site and then react with 1 equiv of NO to liberate N_2O_3 and ferrous heme (eq 12).[203]

$$\left[Fe^{III} - NO_2^{-} \Leftrightarrow Fe^{II} - NO_2\right] + NO \rightarrow Fe^{II} + N_2O_3 \quad (12)$$

Computational and electron paramagnetic resonance spectroscopic evidence suggests that the ferric-nitrite species exhibits significant electron delocalization, giving the heme species partial ferrous-NO_2 radical character.[203,207–209] This radical character would suggest rapid reaction with NO to yield N_2O_3 in a “nitrite anhydrase” mechanism (Figure 2, blue pathway). Given the highly dynamic nature of nitrogen oxide reactions in RBCs, it is entirely possible that both of the above mechanisms (eqs 10 and 12) operate, although these details have yet to be resolved.

Formation of N_2O_3 in RBCs may facilitate export of NO via several pathways. As a small, uncharged molecule, N_2O_3 can readily diffuse across the cell membrane, and the molecule undergoes facile homolytic cleavage to generate NO and NO_2.[187] As described above, N_2O_3 is a powerful nitrosating agent (i.e., NO^+ donor) because of partial $NO^+NO_2^-$ character.[210] Under physiological conditions, N_2O_3 may transfer NO^+ to nucleophilic thiols and amines (eqs 13 and 14).

$$N_2O_3 + RSH \rightarrow RSNO + H^{+} + NO_2^{-} \quad (13)$$

$$N_2O_3 + RR'NH \rightarrow RR'N(NO) + H^{+} + NO_2^{-} \quad (14)$$

Reversible S-nitrosation of β-hemoglobin Cys93 (SNO-Hb) has been proposed as an additional mechanism to extend the lifetime of Hb-derived NO;[211] however, the details of this proposed mechanism are heavily debated. A recombinantly expressed Cys substitution hemoglobin variant, β-Cys93Ala, did not inhibit nitrite-dependent vasodilation in vitro, and an in vivo study showed that genetically engineered mice with the same β-Cys93Ala substitution do not exhibit impaired hypoxic vasodilation.[185,212] While these results demonstrate that SNO-Hb is not a critical intermediate in nitrite-mediated signaling, modifications to β-Hb Cys93, including mutagenic Cys substitution and alkylation using NEM, potentiate nitrite reductase activity by decreasing the heme redox potential and allosterically stabilizing the R-state hemoglobin (vide infra).[213–215] Thus, post-translational SNO-Hb may also enhance nitrite reductase activity and thereby potentiate the rate of nitrite reduction. More generally, post-translational modifications of Cys residues via S-nitrosation may alter protein function in many important physiological and pathophysiological contexts, such as reversible inhibition of proteins in the mitochondrial electron-transfer chain.[216–219]

Allosteric Regulation of Hb-Facilitated Nitrite Reduction.

Fully unliganded hemoglobin exists in the T-state, which exhibits low ligand binding affinity. The binding of ligands, including O_2, CO, and NO, to heme sites within hemoglobin favors an allosteric transition to the high-affinity R-state.[220] These allosteric changes also influence nitrite reduction as the rate constant for deoxyHb increases from 0.1 M^{-1} s^{-1} in the T-state to 6 M^{-1} s^{-1} in the R-state at 25 °C.[194,221–223] Two factors likely contribute to this allosterically induced change in the nitrite reductase activity. First, nitrite reduction (and subsequent heme oxidation) is thermodynamically favored for R-state hemoglobin, which has a lower redox potential than T-state hemoglobin ($E_{1/2}$ vs NHE: HbA^R = 42 mV; HbA^T = 154 mV).[185,194,215] Second, nitrite reduction is kinetically favored for R-state hemoglobin, which possesses a more open heme pocket that allows for facile nitrite binding.

As Hb-mediated nitrite reduction proceeds, NO may bind to a neighboring ferrous heme. NO binding allosterically stabilizes R-state hemoglobin and thereby *increases* the nitrite reduction rate constant of other heme sites within the tetramer. Simultaneously, NO binding *limits* the number of available ferrous heme sites for subsequent nitrite reduction. Under anaerobic conditions, this effect is known as “allosteric autocatalysis” and results in a sigmoidal reaction trace for nitrite reduction.[194,221] Similarly, O_2 binds to deoxyHb under more aerobic conditions, stabilizing the R-state and enhancing nitrite reduction while simultaneously limiting the number of sites where nitrite can react.[221] These opposing ligand-dependent effects give rise to a bell-shaped curve upon estimation of the nitrite reductase activity as a function of O_2 tensions, with maximal activity occurring between 40 and 60% oxyHb saturation (Figure 3).[224,225] Importantly, this range of maximal activity directly coincides with the set point for hypoxic vasodilation in humans, a physiological process in which blood vessels dilate in order to enhance blood flow and match O_2 delivery as hemoglobin desaturates.[226] The coincidence of oxyHb saturation levels for maximal Hb-mediated nitrite reductase activity and the onset of hypoxic vasodilation further supports the hypothesis that bioactivation of nitrite in RBCs contributes to the regulation of vascular tone under physiological conditions in the capillary bed.

Pathways That Propagate Nitrite-Mediated Signaling under Oxygen-Replete Conditions.

In addition to nitrite reduction with deoxyHb, nitrite undergoes a complex series of reactions with oxyHb to generate nitrate and metHb at O_2 tensions near the P_{50} value for hemoglobin (27 mmHg).[227] An initial lag phase is observed when an excess of nitrite is present relative to oxyHb,[228,229] which yields hydrogen peroxide (H_2O_2) and nitrate (eq 15; Figure 2, dashed red pathway).

$$2\mathrm{Hb}\left[\mathrm{Fe}^{\mathrm{II}}-\mathrm{O}_2\right]+2\mathrm{NO}_2^-+2\mathrm{H}^+\rightarrow 2\mathrm{Hb}\left[\mathrm{Fe}^{\mathrm{III}}\right]+\mathrm{H}_2\mathrm{O}_2+2\mathrm{NO}_3^- \quad (15)$$

Several key studies suggest that this initial lag phase involves H_2O_2 but not superoxide ($O_2^{\bullet-}$): catalase, but not superoxide dismutase, inhibits the reaction when added during the initial reaction phase, and kinetic models that incorporate H_2O_2 generation are consistent with experimentally observed reaction traces.[229–231] Reaction of peroxide with metHb results in formation of a ferryl radical cationic $[Fe^{IV}=O)]^{\bullet+}$ species reminiscent of that observed in compound I of cytochrome P450.[229,232] Subsequent reactions of 2 equiv

of nitrite, first with the $[Fe^{IV}=O)]^{\bullet+}$ ferryl radical cation and second with the reduced diamagnetic $[Fe^{IV}=O]$ ferryl intermediate species, result in 2 equiv of NO_2 and metHb in an autocatalytic propagation reaction (eq 16; Figure 2, solid gray pathway).

$$Hb^{\bullet+}\left[Fe^{IV}=O\right]+2NO_2^-+2H^+\rightarrow Hb\left[Fe^{III}-OH_2\right]+2NO_2 \quad (16)$$

Importantly, when the cellular partial pressure of O_2 nears the P_{50} value of hemoglobin, hemoglobin will exhibit partial O_2 saturation. At such physiologically relevant O_2 tensions, nitrite reduction by deoxyHb occurs alongside the nitrite–oxyHb reaction.[232] Cross-reactivity between the products of these two Hb-mediated reactions provides (1) a means to quench propagation of the nitrite–oxyHb mechanism and (2) another pathway for NO escape from RBCs. NO_2, responsible for autocatalysis, oxidizes "inert" ferrous nitrosyl-heme, generated during nitrite reduction, to ferric nitrosyl-heme (eq 17; Figure 2, dashed black pathway).[233,234]

$$Fe^{II}-NO+NO_2\rightarrow Fe^{III}-NO+NO_2^- \quad (17)$$

As described above, ferric heme centers generally exhibit NO dissociation rate constants several orders of magnitude higher than those of ferrous heme centers,[195] and therefore this "oxidative denitrosylation" process leads to facile release of Hb-bound NO.[232]

In summary, numerous physiological data in vitro and in vivo (through human and animal studies) provide evidence that RBC-encapsulated hemoglobin facilitates nitrite-dependent vasodilation and platelet activation via the NO–sGC–cGMP pathway;[176–179,181,186,187,189,235] however, the precise mechanisms that enable this bioactivation are not fully understood. A lingering central question is whether inefficient NO diffusion at the RBC membrane accounts for signaling or if intermediate chemical species are required. The above chemical reactions involving hemoglobin, oxygen, nitrite, NO, NO_2, N_2O_3, and *S*-nitrosothiols, serve as examples of putative pathways that may allow nitrite-derived nitrogen oxides to escape RBCs and participate in the observed downstream signaling. Our investigations all support convergent bioactivation around the P_{50} value of hemoglobin, where (1) NO signaling has been detected experimentally by NO formation, platelet inhibition, vasodilation, and inhibition of mitochondrial respiration and (2) reactions that generate NO, NO_2, and N_2O_3 are likely operative. It is important to note that the prevalence of these different reaction pathways varies under different conditions (i.e., O_2 tension, nitrite concentration and relevant rate constants, pH, membrane localization). Further, there may be undiscovered routes that mediate nitrite-based signaling in RBCs. Fully elucidating the mechanistic details of nitrite signaling in the vasculature and in tissue will provide valuable context for interpretation of the results from clinical trials that employ nitrite therapeutics, described below.

Nitrite Therapeutics.

A growing body of preclinical and clinical studies support the use of nitrate and nitrite therapeutic agents for the controlled delivery of NO. In contrast to NO, which is quickly consumed in the blood ($t_{1/2}$ < 2 ms),[236,237] nitrite persists in circulation long enough

to reach peripheral blood vessels and tissues ($t_{1/2}$ = 30–48 min).[178,180,238,239] In fact, the peripheral vasodilatory effects of therapeutically inhaled NO have been ascribed to more stable NO-derived species, such as S- and N-nitrosated proteins (including SNO-albumin and SNO-Hb),[211,240] nitrated lipids,[241,242] and nitrite,[176] which may form in the pulmonary vasculature during NO inhalation.[222] Hb-facilitated nitrite reduction occurs at low O_2 tensions, and this process offers a complementary pathway to generate NO under physiological or pathological conditions of hypoxia.[161,176,178,243,244] This selective bioactivation provides advantages for nitrite as a NO-generating therapeutic compared to other compounds, such as drug-conjugated NO-releasing moieties, *S*-nitrosothiols, and NONOates, which either undergo premature metabolism before reaching the target tissue or release NO indiscriminately, potentially giving rise to off-target side effects.

By serving as a supplemental source of NO, nitrite may be able to rescue impaired vasodilatory function in patients with hypertension or cardiovascular disease through direct modulation of the NO–sGC–cGMP pathway. Several translational studies have investigated the utility of inhaled, nebulized nitrite in the treatment of PAH associated with heart failure with preserved ejection fraction (PAH-HFpEF).[238,245–247] Acute, inhaled nitrite lowers pulmonary arterial pressure, pulmonary capillary wedge pressure, and pulmonary vascular resistance in several preclinical and clinical studies of animals/patients with PAH-HFpEF.[181,246,248,249] In a recent investigation of five patients with PAH associated with β-thalassemia, the pulmonary arterial pressure was shown to decrease immediately upon nitrite inhalation but returned to basal levels within 15 min of cessation of ventilation.[250]

Given the reversibility of cardiac outcomes observed with inhaled or infused nitrite treatment, numerous preclinical and clinical studies have investigated acute and long-term oral nitrite or nitrate supplementation as a means to achieve improvements in cardiac function in conditions linked to cardiovascular disease.[251,252] As described above, nitrate can be reduced to nitrite by microorganisms in the oral microbiome,[139,253,254] and dietary nitrate supplementation, particularly in beetroot juice and green leafy vegetables, has been utilized extensively as a pharmacological means to increase circulating nitrite levels in humans.[167,244,255–257] Several clinical studies have shown that short- and long-term supplementation with oral nitrate reduces blood pressure in hypertensive patients.[167,179,256,258–261] Contrary to these findings, other studies have shown no nitrate-dependent changes in blood pressure in hypertensive patients,[262,263] although these differences may be due to underpowered study groups, differences in dosing, and variations in participant microbiomes that result in varied nitrate reductase activity and subsequent nitrite bioavailability.[264–266] Our laboratory is currently conducting a placebo-controlled phase II clinical trial to examine how oral nitrite (40 mg three times a day for 10 weeks) effects exercise capacity and hemodynamic outcomes in PAH-HFpEF patients (NCT03015402). For a more complete summary of preclinical and clinical studies that probe the therapeutic potential of nitrite, we direct the reader to a recent review by Kapil et al.[252]

NITRITE-MEDIATED SIGNALING IN ORGANS AND TISSUES

Given its abundance, hemoglobin is likely the primary source of nitrite-derived NO in the vasculature; however, nitrite reductase activity has been observed in a wide

variety of additional metalloproteins.[60,251] Generally, nitrite reduction is facilitated by two metal-containing cofactors: heme and molybdopterin. Like hemoglobin, other heme-containing globin proteins, including myoglobin (Mb),[216,267] neuroglobin (Ngb),[268,269] and cytoglobin (Cygb),[153,270] exhibit nitrite reductase activity (eq 7). Analogous reactivity has been observed in several nonglobin hemoproteins, including but not limited to eNOS,[271] partially unfolded cytochrome *c*,[272,273] cytochrome P450 2B4, and human cystathionine β-synthase.[274,275] Molybdopterins comprise an additional class of redox-active metallocofactors that facilitate nitrite reduction via $Mo^{IV/V}$ and $Mo^{V/VI}$ redox couples. Nitrite reduction occurs more slowly when facilitated by molybdopterin compared to heme, and O_2-sensitive molybdoenzymes, such as XOR, primarily contribute to nitrite bioactivation in tissues where nitrite accumulates at higher concentrations and O_2 tensions are lower.[276,277] Because this Forum Article focuses on hemoprotein reactivity, we direct the reader to other reviews that discuss molybdopterin-facilitated nitrite reduction in greater detail.[278,279]

Mb-Facilitated Nitrite Signaling in Tissue.

Myoglobin facilitates nitrite reduction in a mechanism analogous to that of hemoglobin and likely mediates NO signaling in cardiac and skeletal muscle tissues. The reaction rate constant for deoxymyoglobin (deoxyMb), 5.5 M^{-1} s^{-1} at 25 °C (12 M^{-1} s^{-1} at 37 °C), is comparable to that of R-state hemoglobin.[216,280] NO dioxygenation is also nearly diffusion-limited in oxymyoglobin (oxyMb, second-order rate constant of 4.4×10^7 M^{-1} s^{-1} for equine oxyMb at 20 °C),[200] so myoglobin may act as a NO sink, as well as a NO source. Under normoxic conditions, oxyMb likely scavenges NO, protecting mitochondrial proteins from the inhibition of enzymatic activity.[281] Parallel NO-scavenging roles are proposed for cytoglobin and α-hemoglobin in vascular endothelial cells, where NO dioxygenation likely prevents excessive vasodilation.[152,154,282,283] Under very low O_2 tensions (P_{50} = 3 mmHg for Mb), such as those found in the ventricular walls of the heart, myoglobin may switch from NO scavenger (via dioxygenation) to NO source (via nitrite reductase).[243] Thus, Mb-facilitated nitrite reduction likely contributes to the cardioprotective properties of nitrite.

Emerging Roles of Neuroglobin and Cytoglobin.

Two additional heme-containing globin proteins, neuroglobin and cytoglobin, have emerged in recent years as regulators of NO signaling. The expression patterns of these hemoproteins differ drastically from those of hemoglobin and myoglobin: neuroglobin is expressed at high levels (100–200 μM) in retinal cells and at low levels (~1 μM) in other tissues in the nervous system, gastrointestinal tract, and endocrine organs, while cytoglobin is ubiquitously expressed at low levels.[284–288] Because neuroglobin and cytoglobin are not expressed at millimolar levels in tissues, these recently discovered globins likely do not play a significant role in gas exchange/O_2 transport.

While the specific functions of neuroglobin and cytoglobin in different cellular contexts have not been fully elucidated, in vitro biochemical studies reveal that both proteins facilitate nitrite reduction. Unlike hemoglobin and myoglobin, the distal histidine in neuroglobin and cytoglobin serves as an axial heme ligand, and these proteins exist in equilibrium between distally bound and unbound states.[286,289–291] This labile axial His

ligand dictates small-molecule ligand binding and reactivity as the heme switches from a coordinatively saturated, six-coordinate environment to a coordinatively unsaturated, five-coordinate environment: e.g., a constitutively five-coordinate neuroglobin variant in which the distal His is mutated to a nonheme-coordinating leucine residue (H64L) exhibits nitrite reductase activity 3 orders of magnitude higher than that of the wild-type protein.[268] Interestingly, the distal His binding equilibrium is allosterically regulated by the redox status of two Cys residues outside of the heme pocket. Under oxidizing conditions, a disulfide bridge forms between these two Cys residues (Cys46 and Cys55, which span the CD loop in Ngb; Cys38 and Cys83, which span B and E helices in Cygb), opening the heme pocket and favoring dissociation of the distal His ligand.[268,270,292–296] Consequently, this redox-dependent modulation of heme coordination influences NO and nitrite reactivity in Ngb and Cygb. For example, both proteins exhibit faster nitrite reduction in the disulfide form ($k_{S–S}$ = 0.12 M^{-1} s^{-1} for Ngb and $k_{S–S}$ = 32.3 M^{-1} s^{-1} for Cygb, both at 25 °C) compared to the free thiol form ($k_{S–H}$ = 0.062 M^{-1} s^{-1} for Ngb and $k_{S–H}$ = 0.4–0.63 M^{-1} s^{-1} for Cygb, both at 25 °C),[268,270] suggesting that the cellular redox environment can directly modulate protein reactivity.[295] Protein oligomeric status may also influence ligand binding and reactivity because dimeric cytoglobin bearing intermolecular disulfides exhibits significantly diminished nitrite reductase activity ($k_{S–S(dimer)}$ = 0.26 M^{-1} s^{-1} for Cygb at 25 °C).[270]

Under O_2-replete conditions, cytoglobin likely attenuates NO signaling by facilitating NO dioxygenation. While virtually all oxyferrous hemoproteins may participate in NO dioxygenation reactions in vitro (eq 2), this reaction results in the formation of iron(III) heme, which cannot undergo subsequent NO dioxygenation. This observation suggests that NO scavenging will be limited to stoichiometric reactions in vivo.[297] Importantly, a coupled NADH/cytochrome b_5/cytochrome b_5 reductase system reduces iron(III) heme in cytoglobin,[154] allowing for efficient cytoglobin redox cycling and NO scavenging under physiological conditions.[155] Several in vivo studies suggest that Cygb-mediated NO scavenging in vascular endothelial cells prevents excessive vasodilation at high NO fluxes.[155,282,298] As aforementioned, analogous NO scavenging is carried out by α-hemoglobin in vascular endothelial cells,[152] and the precise interplay between cytoglobin and α-hemoglobin as NO-metabolizing regulators of the vascular tone requires further study.

Nitrite as a Cytoprotectant.

Nitrite-mediated cytoprotection in tissues has been studied extensively in the context of ischemia-reperfusion (I/R) injury, and most of the cytoprotective mechanisms involve bioactivation of nitrite to generate NO.[219] Under ischemic conditions, tissues become hypoxic and may also experience acidosis, leading to favorable nitrite reduction conditions by metalloproteins such as myoglobin.[201] The specific metalloproteins responsible for nitrite reduction likely vary organ to organ based on conditions and expression levels. For example, in the ischemic heart, deoxyMb is likely the primary source of nitrite-derived NO: nitrite reduction in heart tissue was abolished in myoglobin global knockout mice,[299] while allopurinol, a specific inhibitor of the molybdopterin enzyme XOR, only lessened nitrite-derived NO generation in heart homogenates by 10%[216]

In ischemic tissues, nitrite-derived nitrogen oxides primarily exhibit cytoprotective effects by targeting specific mitochondrial proteins.[219] NO can directly nitrosylate the O_2-reducing a_3 heme site in complex IV of the electron-transport chain,[300–302] slowing the rate of O_2 consumption and ameliorating ischemic effects through preservation of high-energy phosphate reserves.[299] As alluded, studies in vivo suggest that Mb-facilitated nitrite reduction is the primary source of NO in the heart: the absence of myoglobin abolished nitrite-dependent cytoprotection in a mouse model of cardiac I/R injury.[299] Ischemic tissue acidosis would promote formation of N_2O_3 (eq 5), which can then modify electron-transport chain proteins, including complexes I and III as well as ATP-synthase, via S-nitrosation. These protein modifications confer cytoprotection during reperfusion by reversibly inhibiting enzymatic activity and curbing ATP synthesis and ROS production.[219,303] Importantly, thiol nitrosation is temporary, and these enzymes recover normal activity over time after reperfusion.[217] Finally, cytoprotection may also be conferred by a NO–sGC–cGMP-dependent mechanism that results in decreased mitochondrial calcium accumulation and ion permeability.[304–306]

Many animal models of I/R injury have demonstrated nitrite-dependent cytoprotection in all major organ systems; however, results from human clinical trials are variable, with nitrite exhibiting clear cytoprotective effects under certain pathological conditions and little protective effects under other conditions. Early pharmacokinetic and toxicity studies demonstrated the feasibility of nitrite infusion in the context of subarachnoid hemorrhage.[180,307] In contrast, intravenous nitrite treatment showed dubious effects in two placebo-controlled phase II clinical trials studying acute myocardial infarction and I/R injury after percutaneous coronary intervention (PCI).[308,309] However, in a follow-up study, Jones et al. also showed that *localized* intracoronary nitrite treatment prior to PCI attenuated immune response.[310] In the context of organ-transplant-induced I/R injury, therapeutic NO inhalation increased plasma nitrite levels and conferred improved organ function and patient outcomes after lung and liver transplantation.[311,312] Recently, a large (N = 1502), placebo-controlled phase II clinical trial assessed the therapeutic potential of nitrite administered to patients after cardiac resuscitation outside of a hospital setting (NCT03452917).[313] In an earlier phase I trial, acute intravenous treatment of sodium nitrite (45 or 60 mg dose) did give rise to an increase in cGMP and nitrated fatty acid levels;[314,315] however, nitrite intervention did not improve survival. Dose-dependent toxicity due to hypotension and methemoglobinemia limited the maximum safe dose for acute nitrite treatment,[180,238] and such dose limitations may explain mixed therapeutic benefits observed in clinical trials. Taken together, these clinical results suggest that dose limitations curb the therapeutic benefit of global nitrite treatment; however, localized administration of nitrite at higher doses may improve outcomes in patients facing acute I/R injury.

“Oxidative” Nitrite Signaling: A Putative NO–sGC–cGMP-Independent Pathway.

Intriguing new preclinical research suggests that nitrite may also exhibit blood-pressure-lowering effects independent of NO, sGC, and cGMP in mesenteric resistance vessels.[316] This hypothesis builds upon the observation that intermolecular disulfide formation at Cys42 in the α-subunit of PKG-1, the downstream target of cGMP, stabilizes a homodimer with enhanced kinase activity.[317,318] By facilitating this disulfide formation, oxidants,

such as H_2O_2 and persulfides (e.g., cysteine persulfide, CysSSH; glutathione persulfide, GSSH), bypass NO, sGC, and cGMP to induce vasodilation and lower blood pressure by directly acting on PKG-1.[319] This H_2O_2-triggered vasodilation is ameliorated in resistance vessels from mice bearing a “redox-dead” Cys-to-Ser substitution that disrupts disulfide formation in PKG-1, and these mice exhibit hypertension in vivo.[318] Recently, Feelisch et al. demonstrated that a single intra-peritoneal dose of nitrite exhibits long-lasting hypotensive effects in mice under normoxic conditions in a cGMP-independent manner consistent with this oxidative activation pathway.[316] Nitrite treatment increased cellular levels of H_2O_2 and persulfide species in mesentery resistance vessels, concomitant with enhanced vasodilation. Nitrite-dependent vasodilation was not observed in the resistance vessels of “redox-dead” C42S mice nor were global nitrite-dependent hypotensive effects. The authors speculate that nitrite may indirectly increase H_2O_2 concentrations due to specific inhibition of catalase;[320,321] however, other chemical and hemoprotein-facilitated nitrite reactions may give rise to oxidizing equivalents in tissue (Figure 2). Specifically, nitrite can react directly with oxyHb to generate H_2O_2 under O_2-replete conditions (eq 16). Further studies are required to unravel the chemical and biochemical pathways that support “oxidative” nitrite signaling in this context.

CONCLUDING REMARKS

NO sits at the interface of inorganic chemistry, physiology, and biomedical research. Central to the interdisciplinary studies of NO are the interactions of this small molecule (and its related nitrogen oxides) with hemoproteins, particularly in the vasculature (Figure 4). Heme serves as the NO-sensing cofactor in the central target of NO signaling, sGC, and as the enzyme active site in NOS-dependent NO biosynthesis. Besides these central hemoproteins, which are dedicated to the canonical NO signaling pathway, many other hemoproteins, such as hemoglobin and myoglobin, facilitate nitrite-mediated signaling through a series of complex oxidative and reductive reactions. These auxiliary hemoprotein reactions unlock a “second axis” of NO signaling: nitrite serves as a stable NO reserve, which can be tapped under conditions of physiological and pathophysiological hypoxia, complementing O_2-dependent NO biosynthesis by NOS enzymes.

The multifaceted chemical reactivity and complex biological signaling pathways regulated by NO have posed many challenges in the development of a thorough understanding of this molecule’s role in human health and disease. However, these complexities have also presented researchers with novel therapeutic strategies that target NO-dependent signaling to treat a range of pathophysiological conditions from cardiovascular disease, organ transplant, infection, and oxidative stress. In particular, nitrite- and nitrate-based therapeutics have shown promise as modulators of NO signaling, and ongoing fundamental and clinical studies are exploring the applicability of localized and systemic nitrite and nitrate supplementation in the treatment of disease.

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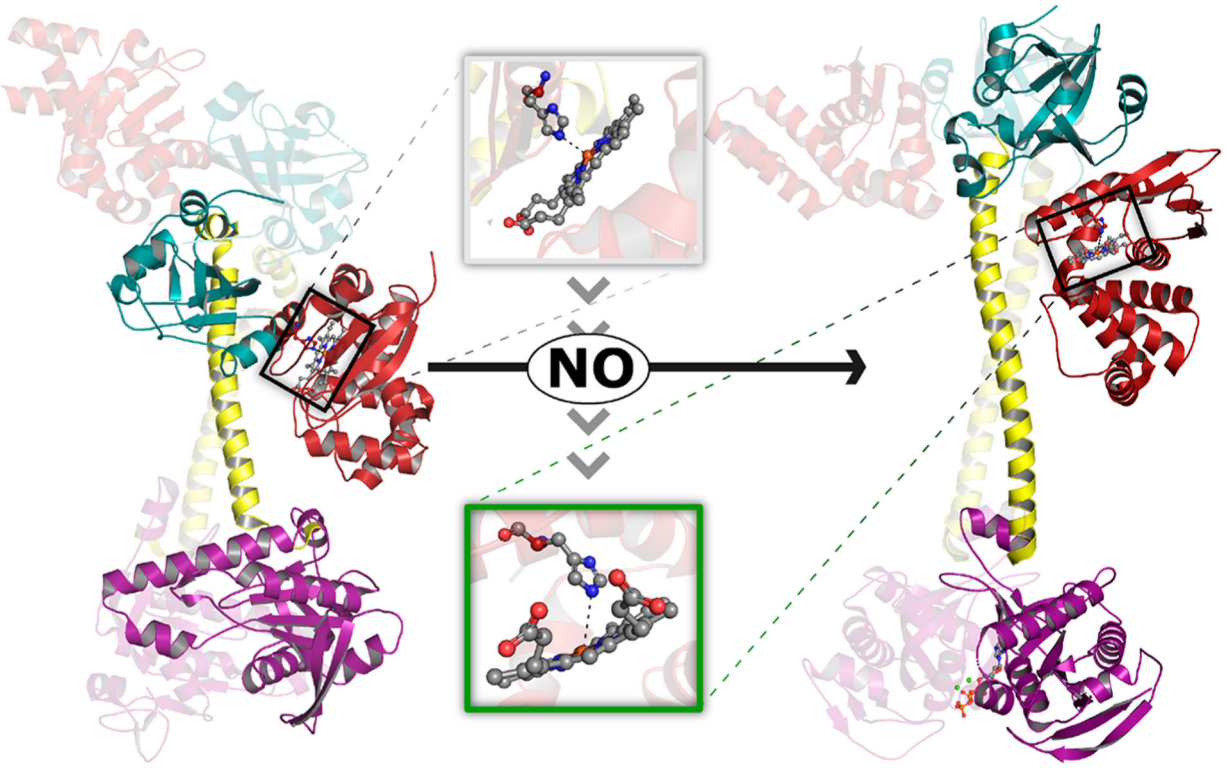

Figure 1.

NO-induced structural rearrangement in sGC. Structural models depicting inactive (left, PDB 6JT1) and active NO-bound (right, PDB 6JT2) sGC were determined using cryo-EM.[30] While the entire heterodimer is depicted for each model, the heme-containing β subunit is highlighted for clarity. Binding of NO to ferrous heme in the β-HNOB domain (red) induces a coil-to-helix transition that extends and reorients the central helices of the CC (yellow). CC elongation is accompanied by rotation of the β-HNOB and core HNOBA (cyan) domains away from the catalytic cyclase domain (purple). Together, these structural changes cause rotation of the relative orientation of the two cyclase domains, increasing the volume of the active site and allowing two Mg^{2+} cations (green spheres) and one GTP molecule (orange ball and stick) to bind at the interface of the catalytic domains. Inset: NO-induced structural changes at the sGC heme. NO binding brings the Fe atom back into the heme plane and is accompanied by a ~0.7 Å increase in the distance between the heme Fe atom and $N^{\varepsilon 2}$ atom of His105. We note that the resolution of the structural data (3.9 and 3.8 Å for inactive and active structures, respectively) precludes in-depth analysis of the heme coordination environment (e.g., the authors do not include heme-bound NO in the active structural model).

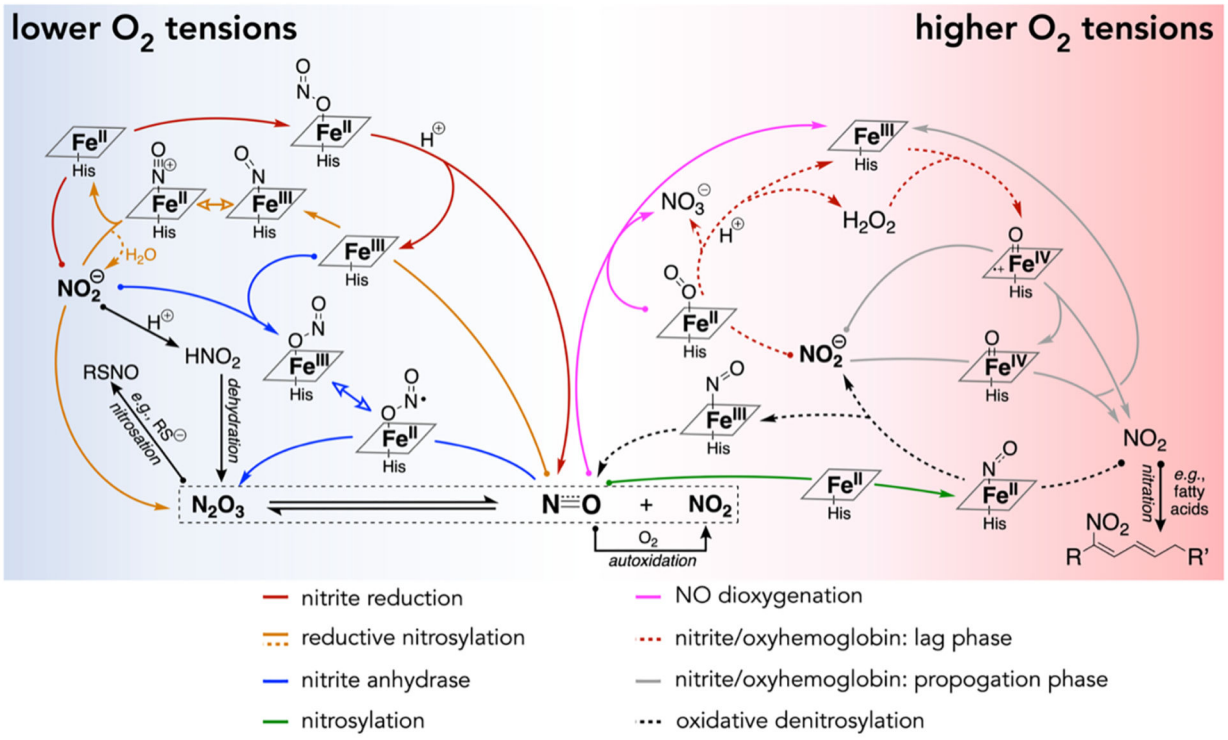

Figure 2.

Compendium of proposed chemical and biochemical pathways that facilitate signaling by NO and NO-related species. Reactions with curved arrows depict processes that are facilitated by hemoproteins. Exact stoichiometries are not shown for clarity but can be found in the text where appropriate.

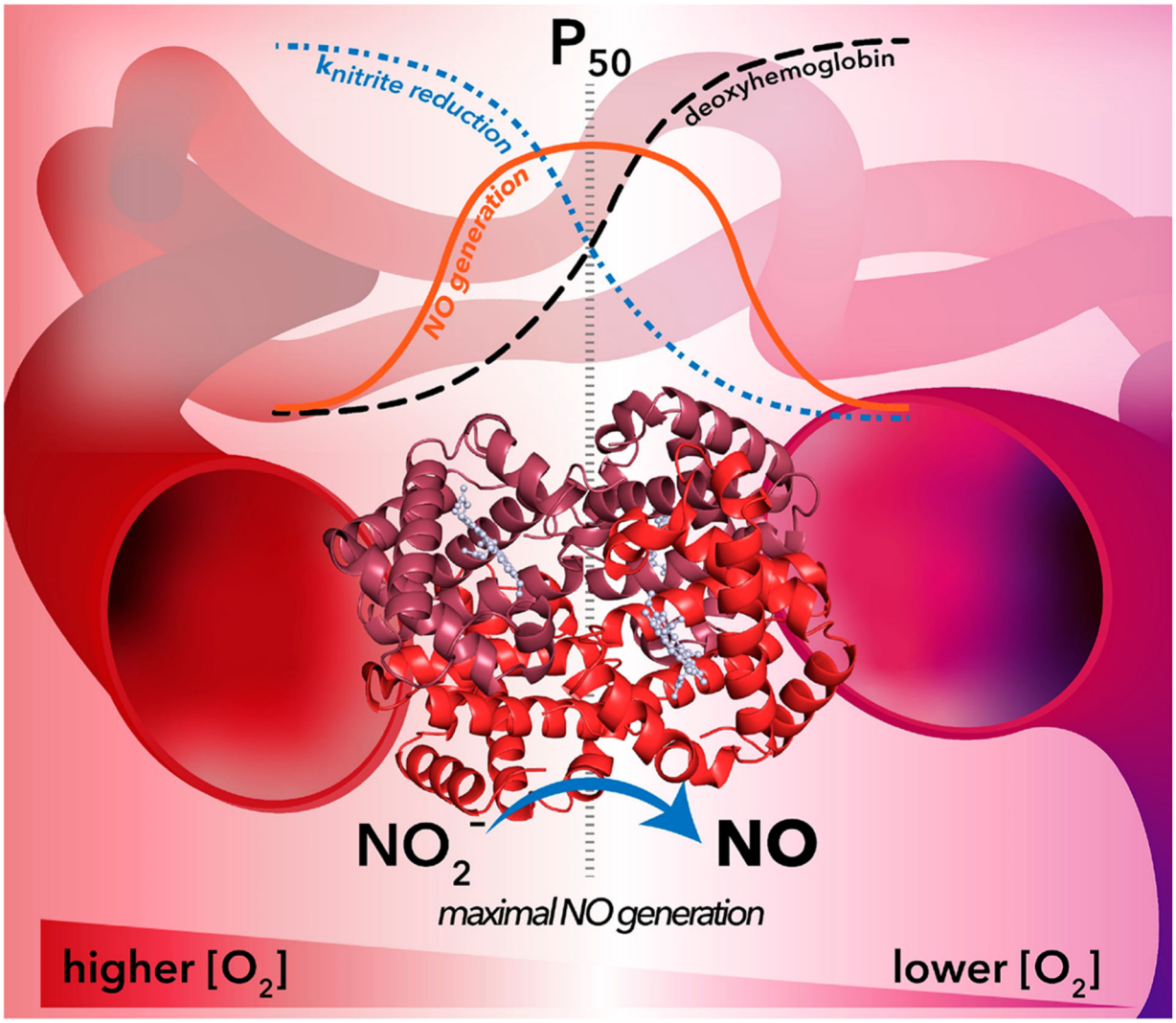

Figure 3.

Maximal nitrite-dependent NO generation coincides with the hemoglobin P_{50} value for O_2 in the vasculature. In RBCs, hemoglobin approaches a maximal nitrite reductase rate constant of 6 M^{-1} s^{-1} (blue dotted line) at O_2 tensions above P_{50} when the majority of hemoglobin is stabilized in the R-state.[194,223] As O_2 tensions fall below P_{50} (moving from left to right across the figure), O_2 dissociates from heme concomitant with an allosteric R-to-T-state transition. At low O_2 tensions, the hemoglobin nitrite reduction rate constant approaches a minimum value of 0.1 M^{-1} s^{-1}.[194,222] The number of deoxyHb sites available for nitrite reduction (black dashed line) mirrors O_2-dependent changes in the nitrite reduction rate constant values: more deoxyHb sites become available as O_2 tensions drop and O_2 dissociates from heme sites. These opposing factors (nitrite reduction rate constant and deoxyHb site availability) give rise to a bell-shaped curve for the observed rate of nitrite-dependent NO generation (orange line) as a function of O_2 tensions in the vasculature. Importantly, maximal nitrite reductase activity occurs at P_{O_2} values between

40% and 60% of the hemoglobin P_{50} value, a range that coincides with the set point of hypoxic vasodilation in humans.[226]

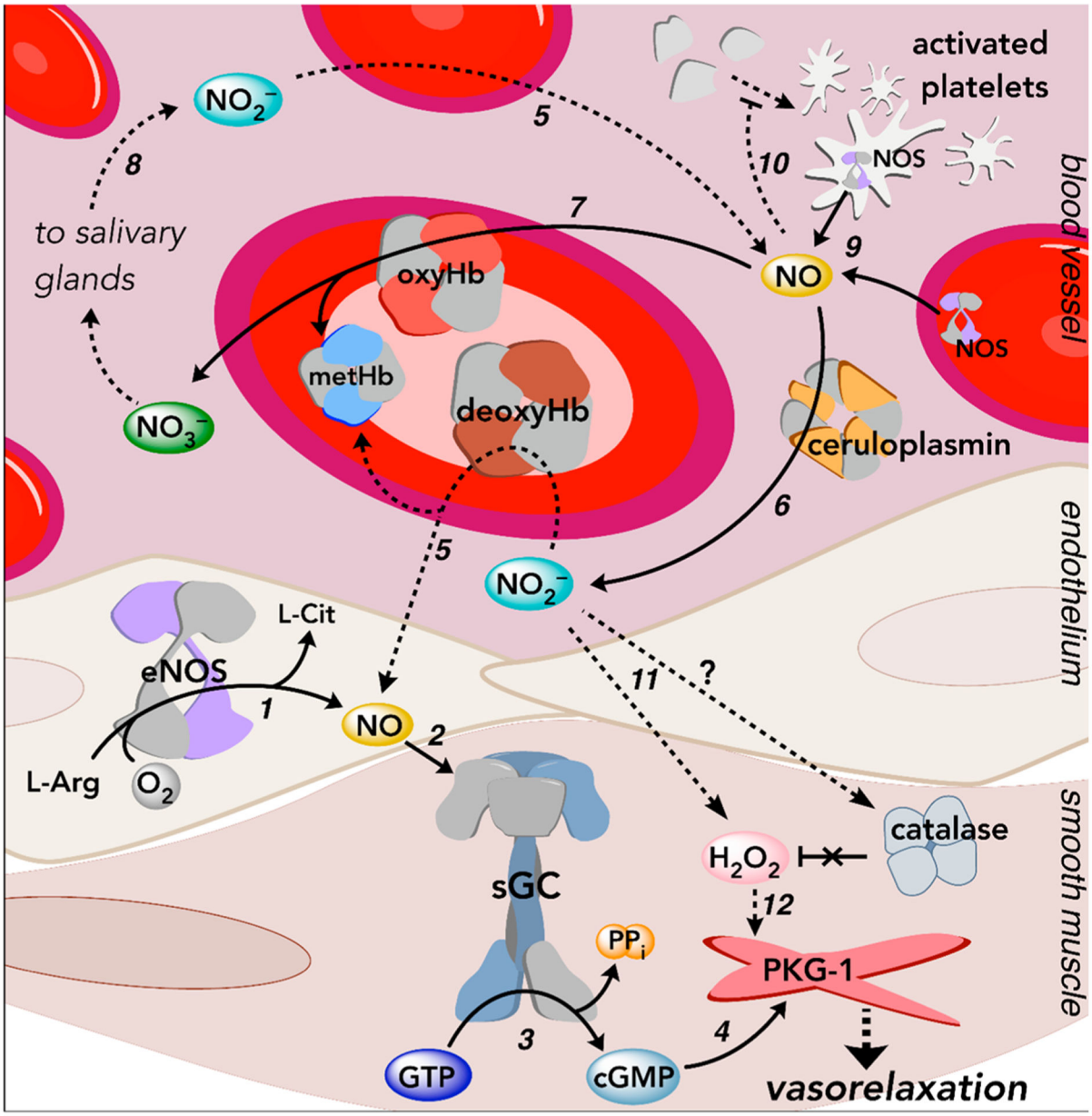

Figure 4.

Overview of NO and nitrite-mediated signaling pathways in the vasculature. Dashed arrows depict multistep signaling processes. (1) eNOS generates NO in the endothelium using L-arginine (L-Arg) and O_2. (2) NO freely diffuses through cellular membranes into neighboring smooth muscle cells and binds sGC. (3) Activation of sGC results in the conversion of GTP to the second messenger cGMP. (4) cGMP binds to and activates PKG-1 in vascular smooth muscle cells, and PKG-1 subsequently phosphorylates downstream targets to regulate physiological processes, such as smooth muscle relaxation (i.e., vasorelaxation). (5) Inorganic nitrite acts as a stable NO reservoir. At low O_2 tensions in the vasculature or under conditions of pathophysiological hypoxia, ferrous deoxyHb reduces nitrite to generate an equivalent of NO and

ferric (met)Hb.[176,178,193,194] Multiple hemoprotein-facilitated reactions regulate NO signaling and prevent overstimulation.[194,203,205,232] (6) The multicopper-containing enzyme ceruloplasmin, found in the plasma, readily oxidizes NO to nitrite,[145] while (7) oxyHb (and other O_2-bound hemoproteins) rapidly oxidize NO to nitrate in a process called NO dioxygenation. (8) Nitrate can be carried throughout the plasma and concentrated in the salivary glands, where nitrate is secreted and metabolized by commensurate bacteria back to nitrite.[141,142] Taken together, these processes (5–8) are collectively part of the nitrate–nitrite–NO pathway, a complementary, O_2-independent route to NO. (9) eNOS is also found in RBCs and platelets, which contribute to regulation of the vascular tone.[322–324] (10) NO, derived from eNOS and/or nitrite reduction, inhibits platelet activation via the canonical sGC pathway.[186–189] (11) Nitrite may increase H_2O_2 levels by directly inhibiting catalase or reacting with hemoproteins under O_2-replete conditions to generate H_2O_2.[229–231] (12) Some evidence suggests that nitrite may therefore also regulate the vascular tone in a NO–sGC–cGMP-independent fashion in mesenteric resistance vessels by facilitating oxidative activation of PKG-1.[316] More details regarding each of these pathways can be found in the main body of the text.