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Forum Review

Heme Proteins and Nitric Oxide (NO): The Neglected, Eloquent Chemistry in NO Redox Signaling and Regulation

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ABSTRACT

The role of nitric oxide (NO) in cellular physiology and signaling has been an important aspect in biomedical science over the last decade. As NO is a small uncharged radical, the chemistry of NO within the redox environment of the cell dictates the majority of its biological effects. The mechanisms that have received the most attention from a biological perspective involve reactions with oxygen and superoxide, despite the rich literature of metal–NO chemistry. However, NO and its related species participate in important chemistry with metalloproteins. In addition to the well known direct interactions of NO with heme proteins such as soluble guanylate cyclase and oxyhemoglobin, there is much important, but often underappreciated, chemistry between other nitrogen oxides and heme/metal proteins. Here the basic chemistry of nitrosylation and the interactions of NO and other nitrogen oxides with metal-oxo species such as found in peroxidases and monoxygenases are discussed. *Antioxid. Redox Signal.* 5, 307–317.

INTRODUCTION

SINCE THE IDENTIFICATION OF NITRIC OXIDE (NO) as the endothelium-derived relaxing factor (EDRF) (41, 50, 51, 71), the physiological functions discovered for this diatomic radical have been expansive. In the vascular system, NO is an important mediator of vascular tone, controlling platelet function, cellular adhesion (76), and extravasation of leukocytes into tissue. NO also modulates neuronal transmission (36), is an essential signaling agent in the immune system (63), influences basic cellular functions such as mitochondrial respiration (8–10), and regulates nutrients and minerals such as iron (42).

In addition to its normal role in modulating physiological functions, NO has also been implicated as a participant in pathophysiological conditions ranging from neurodegenerative and cardiovascular diseases to cancer (59). The proposed mechanisms of tissue injury involving NO are varied and often controversial. Because of its initial association with the deleterious effects of air pollution, NO became regarded as an environmental toxin (79). This became a primary barrier when assigning beneficial properties to NO, and generally the presence of NO was concluded to denote cellular death. However, significant evidence suggests that NO is a powerful antioxidant, protecting cells from injury caused by reactive oxygen species (ROS) (99). Cellular injury may therefore be a result of impaired function or lack of protection due to disruption of NO biosynthesis.

Although the lifetime of NO is considered short-lived under biological conditions (89) and its primary target is soluble guanylate cyclase (sGC), many studies have shown that prolonged exposure at higher NO fluxes will influence longer downstream events. Activation of signal-transduction pathways, such as p53, hypoxia-inducible factor (HIF), nuclear factor-κB (NFκB), JNK-1, and others, has been shown to encompass a NO-mediated regulatory component (11). In addition, NO affects the activity of caspases and cell-cycle proteins like p28 (55). The diverse, often opposing, responses to

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NO biosynthesis make clear the challenges in evaluating and understanding the biology of this diatomic molecule.

The most important determinant of the outcome of NO biosynthesis is the chemical interactions of NO, which are dictated by the local redox environment. Although the chemical reactions in which NO participates are quite varied, the limitations imposed by biological conditions narrow the relevant reactions considerably. The kinetically viable reactions, summarized as the "chemical biology of NO," can be categorized into the two classes of direct and indirect effects (96, 97). Direct chemical reactions are those in which NO binds directly with biological targets. Although NO is a radical, it is relatively inert biologically, interacting primarily with heme proteins and other radicals. However, NO can be activated to higher nitrogen oxides by oxygen or superoxide (O_2^-). The resulting reactive nitrogen oxide species (RNOS) are then able to modify a larger variety of biomolecules through indirect mechanisms. Direct reactions are the basis for most of the biological effects of NO as they are generally quite rapid ($k > 10^6$ M^{-1} s^{-1}) and therefore only require low concentrations of NO. Although the reactions involved in indirect effects may be fast, they require significantly higher concentrations of NO because the reactants involved are usually in low relative concentrations and are therefore competitively scavenged by other species. Indirect effects, as a rule, are limited to areas where high local NO concentrations are sustained for prolonged periods of time.

NO is produced by isozymes of nitric oxide synthase (NOS) (38). Constitutively expressed NOS, particularly in endothelial cells and neurons (83), is thought to generate NO in the submicromolar range for short periods of time (69). Production of NO by inducible NOS, for example in macrophages, is higher and significantly sustained relative to the constitutive isozymes. Therefore, the type of NOS expressed will dictate the chemistry and the ultimate biological outcome of NO production.

Another factor to consider is the proximity of the biological target to the NO source. As NO is highly diffusible, once produced, it will rapidly migrate away from its source of origin, thus producing a local concentration gradient (60). For instance, macrophages producing high levels of NO will induce both direct and indirect effects on adjacent targets, whereas cells further away will experience mostly direct effects as the concentration of NO diminishes. This two-level, concentration-dependent model partially explains the pathology observed under disease conditions resulting in excess NO production or in conditions with depleted levels of NO (39).

Indirect reactions can be further subdivided into nitrosative and oxidative chemistry (96). Nitrosation *in vivo* appears to be mediated primarily by dinitrogen trioxide (N_2O_3), whereas oxidative chemistry can result from formation of a variety of RNOS, including peroxynitrite ($ONOO^-$), nitrogen dioxide (NO_2), and nitroxyl (HNO) (98). It is becoming increasingly evident that the biological and pathological effects differ widely depending on the chemistry that occurs (96). Although both nitrosative stress and oxidative stress originate from NO biosynthesis, they are produced by different chemical mechanisms. Because of this balanced orthogonal relationship, the chemistry of one generally precludes the other (96).

Much of the NO biochemistry literature is concentrated on reactions with O_2^- and O_2 as they relate to stress (24), whereas the interaction of NO and its related RNOS with heme proteins is often overlooked. Although these reactions can be kinetically facile, they may be largely responsible for the majority of the biology of NO, influencing important cellular processes ranging from respiration to pathogen control. In this review, the chemical biology of NO is explored from a metal-oriented perspective.

METAL-BASED CHEMISTRY OF NO AND BIOLOGICAL CONSEQUENCES

Under biological conditions, the kinetically relevant chemistry of NO can be summarized simply as either involving reactions with metal complexes and radicals leading to direct effects, or reactions with O_2 and O_2^- leading to indirect effects. The metal-based reactions are primarily comprised of either simple nitrosylation reactions with metal-oxo species, such as found in the peroxidase and monoxygenase systems, or redox-active metals.

Metal nitrosyl complexes

Nitrosylation involves addition of NO directly to a metal center, or to a radical, to form a nitrosyl complex with no net change in redox state (43, 78).

$$M^{n+} + NO \rightarrow MNO^{n+} \qquad (1)$$

Unfortunately, the term nitrosylation is often used erroneously in the literature to represent the process of thiol modification leading to *S*-nitrosothiols. This mechanism involves either nitrosation (NO^+ donation), generally by N_2O_3, or oxidative nitrosylation in which the thiol is first oxidized to a radical (25). Although oxidative nitrosylation is a potential mechanism for the formation of *S*-nitrosothiols, it is an improbable biological mechanism because thiyl radical reacts with O_2 at a nearly diffusion-controlled rate (1.6×10^9 M^{-1} s^{-1}) (75, 88), and thus O_2 outcompetes NO because of its much higher concentration.

Although metal nitrosyl complexes can be formed with many of the transition metals, the required conditions and stability of the products favor only a few such complexes in biological systems (16). The reaction rate and stability of these products depend on the oxidation state and ligand field of the metal. For example, in aqueous solution, nitrosylation of iron results in stable complex formation for the ferrous but not the ferric state (94).

$$Fe(II)(H_2O)_6^{2+} + NO \rightarrow Fe(II)(H_2O)_5NO^{2+} + H_2O \qquad (2)$$
$$k = 1.42 \times 10^6\ M^{-1}\ s^{-1}$$

When the aqueous coordination sphere is replaced with the metal chelator EDTA, the rate constant for the reaction of NO with ferrous iron increases to $>10^7$ M^{-1} s^{-1} (102).

The rate of nitrosylation of iron porphyrins such as TPPS (tetraphenyl porphyrins) is also enhanced over free iron, but the ligand field allows ready formation of both ferrous and ferric nitrosyl complexes. Again, the facility of formation is significantly higher for the ferrous state as illustrated by a model system (Fig. 1) (45).

$$\mathrm{Fe(III)(TPPS)} + \mathrm{NO} \rightarrow \mathrm{Fe(III)(TPPS)NO} \qquad (3)$$
$$k = 7.2 \times 10^{6}\, M^{-1}\,\mathrm{s}^{-1}$$
$$\mathrm{Fe(II)(TPPS)} + \mathrm{NO} \rightarrow \mathrm{Fe(II)(TPPS)NO} \qquad (4)$$
$$k = 1.8 \times 10^{9}\, M^{-1}\,\mathrm{s}^{-1}$$

Similar trends are observed with heme proteins. The dissociation rates, and thus the stability of the resulting complexes, are also dependent upon the redox state. In general, the rate constant for dissociation of NO from a ferric heme protein exceeds that of the corresponding ferrous state by four orders of magnitude [$k_{\mathrm{off}} > 10\ \mathrm{s}^{-1}$ for ferric (81) and $k_{\mathrm{off}} < 10^{-3}\ \mathrm{s}^{-1}$ for ferrous (54, 82)]. Coupled with the trend toward faster association rate constants for the ferrous state, ferric nitrosyl complexes in heme proteins are, in most cases, reversible, whereas ferrous complexes are stable. However, the protein itself can significantly affect both binding and dissociation of NO due to distal and proximal influences (30).

The most well studied example of nitrosylation of a biomolecule is that of sGC, which leads to membrane translocation and activation of the enzyme (68). This critical regulatory enzyme participates in the control of vascular tone, neurotransmission, and platelet aggregation, as well as numerous other processes. sGC, which converts GTP into cyclic GMP (cGMP), is a heterodimer sequestering a single heme prosthetic group (68). NO activates sGC by binding to the ferrous iron in the heme pocket ($k_{\mathrm{on}} > 10^{5}\ \mathrm{M}^{-1}\ \mathrm{s}^{-1}$) (21, 32, 48, 49, 85). Nitrosylation results in displacement of the iron out of the plane of the porphyrin ring toward NO to such an extent that the axial histidine ligand is decoupling from the heme (84). The subsequent conformational change of the protein improves substrate access to the catalytic site.

One unique feature of the ferrous state of sGC is its low affinity for O_2 relative to NO. Generally, heme proteins in the ferrous state will bind strongly to NO, CO, and O_2. However, it appears that the heme pocket in sGC uniquely minimizes O_2 binding due either to polarity or to the inability of the proximal histidine ligand to adequately bind and stabilize the hexacoordinate $\mathrm{Fe(II)O_2}$ complex (19).

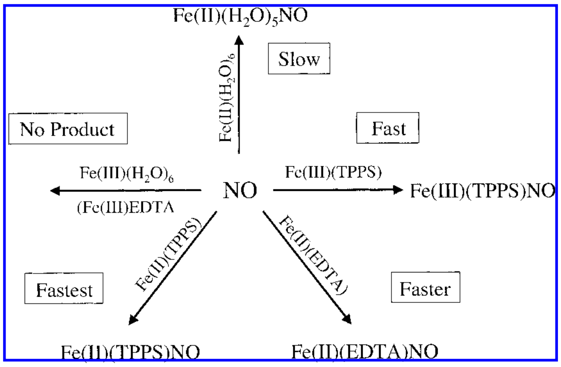

FIG. 1. Comparison between the relative rates of NO reactivity with different redox states and ligand fields of iron.

Another important aspect of heme nitrosyl formation is regulation of cytochrome P450 and other monoxygenases. Unlike sGC, which is activated upon nitrosylation, NO binding potently inhibits monoxygenase function. The presence of axial thiols rather than the usual histidine imidazoles in P450s results in stabilization of the nitrosyl complex in both the ferric and ferrous states, and submicromolar amounts of NO are sufficient to inhibit P450 function in the liver under septic conditions (87).

Regulation of O_2 concentration

Except in special circumstances such as found in sGC, NO will compete with O_2 or peroxide for the metal center. The outcome of this competition will depend on the concentration of each ligand, the relative reaction rates with the metal center, and the stability of each respective complex. Frequently, a ferrous ion is involved, and in general, NO binds more strongly to these centers than O_2.

This competition is critical to O_2 utilization and sensing. Several decades ago, NO was shown to bind reversibly to cytochrome *aa*$_3$ (cytochrome *c* oxidase; complex IV), which is the terminal enzyme in the electron transport chain (5, 8–10, 86). Prevention of O_2 binding not only reduces electron transport, but increases local O_2 tension because O_2 is being continually received, but not consumed, by the tissue (89).

Interestingly, NO has also been shown to enhance stabilization of HIF-1α (11, 80), which usually occurs, as the name suggests, in response to tissue hypoxia. Stabilization of HIF-1α by NO occurs indirectly through nitrosylative inactivation of the non-heme [2-histidine-1-carboxylate iron coordination motif (23)] enzyme prolyl hydroxylase, which tags HIF-1α for degradation through an O_2-dependent hydroxylation (14, 52). As discussed above, most non-heme ferrous nitrosyl complexes are significantly less stable than heme nitrosyl complexes. In these complexes, stabilization is dependent upon the electron-withdrawing characteristics of the coordinated metal. The histidine and aspartate residues around the ferrous iron of prolyl hydroxylase produce a relatively hard ligand field, similar to bipyridine or EDTA, resulting in both facile complex formation and dissociation. This highly labile and easily oxidized nitrosyl complex suggests that local NO levels must be higher to exert an effect on prolyl hydroxylase than on heme proteins. Additionally, the equilibrium between NO and O_2 binding becomes more competitive, and thus more sensitive to the relative concentrations of NO and O_2 (Fig. 2). NO can have a dichotomy of function in that it can increase local tissue oxygenation through interruption of mitochondrial O_2 consumption and concomitantly induce expression of HIF-1α associated factors (*e.g.,* hypoxia-responsive elements). In this manner, NO may transiently fix tissue O_2 levels during rapid hypoxia, but may also ensure a longer term compensatory response if the hypoxia is prolonged.

Regulation of NO concentration

Due to the myriad of effects NO has on biological pathways, numerous consumptive mechanisms, both metal-dependent and -independent, exist for tight regulation and control of NO concentration (Fig. 3). Part of this regulation, which warrants

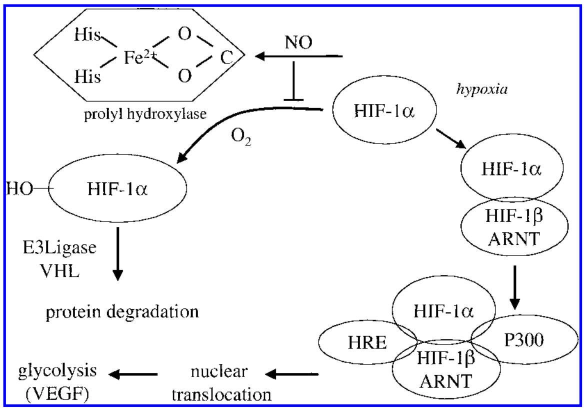

FIG. 2. Mechanisms of HIF-1α stabilization by NO or hypoxia. HIF-1α is stabilized by two mechanisms, NO and/or hypoxia, leading to a common end point: nuclear translocation and initiation of transcription. Under normoxic conditions, HIF-1α is maintained at low basal levels through an O_2-dependent hydroxylation by prolyl hydroxylase. ARNT, aryl hydrocarbon receptor nuclear translocator; HRE, hypoxia-responsive element; VEGF, vascular endothelial growth factor; VHL, von Hippel–Lindau.

particular mention, is the rate of cellular NO consumption, which is directly proportional to the immediate O_2 concentration (89). This dependence provides a feedback mechanism to counterbalance increases in O_2 via mitochondrial inhibition of NO. The concentration interdependence in conjunction with the competition parameters between NO and O_2 binding discussed above and the reversible binding of these ligands provide an autoregulatory mechanism able to respond quickly and transitorily to cellular conditions. Induction of HIF-1α and the consequent changes in cellular metabolism can then be viewed as a necessary compensation by the cell to ensure homeostasis under a variety of conditions.

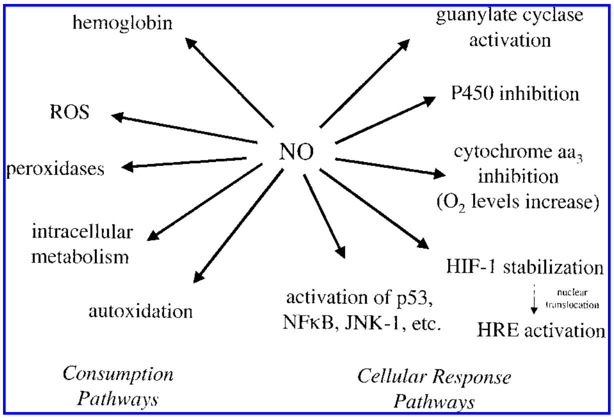

FIG. 3. The multiplicity of biological pathways for NO consumption and cellular responses. HRE, hypoxia-responsive element.

Metal-nitrosyl complexes. Metal-mediated consumption of NO primarily involves heme proteins. Nitrosyl complexes can be subsequently reduced to hydroxylamine and ammonia through electron transfer reactions. This is a common pathway under anaerobic conditions and is especially important in the nitrogen cycle of certain bacteria. Mitochondria can also consume NO by reduction at the cytochrome *c* oxidase site, and NO was initially thought to be directly reduced and thus consumed in this fashion, analogously to O_2 (73). However, the reduction potential for NO was later determined to be too high for direct reduction in mammalian systems (3). Aerobic consumption of NO by cytochrome *c* oxidase has been proposed to occur via a three-electron reduction to form NO_2^- (73).

$$\begin{gathered}\text{Cytochrome } c \text{ oxidase} + NO + O_2 \rightarrow \\ ONO_2^- + H^+ \rightarrow NO_2^- + H_2O\end{gathered} \tag{5}$$

Reaction of NO with metal-O_2 complexes. Reaction of NO with oxyhemoglobin or oxymyoglobin results in oxidation of the heme and production of nitrate.

$$Fe(II)O_2 + NO \rightarrow Fe(III) + NO_3^- \tag{6}$$

Oxidation of NO to NO_3^- is generally considered to be the primary consumptive pathway *in vivo* (95) (Fig. 4). At first approximation, the fast rates [$k = 3 \times 10^7\ M^{-1}\ s^{-1}$ for oxyhemoglobin (61)] and high concentrations of these O_2-binding proteins [4–8 m*M* oxyhemoglobin in blood vessels (61)] should lead to rapid and nearly complete scavenging of NO. The question then arose as to how endothelial-derived NO could activate sGC in adjacent smooth muscle cells when its half-life should be exceedingly short as a consequence of its proximity to a vast heme pool. Lancaster and coworkers resolved this seeming paradox by observing that sequestering oxyhemoglobin within a red blood cell altered the rate-limiting step from diffusion of NO through the globin to diffusion of NO into the cell (61). By having hemoglobin contained within a red blood cell, the rate of its reaction with NO (NO_3^- formation by Eq. 6) is ~1,000 times slower than with the same corresponding amount of free hemoglobin. The resulting longer lifetime of NO in the tissue bed and in the lumen of the blood vessel allows establishment of local NO concentrations sufficient to elicit a response at a target several cell lengths away from the releasing cell.

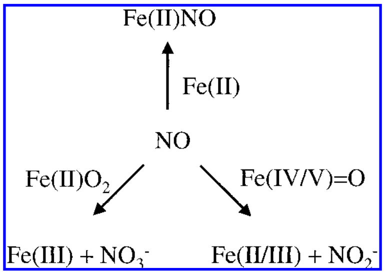

FIG. 4. Metal-mediated consumptive pathways for NO.

Metal-independent consumption of NO. Another important control mechanism for NO involves interaction with ROS. Analogous to O_2 itself, many studies suggest that NO and ROS serve to counter the effects of the other. This balance was first observed by Matsunaga and Furchgott, who showed that removal of superoxide dismutase (SOD) preserved EDRF activity (64).

The rate of the reaction between O_2^- and NO to form $ONOO^-$ is near diffusion control ($k = 4.3–6.7 \times 10^9\ M^{-1}\ s^{-1}$) (18). Scavenging by O_2^- can significantly affect the vasoactive response to NO and thus alter intercellular communication. For instance, stimulation of cGMP formation by NO was shown to prevent apoptotic death in motor neurons (12, 13). Scavenging of NO by O_2^- led to decreased cGMP formation and subsequently to neuronal death (27). Additionally, in a small population of patients with the fatal neurodegenerative disorder familial amyotrophic lateral sclerosis, a single-site mutation in the Cu,Zn superoxide dismutase gene (SOD1) is observed (20). These mutants may be partially responsible for the toxic effects associated with the disease by compromising removal of O_2^-, thereby resulting in increased NO scavenging and reduced cGMP formation.

The NO/O_2^- reaction has been hypothesized to elicit cell death and to propagate many neuronal diseases through formation of the toxic species $ONOO^-$ (91). The resulting chemistry of this reaction is extremely dependent upon the reaction conditions. In the presence of physiological bicarbonate concentrations, $ONOO^-$ is rapidly converted to NO_3^- (62). In addition, $ONOO^-$ reacts with both NO and O_2^-, resulting in less toxic species (90). The tight control of the chemistry of $ONOO^-$ by the relative concentrations of NO and O_2^- leads to the conclusion that SOD plays an important role, not through prevention of the chemistry of $ONOO^-$, but by altering the balance of cellular signaling processes (25).

Reaction of NO with metal-oxo complexes. Consumption of NO and subsequent inhibition of cGMP activity in vessels has been observed upon exposure to hydrogen peroxide (H_2O_2). H_2O_2 does not react directly with NO, and scavenging of NO has been attributed to formation of hypervalent metal-oxo complexes following reactions between hemes and peroxides.

$$\mathrm{Fe(II)} + H_2O_2 \rightarrow \mathrm{Fe(IV)O}\ \textit{or}\ \mathrm{Fe(III)} + H_2O_2 \rightarrow \mathrm{Fe(V)O} \qquad (7)$$

$$\begin{gathered}\mathrm{Fe(IV)O} + \mathrm{NO} \rightarrow \mathrm{Fe(II)} + NO_2^-\ \textit{or} \\ \mathrm{Fe(V)O} + \mathrm{NO} \rightarrow \mathrm{Fe(III)} + NO_2^-\end{gathered} \qquad (8)$$

As these reactions are fast ($k \sim 10^7\ M^{-1}\ s^{-1}$), they likely represent a viable mechanism for consumption of NO and provide an alternate means to balance NO and ROS levels (Fig. 4).

Formation of hypervalent metal-oxo complexes is an integral stage in the catalytic cycles of peroxidases, such as glutathione peroxidase, catalase, heme oxygenase 1, myeloperoxidase, and others (46). Peroxidases can serve as catalytic sites for degradation of both NO and peroxide (Eqs. 7 and 8), which can lead to alterations in the signaling mediated by NO and/or ROS. Additionally, binding of NO can affect the peroxidase activity (37), again demonstrating the duplicity in outcome of NO binding.

METAL-BASED CHEMISTRY OF OTHER NITROGEN OXIDES AND BIOLOGICAL CONSEQUENCES

Reactions with metal-oxo complexes

Other products of NOS, such as the end product NO_2^- and the decoupled intermediate N^G-hydroxy-L-arginine (NOHA), can be oxidized by hypervalent heme species (Fig. 5). Oxidation of NO_2^- results in formation of the strong oxidant NO_2 (93).

$$\begin{gathered}\mathrm{Fe(V)O} + NO_2^- \rightarrow \mathrm{Fe(IV)} + NO_2\ \textit{or} \\ \mathrm{Fe(IV)O} + NO_2^- \rightarrow \mathrm{Fe(III)} + NO_2\end{gathered} \qquad (9)$$

Following a careful comparison, this chemistry has recently been suggested to be the primary source of nitrated tyrosine residues *in vivo* rather than the NO/O_2^- reaction (90). Further, the diffusibility and surprisingly long lifetime of NO_2 in dilute solution, coupled with its differential toxicity toward eukaryotic organisms and mammalian cells (26, 56, 101), may make NO_2 production by Eqs. 8 and 9 an ideal antipathogenic agent during inflammation. In summary, when NO is synthesized for prolonged periods of time, it will be consumed and converted to nitrite by local peroxidase activity. Subsequently, as the nitrite concentration builds up, its oxidation to NO_2 is favored and the resultant pathophysiologic responses ensue.

Oxidation of L-arginine to L-citrulline by NOS proceeds through the intermediate NOHA, and decoupled NOHA can account for as much as 50% of the total product (2). With an oxidation potential of <0.5 V (57), NOHA may serve as an antioxidant. In addition, hypervalent metal-oxo species could oxidize NOHA through a two-electron oxidation resulting in production of HNO (Fig. 5) (31).

$$\begin{gathered}\mathrm{Fe(V)O} + \mathrm{NOHA} \rightarrow \mathrm{Fe(IV)O} + \\ [\mathrm{NOHA} + e^-] \rightarrow \mathrm{Fe(III)} + \mathrm{HNO}\end{gathered} \qquad (10)$$

Although production of HNO *in vivo* is still a major source of debate, it has been found to have unique pharmacological properties, such as modulation of calcium channels (15) and release of the neuropeptide, calcitonin gene-related peptide (72).

Alternative chemistry of NOS

The peroxidase reaction (Eq. 7) in monoxygenases such as NOS may be an alternate mechanism for RNOS production

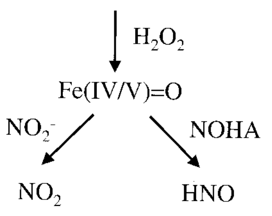

FIG. 5. Oxidation of NO_2^- and NOHA by peroxidase-like hypervalent metal-oxo species to produce NO_2 and HNO.

through a peroxide shunt. Accumulation of the end products NO_2^- and NO_3^- following exposure of NOS to peroxide (66) indicated that oxidation of L-arginine by NOS could in fact be mediated by peroxide. The normal catalytic cycle involves enzymatic reduction of O_2. Peroxide is proposed to form this intermediate directly through electron transfer in a comparable pathway to the peroxide shunt in cytochrome P450 (70).

Although the peroxide shunt may not be the primary function of NOS, it may be an important component of the chemistry of the monomeric form of the enzyme. The monomer-to-dimer ratio varies with specific biological conditions, and in particular with deficiencies in the cofactor tetrahydrobiopterin (92). Both peroxidases and tetrahydrobiopterin-deficient NOS have been proposed to oxidize NOHA to HNO.

The potential for oxidation of NO_2^- and NOHA to give NO_2 and HNO, respectively, through a peroxidase pathway warrants reevaluation of the supposed inactivity of the NOS monomer. This pathway could not only result in production of the oxidative species NO_2 and HNO (Eqs. 9 and 10), but also consume NO (Eqs. 7 and 8), thereby altering the redox balance and possibly facilitating the deleterious effects of NO.

It is conceivable that mitochondrial NOS (mtNOS) can participate in the peroxide shunt pathway. mtNOS, which is located in the inner membrane of the mitochondria, is a truncated form of NOS (Ghafourifar, personal communication; 53). Although initially controversial, the location and functional characteristics of mtNOS and its firm identification as neuronal NOSα has been established by multiple investigators (22, 35) . Transfer of electrons from complex I of the mitochondria is linked to production of NO through oxidation of L-arginine by mtNOS, and inhibition of complex I prevents NO release (33, 34). Production of low, steady-state levels of NO by mtNOS is a basic component of mitochondrial respiration and may also participate in a feedback system, regulating the electron transport chain, cellular O_2 utilization, and intracellular energy stores (40). By reducing complex IV function, NO decreases mitochondrial oxidative phosphorylation and, as mentioned previously, increases tissue O_2 content by decreasing O_2 consumption (Fig. 6A). Mitochondrial membrane polarization is also preserved by NO, likely through its action on mitochondrial K^+ channels (100). At the same time, this regulatory feedback loop is predicted to extend to regulation of Ca^{2+} uptake and release by mitochondria. Activation of mtNOS is dependent on the level of mitochondrial Ca^{2+} (33), which, in turn, is dependent on the cytosolic Ca^{2+} concentration. Ca^{2+} released from the endoplasmic reticulum (ER) is a readily available supply for mitochondria, and the close apposition of ER Ca^{2+} release sites to mitochondria provides a locational advantage (65). NO has been shown to both activate and inhibit Ca^{2+} release channels such as the ryanodine receptor on the ER membrane (28), allowing for strict control. Release of Ca^{2+} from mitochondria is also regulated by NO. At nanomolar levels of NO, Ca^{2+} efflux via the low conductance state of the mitochondrial permeability transition pore (PTP) is decreased (7). Increasing levels of NO, however, increase mitochondrial Ca^{2+} efflux (17, 28, 33, 35, 53), initially preventing overcalcification of the mitochondria (6, 7, 47, 74), but if continued, lead to cytochrome *c* release and potential disruption of cellular Ca^{2+} balance (29, 35). Return of the system to the physiological steady state is brought about by consumption of NO and a decreased effective competition with the rising level of tissue O_2.

The scenario shifts dramatically when mitochondrial complex I is inhibited by agents such as rotenone (Fig. 6B). In this case, mtNOS is likely to serve as a peroxidase. Increased leakage of O_2^- from the electron transport system and its dismutation catalyzed by MnSOD located in the mitochondrial matrix provide a ready source of H_2O_2 for the peroxide shunt. Nitrite would also be available, having been formed from the aerobic consumption of NO by cytochrome *c* oxidase or other reactions, as previously discussed. The resultant production of NO_2 and HNO by the mtNOS (Eqs. 9 and 10) is then likely to initiate a number of reactions that may have very different outcomes to mitochondrial function. Rather than normal physiological feedback control over mitochondrial Ca^{2+} levels and the PTP as seen in the presence of NO, these newly formed RNOS may initiate oxidative or other modifications of the mitochondrial PTP. Oxidation of the PTP causes stabilization into an open configuration and is associated with the loss of mitochondrial membrane potential, efflux of cytochrome *c,* and ultimately cell death (4, 44, 47, 58, 77). Similar functional changes are observed in other types of calcium channels exposed to RNOS. For example, HNO is known to interact with the *N*-methyl-D-aspartate channel in the presence of O_2, stabilizing the inward current by blocking desensitization (15). This effect involves an oxidizing intermediate formed from the reaction of O_2, metals, and HNO (67). As O_2 consumption by the mitochondria is inhibited when the electron transport system is blocked, cellular O_2 content would be high, thus providing sufficient levels of local O_2 for these reactions. The effect of RNOS on calcium channels, although controversial, is further supported by studies in cardiac myocytes. For example, L-type calcium channels in rat cardiac myocytes demonstrates an increased inward current in the presence of NO donors that does not require cyclic AMP (1). Paolocci *et al.* (72) have observed an increased positive inotropy of HNO in cardiac muscles. The extensive nitrotyrosine formation detected in mitochondria treated with complex I inhibitors may also be explained by the formation of NO_2 and other RNOS (Gharfourifar, personal communication). In summary, conversion of mtNOS (92) to a peroxidase may be important in the initiation of mitochondrial changes, which can have major effects on cell survival during pathological events.

CONCLUSION

Direct reactions of NO are dictated by two main characteristics: the oxidation state of the metal and the ligand environment. Reaction of NO with metals and metalloproteins can have either a stimulatory or inhibitory effect on protein activity and may result in consumption of NO. Once NO is synthesized, its ultimate fate and the resultant physiological outcome will vary greatly, depending on the diverse intermediates, end products, and consumptive pathways. Additionally, the seemingly straightforward oxidation of L-arginine by NOS has the potential to generate more than just L-citrulline and NO, de-

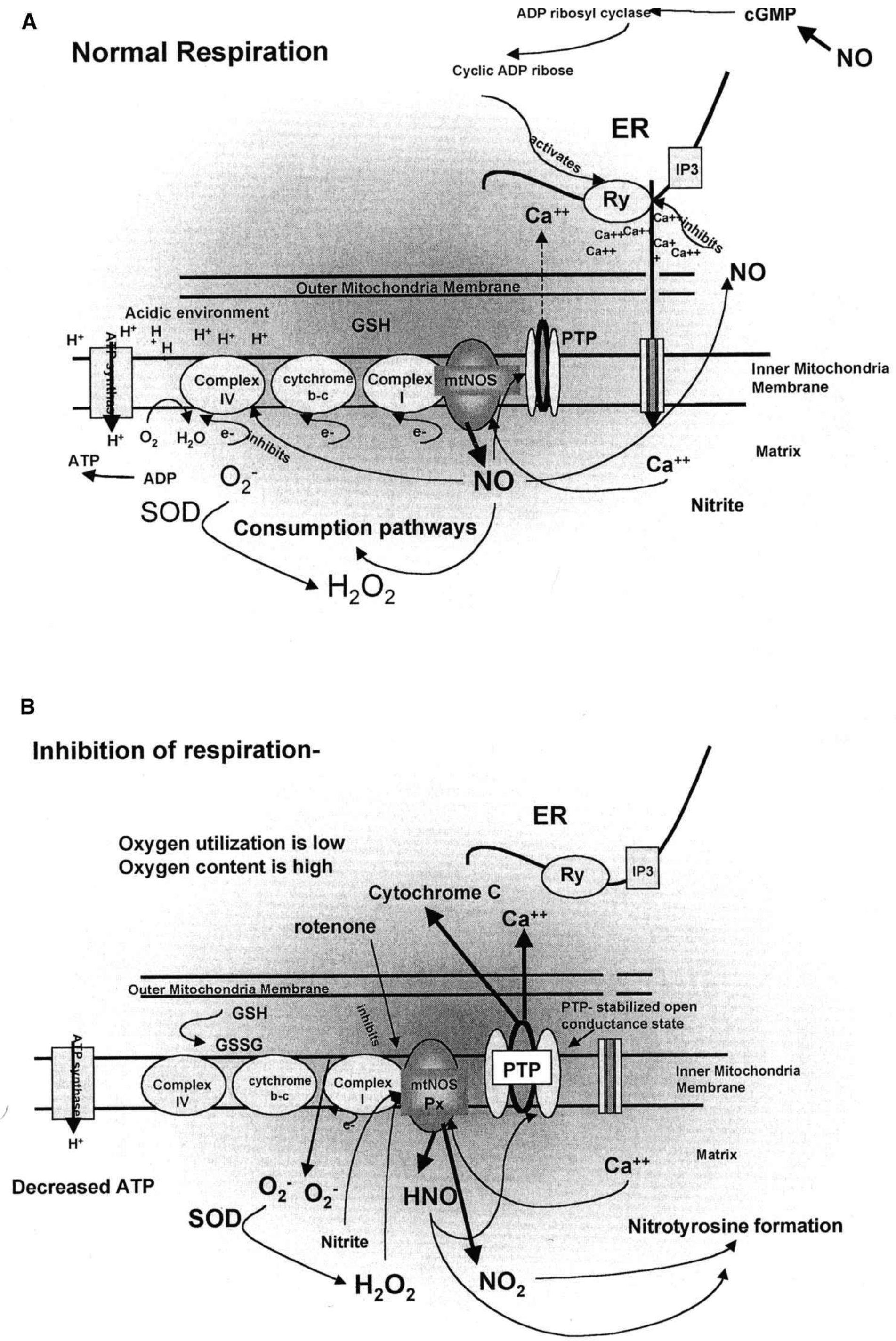

FIG. 6. (**A**) Potential modulation of normal respiration by feedback control of mtNOS and NO levels. (**B**) Generation of alternative products by mtNOS when complex I is inhibited, promoting cytoplasmic calcium imbalance and cell damage. IP_3, inositol trisphosphate.

pending on substrate and cofactor availability and on cellular redox status. Although NO would be preferentially formed from functional intact NOS dimers, the presence of abundant substrates, lack of cofactors, and O_2 or increases in peroxides might favor formation of HNO or NO_2, which may have importance in the regulation of coronary function, neurotransmission, and other pathophysiologic states.

ABBREVIATIONS

cGMP, cyclic GMP; EDRF, endothelium-derived relaxing factor; ER, endoplasmic reticulum; HIF, hypoxia-inducible factor; HNO, nitroxyl; H_2O_2, hydrogen peroxide; mtNOS, mitochondrial nitric oxide synthase; NFκB, nuclear factor-κB; NO, nitric oxide; NO_2, nitrogen dioxide; N_2O_3, dinitrogen trioxide; NOHA, N^G-hydroxy-L-arginine; NOS, nitric oxide synthase; O_2^-, superoxide anion; $ONOO^-$, peroxynitrite; PTP, permeability transition pore; RNOS, reactive nitrogen oxide species; ROS, reactive oxygen species; sGC, soluble guanylate cyclase; SOD, superoxide dismutase.

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