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Review

The Physiological Chemistry and Biological Activity of Nitroxyl (HNO): The Neglected, Misunderstood, and Enigmatic Nitrogen Oxide

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Introduction

It has been over 15 years since the discovery of endogenous nitric oxide (NO) generation in mammalian systems (*1*−*3*). This was a significant event since it represented a fundamentally new paradigm in mammalian cell signaling. Previous to this finding, the idea that a small, freely diffusible, reactive molecule (known more for its toxicity) could be biosynthesized in a highly regulated fashion for eliciting a specific biological function was unheard of and, for some at the time, heretical. Since this discovery, the chemistry, biochemistry, and (patho)-physiology of nitrogen oxide species have become topics of considerable research interest and clinical significance (for example, *4*). Most of the attention has been focused on NO since it is generated directly in mammalian cells by a family of enzymes referred to as the NO synthases (for example, *5*) and is an established cell signaling species capable of regulating, among other things, smooth muscle relaxation and platelet adhesion/aggregation. Many of the signaling properties of NO involve its ability to bind and activate the enzyme soluble guanylate cyclase (sGC) (for example, *6*−*8*). sGC is the primary physiological "receptor" for NO and catalyzes the conversion of guanosine triphosphate to the second messenger cyclic guanosine monophosphate (cGMP). NO activation of sGC involves coordination to a regulatory ferrous heme on the protein resulting in an increase in catalysis and a subsequent increase in intracellular cGMP (leading to, among other things, smooth muscle relaxation).

Along with NO, significant interest also exists for its oxidized congeners nitrogen dioxide (NO_2), peroxynitrite ($ONOO^-$), and nitrite (NO_2^-), among others (Figure 1). This attention is not unwarranted since it is known that NO can decompose to form these oxidized species in certain aerobic, biological environments and there are numerous reports implicating these nitrogen oxides as

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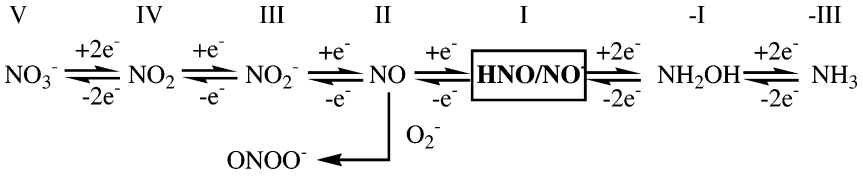

Figure 1. Redox scheme for the nitrogen oxides. Oxidation states of the nitrogen atoms are shown in Roman numerals.

important biological effectors, cellular toxins, and/or cellular signaling agents (for example, *9*). Reduced nitrogen oxide species (with respect to NO) have been relatively ignored by those interested in mammalian nitrogen oxide biochemistry. The disinterest in reduced congeners of NO is likely due to the fact that the biological fate of NO is thought to be primarily oxidative, and until very recently, reports of important biological activity in mammalian tissues associated with reduced NO species were scant. Thus, nitrogen oxide species such as nitroxyl (HNO), the protonated one-electron reduction product of NO (Figure 1), have received much less attention than the more oxidized nitrogen oxides. However, recent and numerous reports of significant and important pharmacology and toxicology associated with HNO have been published (vide infra), sparking great interest in this previously overlooked nitrogen oxide (for example, *10*). In an effort to explain the chemical mechanism(s) associated with the observed biology of HNO, recent work has focused on examining (and/or reexamining) important aspects of HNO chemistry. These studies have resulted in several important revelations regarding the fundamental chemistry of HNO and have changed the way we now think of this molecule in a biological setting. In this short review, we first discuss the physiologically relevant aspects of HNO chemistry with a special emphasis on some recent chemical revelations. We will then follow the discussion of HNO chemistry with a review of the novel and important biology and pharmacology of HNO.

HNO Chemistry

Nomenclature

The use of the term "nitroxyl" is unfortunate since it does nothing to properly describe the structure/molecularity of this species. This term is also ambiguous as it has been used to describe the paramagnetic functional group characterized by an unpaired, delocalized electron associated with an N−O moiety (otherwise referred to as a nitroxide). More descriptive and preferable names for HNO are nitrosyl hydride or hydrogen oxonitrate (*11*), although these terms have not, as yet, gained widespread acceptance. Moreover, nitroxyl has been used to generally describe other isomeric, spin state, and protonation state congeners (vide infra). Herein, we, somewhat regrettably, continue to use the term nitroxyl to specifically describe HNO only because this term has been generally accepted and/or utilized by researchers in this field.

HNO Reactivity and Fundamental Chemical Properties

As compared to the chemistry of other nitrogen oxides, HNO chemistry is significantly more difficult to grasp and less straightforward. The structural simplicity of this triatomic species belies the complexity of some of its fundamental chemical characteristics/properties. Experimentally, HNO is difficult to study since it spontaneously dimerizes to give hyponitrous acid, which dehydrates to give nitrous oxide (N_2O, a commonly used anesthetic agent) and water (reaction 1) (*12*). Detection of N_2O as an end product might serve as a marker for the involvement (or at least the presence) of HNO in biological systems. Indeed, in many studies, the presence of N_2O has been used as a "fingerprint" for HNO (although this is not an absolute proof, vide infra).

$$HNO + HNO \rightarrow HON{=}NOH \rightarrow N_2O + H_2O \quad (1)$$

Thus, unlike all other commonly studied nitrogen oxides such as nitrate (NO_3^-), NO_2^-, NO_2, $ONOO^-$, NO, and hydroxylamine (NH_2OH), HNO cannot be stored or purified/concentrated and is typically studied using donor species that release HNO as a decomposition product (vide infra).

One of the most intriguing and novel features of HNO involves its simple acid−base chemistry. Deprotonation of HNO would generate the corresponding anion, NO^- [typically referred to as the HNO anion and more appropriately termed oxonitrate (−1)]. This reaction appears to be simple and straightforward, but this is not the case. NO^- is isoelectronic with O_2 and likewise has two energetically accessible spin states, singlet ($^1NO^-$) and triplet ($^3NO^-$). Like O_2, the ground state for NO^- is the triplet, with the singlet spin state lying approximately 17−20 kcal/mol higher in energy (the singlet−triplet gap for $^3O_2$ and $^1O_2$ is similar, 23 kcal/mol) (for example, *13*−*15*). Because HNO is a singlet species (all electrons paired), the electronic ground states for the protonated and deprotonated species are different. Thus, considering an equilibrium process between the two ground state species, one can envision deprotonation to occur via two possible pathways: initial deprotonation to generate the singlet species, $^1NO^-$ (in a spin-conserved process), followed by intersystem crossing to give the ground state triplet, $^3NO^-$, or spin conversion that occurs during deprotonation, generating the triplet ground state anion, $^3NO^-$, directly without the intermediacy of the higher energy singlet anion. The latter process is spin forbidden and, therefore, would be expected to occur at a rate far below that of other simple deprotonation events. Studies in the gas phase indicate that the relevant equilibrium is between the singlet HNO species (^1HNO) and the triplet anion, $^3NO^-$ (*16*). Using quantum mechanical calculations to determine the energetics of HNO and NO^- in aqueous solution, the relevant equilibrium was determined to be between ^1HNO and $^3NO^-$, like the gas phase process (reaction 2) (*15*).

$$^1HNO \rightleftharpoons {}^3NO^- + H^+ \quad (2)$$

Determination of the equilibrium constant for reaction 2 will, of course, provide the value for the pK_a of HNO. Unfortunately, determining the equilibrium concentrations of the protonated and unprotonated HNO species is problematic due to, among other things, the self-reactivity of HNO (reaction 1), thus precluding facile experimental determination of the HNO pK_a. Regardless, the pK_a of HNO was originally reported to be 4.7 based on experiments using pulse radiolysis to generate HNO/NO^- (*17*). In these studies, the spin states of the equilibrium species were not mentioned. The pK_a for HNO of 4.7 predicts that the anionic species would be the near exclusive species present in solution at physiological pH and was the exclusively quoted value until just recently. However, several recent studies report significant reaction of HNO with thiols at physiological pH (vide infra), calling into question the validity of the 4.7 pK_a value. Initial theoretical studies by Bartberger et al. (*15*) avoided the experimental pitfalls associated with determining equilibrium HNO concentrations and approximated the pK_a of HNO to be 7.4. This significant upward revision of the generally accepted value provided credence to the involvement of nondeprotonated HNO (as opposed to NO^-) in reactions of biological interest. In this same study, it was suggested that HNO would be an exceptionally reactive, but selective, electrophile reacting with thiols but relatively inert to hydrate formation by addition of water (in stark contrast to, for example, the analogous reactions of aldehydes and activated ketones). Subsequent higher level quantum mechanical studies, utilizing experimental reduction potentials and solvation free energy data for O_2 as a calibrant, along with spectrophotometric and electrochemical measurements by two different groups (*18*, *19*), have adjusted the pK_a of HNO even further from the initial revision. It would appear at the present time that theory and experiment have converged on a pK_a value of 11.4 for the ^{1}HNO/$^3NO^-$ equilibrium, strongly suggesting the dominance of the neutral form at physiological pH.

Because the HNO acid−base equilibrium species possess different electronic spin states (i.e., protonation−deprotonation are spin forbidden), it is expected that the rates of protonation and deprotonation will be extremely slow relative to normal acid−base reactions. Indeed, this appears to be the case although the kinetic barrier existing between interconversion of the equilibrium species has been proposed to be due primarily to nuclear reorganization energies rather than an electron spin conversion (*20*). Thus, if conditions exist for generating $^3NO^-$ in a physiological environment, it may possess a significant lifetime (milliseconds), despite the fact that deprotonation is highly favored. Likewise, deprotonation of HNO will also be a slow process.

Thus far, only the equilibrium partners ^{1}HNO and $^3NO^-$ have been discussed. What about the triplet protonated species and/or the singlet anionic species? Can they have any role in biological HNO chemistry? Protonation of $^3NO^-$ has been proposed to occur on the more electronegative oxygen atom, generating ^{3}NOH (*21*, *22*). ^{3}NOH has been calculated to be approximately 20−23 kcal/mol less stable than ^{1}HNO (*23*, *24*). As mentioned earlier, $^1NO^-$ has been determined to be approximately 17−20 kcal/mol higher in energy than the ground state triplet species, $^3NO^-$. Thus, both ^{3}HON and $^1NO^-$ are biologically inaccessible via thermal processes and are not considered to be biologically relevant.

HNO/NO^- can be generated via one-electron reduction of NO. The physiological relevance of this process hinges directly upon the reduction potential of NO. Unfortunately, reports of the reduction potential for the NO/NO^- couple vary greatly. The existence of catenation reactions between HNO/$^3NO^-$ and NO can confound facile experimental determination of the NO reduction potential since the redox species present will react with each other (reactions 3 and 4).

$$HNO\ (^3NO^-) + NO \rightarrow HN_2O_2\ (N_2O_2^-) \qquad (3)$$

$$HN_2O_2\ (N_2O_2^-) + NO \rightarrow HN_3O_3\ (N_3O_3^-) \qquad (4)$$

Moreover, coordination of NO to electrode surfaces can change the electrochemical potential to reflect more the coordination complex rather than "free" NO. However, two of the more reliable experimental determinations were performed by Ehman and Sawyer (*25*), using chroniopotentiometric/controlled potential coulometric techniques, and Benderskii and co-workers (*26*), using photoelectrochemical measurements. Both groups reported highly negative potentials of approximately −0.8 V (vs NHE throughout) for the NO/NO^- couple (the spin state for the anion not specified). However, a theoretically derived (and frequently quoted) value of +0.39 V for the NO/$^3NO^-$ couple has also been reported (*27*). This significant inconsistency between experiment and theory was reconciled when it was noted that the theoretically derived value for the NO reduction potential was based on an HNO pK_a of 4.7 and, far more importantly, a supposed equilibrium between ^{1}HNO and the singlet form of the anion ($^1NO^-$), in solution (*15*). Because these were both found to be in error, recalculation of the NO/$^3NO^-$ reduction potential using a revised HNO pK_a of 11.4 and considering the proper equilibrium between HNO and $^3NO^-$ gives an approximate value of −0.8 V for the NO/$^3NO^-$ couple (*18*), consistent with the previously reported experimental results. Thus, in a brief span of 1.5 years, a fundamental change has occurred in the understanding of the acid/base and redox chemistry of HNO: Researchers have progressed from the assumption that HNO exists as predominantly an anionic species at physiological pH, easily generated from NO reduction, to the current belief that HNO is the near exclusive species at physiological pH and that reduction of NO to $^3NO^-$ should be difficult under biological conditions.

It should be noted that the reduction of NO to $^3NO^-$ via outersphere electron transfer mechanisms is not likely to be physiologically accessible due to the highly negative potential. However, protonation of $^3NO^-$ to HNO is highly favorable at physiological pH. Thus, as the pH is lowered, a positive shift in the potential to −0.5 to −0.6 V is expected (*18*, *19*). Considering the slightly more favorable potential for the reduction of NO to the protonated HNO species and that the intracellular concentrations of the reactive reductants are likely to be significantly higher than the oxidant (endogenously generated NO), reduction of NO to HNO may, at least in principle, be possible under certain conditions (*28*).

Reaction of HNO with Thiols

As indicated by the initial biological studies (vide infra) and more recent theoretical work, thiols appear to be a

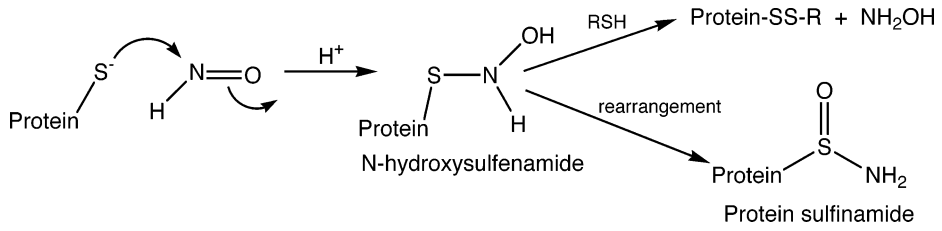

Figure 2. Thiol modification by HNO.

major site of HNO biochemical reactivity. One of the earliest reports of the thiol reactivity of HNO was by Doyle and co-workers (*29*) who proposed that the reaction of thiophenol with HNO generates, as a fleeting intermediate, an N-hydroxysulfenamide (Figure 2). In that study, the N-hydroxysulfenamide was unstable in the presence of excess thiol and the final products were the disulfide and NH_2OH. Further work by Wong et al. (*30*) and Shoeman and co-workers (*31*) supports the idea of an N-hydroxysulfenamide intermediate in the reaction of HNO with thiol species, although this species has yet to be isolated and unequivocally characterized. Wong et al. (*30*) also reported that N-hydroxysulfenamides undergo rearrangement to the corresponding sulfinamide (Figure 2), possibly explaining some of the difficulty in isolating the putative N-hydroxysulfenamide intermediate. Mentioned later in this review, there are numerous examples of HNO reactivity with biological thiol targets. Thus, there appears to be little doubt that thiol proteins or peptides are likely, if not the major, targets for the biological actions of HNO. As shown in Figure 2, the product of the reaction of thiols with HNO can be a disulfide or a sulfinamide. Although the factors that govern the two reaction pathways have not yet been specifically examined, the predominance of one product over the other is likely a function of the concentration of the thiol (or the existence of vicinal thiols in proteins) as well as the chemical nature of the thiol. Significantly, disulfide formation is biologically reversible whereas the generation of a sulfinamide (or the hydrolysis product, the sulfinate) likely represents an irreversible modification.

Reaction of HNO with O_2

One of the most intriguing and enigmatic aspects of HNO chemistry is its reaction with O_2. There is little doubt that HNO (and/or $^3NO^-$) reacts with O_2 (for example, *32*). In the case of the anion, the reaction product is $ONOO^-$ (*32*, *33*). However, the physiological relevance of this reaction is questionable since HNO is the near exclusive species present at physiological pH. Miranda and co-workers have provided evidence that the reaction product of HNO and O_2 is distinct from $ONOO^-$ and that the reaction product(s) are capable of performing oxidation chemistry (*34*, *35*). Whether the reaction of HNO with O_2 is physiologically relevant remains unknown, although initial kinetic studies indicate that it may play a minor role, if any, in mammalian systems (vide infra).

Redox Reactions of HNO

The two-electron reduction potential for the $HNO,2H^+/NH_2OH$ couple at pH 7 is reported to be 0.3 V (*19*), indicating the feasibility of this process under physiological conditions. The individual single electron reduction potentials have been calculated for pH 7 to be 0.1 ± 0.1 for the $HNO,H^+/H_2NO^\bullet$ couple and 0.5 ± 0.1 V for the $H_2NO^\bullet,H^+/NH_2OH$ couple (*36*). These single-electron potentials suggest that the one-electron reduction processes are accessible under biological conditions and, depending on the rates of competing pathways, may even be expected to be a major biological fate.

The negative reduction potentials for the $NO/^3NO^-$ (-0.8 V) and $NO,H^+/HNO$ (-0.5 to -0.6 V) couples indicate that $HNO/^3NO^-$ will be a reasonably good reductant in biological systems. That is, oxidative conversion of $HNO/^3NO^-$ to NO should be easily accessible. A prime example of the one-electron reducing capacity of HNO is seen in its reaction with metals/metalloproteins (vide infra). Moreover, the H−NO bond strength is only about 50 kcal/mol (*15*, *37*), indicating that H-atom abstraction by other radical species is also facile. These properties of HNO predict that it can act as an antioxidant since it will be able to "quench" oxidants either by electron or hydrogen atom donation and, in the process, generate another established antioxidant, NO. Thus, HNO has the potential to be redox active in biological systems (as demonstrated by its propensity to react with thiols), capable of serving as either a reductant or an oxidant.

HNO-Mediated Oxidation Chemistry

Several studies indicate that HNO is capable of causing oxidative damage to DNA (albeit at fairly high concentrations under in vitro conditions) (*38*, *39*). Ohshima and co-workers (*39*) also found that HNO-mediated DNA strand breakage was independent of O_2, precluding $ONOO^-$ or other HNO/O_2 products (vide supra) as oxidants in this system. On the basis of the type of oxidative damage observed and the conditions required to inhibit the oxidation chemistry, hydroxyl radical ($HO^\bullet$) was proposed as the oxidant generated from HNO in these studies. Significantly, $HO^\bullet$ has been detected in a decomposing solution of the HNO donor Angeli's salt (*40*, *41*). The source of $HO^\bullet$ in these chemical studies was proposed to be the result of a homolytic cleavage of *cis*-hyponitrous acid (reaction 5), a reaction proposed initially nearly 40 years ago by Buchholz and Powell (*42*) to explain the existence of a bothersome, albeit minor, radical chain reaction initiated by a decomposing solution of hyponitrous acid.

$$2HNO \rightarrow \textit{cis}\text{-}HON{=}NOH \rightarrow N_2 + 2HO^\bullet \qquad (5)$$

The second-order dependence of HNO on $HO^\bullet$ formation and the fact that homolytic cleavage appears to be a

minor pathway of decomposition makes the biological relevance of reaction 5 questionable.

Another mechanism by which HNO can mediate O_2-independent oxidation chemistry is via the generation of hyponitrite radical ($ONNO^-$). This species is generated from the reaction of HNO/NO^- and NO (reaction 3). Seddon and co-workers (*43*) reported that hyponitrite radical could decompose to generate $HO^\bullet$ (reaction 6), a reaction also proposed earlier by Buchholz and Powell (*42*).

$$N_2O_2^- + H^+ \rightarrow N_2O + HO^\bullet \quad (6)$$

More recently, Poskrebyshev et al. (*44*) reported that hyponitrite radical itself is strongly oxidizing with one-electron reduction potentials of 0.96 and 1.75 V for the $N_2O_2^-/N_2O_2^{2-}$ and $HN_2O_2,H^+/H_2N_2O_2$ couples, respectively. Thus, it appears that HNO itself and HNO/NO mixtures can carry out oxidation chemistry. Whether these processes affect HNO pharmacology and/or HNO physiology (if HNO is physiologically relevant) remains to be seen.

The reaction of HNO with thiols (Figure 2) demonstrates its ability to act as a two-electron oxidant. HNO will also oxidize NADPH by two electrons to give $NADP^+$ (*38*, *45*, *46*). Although NH_2OH would be an expected product, this has not yet been demonstrated. The oxidation of NADPH by HNO occurs in the absence of O_2, precluding participation of $ONOO^-$ or other HNO/O_2-derived oxidants. Because NADPH is considered an obligate two-electron reductant, one-electron oxidized intermediates are not likely to be involved in the oxidation of NADPH by HNO (unless, a potent oxidant such as $HO^\bullet$ is generated). Whether this chemistry is relevant in biological systems remains to be determined.

Reaction of HNO with Metals/Metalloproteins

As mentioned above, $HNO/^3NO^-$ is capable of reducing metals and metalloproteins. For example, HNO will convert ferric myoglobin to the ferrous nitrosyl adduct (reaction 7) (*29*).

$$Fe^{III}Mb + HNO \rightarrow Fe^{II}(NO)Mb + H^+ \quad (7)$$

Hb = myoglobin

HNO can also be trapped similarly using synthetic porphyrins (for example, *47*). These reactions, termed reductive nitrosylation, can occur either via an inner sphere mechanism, whereby direct coordination of HNO to the metal center occurs (preceded or followed by deprotonation), or via an outersphere mechanism involving electron transfer to the metal center followed by coordination of NO to the reduced metal center. The existence of an outersphere process is evidenced by the reaction of HNO with ferricytochrome c, which only produces the corresponding ferrous species and NO (*29*) (reaction 8).

$$Fe^{III}\text{cyt c} + HNO/NO^- \rightarrow Fe^{II}\text{cyt c} + NO + (H^+) \quad (8)$$

HNO is oxidized to NO by ferricyanide, $FeCN_6^{3-}$, a reaction that has been used experimentally to distinguish the biological activities of HNO from those of NO (for example, *38*). The oxidized (cupric) form of CuZnSOD also reacts with HNO to generate NO and the corresponding reduced (cuprous) CuZnSOD (*46*, *48*, *49*) (reaction 9).

$$Cu^{II}ZnSOD + HNO \rightarrow Cu^{I}ZnSOD + NO + H^+ \quad (9)$$

This reaction has been used experimentally to distinguish between the actions of HNO and NO (for example, *50*, *51*). In cases where the biological activity of HNO is increased in the presence of CuZnSOD, the actions are attributed to NO. However, if CuZnSOD decreases the activity of HNO, by converting it to NO, the biological activity is assigned to HNO.

Like NO, HNO forms coordination complexes with metalloproteins. Sulc and co-workers (*52*) report that the reaction of deoxyhemoglobin with HNO gives an HNO adduct, which is thermally stable but decomposes in the presence of oxygen. An HNO−ferrousheme adduct can also be generated via the one-electron reduction of a ferrous−nitrosyl complex (*53*). HNO will react with dioxygen-bound heme proteins (*29)* resulting in a net oxidation of the metal and HNO and reduction of the bound dioxygen (reaction 10).

$$HNO + O_2Fe^{II}Mb \rightarrow NO + HOO^- + Fe^{III}Mb \quad (10)$$

Mb = myoglobin

Thus, redox/coordination chemistry of HNO with metals and metalloproteins is established and, depending on the biological environment (e.g., the proximity and/or concentration of hemeproteins or other metalloproteins) may be an important aspect of HNO biochemistry. The coordination chemistry of HNO has been reviewed recently (*54*).

HNO Kinetics

The likelihood of any of the above-mentioned chemical reactions occurring in a biological system depends on the concentration of the reactants and the rate constants for the reactions. Experimental determination of the rate constants for the reactions of HNO is not straightforward since HNO is a fleeting species that requires donor species for its study. Thus, the concentrations of HNO in kinetic experiments are not easily known. However, using competition kinetics, Miranda and co-workers (*55*) determined the relative rate of the reaction of HNO with a variety of biological reactants. Calibrating the rate constants against the rate constant for HNO dimerization, they found that the relative reactivity toward HNO is oxymyoglobin (1×10^7 M^{-1} s^{-1}) > glutathione (GSH), horseradish peroxidase (2×10^6 M^{-1} s^{-1}) > *N*-acetyl cysteine, CuZnSOD, MnSOD, metmyoglobin, catalase ($3-10 \times 10^5$ M^{-1} s^{-1}) > tempol, ferricytochrome c [$(4-8) \times 10^4$ M^{-1} s^{-1}] > O_2 (3×10^3 M^{-1} s^{-1}). Clearly, the accuracy of these rate constants is entirely dependent on the validity of the HNO dimerization rate constant, which has recently been revised (*19*). The biochemical fate of HNO will be dependent on the cell type and cellular environment (i.e., the presence and concentrations of myoglobin, GSH, etc.). Regardless, the relatively slow reaction with O_2 along with the typically low concentrations of O_2 in cells indicates that the reaction of HNO with O_2 may be relevant only in specific cases, if at all (i.e., lung epithelial cells, etc.). From these kinetic studies, it would appear that HNO quenching by GSH would be a predominant fate for HNO in most cells since the

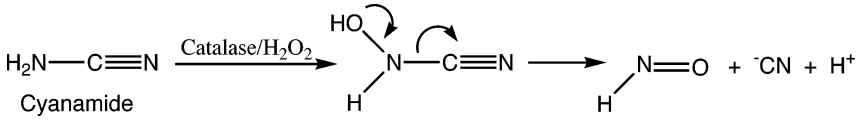

Figure 3. HNO formation from oxidation of cyanamide by catalase/H_2O_2.

concentration of GSH is universally high (1−11 mM in cells) (for example, *56*). Consistent with this idea is the report that significant GSH depletion in mammalian cells by exposure to HNO has been observed (*38*). GSH is the primary "redox buffer" in cells (*56*), and alterations in GSH levels and/or GSH/GSSG ratios can have significant effects on cell biochemistry. However, it remains to be determined whether HNO can specifically alter the activity of thiol-containing enzymes/proteins in a "sea of GSH". It is conceivable that HNO may be capable of selective reaction with target protein thiols if the rate constant for reaction with the target protein is considerably higher than that with GSH and/or the thermodynamics of the reaction with the target protein is far more favorable than with GSH (assuming, of course, a reversible reaction with GSH). Of particular note in this regard are the reports of HNO-mediated inhibition of the thiol enzymes aldehyde dehydrogenase and glyceraldehyde 3-phosphate dehydrogenase (for example, *57*, *58*). One can envision HNO as an "aldehyde mimetic" in these systems, experiencing the same binding and substrate activating forces in the enzyme active site that the normal aldehyde substrate experiences. Such catalytic forces may provide kinetic and/or thermodynamic advantage for reaction of HNO with these enzymes as compared to reaction with other thiols or thiol proteins (i.e., GSH). In this way, HNO may have selective biological targets (e.g., enzymes with aldehyde or carbonyl substrates) and may not be just a general and nonspecific thiol-modifying agent. If this were the case, HNO could be an efficient and selective signaling agent whose signaling properties were the result of regulating only select biochemical processes. Of course, this idea is highly speculative and examining these possibilities, among others, will be an important aspect of future work concerning the biological activity and pharmacological utility of HNO.

Biology, Biochemistry, Pharmacology, and Toxicology of HNO

Endogenous Generation of HNO?

To date, there is no explicit evidence of endogenous HNO biosynthesis in mammalian systems. It may well be that HNO merely possesses important pharmacological properties and is not involved in normal physiological processes and therefore is not specifically biosynthesized. However, the lack of evidence for endogenous HNO generation may also be due to the fact that there is currently no specific and sensitive detector/trap for HNO. Most all assay techniques currently used in purely chemical systems become ambiguous in complex biological systems where redox processes and the presence of other nitrogen oxides cloud interpretation. Thus, the question of whether HNO is an endogenous signaling species remains open and one of the most important issues in the field.

Although endogenous HNO generation in mammalian cells has not been demonstrated, there are numerous in vitro biochemical studies that provide reasonable chemical pathways for this to occur. For example, S-nitrosothiols are well-characterized species formed in biological tissues as a result of NO generation (for example, *59*) and can serve as sources of HNO. S-Nitrosothiols can react with other thiols to generate HNO according to reaction 11 (*30*, *60*).

$$\text{RS-NO} + \text{R}'\text{SH} \rightarrow \text{RSSR}' + \text{HNO} \qquad (11)$$

N-Hydroxy-L-arginine (NOHA), the intermediate in NO biosynthesis from L-arginine (*5*), can also serve as a source of HNO in that nonenzymatic, oxidative degradation of NOHA leads to the generation of HNO (for example, *61*−*64*). Interestingly, NOHA is generated and released by cells in vitro (*65*) and has been found at high levels in plasma in vivo (*66*). HNO generation from L-arginine and/or NOHA via the actions of the NO biosynthesis enzyme (NOS) has also been reported (*50*, *67*) especially in the absence of one of its vital prosthetic groups, tetrahydrobiopterin (*68*, *69*). These reports provide the possibility that NOS is capable of generating HNO depending on the experimental/cellular conditions. Other possible sources of endogenous HNO generation include direct reduction of NO by elements of the electron transport system in mitochondria (*70*, *71*), ubiquinol (*72*), cytochrome c (*73*), manganese superoxide dismutase (*74*), and xanthine oxidase (*75*). Thus, biochemical events have been characterized that may, in principle, result in endogenous HNO generation. Clearly, answering the question of whether HNO is endogenously generated in a controlled fashion for regulating specific physiological functions is an important one and will require the development of sensitive and specific assays for HNO amenable for use in biological systems. Regardless of whether HNO is produced endogenously, there are numerous reports of novel biological activity that may indicate its use as a pharmacological agent (discussed immediately below).

HNO as an Antialcoholic Drug

Some of the earliest studies on the biology of HNO in mammalian systems were by the Nagasawa research group who were examining the actions of the antialcoholic drug cyanamide (*57*, *58*, *76*). The utility of this drug lies in its ability to inhibit aldehyde dehydrogenase, an enzyme involved in the metabolism of ethanol to acetate. Cyanamide has no inherent activity and must be oxidatively bioactivated in order to elicit enzyme inhibition. Oxidation of cyanamide by catalase/H_2O_2 generates an N-hydroxy intermediate that then decomposes to give HNO and cyanide (Figure 3). The inhibition of aldehyde dehydrogenase was then proposed to occur

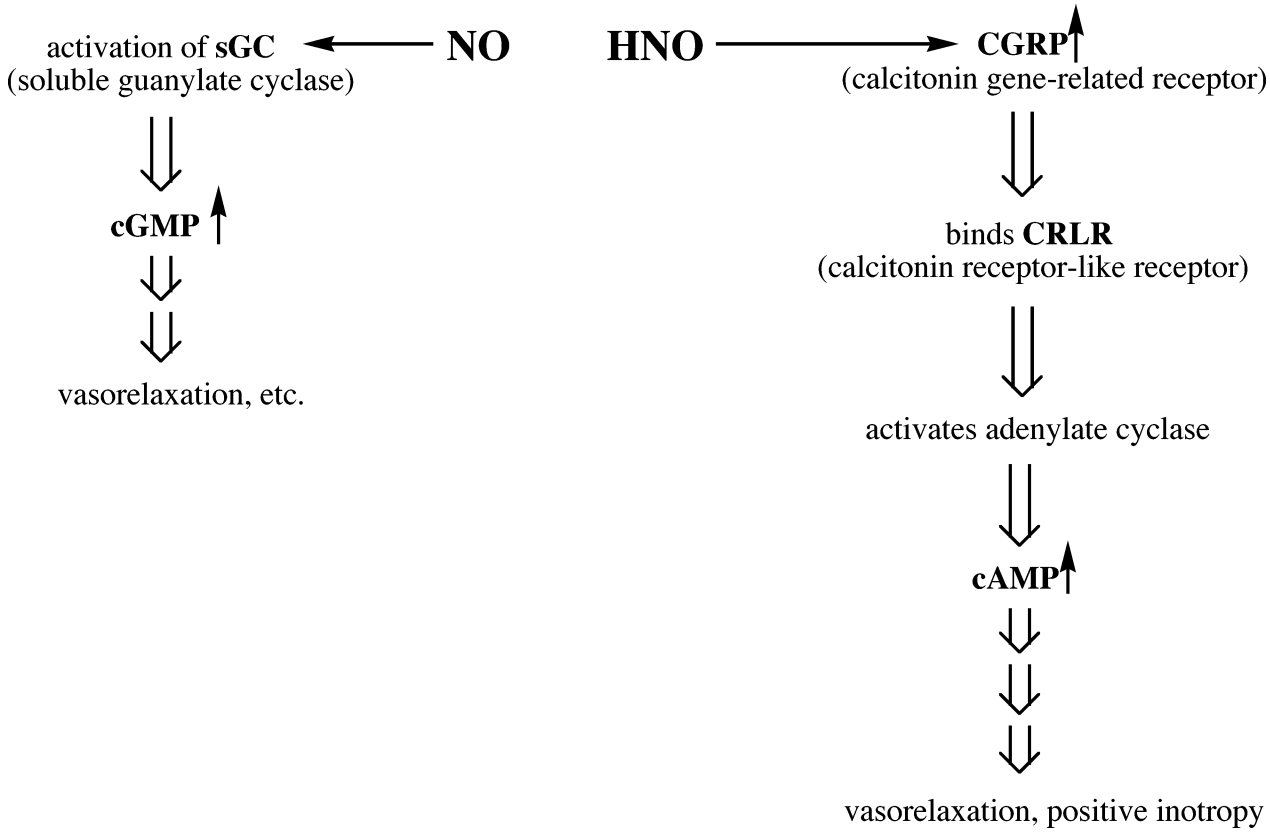

Figure 4. Schematic of the pathways of NO vs HNO signaling.

via reaction of HNO with the active site cysteine thiolate of the enzyme. The reaction between HNO and thiols likely occurs via an initial attack on the electrophilic nitrogen of HNO by thiolate, followed by further reactions depending on the reaction conditions (Figure 2) (*29*, *30*, *60*). These early studies indicated that HNO reacts readily with biological thiols, a reactivity that has been theoretically verified and has become one of the hallmarks of HNO biological chemistry (vide supra). These are the first reports of the biological activity of HNO in mammalian systems and the first to indicate the utility of HNO as a pharmacological agent.

Cardiovascular Actions of HNO

The discovery of endogenously generated NO and its vasorelaxant properties prompted examination of the actions of HNO on vascular tissue. These studies were inspired by the idea that HNO could be generated biologically and was either directly capable of eliciting vasorelaxation or was converted to NO by facile one-electron oxidation resulting on NO-mediated relaxation. For reasons discussed earlier, HNO is typically studied using donor compounds. Currently, the most established and characterized HNO donor amenable for use in biological systems is sodium trioxodinitrate ($Na_2N_2O_3$, commonly referred to as Angeli's salt) (*77*, *78* and references therein). Between pH 4 and pH 8, Angeli's salt decomposes in aqueous buffer solution to generate HNO and NO_2^- with a first-order rate constant of 4.6×10^{-4} s^{-1} (reaction 12) (*79*).

$$N_2O_3^{2-} + H^+ \rightarrow HNO + NO_2^- \qquad (12)$$

Thus, using Angeli's salt, it was reported that HNO has potent vasorelaxant properties (*80*). Interestingly, the relaxation of vascular tissue was increased in the presence of the enzyme copper−zinc superoxide dismutase (CuZnSOD) (*51*). The increase in vasorelaxation by HNO in the presence of CuZnSOD was proposed to be due to oxidative conversion of HNO to NO by the enzyme-bound cupric ion (vide supra). This work alluded to the possibility of utilizing HNO donor species as vasorelaxants.

The uses of HNO as a mediator in antialcoholism therapy or as a vasorelaxant are only two of many of its possible applications as a therapeutic agent. More recently (and probably more importantly from a pharmacological and/or therapeutic perspective), Paolocci and coworkers discovered that administration of HNO (via Angeli's salt) to dogs resulted in an increase in left ventricular contractility with a concomitant lowering of cardiac preload volume and increased venodilation (*81*). Further studies on the effects of HNO on the cardiovascular system found that its actions were independent of NO and cGMP as well as β-adrenergic signaling (*82*). Many of these actions of HNO were mediated by increases in calcitonin gene-related peptide (CGRP). CGRP is an agonist for the calcitonin receptor-like receptor, which, upon CGRP binding, leads to the activation of adenylate cyclase resulting in an elevation of intracellular cAMP (for example, *83*, *84*). Increases in cAMP activate protein kinase A, which phosphorylates L-type Ca^{2+} channels leading to, eventually, vasodilation (Figure 4). Thus, the cardiovascular actions of HNO are, in part, mediated via a cAMP pathway. This is in direct contrast to the signaling actions of NO in the cardiovascular system that are mediated through the activation of sGC

and elevations in cGMP. Therefore, with regards to the cardiovascular system, HNO is not just a precursor for NO and, instead, functions by a separate signaling pathway mediated by cAMP (Figure 4). The signaling actions of HNO and NO have been termed as "orthogonal" due to the fact that they involve distinct second messenger systems (although the physiological outcomes can, at times, overlap) (*55*). Significantly, the specific actions of HNO make it an ideal agent for the treatment of heart failure and, indeed, the pharmacological utility of HNO in this regard has been recognized and discussed (for example, *85*). Currently, the intimate chemical/biochemical mechanism by which HNO elicits a CGRP response is not established and is the topic of ongoing investigations.

HNO and Reperfusion Injury

Nitrogen oxides appear to greatly influence the extent of myocardial reperfusion injury (the tissue injury that occurs when ischemic tissue is resupplied with oxygenated blood). NO has been reported to both exacerbate and attenuate myocardial reperfusion injury (for example, *86*). Exacerbation of injury by NO has been attributed to the simultaneous generation of NO and O_2^-, leading to the formation of the potent oxidant, $ONOO^-$ (reaction 13).

$$NO + O_2^- \rightarrow ONOO^- \quad (13)$$

Protection against reperfusion injury by NO has been proposed to be due to attenuation of leukocyte interactions with endothelial cells. In a recent study comparing the effects of NO and HNO on postischemic myocardial injury, the two nitrogen oxides were found to have opposite actions (*87*). Under the conditions of this study, when the nitrogen oxide donors were given after ischemia but prior to reperfusion, NO was found to be protective whereas HNO exacerbated reperfusion injury. However, later, it was reported that if HNO were given prior to the ischemic event (preconditioning), protection from subsequent reperfusion was observed (*88*). Moreover, the degree of protection granted by HNO appeared to be greater than that observed with equivalent amounts of NO. Thus, HNO was able to precondition myocardial tissue to protect it from subsequent ischemia/reperfusion injury. The biochemical basis for this effect is, as yet, not established.

HNO Regulation of the NMDA Receptor

Consistent with its ability to modify thiol proteins, HNO has been reported to react with a thiol residue on the N-methyl-D-aspartate (NMDA) receptor leading to an attenuation of Ca^{2+} influx (*89*). Because overstimulation of the NMDA receptor has been implicated in the excitotoxicity associated with glutamate, the result of this activity of HNO is a protection against glutamate-based excitotoxicity. However, another lab reported that HNO was capable of blocking glycine-dependent desensitization of the NMDA receptor resulting in a net sensitization of the receptor (*90*). These conflicting reports have not been unequivocally reconciled and are likely due to differences in the experimental design of the two studies (e.g., O_2 levels). Regardless, HNO appears to be capable of modifying the activity of the NMDA receptor in either a positive or a negative fashion.

Thiol Proteins/Peptides as Targets for HNO

Biological thiols are likely targets for the actions of HNO. As previously mentioned, HNO-mediated inhibition of aldehyde dehydrogenase occurs via modification of the active site thiolate. Another example of this is the report that exposure of fibroblasts in culture to the HNO donor Angeli's salt resulted in a dramatic depletion of intracellular GSH levels (*38*). The ability of HNO to disrupt thiol/thiolate-mediated processes is further exemplified in a study by Cook and co-workers (*91*) who examined the biochemistry of the interaction of HNO with the metal responsive yeast transcription factor Ace1. This transcription factor "senses" intracellular copper levels in yeast by binding multiple copper ions via thiolate ligation resulting in the expression of genes protective against copper toxicity. In this way, yeast are able to respond to and protect themselves from high, potentially toxic levels of copper. HNO was found to potently inhibit the copper-dependent activation of Ace1 in an O_2-independent fashion, consistent with a direct interaction of HNO with the Ace1 thiols (as was the case with aldehyde dehydrogenase). Interestingly, NO inhibition of Ace1 activity is O_2-dependent (*92*), indicating distinct pathways of thiol protein disruption by these two nitrogen oxides.

HNO Toxicity

It has become evident that HNO possesses significant pharmacological potential as a treatment for heart failure and alcoholism and could be used as a vasorelaxant and a preconditioning agent for ischemia/reperfusion injury. However, as with all pharmacological agents, there may also be toxicological implications associated with HNO administration. Wink and co-workers reported that HNO, derived from Angeli's salt, is cytotoxic to fibroblasts at millimolar concentrations of the donor (*38*). The observed cytotoxicity was attributed to a number of effects including depletion of cellular GSH and DNA damage. Further studies by others also indicated that HNO could be DNA damaging via the generation of potent oxidants (*39*). Significantly, the oxidation mediated by HNO was found to be O_2-independent (a chemical rationale for these observations was given earlier). HNO toxicity has also been examined in vivo. Intrathecal administration (into the cerebral spinal fluid) of 10 μmol of Angeli's salt to rats has been reported to cause spinal motor neuron injury without affecting sensory neurons (*93*), an outcome similar to that found in amyotrophic lateral sclerosis. Also, direct administration of 400 nmol of Angeli's salt to the substantia nigra compacta region of rats resulted in a diminution of striatal dopamine after 7 days (*94*). Although it is difficult to quantitatively assess the differences in the doses, which exhibit pharmacological benefit (i.e., CGRP response) from those that elicit the observed toxicity (due to the differences in the routes of administration, different animal models, etc.), it appears that the toxicological doses are significantly greater than those that elicit the beneficial pharmacological effects.

For example, the dose of Angeli's salt administered to the entire circulatory system of dogs that elicited a CGRP-mediated response was 10 μg/kg/min for 5−20 min whereas the dose of Angeli's salt that caused motor neuron damage in rats was a bolus 5 mg/kg injected directly into a much smaller volume (cerebral spinal fluid). Thus, HNO can be toxic but the relevance of this toxicity to pharmacological use remains questionable.

Current Use of HNO Donors and Detection/ Trapping of HNO

As mentioned earlier, two of the most prevalent HNO donors utilized thus far to determine the biological actions of HNO are Angeli's salt and cyanamide. These two donors are, however, quite different. Angeli's salt spontaneously releases HNO under physiological conditions, and cyanamide requires oxidative bioactivation (vide supra). They also differ markedly with respect to their byproducts. Angeli's salt generates 1 equiv of NO_2^- for each HNO formed, and cyanamide oxidation leads to the release of 1 equiv of cyanide. Because both of these byproducts have potential biological effects, it is crucial to distinguish between the actions of HNO from NO_2^-, in the case of Angeli's salt, and from CN^-, in the case of cyanamide. Most studies with Angeli's salt account for possible effects of byproducts (and possible impurities) by simply using decomposed solutions in control experiments. Another confounding factor associated with the use of Angeli's salt (or any donor species for that matter) is the possibility that the observed effects are not due to HNO but, rather, to Angeli's salt itself. To address this concern, it is best to utilize multiple, structurally distinct donors that both release HNO. If they all have similar biological effects, it can be reasonably deduced that HNO is the active species. Indeed, the ability of HNO to inhibit aldehyde dehydrogenase and elicit vasorelaxation has been shown to occur with several, distinct donors, implicating HNO as the effector species in these studies (*58*, *80*). As this field grows, there will certainly be a call for the development of new and novel HNO donors.

The unequivocal detection of HNO in biological systems has yet to be accomplished. However, there are several methods that may implicate the presence or fleeting existence of HNO. As mentioned briefly before, the detection of N_2O has been used as evidence for HNO generation since HNO spontaneously dimerizes to hyponitrous acid ($H_2N_2O_2$) that then decomposes to N_2O and water (reaction 1). However, this is not absolute proof of HNO intermediacy since N_2O can be generated in ways not involving free HNO (for example, *64*). Moreover, N_2O formation is second-order in HNO and would require fairly high concentrations for this reaction to be significant. As discussed earlier, HNO reacts readily with thiols. In fact, Pino and Feelisch (*95*) used thiol reactivity as a means of trapping HNO and distinguishing its actions from those of NO. Shoeman and Nagasawa (*96*) reported that nitrosobenzene could trap HNO to give cupferron, a species that complexes copper forming a colored complex. Although this has not yet been exploited for the detection of HNO in a biological system, one can envision water soluble analogues being used as traps/detectors for HNO. However, the efficiency of this trap has not yet been determined.

Clearly, the development of sensitive, specific, and biologically compatible traps/detectors for HNO will be essential in determining whether HNO is physiologically relevant. Moreover, such methodology will be valuable for future investigations distinguishing the specific actions of HNO from those of other nitrogen oxides. The current lack of appropriate traps and detectors for HNO remains one of the biggest obstacles in the study of HNO biology.

Summary

It is clear that HNO possesses unique and a potentially important biological activity that is distinct from NO (and other nitrogen oxides). The "orthogonal" signaling relationship between HNO (acting via cAMP pathways) and NO (acting via cGMP pathways) provides an intriguing scenario in which these two species may be utilized physiologically to control various aspects of, for example, cardiovascular function by independent pathways (*55*). The idea that HNO and NO are naturally occurring signaling partners with distinct mechanisms of action is, in part, dependent on the demonstration of endogenous and regulated HNO generation. Because endogenous production of HNO has yet to be demonstrated, the idea that HNO and NO represent a normal, coordinated, and/or integrated signaling system is purely speculative at this point. Regardless, the pharmacological potential of HNO is clear and warrants continued investigation of its biology and chemistry.

As with NO, the biological utility of HNO lies in its chemistry and, from a purely chemical perspective, HNO is a fascinating molecule with fundamentally unique properties. The biology of HNO is equally fascinating, and it is intriguing to think that it may be an important signaling agent in mammalian systems. At the very least, it possesses important pharmacological properties that may have significant therapeutic applications. However, our current understanding of HNO biochemistry is, at best, scant and inadequate. There is little doubt that thiol proteins can be major targets for HNO, but there are assuredly other potential sites of action as well. Moreover, there remains the question of whether HNO is capable of exhibiting a specific reactivity with select thiol proteins, as opposed to being a nonspecific thiol modifying agent. This question along with delineation of other biochemical mechanisms of HNO biology will undoubtedly occupy many labs for years to come. Furthermore, other profound biological actions of HNO will undoubtedly be discovered as more researchers become interested in this previously unheralded species. We are only in the early stages of understanding both the biology and the biological chemistry of HNO, and there is little doubt that other major discoveries await.

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