Annu. Rev. Pharmacol. Toxicol. 2005. 45:335–55
doi: 10.1146/annurev.pharmtox.45.120403.095959
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NITROXYL (HNO): Chemistry, Biochemistry, and Pharmacology

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Key Words nitric oxide, thiols, calcitonin gene-related peptide, ischemia-reperfusion

■ **Abstract** Recent discoveries of novel and potentially important biological activity have spurred interest in the chemistry and biochemistry of nitroxyl (HNO). It has become clear that, among all the nitrogen oxides, HNO is unique in its chemistry and biology. Currently, the intimate chemical details of the biological actions of HNO are not well understood. Moreover, many of the previously accepted chemical properties of HNO have been recently revised, thus requiring reevaluation of possible mechanisms of biological action. Herein, we review these developments in HNO chemistry and biology.

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INTRODUCTION

The biological activity and biological chemistry of nitrogen oxide species in mammalian systems have received considerable attention over the past 15 years. Interest in this area is primarily the result of the discovery of endogenous generation of nitric oxide (NO) by mammalian cells. Although the focus of much of the past research has been NO, it is becoming increasingly clear that other nitrogen oxides derived in vivo from NO may have significant physiological and/or pathophysiolgical functions. Although significant advances have been made in our understanding of the chemical biology of NO and related/derived nitrogen oxides, such as nitrogen dioxide (NO_2), dinitrogen trioxide (N_2O_3), and peroxynitrite ($ONOO^-$), nitroxyl (HNO) remains the least studied and least understood of all the biologically relevant nitrogen oxides. Despite the original description of HNO more

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than 100 years ago, understanding of the chemistry and biochemistry of HNO has seriously lagged behind other redox nitrogen oxide congeners, even after the discovery of endogenous mammalian nitrogen oxide generation. However, recent reports have indicated that HNO has novel and potentially important biological activity (see below), which prompted numerous labs to investigate the physiological and chemical properties and reactivity of HNO. Much of this recent work has led to redefinition of the fundamental chemistry of this enigmatic species, which aided in partial understanding of the chemistry responsible for the newly discovered biological properties of HNO. In this chapter, we review some of the physiologically relevant chemical properties of HNO and discuss some of its recently discovered biological/pharmacological properties.

FUNDAMENTAL CHEMICAL PROPERTIES OF NITROXYL

From both theoretical and experimental perspectives, nitroxyl has been the topic of numerous studies for more than 100 years. In this review, we concentrate on the chemistry that may be relevant to the biological actions of nitroxyl. For more comprehensive treatments of the pure and applied chemistry of nitroxyl (and related nitrogen oxide species), readers are referred to other excellent reviews (1–3).

Before discussing the details of HNO chemistry, a comment regarding nomenclature is warranted. The term "nitroxyl" is sometimes used to describe a stable radical functional group otherwise referred to as a nitroxide (i.e., $R_2NO\cdot$). However, nitroxyl is also used in the literature to describe the chemical species commonly written as HNO (along with the various spin-state and protonation congeners, see below). Owing to the current widespread use of the term nitroxyl in the literature when referring to HNO (or even NO^-), we will continue to use it in this regard. Another, more appropriate, name for HNO is nitrosyl hydride (4).

One of the first references to HNO in the chemical literature was by Angeli (5), who proposed it as a decomposition product of sodium trioxodinitrate ($Na_2N_2O_3$, Angeli's salt) (Reaction 1):

$$Na_2N_2O_3(\text{Angeli's salt}) + H^+ \rightarrow [NaHN_2O_3] \rightarrow HNO + NaNO_2 \qquad 1.$$

Since then, others have proposed HNO as an intermediate in a variety of chemical and biological processes. For example, HNO has been proposed to be generated during bacterial denitrification (6), released from acid-catalyzed solvolysis of *aci*-nitroalkanes (Nef reaction) (7), the product of the reaction of NO with hydrogen (8), and a product of the reaction of NO with hydroxylamine (9, 10). Direct observation of HNO was accomplished by Brown & Pimentel (11) when they trapped it in an argon matrix during the photolysis of methyl nitrite. HNO has also been generated by pulse radiolysis (12, 13), a technique that has led to the elucidation of some of the fundamental chemical properties of HNO (although some of these properties have been reevaluated and revised recently, see below).

One of the most unique and fascinating aspects of HNO chemistry involves its simple deprotonation. The electronic ground state of HNO is the singlet where all

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electrons are spin-paired (unlike radical species such as NO). Deprotonation of HNO generates nitroxyl anion [more appropriately termed oxonitrate (1-), NO^-]. This species is isoelectronic with dioxygen (O_2) and can exist as an electronic singlet ($^1NO^-$) or triplet ($^3NO^-$), which is analogous to the relationship between singlet O_2 ($^1O_2$) and triplet O_2 ($^3O_2$) (14). The electronic ground state of NO^- is the triplet ($^3NO^-$), which is reported to be approximately 17–20 kcal/mol lower in energy than the first excited electronic singlet state, $^1NO^-$ (15–19). Thus, in the acid-base equilibrium expression for HNO/NO^-, H^+ (Reaction 2), is not straightforward because the electronic ground states of the acid and conjugate base are different. Although it was earlier proposed that HNO deprotonates to the singlet excited state anion (20), $^1NO^-$, which would be followed by intersystem crossing to the ground state triplet species, $^3NO^-$, this is no longer considered the case. Recent theoretical and experimental work indicates that the relevant equilibrium in the acid-base chemistry of HNO/NO^-, H^+ in both the gas phase and in solution is between singlet HNO and triplet NO^- (19, 21–25) (Reaction 2):

$$^1HNO \rightleftharpoons {}^3NO^- + H^+ \qquad 2.$$

Hence, the deprotonation of HNO requires a spin conversion of ground state products to ground state reactants (and vice versa for protonation of the anion). As might be expected, this spin conversion will considerably slow the rate of both deprotonation of HNO and protonation of $^3NO^-$. However, the spin conversion in HNO deprotonation plays only a minor role in the intersystem barrier, with nuclear reorganization representing the majority of the activation barrier (25). Regardless, it is clear that HNO deprotonation and $^3NO^-$ protonation are considerably slower than typical proton transfer processes. This slow process predicts $^3NO^-$ generated at neutral pH will have a significant lifetime (milliseconds), even though its existence relative to the protonated species is unfavorable (25).

Direct experimental determination of the pK_a of HNO by measuring equilibrium concentrations of HNO and/or NO^- is difficult owing to the propensity of HNO to undergo dimerization to hyponitrous acid followed by dehydration to give nitrous oxide (N_2O) and water (26) (Reaction 3). The rate constant for dimerization was originally reported to be near diffusion controlled (26) but has recently been revised to be significantly lower (24).

$$HNO + HNO \rightarrow [HONNOH] \rightarrow N_2O + H_2O\,(8 \times 10^6\,M^{-1}\,s^{-1}) \qquad 3.$$

Thus, equilibrium between HNO and NO^- cannot be achieved at supranano-molar concentrations because HNO can be "siphoned off" to N_2O and H_2O via Reaction 3. Regardless, in a 1970 study where NO^- was generated using pulse radiolysis, a pK_a for HNO of 4.7 was reported (12). This report did not specify the spin states of the relevant equilibrium species, and until recently this was the exclusively quoted value for the pK_a of HNO. Theoretical reevaluation of the HNO pK_a led to a revision of 7.2 (19). Of particular note, this work specifically indicated that the relevant equilibrium in solution, between HNO and $^3NO^-$, was the same as that proposed by Janaway & Brauman for the gas phase (22). Further experimental

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and theoretical work by Shafirovich & Lymar (24) and Bartberger and coworkers (23) provided a consensus agreement that the pK_a of HNO is 11.4.

Bartberger and coworkers (19) noted that the revision of the HNO pK_a required reevaluation of an aspect of NO chemistry as well. An often-quoted reduction potential for the NO/$^3NO^-$ couple was calculated to be 0.39 V (versus NHE throughout) using the assumptions, among others, that the HNO pK_a was 4.7 and the relevant acid-base equilibrium was between HNO and $^1NO^-$ (20). Considering the dramatic revision in the pK_a and the establishment that the relevant equilibrium is between HNO and $^3NO^-$, recalculation of the NO/$^3NO^-$ couple gives a value of -0.8 V (23). This recalculated reduction potential is consistent with experimentally derived values (27, 28), and this previously irreconcilable difference between experiment and calculation can now be explained. Protonation of $^3NO^-$ to HNO will be highly favorable at physiological pH and therefore results in a positive shift in the potential to approximately -0.5 to -0.6V as the pH is lowered (23, 24). These negative values for the one-electron reduction potentials for both the NO/$^3NO^-$ and NO,H^+/HNO couples indicate that direct one-electron reduction of NO to reduced species by an outer sphere electron transfer process is thermodynamically unfavorable and not likely to occur under biological (mammalian) conditions. This idea has, however, been challenged on the basis that if the intracellular concentrations of the reductants and oxidants are considered, a much less negative potential will be realized (29). Moreover, it has been pointed out that the reduction potentials in prokaryotic cells may be capable of reducing NO (29) and may be part of an antipathogenic response of NO.

The discussion of nitroxyl chemistry thus far has focused on HNO and $^3NO^-$. However, a triplet protonated species and a singlet anionic species have been examined in previous studies. Protonation of $^3NO^-$ has been proposed to occur on the more electronegative oxygen atom, generating ^{3}NOH (30, 31). This triplet protonated species has been calculated to be approximately 20–23 kcal/mol less stable than ^{1}HNO (32, 33). Thus, interconverison between ^{1}HNO and ^{3}NOH is biologically inaccessible. As noted earlier, the singlet anionic nitroxyl, $^1NO^-$, has been determined to be approximately 17–20 kcal/mol above the ground state triplet species, which agrees reasonably well with theoretical studies (19). From a biological perspective, the only accessible nitroxyl species are HNO and $^3NO^-$ (which is the reason the chemistry of these has been the focus of discussion). Figure 1 depicts the energy relationships between all of these protonation- and spin-related species.

As is evident from the above discussion, fundamental nitroxyl chemistry is conceptually distinct and, at times, requires one to suspend commonly held chemical dogma/beliefs when thinking about it. When addressing the chemistry of nitroxyl in biological/physiological systems, it will be important to remember the following:

1. The pKa of HNO is approximately 11 and therefore, if formed initially, HNO will be the predominant species present at physiological pH.

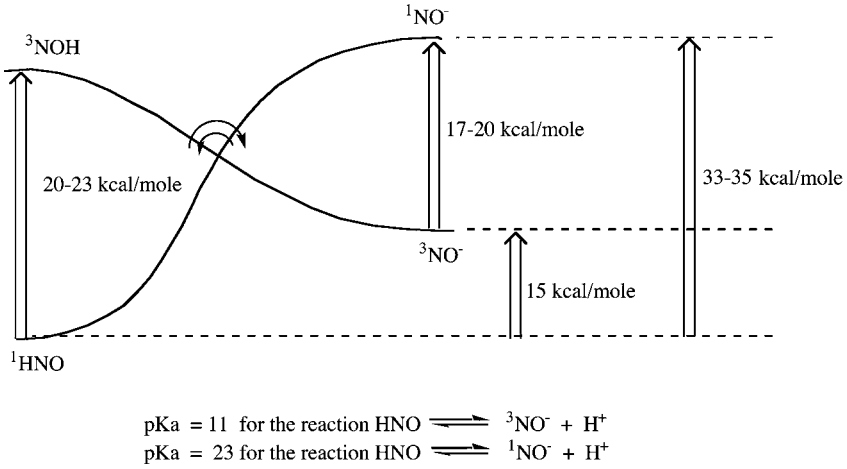

Figure 1 Energetics of the various nitroxyl species.

2. The relevant acid-base equilibrium for nitroxyl is between the singlet protonated, ^{1}HNO, and the triplet anion, $^3NO^-$.
3. The requirement for a spin-state change for nitroxyl protonation-deprotonation means these reactions are slow relative to all other protonation-deprotonation events, which are extremely fast.
4. If circumstances exist whereby $^3NO^-$ is generated/formed, it will have a significant lifetime (milliseconds) because protonation to HNO is slow.
5. The other protonation/spin-state congeners of nitroxyl, namely ^{1}NOH and $^1NO^-$, are biologically inaccessible and irrelevant to most all discussions of biological nitroxyl activity.
6. Generation of HNO or $^3NO^-$ via single-electron reduction of NO by an outer sphere process is not favorable, although it may be possible.

REACTIVITY OF NITROXYL

As already mentioned, an important (and bothersome) reaction of HNO is dimerization with itself followed by dehydration to give N_2O and H_2O (Reaction 3). This propensity to dimerize necessitates the use of donor molecules for most studies of HNO. The ground state triplet anion, $^3NO^-$, reacts with O_2 to generate peroxynitrite, $^-$OONO (34). This reaction (Reaction 4) is isoelectronic with the well-studied reaction of NO with O_2^- (Reaction 5), and both reactions occur at near diffusion-controlled rates (24, 35, 36):

$$^3NO^- + {}^3O_2 \rightarrow {}^-OONO \quad (2.7 \times 10^9\,M^{-1}\,s^{-1}) \qquad 4.$$

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$$NO + O_2^- \rightarrow {}^-OONO \quad (4\text{–}7 \times 10^9\,M^{-1}\,s^{-1}) \qquad 5.$$

Nitroxyl anion generated by pulse radiolytic reduction of NO (spin state not reported) reacts sequentially with NO to give $N_2O_2^-$ and $N_3O_3^-$, the latter species decomposing to N_2O and NO_2^- (12, 13, 37) (Reactions 6, 7, and 8):

$$NO^- + NO \rightarrow N_2O_2^- \quad (k = 1.7\text{–}3.3 \times 10^9\,M^{-1}\,s^{-1}) \qquad 6.$$

$$N_2O_2^- + NO \rightarrow N_3O_3^- \quad (k = 3\text{–}4.9 \times 10^6\,M^{-1}\,s^{-1}) \qquad 7.$$

$$N_3O_3^- \rightarrow N_2O + NO_2^- \quad (k = 87\text{–}330\,s^{-1}) \qquad 8.$$

The existence of Reactions 6–8 preclude the possibility of synthesizing salts of NO^- via direct reduction of NO because any anion formed will rapidly react with excess NO. Sequential reaction of HNO with NO has also been observed (Reactions 9 and 10) with eventual formation of N_2O and HNO_2 (12, 13) (Reaction 11). A rate constant for the reaction of HNO with NO was originally reported to indicate a near diffusion-controlled reaction ($1.7 \times 10^9\,M^{-1}\,s^{-1}$). However, recent reevaluation of the reaction of HNO with NO (Reaction 9), using flash photolysis of Angeli's salt ($Na_2N_2O_3$) for in situ HNO generation, has reported a significantly lower rate constant of $5.8 \times 10^6\,M^{-1}\,s^{-1}$ (24):

$$HNO + NO \rightarrow HN_2O_2 \quad (5.8 \times 10^6\,M^{-1}\,s^{-1}) \qquad 9.$$

$$HN_2O_2 + NO \rightarrow HN_3O_3 \quad (8 \times 10^6\,M^{-1}\,s^{-1}) \qquad 10.$$

$$HN_3O_3 \rightarrow N_2O + HNO_2 \quad (1.6 \times 10^4\,M^{-1}\,s^{-1}) \qquad 11.$$

The catenation reactions of HNO/NO^- with NO may be of some biological/pharmacological interest because both species may be present simultaneously under certain circumstances. Indeed, nitroxyl may be capable of attenuating the actions of NO (and vice versa).

Formation of $N_2O_2^-/HN_2O_2$ (Reactions 6 and 9) has been hypothesized to lead to the generation of the potent oxidant hydroxyl radical (HO·) (13) (Reaction 12):

$$N_2O_2^- \rightarrow N_2O + O^-(\rightarrow HO\cdot) \quad (3.5 \times 10^2\,s^{-1}) \qquad 12.$$

Although this reaction has been proposed to account for some of the oxidative chemistry and/or toxicity associated with nitroxyl (38–40), unequivocal demonstration of this reaction is lacking.

One of the most important and biologically relevant aspects of HNO chemistry is its ability to react as an electrophile with thiols. In fact, this reaction has been used to distinguish between the biology of HNO versus NO because HNO is much more reactive toward thiols compared with NO (41). The electrophilicity of HNO appears to be particularly high for thiols and much less so with oxygen-based nucleophiles (19). The reactivity of HNO with amine nucleophiles has been calculated to be intermediate between thiols and oxygen-based nucleophiles and can be favorable. The initial product of the reaction of HNO with a thiol is an

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N-hydroxysulfenamide (Reaction 13) (42). This intermediate can react further with excess thiol to give hydroxylamine and the corresponding disulfide (Reaction 14) or rearrange to generate a sulfinamide (43, 44) (Reaction 15):

$$\mathrm{HNO + RSH \rightarrow RS\text{-}NHOH} \qquad 13.$$

$$\mathrm{RS\text{-}NHOH + RSH \rightarrow RSSR + NH_2OH} \qquad 14.$$

$$\mathrm{RS\text{-}NHOH \rightarrow [RS^{+}NH + HO^{-}] \rightarrow RS^{+}(OH)NH \rightarrow RS(O)NH_2} \qquad 15.$$

Sulfinamides can hydrolyze to generate ammonia and the corresponding sulfinic acid (43). HNO-mediated oxidation to the disulfide and hydroxylamine (Reactions 13 and 14) represents a biologically reversible process because disulfides are easily regenerated. However, sulfinamide or sulfinic acid formation may represent a process that is either irreversible or more difficult to reverse. To date, there is only one reported example of biological reduction of a sulfinic acid back to the thiol oxidation state (45).

Direct reaction of HNO with thiols represents an HNO reduction process (i.e., HNO serving as an oxidant). Other reports indicate that HNO is a reasonable oxidant, and, indeed, HNO reduction may be a primary fate of HNO in cells. For example, HNO can oxidize NADPH (46–49). This reaction was inhibited by the presence of superoxide dismutase (which converts HNO to NO), indicating that HNO/NO^{-} was the oxidant and not NO. Moreover, NADPH oxidation occurred anaerobically, eliminating the possibility that HNO/O_2 adducts were the oxidizing species. The two-electron reduction potential for the ^{1}HNO, $2H^{+}/NH_2OH$ couple has been reported to be 0.3 V (versus NHE) (24). This favorable potential indicates that reduction of HNO to NH_2OH may be biologically facile and that nitrogen oxide species generated from HNO reduction need to be considered as possible participants in the overall biology of HNO.

Analogous to the reaction of HNO with thiols, reaction of HNO with amines should generate a substituted N-hydroxyhydrazine as an unstable intermediate. It may be expected that loss of water from this species will then lead to the formation of an alkyl diazene (Reaction 16):

$$\mathrm{R\text{-}NH_2 + HNO \rightarrow R\text{-}NH\text{-}NH\text{-}OH \rightarrow RNNH + H_2O} \qquad 16.$$

Alkyl and aryl diazenes are known to be unstable with respect to dinitrogen (N_2) loss, and oxidative decomposition leads to the formation of alkyl radicals (50, 51). The reaction of HNO with amines has not been extensively examined beyond the recently published theoretical treatment (19). However, one study in 1965 reported that reaction of secondary amines with the HNO-donor Angeli's salt led to the generation of dinitrogen and products consistent with radical intermediates (52). Whether HNO release from Angeli's salt was required for this chemistry was not determined, however.

The reaction of nitroxyl with dioxygen has become a topic of considerable interest. As discussed above, there appears to be little doubt that the reaction

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of $^3NO^-$ with O_2 to give $ONOO^-$ is a fast reaction. Of, course, the biological relevance of this reaction is largely dependent on the existence/levels of $^3NO^-$. The pKa of HNO is approximately 11, making equilibrium concentrations of $^3NO^-$ extremely small under physiological conditions. However, as noted above, biological circumstances whereby $^3NO^-$ is made directly (if they exist) could lead to reaction with O_2 because protonation is very slow and, therefore, $^3NO^-$ can have a significant (millisecond) lifetime (25). A more intriguing, provocative, and biologically relevant process is the reaction of HNO with O_2. Although this reaction has been analyzed experimentally and theoretically, it remains a controversial and contentious topic. Several studies by Miranda and coworkers indicate that the reaction of HNO with O_2 results in the generation of a potent two-electron oxidant whose reaction profile is distinct from that of ^-OONO and/or N_2O_3 (53, 54). Interestingly, aerobic decomposition of the HNO-donor molecule Angeli's salt does not generate nitrate (NO_3^-), which would be expected if ^-OONO were the primary nitroxyl-O_2 product (owing to the fact that ^-OONO decomposes to give predominantly NO_3^-) (2). Moreover, a direct and spin-forbidden reaction of HNO with O_2 to generate $HOONO/^-OONO$ would be very slow and highly unlikely (25). Thus, the chemistry and biology of the HNO/O_2 reaction remains one of the most significant and important enigmas in the field of HNO chemistry and biology.

As mentioned above, NO is very difficult to reduce to $^3NO^-$ (indicated by the very low reduction potential for the $NO/^3NO^-$ couple). This means that $^3NO^-$, if formed, can be a one-electron reducing agent. An example of this is the reduction of the cupric form of the enzyme superoxide dismutase (SOD) to the cuprous form by NO^- (49, 55–57) (Reaction 17):

$$NO^- + SODCu^{II} \rightarrow NO + SODCu^{I} \qquad 17.$$

Most of the studies examining the interaction of nitroxyl with SOD were performed prior to the understanding that the primary nitroxyl species present in solution at physiological pH is HNO rather than $^3NO^-$. Thus, all reactions were written as occurring through the deprotonated anionic species. Although this is possible, previously mentioned difficulties in generating significant concentrations of NO^- under biological conditions indicate that coordination of HNO to the metals followed by deprotonation may be an equally likely mechanism for these reactions. HNO can also react with oxidized hemoproteins, such as methemoglobin, to generate the ferrous nitrosyl adducts via reductive nitrosylation (26, 42) (Reaction 18):

$$HNO + HbFe^{III} \rightarrow HbFe^{II}\text{-}NO + H^+ \qquad Hb = \text{hemoglobin} \qquad 18.$$

Interestingly, reduction of a myoglobin-NO adduct ($MbFe^{II}$-NO) results in the formation of a stable HNO-Fe(II) adduct ($MbFe^{II}$-HNO) (58), and more recently it has been demonstrated that HNO can directly ligate deoxyhemoglobin (59).

It is clear that $^3NO^-$ is a strong reductant ($NO/^3NO^-$ $E^0 = -0.8$ versus NHE). The protonated species, HNO, can also be a reasonable reductant under appropriate

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conditions. The H-NO bond strength is only 48–50 kcal/mol (see, for example, 19). This relatively low bond strength indicates that HNO will be a good hydrogen atom donor, and, therefore, can be a good reducing agent for radical species. The product of hydrogen atom abstraction from HNO is NO, which can also react rapidly with other radical species. Thus, it may be expected that HNO can be an efficient radical scavenger via H-atom donation with subsequent generation of another radical scavenger, NO.

To accurately predict which nitrogen oxide species are relevant following biological HNO exposure and which biological targets are affected, it is imperative that the kinetics of the reactions of HNO are known. To this end, Miranda and coworkers (60) used competition kinetics to determine the relative rates of reaction of HNO with a variety of possible chemical/biological reactants and derive approximate rate constants. This study showed that relative reactivity toward HNO is oxymyoglobin ($1 \times 10^7\,M^{-1}\,s^{-1}$) > glutathione (GSH), horseradish peroxidase ($2 \times 10^6\,M^{-1}\,s^{-1}$) > 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 ($3 \times 10^3\,M^{-1}s^{-1}$). Considering the high concentrations of GSH in cells, these results indicate that reaction of HNO with GSH may be a primary fate for cytosolic HNO. However, HNO partitioned into membrane compartments may have a considerably longer lifetime.

NITROXYL DONORS

Dimerization of HNO (Reaction 3) precludes convenient and direct accessibility to HNO for chemical or biological studies. Therefore, most of the studies of HNO chemistry and biology utilize HNO-donor molecules. The best studied, most established and most utilized HNO-donor is sodium trioxodinitrate ($Na_2N_2O_3$), or Angeli's salt (1 and references therein) (Reaction 1). This inorganic salt is fairly stable in base but will spontaneously release HNO between pH 4–8 with a first-order rate constant of $4.6 \times 10^{-4}\,s^{-1}$ (61). Thermal degradation of Angeli's salt can never be used as a source of $^3NO^-$ because conditions for significant HNO deprotonation (strong base) inhibits the release of HNO. At low pH, Angeli's salt becomes an NO-donor, possibly owing to protonation of a relatively nonbasic site, resulting in a different mechanism of decomposition (62). Owing to its ability of release HNO at physiological pH with predictable kinetics, most of the novel biological effects of HNO have been discovered using $Na_2N_2O_3$. However, this compound is limited in that its half-life is only 2.1 min at 37°C (61), making prolonged HNO delivery difficult. Moreover, NO_2^- is released as a coproduct that exhibits its own array of chemistry/biology (see, for example, 63).

Another possible source of nitroxyl is via the decomposition of compounds with the N-hydroxysulfonamide functional group. The best known of these is N-hydroxybenzenesulfonamide (Piloty's acid), which, under basic conditions, decomposes to nitroxyl and benzenesulfinate (Reaction 19):

$$C_5H_6S(O)_2NHOH + HO^- \rightarrow C_5H_6\text{-}S(O)_2NHO^- \rightarrow C_5H_6\text{-}S(O)O^- + HNO \quad 19.$$

The basic conditions required for HNO release allow HNO deprotonation to occur, making these compounds possible sources of $^3NO^-$ (unlike Angeli's salt, which cannot be used as a ready source of $^3NO^-$). Like Angeli's salt, the spin state of HNO initially generated from Piloty's acid is singlet (64). Biological studies using N-hydroxysulfonamides can be troublesome because strongly basic conditions are required for HNO release. N-Hydroxysulfonamides are also readily oxidized by one electron to give the corresponding nitroxide intermediate, which releases NO, not HNO (65). For these reasons, Angeli's salt has been used more extensively for biological studies.

Derivatives of N-hydroxysulfonamides have also been developed as HNO-donors. For example, the Nagasawa lab has synthesized a series of N- and/or O-substituted N-hydroxysulfonamides, which could be activated in biological systems to release HNO (66–71). Similarly, N-hydroxybenzenecarboximidic acid derivatives have also been developed as HNO-donors (72).

HNO can also be generated via a retro-Diels-Alder reaction from acyl- or phosphinoyl-nitroso-diene cycloadducts (73–77). Water-soluble analogs of acyl-nitroso-anthracene adducts, which are amenable to biological studies, have been shown to release the acylnitroso moiety, followed by hydrolysis to give HNO (78).

NITROXYL TRAPS/DETECTION

Studies on the biological actions and biochemistry of HNO are severely hindered by the lack of specific and convenient traps and/or detectors for HNO. Many previous studies relied on the detection of N_2O, which is the ultimate product of HNO dimerization (Reaction 3), as an indication of the intermediacy or existence of HNO. This is, however, equivocal because mechanisms exist whereby N_2O can be generated without the intermediacy of "free" HNO (see, for example, 43). Moreover, HNO dimerization is a second-order process requiring high concentrations of HNO to get significant reaction in the thiol and metalloprotein-rich environment of a cell. Considering the existence of other more likely fates for HNO in biological systems (i.e., reaction with thiols), it is not likely that low levels of HNO in biological systems can be detected via N_2O.

Other traps/detectors for HNO exist. For example, one of the first described traps for nitroxyl is via reaction with tetracyanonickelate [$Ni(CN_4)^{2-}$)] to give the nickel-nitrosyl species (79) (Reaction 20):

$$Ni(CN)_4^{2-} + HNO/NO^- \rightarrow NiNO(CN)_3^{2-} + HCN/CN^- \quad 20.$$

However, this is a pH-dependent (only occurs at high pH) and inefficient process and unlikely to be useful in biological systems.

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Deoxymyoglobin efficiently reacts with HNO to form the HNO-Fe(II) complex (59) (Reaction 21). However, this complex decomposes in the presence of O_2 to give Fe(III) myoglobin, thus limiting its utility as an HNO detector in biological systems. Further, the aerobic reaction of NO and deoxymyoglobin will also produce Fe(III) myoglobin, complicating identification of the reacting nitrogen oxide:

$$\mathrm{MbFe^{2+}} + \mathrm{HNO} \rightarrow \mathrm{MbFe^{2+}\text{-}HNO} \qquad 21.$$

HNO can also be trapped and detected via reaction with nitrosobenzene (44). This reaction generates cupferron (N-nitroso-N-phenylhydroxylamine) that can chelate the cupric ion to form a colored complex (Reaction 22):

$$\text{Phenyl-NO} + \mathrm{HNO} \rightarrow \text{Phenyl-NH(OH)NO} \rightarrow \text{chelates } \mathrm{Cu^{2+}} \qquad 22.$$

Unfortunately, nitrosobenzene lacks sufficient water solubility for this assay to be useful in biological systems. Although the generation of water-soluble analogs of nitrosobenzenes may prove useful in the future for biological studies, the intrinsic activity of nitroso compounds must be carefully evaluated because, for example, nitroso compounds are known to bind to and inhibit hemeproteins (80) as well as being subject to redox processes.

There are other published reports of the trapping/detection of HNO using metal complexes and metalloproteins. For example, HNO can be directly trapped using synthetic ferric porphyrins (81) as well as ferric hemoglobin and myoglobin (see, for example, 82) giving the ferrous nitrosyl complex (Reaction 23):

$$\text{Porphyrin-}\mathrm{Fe^{3+}} + \mathrm{HNO} \rightarrow \text{Porphyrin-}\mathrm{Fe^{2+}}\text{-NO} + \mathrm{H^+} \qquad 23.$$

Although this represents an efficient trap for HNO, the products of these reactions can also be generated by reaction with NO via a more involved series of reactions (see, for example, 83) (Reactions 24 and 25):

$$\text{Porphyrin-}\mathrm{Fe^{3+}} + \mathrm{NO} + \mathrm{H_2O} \rightarrow \text{Porphyrin-}\mathrm{Fe^{2+}} + \mathrm{NO_2^-} + 2\mathrm{H^+} \qquad 24.$$

$$\text{Porphyrin-}\mathrm{Fe^{2+}} + \mathrm{NO} \rightarrow \text{Porphyrin-}\mathrm{Fe^{2+}}\text{-NO} \qquad 25.$$

Thus, detection of a ferrous nitrosyl complex from a ferric detector species is not an explicit indication of the presence of HNO.

BIOLOGICAL GENERATION OF NITROXYL

Thus far, there has been no unequivocal evidence for the endogenous generation of HNO in mammalian systems. This may be due, however, to inefficient or nonspecific detection systems for this elusive species. Regardless, chemical and biochemical processes have been characterized that allow for the possibility, if not probability, of endogenous HNO formation. For example, the S-nitrosothiols can

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react with other thiols to generate HNO according to Reaction 26 (43, 84):

$$\text{RS-NO} + \text{R}'\text{SH} \rightarrow \text{RSSR}' + \text{HNO} \qquad 26.$$

Another source of endogenous nitroxyl is via the oxidative degradation of the NO biosynthesis intermediate, N-hydroxy-L-arginine (NOHA), which can be released at high levels by some cells both in vitro (85) and in vivo (86). NOHA is easily oxidized to give nitroxyl (see, for example, 87–90). Nitroxyl may also be generated from L-arginine and/or NOHA by the action of the nitric oxide biosynthesis enzymes (NOS) (91, 92), especially when it is deplete of one of its prosthetic groups, tetrahydrobiopterin (93, 94). This work provides the possibility that NOS is capable of generating HNO depending on the experimental/cellular conditions. Nitroxyl generation has also been reported to occur via the interaction of NO with elements of the electron transport system in mitochondria (95, 96) from reaction with ubiquinol (97), cytochrome c (98), manganese superoxide dismutase (99), and xanthine oxidase (100). Thus, biochemical events have been characterized that can result in endogenous HNO generation. Although purely speculative at this time, it is also possible that an HNO-synthase enzyme exists and remains to be discovered if and when a specific and sensitive HNO-detection system is developed.

NITROXYL PHARMACOLOGY/TOXICOLOGY

It remains uncertain whether HNO is endogenously generated in mammalian cells. Therefore, the question of whether HNO is an endogenous signaling/effector species or simply a metabolite of NO remains open. However, numerous studies indicate that exogenous HNO administration results in interesting, novel, and potentially important pharmacology and toxicology. Some of the earliest studies of the biological activity of HNO reported that nitroxyl can be a potent vasorelaxant (see, for example, 101). Because NO is known to elicit vasodilation via activation of the enzyme soluble guanylate cyclase (sGC) (see, for example, 102), it is possible that HNO was being converted to NO in these experiments. This seems especially likely because HNO itself has been reported to be incapable of activating sGC (103). It should be noted that this study was performed using an in vitro preparation of the enzyme in the presence of the thiol dithiothreitol. As discussed above, thiols can react rapidly with HNO precluding interaction with the enzyme. Thus, possible HNO-mediated sGC activation needs reinvestigation.

The ability of HNO to react with thiols predicts that it will be capable of disrupting thiol proteins. The Nagasawa group was one of the first to demonstrate this as they observed inhibition of the enzymes aldehyde dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase when exposed to HNO donors (67, 104, 105). The finding that aldehyde dehydrogenase was inhibited by HNO was the result of the elucidation of the mechanism of action of the alcohol deterrent drug cyanamide by the Nagasawa lab. They found that cyanamide could be hydroxylated by the enzyme catalase, which resulted in the formation of an unstable

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N-hydroxycyanamide intermediate. Decomposition of the N-hydroxycyanamide resulted in the release of HNO along with hydrogen cyanide (67, 105). Thus, in vivo, cyanamide can serve as a prodrug for HNO, and indeed, the utility of cyanamide as an antialcohol drug is due to its ability to release HNO after bioactivation. More recently, HNO was found to disrupt the activity of a copper-sensing yeast transcription factor, presumably via disruption of metal binding thiolate moieties (106). It should be noted that the ability of HNO to disrupt the actions of intracellular thiol proteins need not occur via direct interaction with the protein thiols. HNO has been shown dramatically lower cellular thiol levels (i.e., GSH) (46), an effect that will alter the redox status of the cell and, subsequently, may alter the activity of redox-sensitive thiol proteins. Poly(ADP-ribose) polymerase (PARP), a protein that contains two zinc-finger motifs and that is involved in initiating DNA repair, is also inhibited by HNO (107), as is mitochondrial respiration, presumably via reaction with critical thiol residues present in complex I and II (108).

The toxicity of HNO has been examined in a variety of systems. Using in vitro clonogenic assays, HNO was found to be toxic to fibroblasts via mechanisms not involving conversion to NO (46). In this study, HNO in the presence of O_2 resulted in dramatic decreases in GSH levels and DNA strand breakage. Although it is possible that HNO (or NO^-) can react with O_2 to generate the oxidant $ONOO^-$, as mentioned previously, it is reported that the reaction of HNO with O_2 generates an oxidant that is not $ONOO^-$ and possesses a slightly different oxidation profile (53, 54). This topic remains controversial. However, it is clear that the reaction of HNO with O_2 is capable of generating an oxidizing species that has the potential to react with and alter biological macromolecules. In contrast to $ONOO^-$, the oxidant generated from HNO and O_2 is capable of readily entering cells and reacting with intracellular species (109). These reports suggest that there are fundamental differences in the reaction pattern between HNO/O_2 and NO/O_2^-. The Ohshima lab has also described the ability of HNO to cause DNA damage (39, 40).

Nitroxyl greatly exacerbates ischemia reperfusion injury when administered during reperfusion, whereas NO has the opposite effect (110). Interestingly, HNO given prior to ischemia offers protection against subsequent reperfusion damage (111). The exacerbation of ischemia reperfusion injury was shown to be due to increased neutrophil infiltration (110). In a recent and related study, Takahira and coworkers (112) proposed that neutrophil infiltration in ischemia/reperfusion injury may actually be due to endogenous HNO generation and that the protective effects of dexamethazone may be the result of an inhibition of HNO generation.

Nitroxyl has been reported to attenuate the activity of the NMDA receptor by modifying a critical thiol residue, leading to a decrease in Ca^{2+} influx (113). This process was proposed to provide protection from neuroexcitotoxicity. In another study of the effect of HNO on the NMDA receptor, it was found that HNO blocked glycine-independent desensitization of the receptor (114). This observation is in contrast to the findings of Kim et al. (113), which may be partially explained by differences in experimental design because the levels of O_2 may be an important

factor in the effect of HNO. That is, under hypoxic conditions, HNO appears able to decrease steady-state ion currents, whereas under normoxic conditions blockade of glycine-independent desensitization occurs, leading to ion current enhancement (114). Thus, the ultimate effect of HNO on the NMDA receptor can be a function of cellular/tissue O_2 levels.

One of the most recent and provocative pharmacological actions of nitroxyl appears to be as a unique cardiovascular agent. Paolocci and coworkers (115) have reported that HNO increases left ventricular contractility while lowering cardiac preload and diastolic pressure without an increase in arterial resistance. This novel activity is likely responsible for much of the recent attention given to HNO (see below). The actions of HNO on the vascular system have been found to be mediated by calcitonin gene-related peptide (CGRP). Significantly, the effects of HNO were unaffected by β-receptor blockade and additive to those of the β_1-selective agonist dobutamine, indicating that the effects of HNO are independent of β-adrenergic signaling (60, 116). Also, HNO administration to animals does not result in an increase in cGMP, indicating that the vascular effects were not due to enhancement of levels of this second messenger. CGRP is a broadly distributed neuropeptide found in many cardiovascular tissues and is the most potent vasorelaxant currently known, with established positive inotropic effects on the human heart (see, for example, 117, 119). The actions of CGRP occur through activation of the calcitonin-receptor-like receptor (CRLP), which leads to activation of adenylate cyclase and elevation of intracellular cAMP (see, for example, 118, 119). Increases in cAMP results in PKA activation followed by phosphorylation of L-type Ca^{2+} channels and, eventually, vasodilation. Thus, the actions of HNO, at least with regard to the cardiovascular system, appear to occur primarily through a cAMP-mediated pathway. This is in contrast to NO, whose actions in the cardiovascular system are mediated by cGMP. This fundamental difference in signaling indicates that HNO is not merely converted to NO and that the two species have "orthogonal" signaling pathways (60).

The ability of nitroxyl to elicit CGRP-mediated responses in vivo makes it a candidate for the treatment of heart failure because it will increase heart contractility while decreasing vascular resistance. As pointed out by Feelisch (119), with the current lack of selective CGRP-mimetics and the increasing interest in inodilators, the potential for HNO-donors to be developed as drugs to be used in heart failure is significant. Of course, it needs to be realized that these ideas are in the early stages of development; much more work needs to be done before the therapeutic utility of HNO and HNO-donor drugs can be properly evaluated.

SUMMARY

Recent reevaluation of some of the fundamental chemical properties and reactivity of HNO provides a basis to begin to understand the chemistry responsible for some of its novel and potentially important biology. However, a clear

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understanding of the chemistry and specific biological targets associated with the physiological/pharmacological actions of HNO remains to be established and is an area of extreme interest/need. It is clear that nitroxyl possesses biological properties unique from those of other nitrogen oxides and that may be of significant physiological/pharmacological importance. The idea that the actions of HNO are, in part, mediated through cAMP, whereas NO regulates through cGMP is intriguing and represents an interesting physiological paradigm whereby redox nitrogen oxide congeners have orthogonal signaling properties. Whether this redox nitrogen oxide system is physiologically important is a question that remains and is contingent upon, among other things, determining whether HNO is generated endogenously, and, if so, under what conditions.

The utility of HNO as a possible therapeutic agent, for example, in the treatment of heart failure or as a preconditioning agent to prevent ischemia-reperfusion injury needs to be reconciled with its possible toxicity. Of course, this is not an uncommon situation, as the same issues are important for the development of NO as a therapeutic agent. However, it is worth noting that although HNO donors at millimolar levels have significant toxicity (46), animal studies have shown that long-term administration of the HNO donor Angeli's salt is very well tolerated, with an LD_{50} well above 130 mg/kg with no observable carcinogenesis (120). This concentration is greater than 10,000 times that which has been shown to demonstrate beneficial cardiovascular effects. Regardless, HNO can now take its place among other nitrogen oxides and oxygen-derived species as an important signaling/effector species possessing novel and possibly useful pharmacology as well as toxicological properties. Considering that many of the major discoveries of the physiological chemistry and biology of nitroxyl are relatively recent, it may be expected that many more interesting and important discoveries await.

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Annual Review of Pharmacology and Toxicology
Volume 45, 2005

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