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Review

The chemistry of nitroxyl (HNO) and implications in biology

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Abbreviations: BH_4, tetrahydrobiopterin; cGMP, guanosine cyclic 3′, 5′-monophosphate; CGRP, calcitonin gene related product; cyt *c*, cytochrome *c*; deoxyMb, deoxymyoglobin; DETC, diethyldithiocarbonate; DTT, dithiothreitol; EDRF, endothelium-derived relaxing factor; EPR, electron paramagnetic spectroscopy; FAD, flavin-adenine dinucleotide; ferricyt *c*, ferricytochrome *c*; ferrocyt *c*, ferrocytochrome *c*; FMN, flavin mononucleotide; GSH, reduced glutathione; GSNO, *S*-nitrosoglutathione; Hb, hemoglobin; HbNO, nitrosohemoglobin; HNO, nitroxyl; HRP, horseradish peroxidase; LD_{50}, lethal dose 50%; Mb, myoglobin; MbNO, nitrosyl myoglobin; MbO_2, oxymyoglobin; metHb, methemoglobin; metMb, metmyoglobin; NAD, nicotinamide adenine dinucleotide; NADH, reduced NAD; NADPH, reduced nicotinamide adenine dinucleotide phosphate; NHE, normal hydrogen electrode; NMDA, *N*-methyl-D-aspartate; NO, nitric oxide; NO^-, nitroxyl anion; NO_2^-, nitrite; NO_3^-, nitrate; NOHA, N^G-hydroxy-L-arginine; NOS, NO synthase; PARP, poly ATP-ribosyl polymerase; RNOS, reactive nitrogen species; ROS, reactive oxygen species; sGC, soluble guanylyl cyclase; SOD, superoxide dismutase; TMPyP, meso-tetrakis(*N*-methylpyridinium-4-yl)porphyrinato

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Abstract

Over the past century, HNO research has evolved from fundamental physical examinations to elucidation of interactions in atmospheric, industrial and bacterial processes. Most recently, the HNO literature has been primarily concerned with the pharmacological effects and potential physiological functions of HNO in mammalian systems. The chemistry of HNO is inordinately complicated for a triatomic molecule. Further, the rapid self-consumption of HNO through dehydrative dimerization impedes detection and necessitates in situ production. This review provides a detailed discussion of the most common donors of HNO and of the current understanding of the aqueous chemistry of HNO and the synthesis, consumption and reactivity of HNO in a cellular environment, as ascertained with these donors. Additionally, the consequences of the molecular interactions of HNO on physiology are described, and a comparison is made to NO in terms of cellular signaling and pharmacological potential.

Keywords: Nitroxyl; Nitric oxide; Chemical biology; Donor compounds; Detection; Angeli's salt

1. Introduction

Postulation of the formation of HNO (variously called nitroxyl, nitrosyl hydride, hydrogen oxonitrate (IUPAC), nitroso hydrogen, monomeric hyponitrous acid) as an intermediate in a variety of thermal and photochemical reactions dates from the early 1900s [1,2]. As a triatomic molecule, HNO has been the subject of extensive experimental and theoretical analysis of structure, spectroscopy and chemical dynamics ([3–6] and references therein). This fundamental research evolved into investigation of the intermediacy of HNO in combustion of nitrogen-containing fuels, in atmospheric and interstellar chemistry and in bacterial denitrification. The recent HNO literature has been primarily concerned with the pharmacological effects and potential physiological functions of HNO in eukaryotes. This review focuses on the chemical mechanisms of HNO under physiological conditions and on the consequences of these reactions in mammalian biology and extends the information provided by past reviews [7–13].

The caveat must be imparted here that the production, detection and investigation of the chemistry of HNO is severely complicated by high reactivity, such that the gas phase and solution literature has been controversial at nearly every stage of experimental sophistication including recent biological investigations. This review presents the current understanding of the chemical biology of HNO, which will no doubt be modified and expanded considerably as the field matures.

A major impediment to the understanding of HNO chemistry is the rapid dehydrative dimerization of HNO, which produces nitrous oxide through the transient hyponitrous acid [14,15].

$$2\mathrm{HNO} \rightarrow [\mathrm{HONNOH}] \rightarrow \mathrm{N_2O} + \mathrm{H_2O} \quad (1)$$

This reaction has been studied both experimentally and theoretically since the 1960s, and numerous values for the rate constant have been offered (reviewed in [10,16]). The accepted value, determined by flash photolysis techniques at room temperature, is $8 \times 10^6\ \mathrm{M^{-1}\,s^{-1}}$ [17], recently revised from $2 \times 10^9\ \mathrm{M^{-1}\,s^{-1}}$ [18]. Although this reaction provides N_2O as an indirect marker of HNO formation, direct detection of HNO is inherently challenging, and the in situ use of reductive techniques or donor compounds is generally required in the study of HNO.

2. HNO donors

HNO has been suggested to be a probable intermediate of a variety of oxidation processes involving nitrogen-containing compounds [19]. This is well illustrated by the extensive number of proposed mechanisms for HNO formation, which encompasses both inorganic and organic reactions and over a century of literature (reviewed briefly in [9,20,21]). Inorganic pathways, for example the reaction of hydrogen with nitric oxide (NO) [22,23] or aerobic photolysis of ammonia [24], have significant atmospheric importance. Processes leading to HNO elimination from organic precursors include acid-catalyzed solvolysis of *aci*-nitroalkanes (Nef reaction [25,26]), nitrosative cleavage of tertiary amines [27], retro Diels–Alder reactions [28–30] and decomposition of organophosphorous compounds [31–33].

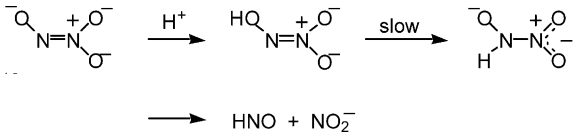
Synthesis of HNO for experimental use has most commonly been achieved by either photochemical or thermal decomposition of organic compounds, such as nitromethane [3] or *N*-hydroxybenzensulfonamide (Piloty's acid, benzensulfohydroxamic acid) [34], or the inorganic salt $Na_2N_2O_3$ (Angeli's salt, sodium salt of trioxodinitrate, α-oxyhyponitrite, hyponitrate, *N*-nitrohydroxylamate or trioxodinitrate(N–N) (2−) (IUPAC)) [35]. Of the available HNO donors, Angeli's salt has been studied most extensively in terms of structure, thermodynamics and decomposition mechanism. Further, the majority of the available data on the aqueous solution chemistry and pharmacological effects of HNO have been obtained with Angeli's salt. The primary organic donors of HNO are Piloty's acid ($C_6H_5SO_2NHOH$) and more recently synthesized sulfohydroxamic acid derivatives, such as hydroxylamine-*N*-sulfonic acid ($HOSO_2NHOH$) [36] and *N*-hydroxymethanesulfonamide (methanesulfohydroxamic acid (MSHA), methylsulfonylhydroxylamine; CH_3SO_2NHOH) [37]. Nagasawa and coworkers have synthesized several series of N- and/or O-substituted analogs of

Piloty's acid as prodrugs that release HNO following initiation of decomposition by enzymatic ester hydrolysis (see for instance [38–43]). The synthetic methodologies and applications of Angeli's salt and Piloty's acid, which are both commercially available, have been recently described [12,21].

The rate of the first order [44] thermal decay of Angeli's salt is pH-dependent [45,46] in a manner reflective of the pK_as of 2.5 and 9.7 for oxyhyponitrous acid, $H_2N_2O_3$ [47]; decomposition is essentially pH-independent from pH 4 to 8 ($6.8 \times 10^{-4}\,s^{-1}$ at 25 °C and 4–5 $\times 10^{-3}\,s^{-1}$ at 37 °C [48,49]) but is substantially accelerated below pH 3 and diminished above pH 9. The decomposition rate is readily monitored by loss of the maximum at 250 nm (ε = $8000\,M^{-1}\,cm^{-1}$ [45,48,49]) for the oxyhyponitrite dianion, $N_2O_3^{2-}$, or 237 nm (ε = $5500\,M^{-1}\,cm^{-1}$ [45] or $6100\,M^{-1}\,cm^{-1}$ [49]) for the monobasic species, $HN_2O_3^-$.

Crystallographic analysis [50] demonstrated that Angeli's dianion is planar with a N=N double bond, $[ON(1)=N(2)O_2]^{2-}$. Raman and ^{15}N NMR spectroscopy [51,52] established that the dianion is protonated at the nitroso nitrogen, N(1). The position of the second proton in the free acid has not been determined spectroscopically.

The generally accepted decomposition mechanism of Angeli's salt above pH 4 involves protonation of the dianion followed by tautomerization and heterolytic cleavage of the N–N bond to produce HNO and nitrite [2,45,48,53–55].



(2)

^{15}N labeling revealed that nitrite originates from N(2) [53] while HNO is derived exclusively from N(1) [45]. Angeli's salt decomposition is modestly inhibited by excess nitrite, in a pH-dependent manner, suggesting the reversibility of Eq. (2) [54,56].

A recent theoretical exploration of the decomposition mechanism of Angeli's salt [57] predicted that the most thermodynamically stable monoanionic isomer was indeed protonated at the oxygen of the nitroso group. This species was calculated to be stable toward heterolytic cleavage to HNO and nitrite by 16.4 kcal/mol, with a 53.8 kcal/mol barrier. The tautomer protonated at the nitrogen of the nitroso group, N(1), was computed to lie 5.3 kcal/mol higher in energy, but heterolytic cleavage was predicted to be favorable by 3.8 kcal/mol, with an overall barrier for N–N cleavage of 13.1 kcal/mol. These quantum mechanical calculations provide solid support for Eq. (2).

Decomposition of Angeli's salt in concentrated acid has been known since the early studies to produce NO as the only nitrogen-containing end product [44,58]. The alteration in product formation occurs at pH 3 [45] (or at higher pH in the presence of excess nitrite [48]), and although the low pH mechanism has been examined to various degrees by numerous researchers, it has yet to be fully elucidated.

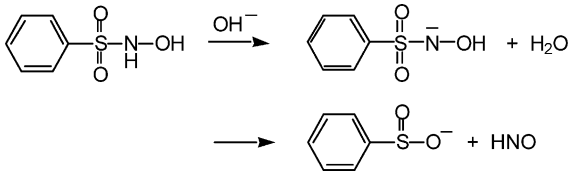
The second acceleration of the decomposition rate of Angeli's salt below pH 4 could be logically assumed to result from a further reduction in stability upon diprotonation (pK_{a1} for $H_2N_2O_3$ of 2.5 [47]). However, the free acid has been suggested to in fact have a substantially higher stability than the monoanion [48] due to conversion from N(1)- to O-protonation [52]. The stability toward decomposition of the most thermodynamically stable diprotonated species was corroborated recently by quantum mechanical calculation [57]. With this basis, the proposal [48] has been made that the mechanism of decomposition (Eq. (2)) is unaltered at lower pH and that NO is a secondary product arising via reaction of nitrous acid (HNO_2), which has a pK_a of 3.5, with H_2NO_3 through a free radical chain mechanism. The elevated rate and altered end product at low pH would thus be a function of protonation of nitrite to produce a new reactant. Theoretical analysis [57] has instead predicted that NO results from tautomerization of the diprotic species to a higher energy water:NO dimer structure followed by dissociation. The thermodynamics of the free radical chain mechanism were not examined. The reader is directed to the original references for more comprehensive discussions [45,48,54,56,57,59].

Production of NO from thermal decomposition of Angeli's salt is likely to only have physiological relevance in the stomach. This may be significant for oral delivery of HNO donors, although procedures exist to deliver drugs beyond the gastric region. Angeli's salt can also produce NO by direct oxidation, for example by hexaammineruthenium(III) or hexacyanoferrate(III) under alkaline conditions [60,61].

$$N_2O_3^{2-} + M^{3+} \rightarrow NO + NO_2^- + M^{2+} \quad (3)$$

Near neutral pH, however, Angeli's salt is generally considered to be an HNO donor of high utility due to the ease of handling and well-behaved decomposition kinetics.

Piloty's acid shares many similarities with Angeli's salt, including the pH-dependence of the first order decomposition rate due to varied availability of the monobasic reactant (pK_a = 9.29 [62]) and the reversibility of decomposition [20,62]. In keeping with the protonation status of the starting material, Piloty's acid decomposes through a base-catalyzed deprotonation mechanism followed by S–N bond heterolysis [1,20].



(4)

Analogously to Angeli's salt, the protonation change is suggested to occur at the nitrogen that is released as HNO [20,63,64], thus, alterations in protonation state lead in both compounds to destabilized anions.

The rate constant for decomposition of Piloty's acid at pH 13 ($4.2 \times 10^{-4}\,s^{-1}$ at 25 °C and $1.8 \times 10^{-3}\,s^{-1}$ at 35 °C [20,62]) is similar to that for Angeli's salt at pH 4–8, providing convenient methods to investigate the chemistry of HNO

at both neutral and highly alkaline pH. However, Piloty's acid is readily oxidized to the corresponding nitroxide, which then releases NO rather than HNO [65].

$$C_6H_5SO_2NHOH + [O] \rightarrow C_6H_5SO_2NHO^{\bullet} \rightarrow C_6H_5SO_2H + NO \quad (5)$$

This reaction is significant at neutral pH, where thermal decomposition is slow. Consequently, to function as an HNO donor, Piloty's acid must be utilized under anaerobic conditions in a reducing environment. In fact under physiological conditions, oxidation of Piloty's acid to NO (Eq. (5)) is suggested [65] to be the primary degradation mechanism with little contribution from the comparatively slow thermal decomposition to HNO (Eq. (4)).

This comparison of Angeli's salt and Piloty's acid demonstrates primary chemical similarities; however, the slow rate of decay at neutral pH and the susceptibility of Piloty's acid to oxidation reveals the superior suitability of Angeli's salt for use as a biological HNO donor.

3. The fundamental chemistry of HNO

Deprotonation of HNO produces the conjugate base NO^- (nitroxyl anion, oxonitrate (1−) (IUPAC), nitroside anion), which is isoelectronic to molecular oxygen [66]. Like O_2, NO^- has a triplet ground state and a singlet lowest excited state with a measured (see [67–70]) and calculated [71,72] energy gap of 16 and 16–21 kcal/mol, respectively. Ground state HNO, however, is a singlet [73,74] and has been determined by photoelectron spectroscopy and chemiluminescent detection to lie 18–19 kcal/mol below the triplet lowest excited state (see [75,76]). HNO, which is bent with a bond angle of 109° [3], has been the subject of numerous theoretical studies including calculations of global potential energy surfaces (for example, [5,77]).

The different spins significantly complicate the acid–base relationship of HNO and NO^-. The expectation has often been that deprotonation of ^{1}HNO would produce $^1NO^-$, which would then rapidly intersystem cross to $^3NO^-$, similarly to O_2 (4 μs lifetime for $^1O_2$ in water [78]). Protonation of $^3NO^-$ has been postulated to occur at the more electronegative oxygen atom to give NOH, which is ground state triplet [73,79], rather than the HNO isomer [79,80]. The only direct experimental evidence for the existence of NOH was obtained through detection of a low yield IR band in a solid argon matrix following microwave discharge into a H_2/NO/Ar mixture [81]. In contrast, HNO was observed spectroscopically in the gas phase and in an argon matrix 40 years earlier [3,4].

The energy gap between the ^{3}NOH and ^{1}HNO isomers is estimated at 20–23 kcal/mol [5,77,82] with isomerization barriers ranging from 17 to 70 kcal/mol depending on the direction of isomerization and the spin states involved (i.e., isomerization between varied combinations of excited and ground states) [77,83–85]. Direct conversion of ground state HNO to NOH (70 kcal/mol to ^{1}NOH) is thermally inaccessible under biological conditions. However, discrete deprotonation of HNO and NOH could lead to acid–base equilibria as convoluted as:

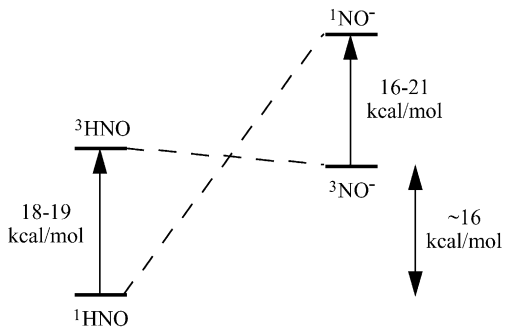
$$^1HNO \overset{K_{a1}}{\rightleftharpoons} {}^1NO^- \rightarrow {}^3NO^- \overset{K_{a2}}{\rightleftharpoons} {}^3NOH \rightarrow {}^3HNO \rightarrow {}^1HNO \quad (6)$$

($\Delta G = -16$ kcal/mol for $^1NO^- \rightarrow {}^3NO^-$; $\Delta G = -3$ kcal/mol and $\Delta G^{\ddagger} = 31$ kcal for ^{3}NOH → ^{3}HNO; and $\Delta G = -18$–19 kcal/mol for ^{3}HNO → ^{1}HNO). However, this possibility has generally been ignored in favor of the assumption that the ^{1}HNO/$^3NO^-$ relationship is that of a conventional conjugate pair or at worst involves $^1NO^-$.

Janaway and Brauman [86] observed gas phase, spin-forbidden proton transfer from a variety of acids to $^3NO^-$. In this system, proton transfer was suggested to occur through a concerted step, rather than involving discrete inner-system crossing, as a result of the very low energy barrier of singlet-triplet potential energy curve crossing near the potential wells for HA·$^3NO^-$ and A^-·^{1}HNO. This relationship is shown simplistically in Scheme 1. The depth of the singlet and triplet potential energy wells will depend upon the acid, but the favorability of curve crossing is apparent even without the addition of wells. From this analysis [86], Eq. (6) can be simplified to exclude $^1NO^-$ (the involvement of NOH was not considered).

$$^1HNO \overset{K_{a1}}{\rightleftharpoons} {}^3NO^- \overset{K_{a2}}{\rightleftharpoons} {}^3NOH \rightarrow {}^3HNO \rightarrow {}^1HNO \quad (7)$$

In the Janaway–Brauman study [86], the efficiency of proton transfer from the acid to NO^- was described as being a function of the relative rates of proton transfer and dissociation of the HA·$^3NO^-$ intermediate complex, rather than being encounter controlled, as is the usual case. The rate of proton transfer from the acid to NO^- was too slow to measure in both endothermic (e.g., methanol) and exothermic (e.g., small acids such as H_2S) systems, which would be expected to be similar to water. The rate constant for proton transfer to $^3NO^-$ was estimated to be 2×10^5 s^{-1} using larger acids such as $(CH_3)_3CSH$, which had higher, measurable transfer efficiencies due to longer lived intermediates ($k_{dissociation}$ <



Scheme 1.

$10^6\ s^{-1}$). A similar comparison of transfer of a proton from HNO to a series of bases has not been published.

The different ground states of HNO and NO^- were central in the discernment of the mechanism of HNO formation from Angeli's salt (and Piloty's acid [20]). Angeli originally proposed that decomposition of Angeli's salt produces NOH [2]; however, HNO production was later favored due to the decisive lack of experimental evidence for NOH. The determination that Angeli's salt decomposition (Eq. (2)) is reversible [54] verified that ^1HNO is the initial product due to the requirement for microscopic reversibility of ground state $^1NO_2^-$ with a species of comparable spin. Further consideration of spin states led to the suggestion of tautomerization to a N–N single bond structure prior to heterolytic cleavage (Eq. (2) [54,55]). Stabilization of this N–N tautomer by cytochrome P450 was suggested to explain the 100-fold acceleration of Angeli's salt decomposition in the presence of the metalloprotein [87].

Dissimilar rates of dehydrative dimerization (Eq. (1)) were observed subsequent to decomposition of Angeli's salt compared to hydrogen atom abstraction from hydroxylamine (NH_2OH) by NO [88]. The accelerated formation of N_2O from the latter reaction was proposed [88] to originate from production of either ^3NOH or ^3HNO rather than ^1HNO [89,90].

$$NO + NH_2O^- \rightarrow {}^3NOH/{}^3HNO + NHO^- \quad (8)$$

Dimerization of both HNO and NOH has been suggested to result in the same transient, HONNOH (Eq. (1)) [79], and therefore the disparate rates of N_2O formation may be a function of transient isomerization. Alternatively, this may be a result of augmented dimerization for $^3NO^-$ compared to HNO. Despite the unresolved mechanistic questions, these comparisons of different donor systems [89,90] demonstrated that the chemistry of HNO is not equivalent to that of NO^-.

The distinct chemistry of the two spin states was also observed during photolytic and thermal degradation of Angeli's salt. Indirect evidence for $^3NO^-$ production during steady-state photolysis of Angeli's salt in alkaline solution [55] was obtained via spectrophotometric observation of peroxynitrite [91].

$$^3NO^- + {}^3O_2 \rightarrow ONOO^- \quad (9)$$

This reaction is isoelectronic to the nearly diffusion controlled reaction of NO and superoxide ($4–7 \times 10^9\ M^{-1}\ s^{-1}$ [92,93]).

$$NO + O_2^- \rightarrow ONOO^- \quad (10)$$

Formation of $ONOO^-$ from Eq. (9) was quantitative in strongly alkaline solution [17] but was not apparent near neutral pH [55]. In contrast, thermal degradation of Angeli's salt in aerobic solution did not produce detectable $ONOO^-$, indicating that photolytic decomposition initiated thermally inaccessible pathways.

Shafirovich and Lymar [17] analyzed the kinetics involved in formation of the chromophore $ONOO^-$ (302 nm, ε = $1670\ M^{-1}\ cm^{-1}$ [94]) via Eq. (9) by utilizing UV laser flash photolysis to decompose Angeli's salt and thus eliminating the inherent constraint of thermal decomposition of the donor compound in which heterolysis of the parent is rate-limiting. Rather than initially producing ^1HNO from the monoanion as during thermal degradation (Eq. (2)), photolysis of Angeli's salt at high pH was proposed to induce heterolytic cleavage of the dianion to form the deprotonated singlet.

$$N_2O_3^{2-} + h\nu \rightarrow {}^1NO^- + {}^1NO_2^- \quad (11)$$

The extremely high basicity of $^1NO^-$ ($pK_a \sim 23$) was suggested to lead to immediate protonation.

$$^1NO^- + H_2O \rightarrow {}^1HNO + OH^- \quad (12)$$

In agreement with Janaway and Brauman [86], the reverse reaction, rather than resulting in a simple equilibrium, is proposed [17] to produce ground state NO^- as the rate-limiting step,

$$^1HNO + OH^- \rightarrow {}^3NO^- + H_2O \quad (13)$$

with subsequent, rapid autoxidation to $ONOO^-$ (Eq. (9)). The sequence of Eqs. (11–13) and (9) demonstrates the remarkable inhibition of proton transfer in Eq. (13) to such an extent as to become rate-limiting, due to the thermodynamic barrier for spin-forbidden reactions. Additionally, the dependence of the product on the spin- and protonation-state of the reactant is revealed.

Photolytic cleavage of Angeli's salt [17] in neutral solution proceeded by the same mechanism as thermal decomposition (Eq. (2)), in keeping with the monoanion as the principal solution species (pK_{a2} of 9.7 for $H_2N_2O_3$ [47]). However, the high-energy system utilized also produced excited state products, ^3HNO and $^3NO_2^-$ (the possibility of ^3NOH formation was not considered). ^3HNO is expected to rapidly deprotonate (pK_a of -2 [72]),

$$^3HNO + H_2O \rightarrow {}^3NO^- + H_3O^+ \quad (14)$$

such that $ONOO^-$ formation (Eq. (9)) is rate-limiting and independent of OH^- as well as O_2 under the experimental conditions utilized (air- and O_2-saturated solution, 0.3–1.3 mM O_2; $ONOO^-$ was not observed under argon) [17].

Kinetic analysis under varied alkalinity [17] provided rate constants for the base-dependent deprotonation of HNO (Eq. (13), $5 \times 10^4\ M^{-1}\ s^{-1}$), for $ONOO^-$ formation (Eq. (9), $2.7 \times 10^9\ M^{-1}\ s^{-1}$) and for HNO dimerization (Eq. (1), $8 \times 10^6\ M^{-1}\ s^{-1}$). These values are currently preferentially cited. From these measurements, the equilibrium constant and the reverse rate constant for Eq. (13) were estimated at $400\ M^{-1}$ and $1 \times 10^2\ s^{-1}$, respectively. This rate constant for proton transfer to NO^- ($1 \times 10^2\ s^{-1}$) is substantially lower than the gas-phase estimation by Janaway and Brauman ($2 \times 10^5\ s^{-1}$ [86]); however in either case, an appreciable lifetime for $^3NO^-$ is suggested ($\tau \sim$ ms), while $^1NO^-$ can be

expected to have no applicability to the thermal chemistry [95]. Ancillary evidence for a significant lifetime for HNO was obtained by time-resolved IR techniques following photolysis of 3,5-diphenyl-1,2,4-oxadiazole-4-oxide to benzoyl nitroside in acetonitrile and dichloromethane [95]. HNO was observed directly at 2650 cm^{-1} (N–H vibrational frequency), which is consistent with gas phase studies [96].

With the exclusion of $^1NO^-$ involvement, simplification of Eq. (7) to a conventional acid–base relationship between ^1HNO and $^3NO^-$ alone depends on the values of K_{a1} and K_{a2} as well as the forward and reverse rate constants involved. In 1970, Gratzel et al. [97] examined the chemistry of NO^- via the reduction of NO by the hydrated electron generated during pulse radiolysis and reported a pK_a for HNO of 4.7 (spin states not specified). Based on this value as well as estimates of the H–NO bond dissociation energy and the aqueous solvation energy of HNO and the assumption that the relevant acid–base equilibrium is between ^1HNO and $^1NO^-$, the standard potentials of the NO/$^3NO^-$ and NO/$^1NO^-$ couples were derived to be 0.39 and −0.35 V (1 M versus NHE listed throughout), respectively [98].

Despite numerous experimental references for the NO/$^3NO^-$ couple potential, ranging from 0.4 to −1 V [99–104], the derived value of 0.39 V was most commonly cited. Accordingly, HNO was often assumed to be a short-lived species at physiological and higher pHs, and Angeli's salt was considered to be effectively a $^3NO^-$ donor. Additionally, reduction of NO was expected to be highly favorable compared to reduction of O_2 (−0.16 V [105]), in which case the chemistry of NO would be predicted to be easily shunted to the chemistry of $ONOO^-$ by a variety of mild reducing agents.

$$NO \xrightarrow{e^-} {}^3NO^- \xrightarrow{O_2} ONOO^- \quad (15)$$

The facile reduction of NO compared to O_2 would also be expected to predicate evolution of an enzyme analogous to superoxide dismutase (SOD) to accelerate removal of NO^- through dimerization (Eq. (1)).

Data in opposition of these axioms have been accumulating for the past decade. For instance, oxidation of reduced methyl viologen by O_2 is 100-fold faster than by NO [8], indicating a considerably lower reduction potential for NO than 0.39 V. Further, the annual publication rate for studies showing distinct effects of NO and HNO donors in biological systems is increasing (see Section 5.1), suggesting that facile redox conversion does not occur.

These observations led to detailed examination of the chemistry of Angeli's salt under physiological conditions [106,107]. The central objective was to determine if similar modifications arose in neutral solution from decomposition of Angeli's salt and synthetic $ONOO^-$, as would be expected from the pK_a for HNO determined by pulse radiolysis (4.7 [97]) and the near-diffusion controlled reactions for Eq. (9) ($2.7 \times 10^9\ M^{-1}\ s^{-1}$ [17]) and Eq. (10) ($4–7 \times 10^9\ M^{-1}\ s^{-1}$ [92,93]). Instead, Angeli's salt and synthetic $ONOO^-$ exhibited dramatically distinct profiles of one-electron oxidation, nitration, hydroxylation, cytotoxicity, DNA cleavage, dependence upon CO_2, and reactivity with a variety of biomolecules, especially those containing thiols, although the two-electron oxidative properties were similar [106,107].

The inconsistencies with the accepted pK_a for HNO and the observed chemistry of HNO donors led to reevaluation of the reduction potential of NO and consequently of the pK_a for HNO. Bartberger et al. [108] utilized a variety of techniques including quantum mechanical calculations, cyclic voltammetric analysis and spectrophotometric observation of viologen reduction during Angeli's salt (Eq. (2)) and Piloty's acid (Eq. (4)) decomposition to corroborate the potential for the NO/$^3NO^-$ couple of −0.8 V reported previously by both Ehman and Sawyer [99] and Benderskii et al. [100]. The potential for the NO/$^1NO^-$ couple was estimated at −1.7 V, which again indicates the inaccessibility of $^1NO^-$ under biological conditions. From these potentials, the pK_as for HNO deprotonation to $^3NO^-$ and $^1NO^-$ were derived to exceed 11 and 23, respectively. Similar values were calculated by Shafirovich and Lymar [17].

Protonation of $^3NO^-$ to HNO will be thermodynamically favorable in aqueous solution, thus resulting in a positive shift in the NO/$^3NO^-$ couple potential from −0.8 to −0.5 V at physiological pH [17,108]. Still such a negative potential is in accord with the numerous examples of the dissimilarity of the chemistry of NO and $ONOO^-$ in chemical and biological systems as well as the detection of free and complexed NO in vivo. Therefore, NO should be regarded as quite stable to outer-sphere one-electron reduction to NO^- in mammalian systems due to a nearly biologically inaccessible reduction potential and to the higher concentration of O_2, which is substantially more favorable to reduce (−0.16 V [105]). It should be mentioned that the potentials in certain prokaryotes may be capable of reducing NO [109], however, in mammalian cells the thermodynamic barrier to reduction allows the chemistry of NO to be the basis for multiple biological functionalities.

Of course, the highly unfavored reduction potential for NO results in highly favored oxidation of NO^-, suggesting that NO^- should not exhibit distinct chemical or biochemical effects in aerobic solution.

$$NO^- + O_2 \rightarrow NO + O_2{}^- \rightarrow ONOO^- \quad (16)$$

The nearly diffusion controlled reaction of NO^- and O_2 (Eq. (9), $2.7 \times 10^9\ M^{-1}\ s^{-1}$ [17]), monitored by time resolved techniques, indicates that a discrete electron transfer step is not required for $ONOO^-$ formation; however the resulting chemical profile would not be altered. Indeed, aerobic oxidation of NH_2OH (Eq. (8)) results in production of $ONOO^-$ (Eq. (9)) [91]. In contrast, decomposition of Angeli's salt produces detectable $ONOO^-$ only during decomposition in highly alkaline solution [55].

This pH-dependence of $ONOO^-$ observation from Angeli's salt has been suggested to be a function of the rate of $ONOO^-$ decomposition, which proceeds through perox-

ynitrous acid, ONOOH [110]. However, since Angeli's salt initially releases HNO above pH 4 (Eq. (2)), deprotonation of HNO to NO^- must be considered in the formation of $ONOO^-$ by Eq. (9). With a $pK_a > 11$, the HNO to NO^- ratio will exceed 10^4:1 at neutral pH but will slightly favor NO^- at pH 12. Divergent abundance coupled with a markedly inhibited rate of $ONOO^-$ decomposition rate would be a reasonable explanation for a standard acid–base pair engaged in extremely rapid proton exchange such that an equilibrium is reached. However, the highly unusual slowness of proton transfer in either direction (Eq. (13), $k_f = 5 \times 10^4\ M^{-1}\ s^{-1}$ and $k_r = 1 \times 10^2\ s^{-1}$ [17]) dictates that any resulting chemistry is actually restricted by these rate constants rather than the pK_a.

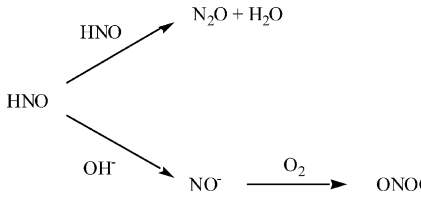
Remarkably, the calculated equilibrium constants [17,108], although they may be of higher accuracy than the experimental value due to the inability to achieve an equilibrium concentration of NO^- experimentally (see Section 4.3) [97], have little relevance to the solution chemistry of the HNO/NO^- system. Ultimately, the chemistry that results from HNO produced by Angeli's salt, Piloty's acid or any other source is dependent upon the relative rates of dimerization, deprotonation and autoxidation. Further, catalysis of HNO deprotonation by OH^- (Eq. (13), $5 \times 10^4\ M^{-1}\ s^{-1}$ [17]) dictates that the competition between consumption pathways will be pH-dependent.

In anaerobic neutral solution, HNO deprotonation (Eq. (13), $k_{obs} \sim 5 \times 10^{-3}\ s^{-1}$ [17]) will not be kinetically competitive with dimerization (Eq. (1), $8 \times 10^6\ M^{-1}\ s^{-1}$ [17]) until the HNO concentration declines to the low nanomolar range. The 10^5-fold acceleration of HNO deprotonation induced by elevating the pH from 7 to 12 ($k_{obs} \sim 5 \times 10^2\ s^{-1}$ [17]) brings the competitive limit into the high micromolar range. The slow back reaction of NO^- to HNO (Eq. (13)) is not pH-dependent ($1 \times 10^2\ s^{-1}$) and will only out-compete dimerization at <1 μM, assuming identical rate constants for NO^- and HNO dimerization, although NO^- dimerization has been suggested to be accelerated [88]. As a result, an equilibrium between NO^- and HNO is likely to be established only at submicromolar concentrations in alkaline solution (pH > 9) and in the absence of other consumption pathways. Further, the inability to observe $ONOO^-$ during decomposition of Angeli's salt near neutral pH is not a function of proton-mediated acceleration of $ONOO^-$ degradation beyond the detection limit but due to inhibition of base catalyzed proton transfer (Eq. (13)) and thus formation of $ONOO^-$ (Eq. (9)).

Due to the different spin states, the reaction of HNO with O_2 is slow ($\sim 3 \times 10^3\ M^{-1}\ s^{-1}$ [111]; $\ll 3 \times 10^5\ M^{-1}\ s^{-1}$ [17]; $\sim 8 \times 10^3\ M^{-1}\ s^{-1}$ [109]; gas phase, $4 \times 10^3\ M^{-1}\ s^{-1}$ [19]) such that HNO autoxidation will become kinetically relevant in the high nanomolar range. Decomposition of Angeli's salt at neutral pH consumes O_2 [112], indicating that the flux of HNO produced is appropriate for competition between dimerization and reaction with O_2. Competition studies determined that the O_2 pathway was quantitative below 2 μM Angeli's salt and competitive to 30 μM Angeli's salt [111].

The concentration of Angeli's salt cannot be directly equated to the HNO concentration since thermal decay provides a nonsteady-state profile. Methods to quantify this profile are not currently available. Further, the maximal HNO concentration will be altered by the available consumption pathways, for instance by addition of O_2 or other scavenging species. This is clearly demonstrated by the dependence of the rate of O_2 consumption upon the Angeli's salt concentration in closed systems [112,113]. Therefore, the importance of careful consideration of the relationship of the HNO limits from this analysis to experimental donor concentrations cannot be overemphasized.

The nearly diffusion-controlled reaction between NO^- and O_2 (Eq. (9), $3 \times 10^9\ M^{-1}\ s^{-1}$ [17]) will completely inhibit reprotonation and dimerization of NO^- such that $ONOO^-$ is formed quantitatively. The pH-dependent partitioning of HNO to deprotonation and dimerization will determine the yield of $ONOO^-$, with equal distribution near pH 12 [17].



(17)

From this analysis, assuming standard conditions of use, Angeli's salt (pH 3–9) is exclusively an HNO donor while Piloty's acid (pH > 9) can be considered to be ultimately a NO^- donor in anaerobic solution due to the pH-dependent decomposition rates of both donors. In the presence of air, a concentration-dependent contribution from the O_2 product can be expected from Angeli's salt whereas Piloty's acid will be a $ONOO^-$ donor, assuming no contribution from autoxidation of Piloty's acid itself (Eq. (5)). This pH-dependent paradigm should be extendable to any nitroxyl-donating system regardless of protonation status of the initial species.

Further, the equilibria in Eqs. (6) and (7) likely have little consequence to the solution chemistry of HNO and NO^-. Instead, for HNO donors decomposing either thermally or photochemically, the pertinent reactions,

$$^1HNO \rightarrow {}^3NO^- \quad \text{(high pH only)} \tag{18}$$

$$^3HNO \rightarrow {}^3NO^- \tag{19}$$

$$^1NO^- \rightarrow {}^1HNO \tag{20}$$

are generally unidirectional, either due to a slow back reaction relative to other consumption pathways or to extremely high acidity or basicity. The involvement of ^3NOH is unknown but is expected to be minimal at best.

The above assessment only considered simple decomposition conditions. The involvement of the O_2 product in neutral solution and NO^- or $ONOO^-$ in high alkalinity may be drastically affected in a more complex environment including

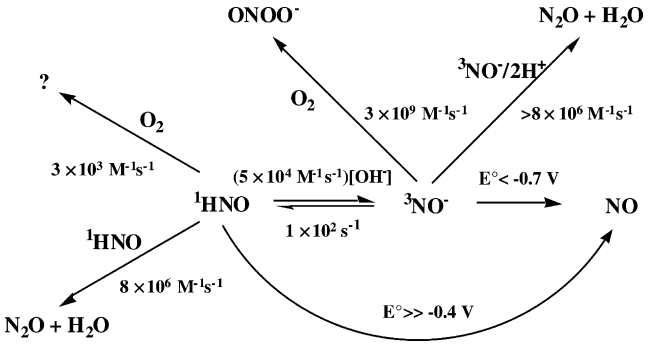
additional species that react directly with HNO. Under these conditions, the rate of the reaction of HNO with O_2 or OH^- may be comparatively slow to other consumption pathways, hence providing additional restrictions on formation of the HNO/O_2 or NO^-/O_2 product.

Piloty's acid is sensitive to air-oxidation [65,112], and the favorability of NO^- oxidation may contribute to NO formation at high pH. The insensitivity of NO formation to the atmospheric environment during Angeli's salt decomposition [65] is likely a consequence of the relatively high reduction potential of NO to HNO compared to NO^-. The thermodynamic barriers preventing fast proton transfer dictate that both HNO and NO^- are oxidized directly to NO by distinct pathways.

$$^3NO^- - e^- \rightarrow NO \quad (21)$$

$$^1HNO - e^- \rightarrow NO \quad (22)$$

Comparative chemical reduction analysis with Angeli's salt and Piloty's acid and a series of viologens placed the standard electrode potentials of the NO, H^+/HNO and NO/NO^- couples above −0.4 V and below −0.7 V, respectively [108]. These potentials suggest that reduction of NO will result in HNO not NO^-, but the reversibility of either Eqs. (21) or (22) is not definitively known. However, these potentials do suggest that under biological conditions the chemical reactivity of HNO will be electrophilic rather than redox in nature while NO^- will function as a strong reductant. In fact, the cited reevaluations of the pK_a of HNO and the reduction potential of NO [108,17] were in part due to the repeated observations of electrophilic rather than nucleophilic modifications consequent to HNO donor decay. In summary, the pertinent reactions of HNO and NO^- are shown in Scheme 2.



Scheme 2.

4. The reactivity of HNO

The literature on the chemistry of HNO includes multiple examples of reactivity toward nucleophiles, oxidants and metalloproteins. Bartberger et al. [72] used quantum mechanical calculations to predict that HNO will primarily react with soft nucleophiles, such as amines and thiols. It is these reactions as well as association with metalloproteins, and potentially consumption by O_2 or NO, that are the most pertinent biologically due to cellular abundance and kinetic parameters.

4.1. Metals

4.1.1. HNO complexes

Few examples of stable HNO complexes exist, particularly compared to the vast number of nitrosyl complexes, which include the formal oxidation states of NO, NO^+ and NO^-. The first HNO complex, $Os(PPh_3)_2(CO)(HNO)Cl_2$, was reported in 1970 upon exposure of HCl to $Os(PPh_3)_2(CO)(NO)Cl$ [114], and the X-ray crystal structure was published in 1979 [115]. Recent interest has resulted in isolation of additional examples of HNO complexes, and the structures of three similar complexes have been reported [(Ru(HNO)(2,6-bis(2-mercapto-3,5-di-tert-butylphenylthio) dimethylpyridine) [116], $ReCl(CO)_2(PR_3)_2(HNO)$ [117] and $IrHCl_2(PPh_3)_2(HNO)$ [118]].

Preparative routes generally involve protonation, hydride addition or reduction of a coordinated nitrosyl [116,117,119–127]. Farmer and coworkers also have described the first synthesis of an HNO complex directly as a result of exposure to a donor compound [128]. Lee and Richter-Addo have also observed the HNO adduct of a heme model complex (a ruthenium porphyrin [129]).

An early detection method for nitroxyl production involved nitrosylation of nickel cyanide [130–132] to form a deep violet product. The reaction only occurs under basic conditions (pH > 9), indicating direct displacement of CN^- by NO^-.

$$Ni(CN)_4^{2-} + NO^- \rightarrow Ni(CN)_3NO^{2-} + CN^- \quad (23)$$

This illustrates the expected higher reactivity of NO^- compared to HNO [88,90] and the difficulty in preparing HNO complexes.

4.1.2. Reductive nitrosylation

Oxidized metal nitrosyl complexes tend to be less stable than the corresponding complex with the reduced metal (see [133–136]). Formation of these higher stability complexes from the oxidized starting material can result from reductive nitrosylation of the metal by NO in the presence of a nucleophile. This reaction was initially demonstrated for synthetic iron porphyrin complexes [137–139] and was later extended to heme proteins [140–142].

$$Fe(III) + NO + ROH \rightarrow Fe(II) + RONO + H^+ \quad (24)$$

$$Fe(II) + NO \rightarrow Fe(II)NO \quad (25)$$

In contrast, reductive nitrosylation by HNO is a single-step reaction [59,143], originally observed upon exposure of metmyoglobin (metMb) or methemoglobin (metHb) to

Angeli's salt [144].

$$\mathrm{Fe(III) + HNO \rightarrow Fe(II)NO + H^+} \quad (26)$$

The mechanism is suggested to involve association of HNO rather than outer-sphere electron transfer, as indicated by the isosbestic behavior of the reaction in air [145].

Reductive nitrosylation of oxidized metals such as Fe(III) and Cu(II) is one of the most important reactions of HNO. For instance, the reaction of HNO with metMb is the most common method used to detect HNO. This utility does not extend to quantitation of HNO since complete conversion to MbNO requires an excess of Angeli's salt [18], presumably due to competition with dimerization (Eq. (1)). Further, the ferric species is slowly regenerated in aerobic solution. MetHb is not generally used for this purpose due to the complications of allosteric interactions [59] and competitive binding of HNO to the β-93 cysteine residues [143].

Reductive nitrosylation of a synthetic ferric porphyrin by Angeli's salt was estimated to proceed with a rate constant of $1 \times 10^7\ \mathrm{M^{-1}\,s^{-1}}$ [146]. However, this value was based on the HNO dimerization constant of $8 \times 10^9\ \mathrm{M^{-1}\,s^{-1}}$ [59], which is now regarded to be nearly 1000-fold lower [17]. Recalculation gives an HNO/ferric porphyrin rate constant of $3 \times 10^5\ \mathrm{M^{-1}\,s^{-1}}$, which is comparable to that for metMb ($8 \times 10^5\ \mathrm{M^{-1}\,s^{-1}}$ [111]). For comparison, the association of NO with iron is substantially hindered by the protein (e.g., $2 \times 10^7\ \mathrm{M^{-1}\,s^{-1}}$ for Mb [147] compared to $1 \times 10^9\ \mathrm{M^{-1}\,s^{-1}}$ for Fe(II) porphyrins [148]), suggesting that diffusion of NO to the active site is rate-limiting. Conversely, the rate-limiting step for reductive nitrosylation of metMb appears to be addition of HNO to the metal with little structural influence from the protein.

Other ferric proteins including cytochromes and peroxidases also undergo reductive nitrosylation by HNO [87,135,142,143,149–151], indicating lack of specificity toward nitrogen (histidine), sulfur (cysteine or methionine) and possibly oxygen (tyrosine) as the proximal protein ligand to iron, *trans* to the HNO binding site. However, the protein environment around this site can have significant impact on the kinetics of reductive nitrosylation. For example, the iron in cytochrome *c* (cyt *c*) is axially bound by both histidine 18 and methionine 80 [153]. Thus, HNO must displace an axial ligand, which results in a 20-fold decrease in the rate constant ($4 \times 10^4\ \mathrm{M^{-1}\,s^{-1}}$ [111]) compared to metMb ($8 \times 10^5\ \mathrm{M^{-1}\,s^{-1}}$ [111]), which has an open binding site. The proximity of histidine 18 and methionine 80 in cyt *c* also affects the stability of the resulting ferrous nitrosyl complex ([134,142,152]) through inhibition of the rate of geminate recombination such that Fe(II) and free NO are the products of reductive nitrosylation [143,154].

Free NO is also a product of the interaction of Cu,Zn SOD and HNO, although the reaction has been suggested to be reversible [155,156].

$$\mathrm{Cu(II) + HNO \rightleftharpoons Cu(I) + NO + H^+} \quad (27)$$

The formation of HNO from the association of reduced SOD with NO was indicated by scavenging by metMb (Eq. (26)) [155]. The unfavorability of electron transfer from reduced SOD to NO has been proposed to be surmounted by H^+ donation to NO from SOD [155], although this explanation seems unlikely. Recycling of Cu(I) SOD has also been suggested to occur through the intermediacy of HN_2O_2 [157].

$$\mathrm{HNO + NO \rightleftharpoons HN_2O_2}, \quad k = 5.8 \times 10^6\ \mathrm{M^{-1}\,s^{-1}}\ [17] \quad (28)$$

$$\mathrm{HN_2O_2 + Cu(I) + H^+ \rightleftharpoons H_2N_2O_2 + Cu(II)} \quad (29)$$

The product, $H_2N_2O_2$, is the dimer of HNO, which would rapidly dehydrate to N_2O (Eq. (1)). The forward reaction of Eq. (27) is applicable for aqueous copper as well [112].

Dissociation of free NO from cyt *c* and Cu,Zn SOD could be a result of either outer-sphere electron transfer or an addition–elimination reaction. Reduction of ferricyt *c* and the SOD mimic, Tempol, a nitroxide, by HNO is approximately two orders of magnitude slower [111,157] than by O_2^- ($4 \times 10^6\ \mathrm{M^{-1}\,s^{-1}}$ [158]). Assuming that these reactions proceed through outer-sphere electron transfer suggests that the potential for the $NO,H^+/HNO$ couple is considerably more positive than that of the O_2/O_2^- couple (−0.16 V [105]). This supports prior indications that HNO is at best a mild reducing agent [108], and therefore is unlikely to be converted to NO through simple outer-sphere electron transfer under biological conditions.

Rather, HNO is expected to first bind to the metal, resulting in reductive nitrosylation (Eq. (26)) with the stability of the consequent nitrosyl complex dictated by the protein and metal. For instance, inhibition of NO association by the axial ligands of cyt *c* will result in diffusion of NO away from the protein. For SOD [111,157], the d^{10} configuration for Cu(I)NO would result in rapid dissociation of NO. Consequently, conversion of HNO to NO is suggested to not be an incidental redox process but to require association, likely primarily with metal complexes. The expectation that reductive nitrosylation (Eq. (26)) will lead to enzyme inhibition [145] has been demonstrated with cytochrome P450 [159], horseradish peroxidase (HRP) [151] and with the mitochondrial respiration complexes I and II [160].

4.1.3. Fe(II) complexes

The relative stability of ferrous compared to ferric nitrosyl complexes is widely recognized, particularly for macrocylic ligands and has been reviewed frequently (for instance, for proteins see [133,134]). The ferric species exhibit greater stability only in rare cases, such as cyt *c* in which NO binding is affected by protein interactions. The prevalence for reductive nitrosylation (Eq. (26)) does not allow a similar comparison for HNO. However, the association between HNO and Fe(II) is generally considered to be weak. For instance, HNO from Angeli's salt does not bind detectably to ferrocyt *c* [143,144].

The reaction of Hb with Angeli's salt produces HbNO and metHb, with HbNO the sole product at high ratios of Angeli's

salt (>4:1 [143,144,161]).

$$2Fe(II) + HN_2O_3^- \rightarrow 2Fe(II)NO + OH^- \quad (30)$$

The two-fold higher rate constant ($1.4 \times 10^{-3}\,s^{-1}$ at pH 7, 25 °C [144]) compared to the decomposition rate of Angeli's salt is consistent with the stoichiometry. The initial interaction with Hb is somewhat controversial, and the mechanism remains to be fully elucidated. The overall reaction indicates that nitrite from Angeli's salt decomposition (Eq. (2)) is consumed. In fact, the product ratios depend on Angeli's salt, protein and nitrite, with little product observed at low Angeli's salt to heme ratios in nitrite-free buffer [144]. This suggested the involvement of HNO-catalyzed heme oxidation by nitrite [143], ultimately resulting in Eq. (30).

$$HN_2O_3^- \rightarrow HNO + NO_2^- \quad (31)$$

$$Fe(II) + HNO \rightleftharpoons Fe(II)HNO \quad (32)$$

$$Fe(II)HNO + NO_2^- + H^+ \rightarrow Fe(III) + NO + HNO + OH^- \quad (33)$$

$$Fe(II) + NO \rightarrow Fe(II)NO \quad (34)$$

$$Fe(III) + HNO \rightarrow Fe(II)NO + H^+ \quad (35)$$

Direct oxidation of Hb by nitrite is not competitive ([162]; 15-fold slower [161]), however the involvement of nitrite was demonstrated by unique spectral changes at pH 7 (HbNO and metHb) and 8 (proposed to be HbHNO) [143]. In nitrite-free buffer the production of HbNO by Eq. (30) was both slow and low yield. However, in blood where Hb will rapidly scavenge HNO and nitrite is readily available, this reaction may be of consequence [163].

Farmer and coworkers have suggested that electrochemical and chemical reduction of MbNO and the exposure of Mb to Angeli's salt produces thermally stable MbHNO [123,124,128]. Interestingly, the spectral changes induced by reaction of Angeli's salt with Hb at pH 8 [143] were similar to those for reduction of MbNO at pH 10 [16] while the products from Angeli's salt with Hb at pH 7 [143] or Mb at pH 8 [128] were spectrally similar. The latter spectra are proposed [128] to arise from mixtures of MbNO and MbHNO rather than metMb. Product identity, physiological relevance and the contribution of nitrite in the reaction of HNO with deoxyglobins remain to be determined.

The reaction of Angeli's salt with reduced *E. coli* cytochrome *d* is suggested to form a stable $Fe(II)NO^-$ species although quite slowly [164]. Certain proteins may therefore inhibit reductive nitrosylation. Additionally, specific proteins may have the reducing capacity to convert NO to NO^-, which likely is important for bacterial denitrification [165,166]. Interestingly, yeast ferrocyt *c* was shown to be oxidized by NO to nitrosylated ferricyt *c* [145]. The release of HNO (or possibly NO^-) was determined by trapping with metMb (Eq. (26)) and may be a function of the cysteine that is not present in the horse protein or of the presence of nitrite. In fact, mammalian cyt *c* is suggested to not have this functionality (unpublished results). Regardless of whether this reaction is in fact specific to prokaryotes, the rate is likely too slow to account for significant consumption of NO in mammalian cells.

Generally, metMb and deoxyMb are considered to be specific traps for HNO and NO, respectively [167]. However, products can be easily discerned spectrophotometrically during anaerobic exposure of either nitrogen oxide to metMb (Eq. (26) for HNO and Eq. (36) for NO).

$$Fe(III) + NO \rightleftharpoons Fe(III)NO \quad (36)$$

Under aerobic conditions, both products, Fe(II)NO from Eq. (26) and Fe(III)NO from Eq. (36), are converted back to the starting material, Fe(III). For Fe(III)NO this occurs simply due to dissociation and autoxidation of NO. For the more stable Fe(II)NO, the mechanism is as follows (for further discussion see [134]):

$$Fe(II)NO \rightleftharpoons Fe(II) + NO \quad (37)$$

$$Fe(II) + O_2 \rightleftharpoons Fe(II)O_2 \quad (38)$$

$$Fe(II)O_2 + NO \rightleftharpoons Fe(III) + NO_3^- \quad (39)$$

A similar scenario exists for deoxyMb as excess protein also provides an identical end point, MbNO, following exposure to HNO (Eq. (30)) or NO (Eq. (25)). Hence, these assays must be utilized with careful consideration to experimental conditions and timing of spectral observation. Despite these complications, metMb and deoxyMb are extremely useful for the detection of HNO and NO, respectively.

4.1.4. *Fe(II)O_2 complexes*

The different reactivity of HNO and NO toward ferric species (Eqs. (26) and (36)) is not observed with $Fe(II)O_2$ complexes. Rather, oxyhemes are oxidized to the ferric state by both NO (Eq. (39), $4 \times 10^7\,M^{-1}\,s^{-1}$ at 25 °C [168,169]) and HNO [143,144].

$$2Fe(II)O_2 + HN_2O_3^- \rightarrow 2Fe(III) + NO_3^- + NO_2^- \quad (40)$$

Eq. (40) is not balanced in oxygen, due in part to the lack of complete end product characterization. Doyle et al. [143] originally proposed the intermediacy of NO and the formation of peroxide.

$$Fe(II)O_2 + HNO \rightarrow Fe(III) + NO + HO_2^- \quad (41)$$

$$Fe(II)O_2 + NO \rightarrow Fe(III) + NO_3^- \quad (42)$$

This mechanism accounts for the observed end products, metMb and nitrate, but does not consider the fate of peroxides in the presence of metMb, which results in ferryl π cation radical formation (reviewed in [170,171]). The isosbestic behavior [143] does not support formation of stable species other than metMb. An alternative, hypothetical consideration is reduction of HNO to H_2NO rather than oxida-

tion to NO, with rapid dissociation of the resulting $Fe(III)O_2$ complex:

$$Fe(II)O_2 + HNO \rightarrow Fe(III) + H_2NO + O_2 \quad (43)$$

$$Fe(II)O_2 + H_2NO \rightarrow Fe(III) + NH_2OH + O_2 \quad (44)$$

Regardless of the mechanism, this section again demonstrates that an assay once considered to be specific for NO can instead indicate the presence of HNO. In fact metHb formation was observed after exposure of whole blood to either Angeli's salt or a variety of NO donors [172].

4.2. Sulfur

One of the most biologically significant and facile reactions of HNO is with thiols. Doyle et al. [143] originally determined that in 40% aqueous acetonitrile, thiophenol is converted to phenyl disulfide with a 98% yield at equimolar ratios with Angeli's salt.

$$C_6H_5SH + HN_2O_3^- \rightarrow C_6H_5SNHOH + NO_2^- \quad (45)$$

$$C_6H_5SH + C_6H_5SNHOH \rightarrow C_6H_5SSC_6H_5 + NH_2OH \quad (46)$$

This yield suggests that the association of HNO with thiols significantly exceeds the rate of HNO dimerization (Eq. (1)). In the same study, the β-93 cysteines of Hb were found to react reversibly with HNO, indicating applicability to aqueous solution.

The thiol reactivity in Eqs. (45) and (46) was in fact later expanded to encompass biologically relevant thiols, such as reduced glutathione (GSH) [173] and dithiothreitol (DTT) [174]. In addition the thiol end product was found to be condition-dependent since under low reduced thiol conditions the *N*-hydroxysulfenamide intermediate rearranged to the sulfinamide and then finally to the sulfenic acid [173,175].

$$RSH + HNO \rightarrow RSNHOH \rightarrow RS(O)NH_2 \rightarrow RSOOH \quad (47)$$

A greater applicability of these reactions to biology became apparent with the determinations that L-cysteine is useful to discriminate between HNO and NO in bioassays [176] and that HNO inhibits aldehyde dehydrogenase [177] by interacting with an active-site thiol [178]. This latter observation by Nagasawa and coworkers led to the development of a large series of prodrugs of HNO to elicit this response in vivo (see for instance [38–43]).

Additional examples of inhibition of enzymes containing critical-thiols have since been observed, for instance the zinc finger protein poly(ADP-ribose) polymerase (PARP) [179], which is involved in initiating DNA repair, and the yeast copper thiolate transcription factor Ace1 [180]. Kim et al. [181] also proposed that the activity of the *N*-methyl-D-aspartate (NMDA) channel is attenuated by association of HNO with critical thiols, resulting in RSNHOH or possibly disulfide formation. These examples illustrate the diverse environments that accommodate association of HNO with thiols.

4.3. Nitrogen

The propensity for HNO to react with other nucleophilic nitrogen oxides has been recognized for decades. For instance, HNO reacts with both nitrite (reverse of Eq. (2) [54,56]) and hydroxylamine [88,89].

$$NH_2OH + HNO \rightarrow N_2 + 2H_2O \quad (48)$$

The reactivity of HNO with other amines has not been investigated in detail experimentally, although a theoretical treatment by Bartberger et al. [72] predicts that the nucleophilic addition of amines to HNO will be highly thermodynamically favorable in solution.

In the early 1970s Gratzel et al. [97] and Seddon et al. [182,183] utilized pulse radiolysis of aqueous NO solutions to study the chemistry of NO^-. These experiments were necessarily conducted in the presence of a large excess of NO, which complicated determination of the pK_a of HNO but also allowed observation of polymerized species:

$$NO^- + NO \rightleftharpoons N_2O_2^-, \quad k_f = 2 \times 10^9\,M^{-1}\,s^{-1}, \quad k_r = 3\text{–}6 \times 10^4\,s^{-1} \quad (49)$$

$$HNO + NO \rightleftharpoons HN_2O_2, \quad k_f = 2 \times 10^9\,M^{-1}\,s^{-1}, \quad k_r = 8 \times 10^6\,s^{-1} \quad (50)$$

$$N_2O_2^- + NO \rightarrow N_3O_3^-, \quad k = 3 - 5 \times 10^6\,M^{-1}\,s^{-1} \quad (51)$$

$$N_3O_3^- \rightarrow N_2O + NO_2^-, \quad k = 87\text{–}330\,s^{-1} \quad (52)$$

Shafirovich and Lymar [17] recently reexamined the reactivity of HNO/NO^- with NO by flash photolysis of Angeli's salt in NO saturated solutions. The rate constant for Eq. (49) determined by this technique was equivalent to the pulse radiolysis value; however the rate constant for the reaction of HNO with NO (Eq. (50)) was more than 20-fold lower ($6 \times 10^6\,M^{-1}\,s^{-1}$), possibly due to different reactant spin states. Additional kinetic parameters for protonated species were also obtained by this method:

$$HN_2O_2 + NO \rightarrow HN_3O_3, \quad k = 8 \times 10^6\,M^{-1}\,s^{-1} \quad (53)$$

$$HN_3O_3 \rightarrow N_2O + HNO_2, \quad k = 2 \times 10^4\,M^{-1}\,s^{-1} \quad (54)$$

These reactions abate the chemistry of HNO/NO^- in solution and provide an additional path to N_2O formation other than HNO dimerization (Eq. (1)). The coexistence of HNO and

NO is certainly conceivable in a cell, although at low concentrations. Thus, Eqs. (49)–(54) may provide an additional consumption pathway for both nitrogen oxides.

4.4. Molecular oxygen

Aerobic decomposition of Angeli's salt consumes O_2 [106,112,113] without appreciable formation of NO [65,176,184–186]. In alkaline solution, this is clearly a result of the reaction of NO^- with O_2 to produce $ONOO^-$ (Eq. (9)). In neutral solution, however, autoxidation of HNO primarily produces nitrite rather than nitrate [187], suggesting that $ONOO^-$ is not a consequential product [188]. In the 60 years since this observation, in situ to in vivo studies have conclusively demonstrated that aerobic HNO is not synonymous to $ONOO^-$ [17,55,106,107,157,189–194]. Despite recent experimental and computational efforts by several groups, the structure of the product from the spin forbidden reaction between HNO and O_2 is currently unknown. The mechanism, which requires further investigation, may involve either direct, spin-forbidden association of O_2 and HNO or nucleophilic addition of HNO to a solvent or target molecule followed by reaction with O_2 with this HNO adduct.

Outer-sphere electron transfer between HNO and O_2 is not indicated since SOD concentrations far in excess of catalytic amounts were required to inhibit oxidation (i.e., by Eq. (27)) [157]. The lack of a discrete electron transfer step is consistent with the common theme in HNO reactivity of HNO of association rather than outer-sphere electron transfer mechanisms (e.g., reductive nitrosylation, Eq. (26)). Further, the reaction of NO^- with O_2 (Eq. (9)) has been suggested to be associative [17].

The product of the HNO/O_2 reaction is a strong two-electron oxidant and hydroxylating agent but does not appreciably participate in radical chemistry such as one-electron oxidation or nitration of phenols [106]. However, although anaerobic production of HNO results in reduction of ferricyt *c*, ferrocyt *c* is oxidized by HNO in aerobic solution [157]. This indicates that the HNO/O_2 product can induce one-electron oxidation in certain situations. The HNO/O_2 product is also cytotoxic at low millimolar concentrations and induces DNA strand breaks [107,189,195,196]. The involvement of HNO or the HNO/O_2 product can be distinguished by scavenging of the oxidant with urate [106], which does not react with HNO, or by comparing the effects of aerobic and anaerobic conditions.

4.5. Detection methods

Although specialized spectroscopic techniques can be utilized to detect HNO (for example, [3,4,95,197,198]), at present, all common detection methods are indirect (reviewed in [8]). These include spectrophotometric detection by trapping with transition metals (Eq. (26)), with highest applicability of metMb or synthetic analogs, detection of N_2O (Eq. (1)) by gas chromatography, particularly with use of ^{15}N labeled donor complexes, and trapping to produce EPR active species [199–201]. Although detection of N_2O has been frequently utilized to indicate HNO production in solution, this technique is less specific and convenient in bioassays, where N_2O can be formed without the intermediacy of HNO (see for example Eqs. (49)–(52) and [173]) and where collection of N_2O can be problematic. Further, HNO consumption in vivo will likely occur to a large extent by pathways other than second order dimerization (e.g., by GSH and metal or critical-thiol proteins). Although the high reactivity of HNO with thiols (Eqs. (45)–(47)) likely precludes detectable formation of N_2O, it provides an additional indication of HNO formation in an inhibitory role. Consequently, in all assays the susceptibility to signal quenching by thiols such as GSH should be determined.

Other indirect detection methods for HNO include the conversion of HNO to NO, by for example ferricyanide, coupled with chemiluminescence or electrochemical analysis or the oxidation by the HNO/O_2 product of dihydrorhodamine to the fluorescent dye rhodamine [106]. In the latter case, both GSH and urate can be utilized as scavengers of HNO and the HNO/O_2 product, respectively. In organic solvents, HNO reacts with nitrosobenzene to form the colorimetric indicator cupferron (*N*-nitrosphenylhydroxylamine) [202].

Although many of these assays utilize biomolecules or have been adapted for in vitro use (for example see [176,194]), the field is severely hampered by the lack of efficient, highly specific or direct methods of HNO detection. Consequently, there is at present no unequivocal evidence for the endogenous generation of HNO in mammalian systems despite the identification of both potential biosynthetic pathways and likely biotargets (discussed in Section 5).

4.6. Kinetics

The potential HNO scavenging pathways in a cellular environment are extensive and include reaction with oxidized, reduced and oxygenated metals, thiols and a large variety of one-electron oxidants, some of which have quite low potentials. Chemical and biochemical agents that scavenge HNO and the resulting nitrogen end products are listed in Table 1 (where applicable references indicate detection of products containing the HNO nitrogen, generally by chemiluminescence, NO-sensitive electrode or bioassay). The consumption reactions are varied, but many lead to the formal oxidation of HNO to NO.

Several analyses [109,111,157] have been performed to determine the relative rates of HNO toward common biomolecules in order to begin to ascertain the likely consumption pathways of HNO in a cellular environment. Estimated rate constants (Table 2) were calculated from the relative reactivates of the biomolecules toward HNO (derivations provided in [109,111,157]).

Table 1
HNO scavenging pathways

Reactant	Nitrogen product	Reference
SOD	NO	[112,155]
metHb/metMb	NO	[18,144,184]
ferricyt *c*	NO	[143,184,203]
MbO_2	NO/NO_3^-	[143,144]
Mb	MbNO	[143,144,161]
Mb	MbHNO	[128]
cytochrome P450	NO	[87,149,204]
HRP	NO	[205]
ceruloplasmin	NO	[203]
MnTMPyP (SOD mimic)	NO	[203]
$Cu(OAc)_2$, $CuSO_4$	NO	[112,186,203]
ferricyanide	NO	[65,189]
NO	N_2O	[97,183]
HNO	N_2O	[15]
NO_2^-	$HN_2O_3^-$	[54,56]
O_2	?	[106,112,113]
ascorbate	?	[106]
thiols	RSNHOH, NH_2OH, $RSONH_2$	[143,173–175]
S-nitrosothiols	NO	[173]
NH_2OH	N_2	[88,89]
FAD	NO	[112]
FMN	NO	[206]
NADPH	Possibly NH_2OH	[156,189,192]
quinines	NO	[113]
flavonoids	NO	[207]
methylene blue	NO	[184]
nitro blue tetrazolium	NO	[184]
Tempol	NO	[189]

These analyses, although far from comprehensive, indicate that while HNO reacts with a large number of purified biological species, cellular consumption will primarily involve reduction of ferrihemes or oxidation of thiols and oxyhemes due to the high rate constants and cellular concentrations. The higher rate constant for GSH compared to *N*-acetyl-L-cysteine may indicate a dissimilar affinity of HNO for the cytoplasmic antioxidant GSH compared to protein thiols. Further, oxidant formation by reaction with O_2 is expected to be minimal in the presence of GSH or metalloproteins.

Table 2
Derived rate constants for HNO consumption by biomolecules (37 °C)

Biomolecule	k ($M^{-1}\,s^{-1}$)	Reference
ferricyt *c*	4×10^4	[111]
	2×10^4[a]	[157] (23 °C)
metMb	$6–8 \times 10^5$	[18,111]
MbO_2	1×10^7	[111]
catalase	3×10^5	[111]
HRP	2×10^6	[111]
Mn SOD	7×10^5	[111]
Cu,Zn SOD	$0.7–1 \times 10^6$	[111]
	9×10^4[a]	[157] (23 °C)
Tempol	8×10^4	[111]
O_2	3×10^3	[111]
	1×10^4	[109] (23 °C)
GSH	2×10^6	[111]
N-acetyl-L-cysteine	5×10^5	[111]
NO	6×10^6	[17]
NO_2^-	1×10^5	[109] (23 °C)

[a] Recalculated value based on a rate constant for dimerization of $8 \times 10^6\,M^{-1}\,s^{-1}$ [17].

5. The biology of HNO

Until the mid 1980s, biological interest in nitrogen oxides was primarily centered on the elucidation of bacterial nitrogen fixation and denitrification mechanisms. In humans, attention to nitrogen oxides was generally limited to environmental concerns, for example exposure from air pollution and cigarette smoke or from leaching of fertilizers into ground water. However, vasodilation was determined in 1980 to be actively mediated by an unidentified species [208], which was labeled the endothelium-derived relaxing factor (EDRF) [209]. Quite surprisingly, numerous chemical and biological comparisons led to the conclusion that NO is the EDRF [210–217]. Vascular tone is controlled by diffusion of NO into the smooth muscle cells that surround blood vessels. Here, NO activates the heme protein soluble guanylyl cyclase (sGC) leading to accelerated conversion of GTP to cGMP, which initiates a cascade event cumulating in relaxation [218].

NO has since been implicated as a mediator of nearly every major mammalian physiological system (reviewed extensively in [219]). For instance, NO controls platelet function, cellular adhesion and infiltration of leukocytes into tissue [220–223], modulates neuronal transmission [224], is an essential signaling agent in the immune system [225], influences basic cellular functions such as mitochondrial respiration [226,227] and regulates nutrients and minerals such as iron [228].

In addition to its normal role in controlling numerous physiological functions, NO has also been implicated as a participant in pathophysiological conditions ranging from neurodegenerative and cardiovascular diseases to cancer (reviewed in [229–232]). The proposed mechanisms of tissue injury involving NO are varied and often controversial. However, significant evidence suggests that NO is a powerful antioxidant, protecting cells from injury caused by reactive oxygen species (ROS) (reviewed in [233]). Cellular injury may therefore be a result of impaired function or deficient protection due to disruption of NO biosynthesis.

Cellular damage is also directly mediated by higher nitrogen oxides such as $ONOO^-$ and dinitrogen trioxide (N_2O_3). These reactive nitrogen oxide species (RNOS) are formed by the reaction of NO with O_2^- or O_2 and modify biomolecules through oxidative, nitrative and nitrosative mechanisms [234,235]. In this light, elucidation of the chemical biology of NO begins with enzymatic formation of NO by NO synthase (NOS), progresses to the reactivity of NO itself, continues with the modifications by other RNOS formed from NO and concludes with the effects of nitrogen oxide-induced biomolecule alterations on physiology and pathophysiology.

The vast majority of the literature on this subject is concentrated on NO and its oxidized products. However, reduced species, particularly HNO, have been of interest

from the original studies attempting to determine the identity of the EDRF. The observation of certain dissimilarities (see [236]) between the effects exerted by NO or NO donors and the EDRF led to speculation that this species was more appropriately assigned as HNO. In a similar fashion, serious consideration has been given to the oxidation of N^G-hydroxy-L-arginine (NOHA), the intermediate formed during NOS catalysis, leading to HNO production with subsequent oxidation to NO [112,184]. These conclusions are minority opinions but have led to numerous in vitro, in vivo and ex vivo comparative studies of NO and HNO donors, most commonly in vasorelaxive assays.

Donor compounds of NO [9,237] have been commonly used in solution and in bioassays both due to the convenience of use compared to aqueous solutions of NO or enzymatic production and to the ability to release NO with controlled rates, in simulation of NO biosynthesis by NOS. In this regard, the availability of donor compounds has been invaluable to the elucidation of the biological properties of NO. Accordingly, the necessity for chemical production of HNO (Eq. (1)) is not necessarily an experimental impediment as the use of donors facilitates direct comparison of the effects of exposure to NO or HNO. However, it should be reiterated that study of the biological effects of HNO to date has required exposure to donor systems and that endogenous production of HNO has not been demonstrated.

5.1. Biological effects

5.1.1. Vasoactivity assays

Angeli's salt (Eq. (2)), Piloty's acid (Eqs. 4 and 5) and the clinical alcohol consumption deterrent cyanamide, which is bioactivated to HNO [177,238],

$$H_2NCN \xrightarrow{\text{catalase}/H_2O_2} [HONHCN] \rightarrow HNO + HCN \qquad (55)$$

all result in vasorelaxation when administered intraperitoneally or intraarterially to mice or rats [199] or in rodent arterial/aortic ring assays [38,112,155,184,199]. The vasoactivity, which was accompanied by increased cGMP production [184], was generally less effective than that elicited by NO donors but was both prolonged and substantially (up to 30-fold) enhanced by SOD [112,155] and moderated by L-cysteine [176]. Such results were proposed to indicate that HNO was converted to NO in vivo and that HNO was an intermediate form of EDRF [38,155,184,239]. Similar experiments have led to speculation that HNO is also the nitrergic transmitter in nonvascular smooth muscle [186,240,241].

The initiation of vasorelaxation by HNO donors instigated in vitro examination of the effects of these donors on sGC function. In fact, a role for HNO in sGC activation is thematic in these studies, which commonly either involved measurement of cGMP levels or determination of vasorelaxive potency in the presence of sGC inhibitors [38,112,176,184,186,190,239,241–243]. The question of direct activation of sGC was addressed by Dierks and Burstyn [236], who exposed partially purified sGC to donors of NO, NO^+ and HNO (Angeli's salt and Piloty's acid). The resulting observation of enhanced cGMP formation only upon introduction of NO has been the cornerstone for interest in the mechanisms of tissue-dependent oxidation of HNO to NO. There is a possibility, however, that HNO was scavenged by the 5 mM DTT present in the buffer. Conclusive evidence would have to be obtained in thiol-free buffer preferably with spectrophotometric analysis of HNO complex formation or reductive nitrosylation.

Since release of NO during decomposition of Angeli's salt, cyanamide or Nagasawa's HNO prodrugs was demonstrated to not be appreciable in situ [38,184], the vasoactivity induced by these HNO donors was suggested to be a result of oxidation of HNO to NO in tissue. Fukuto et al. [112] demonstrated that HNO could be easily converted to NO in biological systems by a variety of agents including SOD, metHb and flavins. The amount of SOD required to convert HNO to NO was later determined to be supracellular [203]. Interestingly, coinfusion of Angeli's salt and the EPR trap diethyldithiocarbonate (DETC) suggested that HNO was only minimally oxidized to NO in vivo (<5% [199]).

The presence of advantageous metals, particularly copper [112,203] (Eq. (27)), does lead to a small amount of NO production during Angeli's salt decomposition. Although NO formation in situ is completely abolished by EDTA [203], the vasorelaxant response to Angeli's salt is not attenuated [176]. Other metal chelators, such as diethylenetriaminepentaacetic acid (DTPA) or the EPR trap DETC [176], should thus be utilized instead of EDTA as metal complexes of EDTA remain redox active. The physiological concentration of free metals is extremely low, hence, in vivo conversion of HNO to NO should be negligible by this pathway. Currently, the mechanism leading to the vasoactivity of HNO donors remains uncertain.

5.1.2. Pharmacological effects

Comparisons of NO and HNO donors in nonvasoactive assays have nearly universally demonstrated that the physiological properties of these two nitrogen oxides are discrete (recently reviewed in [13,111,244]). Early in vitro studies included observations of redox specific toxicity toward a food spoilage organism, with HNO (from Angeli's salt or Piloty's acid) presumably interacting with an Fe–S cluster [245], and orders of magnitude enhanced cytotoxicity toward lung fibroblasts for Angeli's salt compared to NO donors [189]. The low millimolar toxicity of Angeli's salt, determined by clonogenic assay [189], was comparable to alkyhydroperoxides. In fact, Angeli's salt enhanced oxidative stress by peroxides while NO was protective under these conditions [246]. The cytotoxicity of Angeli's salt [189] was dependent upon an aerobic environment and was exacerbated by chemical depletion of cellular GSH. This study provided the first indication that HNO could affect cellular functions by altering the redox status of the cell. In this fashion, HNO can affect the activity of enzymes containing critical thiols by either direct

association or by scavenging of GSH. Conversely, GSH provides significant protection for these proteins against HNO toxicity.

The aerobic dependence of the cytotoxicity of Angeli's salt suggests a role for the unidentified product of the HNO/O_2 reaction. Alternatively, an interaction of HNO may become more favorable during cellular metabolism under aerobic conditions (e.g., inhibition of respiration or modification of calcium channels). Aerobic exposure to Angeli's salt damaged DNA both with purified plasmids and in vitro through induction of single and double strand breaks as well as base modifications (8-oxo-2′-deoxyguanosine formation) [107,189,195,196,207]. This may in part be to the Haber–Weiss reactivity of HNO,

$$HNO + M^{n+} \rightarrow NO + M^{(n-1)+} \quad (56)$$

and subsequent facilitation of oxidative damage by the Fenton reaction [196].

$$M^{(n-1)+} + H_2O_2 \rightarrow MO + H_2O \text{ or } M^{n+} + {}^{\bullet}OH + OH^- \quad (57)$$

Additionally, plasmid strand breakage is alleviated by urate (unpublished results), indicating participation of the HNO/O_2 product.

These early in vitro experiments suggested that the discrete toxicological properties of HNO and NO originate from distinct cellular chemistry. For HNO, cytotoxicity may be partially mediated by the nuclear nick sensing enzyme PARP, which when activated by induction of strand breaks can lead to cellular necrosis by depletion of NAD, and consequently of ATP [247]. Interestingly, PARP itself, which is a zinc finger protein, is inhibited by Angeli's salt [179]. Further, Angeli's salt stimulated neutrophil migration [190], which protects against infection but also induces inflammation. A concomitant increase in cGMP was detected, suggesting again that HNO was converted intracellularly to NO. Stimulated migration was maximal at 40 nM Angeli's salt and was independent of O_2 and inhibited by thiols. The observation of cGMP-independent effects as well indicated the possibility of interactions of HNO with critical metals or thiols, which may modify the redox environment at the endothelial surface to increase susceptibility to infiltration.

The enhancement of oxidative stress and neutrophil infiltration elicited by Angeli's salt in vitro [189,190] was also observed in vivo in rabbit hearts [185]. Tissue damage elicited by 45 min of interrupted blood flow (ischemia) and 3 h of reperfusion was exacerbated by infusion of Angeli's salt (1–3 μmol/kg for 1 min) just prior (5 min) to the onset of reperfusion. In contrast, NO proved to be protective against reperfusion injury in this system. Although it is tempting to attribute the enhanced injury by Angeli's salt to chemical insult, the tissue experienced 1000-fold less Angeli's salt than necrotic levels in vitro (∼2 mM [189]). In fact, Angeli's salt is well tolerated in animals under normal conditions with an LD_{50} of 140 mg/kg [248]. Instead of direct necrosis, Angeli's salt facilitated [185] inflammatory damage, mediated by neutrophil recruitment, and oxidative damage, which arises from induction of reduced intracellular conditions during ischemia and subsequent reduction of O_2 to O_2^- and H_2O_2 during reperfusion.

Pagliaro et al. [249] demonstrated in isolated rat heart preparations (Langendorff model) that administration of Angeli's salt prior to induced ischemia resulted in a substantial reduction of infarct area following reperfusion. This result was in direct contrast to the increased damage observed in rabbit heart with Angeli's salt infused just prior to reperfusion [185]. Thus, simply altering the timing of exposure to HNO provides a remarkable disparity in the elicited response. That HNO imparted a greater magnitude of myocardial protection than NO in the pre-ischemia study [249] suggests the existence of species-dependent, pro-oxidative and/or nitrosative stress-related triggers. Further, this study indicates that a compound that releases HNO under normoxic conditions but converts to a NO donor during ischemia may be pharmacologically beneficial in treatment of myocardial infarction or stroke.

Nitrogen oxides have also been determined to influence neuronal function, particularly through manipulation of NMDA receptor/channel response [113,181,250–252] and consequent inward flux of calcium (see [113] and references therein). The observed responses have revealed several important aspects to the relationship between NO and HNO. Under aerobic conditions, long term exposure to millimolar NO donors attenuated glutamate-stimulated calcium influx through the NMDA ion pore [251], possibly through *S*-nitrosation of a thiol residue. The high concentration of NO required suggests that this mechanism may be mediated by stimulation of inducible NOS but not by neuronal or endothelial NOS, which produce substantially lower amounts of NO.

In contrast to the high NO effect, short term, pulsed aerobic infusion of NO donors potentiated calcium influx with an enhanced effect exhibited under hypoxia [113]. Substitution of Angeli's salt in the perfusate produced similar augmentation aerobically, but channel response was attenuated by HNO under hypoxia. This suggests a potential pharmacological treatment for reduction of neuronal damage, for instance during stroke. Potassium channels in nitrergic nerves [241] and resistive arteries [243] have also been suggested to be divergently regulated by HNO (Angeli's salt) and NO.

Other distinct biological effects of HNO include enhanced blood-brain barrier disruption [253] and neutrophil infiltration during renal ischemia/reperfusion, perhaps by enhancing expression of adhesion molecules [254], compared to NO donors. Further, Piloty's acid but not NO donors induced the release from rat aortic strips of the calcitonin gene related product (CGRP) [255], which is a small non-cGMP vasoactive neuropeptide located in peptidergic neurons [256].

The ischemia-reperfusion studies in rabbit [185] and rat [249] hearts led to intensive investigation of the cardiovascular properties of HNO from Angeli's salt (recently reviewed

in [13,244]). Paolocci et al. [257] determined that the vascular effects of Angeli's salt in conscious dogs were limited to the venous side of the circulatory system unlike NO donors, including nitroglycerin and amylnitrates, which are systemic hypotensive agents. Maintaining arterial vascular tone while specifically reducing venous tone allows blood to flow through organs without a pooling effect that is often deleterious in hypotensive crisis [258]. Additionally, Angeli's salt was determined to enhance contractility even in failing hearts [257,259].

Clinical treatment of congestive heart failure currently involves administration of a β-agonist, which targets the β-adrenergic channel of the cardiac myocyte to increase calcium influx and consequently stimulate muscle contraction and cardiac output (reviewed in [260]), and nitroglycerin, which is a vasodilator and thus reduces resistance to the heart [261]. Unfortunately, the effects of β-agonists are counteracted by NO donors [262]. However, not only does Angeli's salt preferentially reduce venous pressure, the increased cardiac output is additive with the β-agonist dobutamine [257,259]. The favorable vascular effects and increased contractility suggest that HNO donors may provide a novel class of vasodilators and heart failure treatments (commented upon recently in [263]).

Paolocci et al. [257] investigated the chemical origin for the differing cardiovascular effects observed for HNO and NO donors. As expected, infusion of NO resulted in elevated cGMP levels in plasma. Conversely, exposure to Angeli's salt released CGRP, as was observed previously following exposure of rat aortic strips to Piloty's acid [255]. Soluble GC is activated at very low concentrations of NO (<100 nM [264]), and the observation of different signaling agents from HNO and NO suggest that these redox siblings do not interconvert in blood. Thus, this in vivo study indicates that the observation of cGMP in vitro (Section 5.1.1) may be artifactual with HNO converted to NO by exogenous sources or due to release of normally sequestered species during tissue preparation.

Release of CGRP from peptidergic neurons leads to activation of adenylate cyclase and elevation of intracellular cAMP [256,265]. Interestingly, cAMP can induce vasodilation by a discrete cascade event to that of cGMP, although both cyclonucleotides induce phosphorylative activations. Consequently, observation of vasodilation by HNO donors may not be a specific indicator of oxidation of HNO to NO. Hence, the in vitro vasorelaxive studies in Section 5.1.1 may require additional analysis with the inclusion of assessment of CGRP levels. Beyond providing a potential explanation for the vasoactivity of HNO donors, CGRP offers the first in vivo marker of HNO. Further, elevation of CGRP by HNO and of cGMP by NO definitely demonstrates that these two nitrogen oxides initiate discrete signaling pathways in vivo.

5.2. Endogenous production of HNO

The cardiovascular properties [249,257,259] and regulation of the NMDA channel [113] by HNO has led to considerable interest in the pharmacological utility of compounds that release HNO (for instance, see [263]). These results coupled with earlier studies suggesting that HNO is an intermediate form of the EDRF [38,155,184,239] and is the nitrergic transmitter [240] have also led to speculation of a physiological role for HNO as an alternative signaling agent to NO. Certainly, the unique biological signatures of HNO and NO suggest the potential for control of physiological processes in a condition-dependent manner through specific regulation of proteins containing critical metals or thiols. The question thus arises as to the possible endogenous sources of HNO.

Numerous biomolecules can be utilized as HNO donors in situ. For example, the reaction of NH_2OH with NO produces HNO, although slowly ($5 \times 10^{-3}\,M^{-1}\,s^{-1}$ at 25 °C, pH 7.3 [88,89]).

$$NO + NH_2OH \rightarrow HNO + NHOH \quad (58)$$

$$NHOH + NO \rightarrow N_2O + H_2O \quad (59)$$

Similarly, conversion of NO to HNO is facilitated by *N*-hydroxyguanidine [266], reduced flavins [112] and ubiquinol [267]. Even aqueous Fe(II) has been suggested to reduce NO to HNO [268,269],

$$Fe(II) + NO + H^+ \rightarrow Fe(III) + HNO \quad (60)$$

although the concentration of free Fe(II) will be extremely low under normal physiological conditions.

The reaction of *S*-nitrosthiols with excess thiols results in release of HNO [261,270–272].

$$RSNO + RSH \rightarrow RSSR + HNO \quad (61)$$

The slow reaction rate ($8.3 \times 10^{-3}\,M^{-1}\,s^{-1}$ [272]) and dependence upon thiol proximity [271] would establish a competition between RSNO (Eq. (61)) and HNO (Eq. (47), $\sim 1 \times 10^5\,M^{-1}\,s^{-1}$) for the reduced thiol. As a result, in a sea of low molecular weight thiols such as GSH, any HNO produced by Eq. (61) is likely to be consumed near the point of origin. However, this reaction may be important in direct protein thiol/nitrosothiol interactions, for example in proteins containing vicinal thiols. This dependence on reaction conditions exemplifies the necessity for caution in extrapolation of in situ or in vitro results to in vivo situations.

A significantly more complicated reaction scheme for the reactivity of GSH with *S*-nitrosoglutathione (GSNO) has also been suggested [273] and does not include formation of free HNO. This discrepancy has yet to be resolved. Alternative reductants have also been suggested to generate HNO from RSNOs [206],

$$GSNO + NADH \rightarrow NAD^+ + GS^- + HNO \quad (62)$$

indicating a crucial dependence on cellular conditions and compartments.

Despite the ready occurrence of the above reactions in situ with prepared or purified biomolecules, in general the rate constants and low reactant concentrations dictate that

these reactions are likely to be at best of minor significance in vivo. Of potentially greater consequence is the production of HNO during turnover of NOS. The speculation that the initial nitrogen oxide produced by NOS is HNO rather than NO [184,274–276] resulted from the determination that L-arginine is oxidized to L-citrulline by NOS in a two-stage process involving the intermediacy of NOHA [277,278]. Chemical analysis has shown that NOHA, which can be uncoupled from NOS at high levels both in vitro [279] and in vivo [280], can be oxidized to release HNO by a variety of oxidants [274,281] including NO [266]. Further, low levels of N_2O and a transient ferrous nitrosyl center were observed during anaerobic oxidation of NOHA by inducible NOS [276]. Observation of these markers of HNO (Eqs. (1) and (26)) only under anaerobic conditions suggested that HNO is either competitively scavenged by O_2 or more likely is not released due to attack of the heme center, $Fe(III)NO^-/Fe(II)NO$, by O_2.

The biosynthesis of NO from NOS is well established. For instance, NO can be detected by chemiluminescence during turnover of purified inducible or neuronal NOS [275]. However, in this assay, the end product ratio of nitrite/nitrate to L-citrulline was equimolar, but the rate of L-citrulline formation exceeded that of NO production unless SOD was present. The recovery of NO standards proved to be SOD-independent, indicating that rather than being a sink for O_2^- produced by cofactor autoxidation or by NOS itself, the function of SOD was to convert HNO to NO (Eq. (27)) in a reactant rather than catalyst role. Schmidt et al. [282] suggested that this reaction was in fact essential for NO formation, particularly in the absence of the cofactor tetrahydrobiopterin (BH_4).

The role of BH_4 in NOS catalysis became apparent with the detection of elevated NO formation in complete compared to BH_4-free media [149,276,282,283]. These observations led to speculation that BH_4 is required for the enzyme to serve as an NO rather than an HNO synthase. This functionality was further defined by Adak et al. [284], who determined that both L-arginine and NOHA could be utilized as substrates of NOS in BH_4-free systems, but that the efficiency was highest for the neuronal isoform. These researchers did not detect NO by chemiluminescence unless BH_4 was present and observed that the oxidation state of the iron nitrosyl complex produced during NOS turnover was BH_4-dependent. These results led to the proposal that the function of BH_4 is to enable NOS to generate NO instead of HNO. There is less specificity in other cofactors. For example, the enzymatic conversion of NOHA to L-citrulline can be supported by either H_2O_2 or NADPH [149,276,283].

It is clear that NOS primarily releases NO. However, HNO can be produced by the oxidation of NOHA, either uncoupled [274,281] or coupled to NOS [276,283], or of L-arginine, under low NOS cofactor conditions [149,276,282,284]. Although this has yet to be observed in vivo, the divergent physiological properties observed following exposure to NO and HNO donors suggest an intriguing condition-dependent dimension to signal transduction and cellular regulation. The utility of HNO produced by NOS as a signaling agent would require that association with a biotarget is kinetically competitive with the back reaction of ferric NOS with HNO (Eq. (26)). Since endothelial and neuronal NOS activity is associated with translocation to specific sites in cellular membrane this remains a possibility.

The pharmacological production of NO has wide ranging ramifications for human health. For example, the vasodilator properties of nitroglycerin arise through metabolism to NO [261]. Similarly, drug metabolism may also lead to HNO production, potentially site-specifically, as has been indicated for the clinical alcohol deterrent cyanamide (Eq. (55)) and the sickle cell disease treatment hydroxyurea [177,285,286].

5.3. The chemical biology of HNO

The distinct effects of HNO in the cardiovascular system suggest that HNO may not only be an important pharmacological agent but also support prior suggestions that HNO is an endogenous component in the control of biological functions (see Section 5.2). The recent efforts to elucidate the chemistry and kinetics of HNO in a cellular environment (see Section 4.6) have begun to provide insights into the unique biological functions of HNO and the mechanisms by which HNO and NO may be distinguished.

The kinetic viability of the reaction of HNO with O_2 is of primary concern due to the high cytotoxicity induced by aerobic decomposition of Angeli's salt compared to chemical donors of NO [189]. The rates of autoxidation of both NO [287] and HNO [109,111] are slow relative to reactions with other cellular components. For instance, HNO reacts 1000-fold faster with GSH (Table 1), which is found at 1–10 mM in the cytosol [288]. The low rate constant and concentration of O_2 relative to other more abundant biomolecules will severely limit the in vivo formation of the deleterious oxidant from the association of HNO with O_2. In the event this oxidant does form, for instance in cellular membranes in which HNO and O_2 have higher solubilites [194,289], its diffusion to targets such as DNA will be further impeded by additional scavengers such as urate [106]. Consequently, the oxidative insult observed in vitro at millimolar concentrations of HNO donors [189] is predicted to be minimal upon pharmacological administration of HNO in vivo. Further, the low probability of significant autoxidation suggests that HNO will primarily function as a signaling agent, similarly to NO.

The high efficiency of scavenging of HNO by GSH due to relative rate constant and cellular concentration is not only important as a protective mechanism but also provides a means of differentiating HNO from NO [176]. Unlike HNO, NO must first be converted to higher nitrogen oxides such as N_2O_3 to interact significantly with thiols. Certainly, *S*-nitrosation of thiols by an NO autoxidative mechanism [287] is relatively quite slow compared to the association of HNO with thiols (Table 1, Eqs. (45)–(46)), and the products can be distinguished.

From similar comparisons [109,111,157], the primary cellular targets for HNO have been suggested to be thiols and oxidized metals while NO is thought to principally interact with other free radicals and with reduced metals. The varied reactivity as a function of the redox state of metals is nicely illustrated by Eq. (27). However, the most prevalent metal targets in vivo will be heme proteins, due to relative rate constants and concentrations. With hemes, the products of the reactions of HNO with the oxidized iron (Eq. (26)) and NO with the reduced iron (Eq. (25)) will lead to an identical product, the Fe(II) nitrosyl complex. This may have significant relevance to biology in that the same recycling system can be utilized to bring the metal to the appropriate resting state.

There is some overlap in the reactivity of HNO and NO, for instance toward metal-oxo species (Eqs. (40) and (42)). Further, NO of course does react with oxidized heme (Eq. (36)). However, the general tendency for ferrous nitrosyl complexes to exhibit substantially higher stability than the corresponding ferric species is well established (reviewed in [133–135]). Exceptions such as cyt *c* [143,154] tend to have reduced binding affinities due to protein interactions. Thus, the reaction of NO with ferric hemes in vivo will ordinarily be transitory and therefore of small significance compared to the more stable ferrous nitrosyl analogs.

Investigation of the reactivity of HNO with heme proteins has been minimal compared to NO. The generalization that HNO preferentially reacts with ferric heme stems from early studies with Hb, Mb [59,143,144] and sGC [236]. For example, the activity of sGC, which has a ferrous resting state, was determined to be enhanced by NO [290] and unaffected by HNO [236]. The possibility exists that further analysis may demonstrate that HNO is able to activate purified sGC, since 5 mM DTT was present in the assay buffer. However, the experimental conditions utilized are applicable to cellular concentrations of thiols. Thus, regardless of the outcome of future experiments with purified sGC, the reactivity of HNO toward this cytosolic protein in vivo will be severely restricted by the high intracellular concentration of GSH. Consequently, both molecular targeting at the protein level, by valence state in this case, and the cellular location dictate that elevated cGMP production from sGC will only be consequent to NO exposure. This conclusion is substantiated by the existence of the distinct plasma markers, CGRP for HNO and cGMP for NO [111,257].

The reactivity of HNO toward GSH will in fact inhibit the association of HNO in vivo with most cytosolic targets. For example, although HNO is readily converted to NO by purified SOD (Eq. (27)) and the rate constants for SOD and GSH are similar (Table 2), the relative concentrations of both species in the cytosol (1–10 mM GSH [288] and 10 μM Cu,Zn SOD [291]) indicate that HNO will preferentially be scavenged by GSH. Thus, unlike NO, the high reactivity of HNO will inhibit diffusion such that HNO will in general have to be produced in close proximity to the target.

Compartmentalization of molecular targets may provide an additional means to promote reactivity by sequestering the target from cytosolic scavengers such as GSH. In this regard the ability of HNO, administered intravenously at low concentrations (<10 μM) [257], to diffuse through cell membranes and initiate neuronal release of CGRP is quite intriguing. Exocytosis of CGRP from nonadrenergic/noncholinergic neurons is regulated by calcium influx through voltage-gated channels [292]. HNO may stimulate neuropeptide release by interacting directly with the channel, most likely through binding to a thiol or metal. This is an attractive proposition since the chemical modification would occur at the membrane where HNO scavengers such as GSH are low and to which HNO will partition [194].

Compartmentalization within cellular organelles, particularly mitochondria is also intriguing. The mitochondria have a high density of cellular membranes and have been suggested to possess a unique isoform of NOS [293]. Further, the electron transport chain may provide a sufficient potential to reduce NO to HNO. In fact, mitochondrial interconversion of HNO and NO has been suggested [145,160,294–296], for example by coenzyme Q/ferricyt *c* [297], unstable quinines [297] or Mn SOD [298]. Reductive nitrosylation of Mn SOD, cytochromes or other heme proteins or interaction of HNO with quinines may lead to production of NO. The mechanisms for mitochondrial reduction of NO to HNO are less apparent and require further investigation.

The chemical biology of HNO is of higher complexity than that of NO. For instance, the primary endogenous source of NO is NOS while investigations with purified biomolecules point to the possibility for multiple biosynthetic routes to HNO (see Section 5.2). Further, the reactivity of HNO (Table 1) is substantially more multifaceted than that of NO, leading to the potential for both a more diverse signaling functionality and an elevated toxicity. However, this varied reactivity also imparts numerous control mechanisms, for example through facile scavenging of HNO by GSH, ascorbate and urate [106], and consequently, considerably limits the diffusion range of HNO compared to NO [299,300]. The concentrations of HNO in vivo, from pharmacological or endogenous sources, are unlikely to exceed the cellular capacity for protective scavenging, unlike the in vitro systems in which millimolar Angeli's salt was cytotoxic and induced DNA damage [107,189,195,196]. Thus, HNO has the potential to function as a more extensive, yet tightly controlled, signaling agent than the free radical NO.

In the event that HNO is produced endogenously for signal transduction, the condition-dependent responses to nitrogen oxides can be envisioned to be vital to cell physiology. For instance since neuronal NOS is attached to the NMDA receptor in certain neurons [301], the disparate response of this calcium channel to NO and HNO, observed in vitro [113], may provide a mechanism for regulation of neuronal function by NOS under varied conditions. Under normal circumstances, low levels of NO promote calcium influx through the pore, thus regulating normal metabolism [113]. Conversely, high

fluxes of NO from activated leukocytes signal channel closure, potentially reducing damage from the immune response [251]. Tissue initially experiencing hypoxia also often produces a burst of NO from endothelial NOS in an attempt to reestablish normal blood flow by vasodilation [302]. However, as cofactors and substrates for NOS diminish with continued ischemia, neuronal NOS may convert to an HNO synthase [284]. This alteration could be critical for cell survival since NO enhances calcium influx during hypoxia in vitro [252], which upon reperfusion would aggravate deleterious processes, while HNO induces channel closure, which protects against damage upon restoration of blood flow. Reperfusion would induce channel opening and would reestablish NO synthesis, again promoting normal function.

The responses of the NMDA channel to nitrogen oxide exposure are rapid and transitory, thus indicating interaction with a metal rather than a covalent modification [113]. The involvement of a loosely bound metal is supported by channel closure in the presence of metal chelators and rapid function return with chelator-free perfusate [113]. It is therefore likely that the distinct responses of the NMDA channel toward NO and HNO are due to different reactivity with the target, although the possibility of the existence of several targets has not been addressed experimentally.

HNO may interact similarly with other calcium channels, for instance the voltage-gated channel that controls neuronal exocytosis of CGRP [257,292]. The exact nature of the interaction of HNO with these channels and whether modifications that elicit CGRP release are strictly pharmacological or physiological as well remain to be determined. Despite the need for further investigation, these calcium channel examples illustrate the potential for a considerable signaling role for HNO given the abundance and functional diversity of proteins containing critical thiols or metals.

6. Summary

Over the past 100 years, HNO research has evolved in stages from basic physical examinations to elucidation of interactions in atmospheric and industrial processes and finally to understanding the functions in prokaryotic and now eukaryotic systems. The complications experienced in the original chemical characterizations of HNO, due to the short half-life, foreshadowed the controversial nature of the HNO/NO^- field as a result of inordinately complicated chemistry for a triatomic molecule. However, it is this chemistry, for example a weak NO, H^+/HNO reduction potential, high electrophilicity, slow autoxidation and inertness to a standard conjugate acid–base relationship with NO^-, due to extremely slow, spin-forbidden proton exchange coupled with fast dimerization, that provides the potential for use of HNO as a unique cellular signal.

The divergent physiological properties of HNO and its redox sibling NO in mammalian systems stem from the low propensity for redox exchange and the unique chemistry of each species: high reactivity of HNO toward thiols and oxidized metals and of NO with other radicals and with reduced metals. Interestingly, the scavenging mechanisms for HNO far surpass those of the free radical NO. Consequently, the sphere of influence of NO is likely to be substantially larger than that for HNO. In a biological setting, this provides the ideal mechanism to produce a site-specific response while providing numerous pathways to prevent uncontrolled modifications. These intrinsic safeguards present the opportunity to develop novel pharmaceutics with high specificity and minimal side effects for treatment of diseases or conditions that have a dependence upon proteins with critical metals or thiols.

The next iteration of the HNO field will likely involve more comprehensive evaluations of the biomolecular targets, including sGC, the mechanisms for endogenous formation and the physiological consequences of reductive nitrosylation and thiol oxidation. Sensitive, specific detection methods and a larger variety of HNO donors than Angeli's salt and Piloty's acid, which were first synthesized in the late 1800s, will be required to advance this promising area of biomedical research. Lastly, as was recently the case for NO, the role of the reduced products of HNO requires investigation.

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