The Chemistry and Biology of Nitroxyl (HNO): A Chemically Unique Species with Novel and Important Biological Activity

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Introduction

In the late 1980s, it was reported that mammals synthesize nitric oxide (NO) to elicit vasorelaxation.[1–3] This significant finding represents a fundamentally new paradigm in cell signaling whereby a small, low-molecular-weight, normally gaseous species (in its pure form at room temperature and pressure) is biosynthesized specifically for the purposes of cell signal transduction. Since that watershed discovery, there has been significant interest in nitrogen oxide biology and chemistry. It is now known that nitric oxide, and other related nitrogen oxides, play key roles in a variety of mammalian physiological and pathophysiological processes. For example, nitrogen oxides are also biosynthesized in the central nervous system, during an immune response, and in mitochondria (although the mechanisms and roles of nitrogen oxide production in these other systems are not well defined).[4] Along with NO, other related/derived nitrogen oxide species have become the subjects of research interest and are proposed to have biological significance. In this regard, the biology and chemistry of peroxynitrite ($ONOO^-$), nitrogen dioxide (NO_2), and dinitrogen trioxide (N_2O_3), all species derived from reactions of NO with oxygen and oxygen-derived species, have been examined in detail and implicated in numerous physiological/pathophysiological processes.[5] Reduced nitrogen oxides (relative to NO) such as hydroxylamine (NH_2OH) have received much less recent attention but have been studied in the past with regards to their biological activity/toxicity.[6] Most nitrogen oxides have been examined previously to the extent that the mechanisms of their biological actions can be gleaned from what is known about their chemical properties and reactivity. However, among all nitrogen oxides, the one-electron reduced NO species, nitroxyl (HNO), remains poorly understood and inadequately studied. This species, however, has garnered much recent attention because of reports of its unique and potentially important biological activity (vide infra). Herein, we briefly review some of the recent discoveries regarding the unique biological actions of HNO and then discuss some recent and profound revelations about the novel chemistry of this enigmatic nitrogen oxide species.

HNO Biology

It is well established that nitrogen oxides such as NO, NO_2, and $ONOO^-$ can be generated in mammalian systems under certain conditions. However, it is unknown whether HNO is endogenously generated since there has been no unequivocal demonstration of its production in mammalian cells (discussed in more detail later). Therefore, the question of whether HNO is an endogenous signaling/effector species, akin to NO, remains unanswered. Regardless, several studies demonstrate potentially important biological activity associated with pharmacological administration of HNO. Some of the earliest studies in this regard found HNO to be a fairly potent vasorelaxant, possibly serving as a precursor to NO.[7] At about the same time, Nagasawa and co-workers reported that HNO was a potent inhibitor of the enzyme aldehyde dehydrogenase and had the potential to be developed as a treatment for alcoholism.[8,9] The mechanism by which HNO inhibits aldehyde dehydrogenase was proposed to be through reaction with the active-site thiolate, leading to covalent modification and loss of catalysis.[10] This was one of the earliest demonstrations of the thiophilic reactivity of HNO (vide infra), a property of HNO that might be very important to its general biological activity. Other thiol proteins are also susceptible to disruption by HNO. For example, HNO will inhibit the activity of metal-binding transcription factors in yeast, again presumably through reaction with the thiolate functional groups on the protein.[11] These studies in the whole-cell yeast system indicate the capacity of HNO to cross cell membranes and access intracellular space. The ability of HNO to generally affect thiol systems in cells was also demonstrated by Wink and co-workers when they reported that exposure of fibroblasts to HNO resulted in a dramatic depletion of the most prevalent intracellular thiol species, glutathione.[12] The effects of intracellular glutathione depletion can be dramatic since it is, among other things, responsible for maintaining protein thiols in their reduced form.

Ischemia-reperfusion injury is a phenomenon whereby ischemic (oxygen-deplete) tissues are greatly damaged when the oxygen supply (reperfusion of blood) is reintroduced. The etiology of this injury is not firmly established but probably in-

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volves the generation of "reactive oxygen species" (ROS) such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2) during the reperfusion event.[13] Nitric oxide is known to protect against reperfusion injury, possibly by scavenging O_2^-. However, HNO, administered by using the HNO donor Angeli's salt (vide infra), greatly exacerbates ischemia-reperfusion injury when administered *during* reperfusion.[14] The exacerbation of ischemia-reperfusion injury by HNO is reported to be due to increased neutrophil infiltration. Moreover, Takahira and co-workers propose that neutrophil infiltration in ischemia-reperfusion injury might be due to the endogenous production of HNO, which can be ameliorated by the administration of the anti-inflammatory drug dexamethazone.[15] Interestingly, when HNO is given *prior* to ischemia, protection against subsequent reperfusion damage is observed.[16]

Overactivation of the glutaminergic *N*-methyl-D-aspartate (NMDA) receptor is involved in the excitotoxicity associated with glutamate. Nitroxyl is protective against this type of toxicity as it is able to attenuate the activity of the NMDA receptor by modifying a critical thiol residue, exemplifying the ability of HNO to react with protein thiols, leading to a decrease in Ca^{2+} influx.[17] However, another study reports that HNO is capable of increasing Ca^{2+} influx by blocking a desensitization pathway.[18] These dichotomous reports might be the result of differences in the experimental conditions of the studies and remain to be reconciled. Regardless, it is clear that HNO can dramatically alter the activity of the NMDA receptor and the nature of these actions is likely to be dependent on cellular/experimental conditions.

Much of the recent excitement in the chemistry and biology of HNO was the result of a report by Paolocci and co-workers regarding the effects of HNO on failing hearts. They made the provocative and pharmacologically important discovery that HNO has the somewhat unique ability to increase left ventricular contractility and, at the same time, to lower cardiac preload and diastolic pressure without increasing arterial resistance.[19,20] The ability of nitroxyl to act in this regard makes it an ideal candidate for treating heart failure since it will increase heart contractility while decreasing vascular resistance.[21] These effects are independent of β-adrenergic signaling and cGMP and are, instead, mediated by elevated levels of calcitonin gene-related peptide.[20,22] The activity of calcitonin gene-related peptide is the result of its ability to activate the calcitonin-receptor-like receptor. This is an adenylate cyclase-coupled receptor system and, therefore, leads to an elevation of intracellular cAMP.[23,24] This indicates that HNO can act (at least in the vascular system) through a cAMP signal transduction pathway, which is fundamentally distinct from NO whose vascular activity is due to elevation of cGMP. This "orthogonal" relationship between HNO and NO indicates that the actions of HNO are not merely due to its oxidative conversion to NO.[22]

Endogenous HNO generation

Based on the discussion above, it is tempting to speculate that the unique signaling properties of HNO and its possible "orthogonal" relationship with NO are part of an intricate and important endogenous signal transduction system. However, it needs to be emphasized that there has, to date, been no unequivocal demonstration of the endogenous production of HNO in mammalian systems. Of course, this does not necessarily detract from the potential pharmacological importance of HNO. The lack of evidence for endogenous HNO generation might be due to the fact that there is currently no efficient, specific and/or sensitive detection method amenable for use in biological systems. Difficulties in developing appropriate detection systems are, to a large part, due to its inherent chemistry and fleeting nature (vide infra). Regardless, several reports allude to the possibility of biological HNO generation and chemical/biochemical processes have been characterized which allow for the possibility, if not probability, for endogenous HNO formation. These processes are discussed below.

It is generally established that *S*-nitrosothiols can be formed in mammalian systems from the reaction of protein or peptide thiols with endogenously generated nitrogen oxide species.[25] Although there is no consensus agreement as to the mechanism(s) by which *S*-nitrosothiols are formed (or their biological relevance), there appears to be little doubt that they are prevalent. Accepting this, one possible mechanism for the endogenous generation of HNO is the reaction of an *S*-nitrosothiol with another thiol species [Eq. (1)].[26,27]

$$RS{-}NO + R'{-}SH \rightarrow RSSR' + HNO \qquad (1)$$

The reaction of a thiol with an *S*-nitrosothiol can have an alternative outcome as well. Equation (1) depicts a process whereby thiol attack occurs on the sulfur atom of the *S*-nitrosothiol to give the corresponding disulfide and HNO. Alternatively, the attacking thiol can react at the nitroso function, resulting in a simple trans-nitrosation process (for example, the transfer of a nitrosonium equivalent from one thiol to the other).[28] The factors that govern the relative rates of these two competing processes have not yet been thoroughly investigated.

Nitric oxide is synthesized by a family of enzymes referred to as the nitric oxide synthases.[29] They are capable of converting L-arginine to NO and L-citrulline via an intermediate *N*-hydroxy-L-arginine. A number of chemical studies indicate that HNO can be generated from oxidative degradation of *N*-hydroxy-L-arginine.[30–32] This has the potential to be a physiologically relevant process since *N*-hydroxy-L-arginine has been detected at significant levels (up to 20 μM) in plasma[33,34] and is released by some cells in vitro.[35] HNO might also be generated by nitric oxide synthase directly[36,37] especially when it is deplete of one of its prosthetic groups, tetrahydrobiopterin.[38,39]

Since HNO is the one-electron reduction product of NO, it is possible that simple reduction of NO can result in endogenous HNO formation. Indeed, it has been proposed that the reduction of NO can occur in mitochondria[40,41] or by reaction with ubiquinol,[42] cytochrome c,[43] manganese superoxide dismutase,[44] and xanthine oxidase.[45] Clearly, an important factor in the generation of HNO from biological NO reduction might be

the reduction potential of NO. Recent and significant work regarding the NO reduction potential will be discussed later.

Like NO, HNO possesses unique and potentially important biological activity. Although it is not known whether the actions of HNO are part of a normal, endogenous signaling system, the potential pharmacological importance of HNO necessitates further investigation of the intimate details of its mechanism(s) of action. To date, the chemical mechanism by which HNO elicits its potentially beneficial effects for the treatment of heart failure or ischemic-reperfusion injury is wholly unknown. Thus, the question remains: What is the chemistry of HNO responsible for its observed biological activity? In the following section, we discuss some of the fundamental chemical properties and reactivity of HNO, some of which has only recently come to light. The chemistry discussed below serves only as a starting point in our attempt to provide chemical rationale for the unique and important biology of HNO.

The Physiological Chemistry of HNO

Before embarking on a discussion of the chemistry of HNO, it is worthwhile to briefly address nomenclature. The term "nitroxyl" is, at best, ambiguous and confusing. It does little to describe the structure or molecular makeup of this species. In fact, a literature search of this term will reveal that it is used at times to describe not only HNO but also the functional group characterized by a stable unpaired electron otherwise called a nitroxide (e.g. $R_2NO^{\bullet}$). A more appropriate, less ambiguous, and more descriptive moniker for HNO is "nitrosyl hydride".[46] However, the use of the term "nitroxyl" to refer to HNO is entrenched in both the chemical and biological literature. Thus, we continue to use this term so as not to add to the confusion and to remain consistent with most of the current literature on this topic. A second issue is that "nitroxyl" is used to describe both HNO and the conjugate base, NO^-, which are in equilibrium in basic media (vide infra).

The chemistry of nitroxyl has been a topic of discussion for over 100 years. The first recorded mention of nitroxyl was as a proposed product of the decomposition of sodium trioxodinitrate ($Na_2N_2O_3$), also known as Angeli's salt [Eq. (2)].[47]

$$Na_2N_2O_3 + H^+ \rightarrow [NaHN_2O_3] \rightarrow HNO + NaNO_2 \qquad (2)$$

However, experimental validation of the actual existence of HNO was not obtained until 1958 when Brown and Pimentel observed it spectroscopically from the photolysis of methyl nitrite in an argon matrix.[48] Subsequent reports described the chemistry of HNO, and other related nitrogen oxides, generated by pulse radiolysis.[49,50] These studies resulted in the elucidation of some of the fundamental chemical properties of HNO (although some of these properties have been re-evaluated and revised recently, vide infra). The generation of HNO from Angeli's salt has been established by a series of excellent studies[51] and has recently been examined theoretically.[52] Indeed, this salt has been used extensively in the discovery of many of the biological actions of HNO.

At first glance, HNO appears to be a simple triatomic species requiring no special consideration. However, HNO is a unique species with novel and atypical chemistry and most researchers are unfamiliar with this simple species. While simple nitrogen oxides such as nitrate (NO_3^-), nitrite (NO_2^-), nitrogen dioxide (NO_2), nitric oxide (NO), and hydroxylamine (NH_2OH) have received much attention in the literature, nitroxyl is a relative stranger. This has to do with the inherent instability of HNO (vide infra), making experimentation difficult, and the previous lack of evidence for any biological relevance. However, prominent discoveries of the importance of nitrogen oxide chemistry in mammalian (patho)physiology as well as the previously mentioned reports of the novel biological activity of HNO specifically have prompted many laboratories to attempt to further define the inherent chemistry of HNO.

Even the simplest of all processes involving HNO, deprotonation, is relatively complex. The electronic ground state of HNO is singlet where all electrons are spin-paired, the usual case for stable molecules. However, deprotonation of HNO generates an anion (often referred to as nitroxyl anion or, more appropriately termed, oxonitrate (1-), NO^-), which is isoelectronic with dioxygen (O_2). As for O_2, NO^- has two low-energy spin states, singlet ($^1NO^-$) and triplet ($^3NO^-$). The ground state of NO^- is the triplet, $^3NO^-$, just like O_2. The excited singlet spin state is approximately 17–20 kcal mol^{-1} higher in energy,[53] similar to the energy gap (23 kcal mol^{-1}) in O_2. This means that the electronic ground states for the protonated and deprotonated nitroxyl species are different. This is a unique situation since most all other simple protonation–deprotonations occur without a change in the nature of the electronic ground states of the products (the high-energy protonation, $^3O_2 \rightarrow HOO^+$ would be analogous). Simple deprotonation of HNO can conceivably occur via two distinct pathways: 1) deprotonation of singlet HNO to initially give $^1NO^-$, followed by intersystem crossing to the triplet ground state, $^3NO^-$ or 2) deprotonation of singlet HNO directly to the triplet species, $^3NO^-$, without the intermediacy of the singlet species. It was earlier proposed that HNO deprotonates via pathway 1 through the intermediacy of the excited state anion.[54] However, this is no longer considered to be the case. Recent theoretical and experimental work finds that the relevant equilibrium is between the singlet protonated species and the triplet anion [Eq. (3)].[53,55–59]

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

Thus, protonation–deprotonation of nitroxyl represents a spin-forbidden processes and would be expected to be considerably slower than normal acid–base reactions. This is indeed the case, although the spin conversion might play only a minor role in the intersystem barrier as it is proposed that nuclear reorganization energies represent most of the activation barrier.[59]

It is now established that deprotonation of HNO (or protonation of $^3NO^-$) represents a unique process as it requires a spin state conversion; but what is the pK_a of HNO? Direct experimental determination of the HNO pK_a is confounded by the

fact that HNO can dimerize–dehydrate to give N_2O and water [Eq. (4)].[58,60]

$$HNO + HNO \rightarrow [HONNOH] \rightarrow N_2O + H_2O \quad (8 \times 10^6\ M^{-1}\ s^{-1}) \quad (4)$$

The existence of this reaction prohibits establishment of a chemical equilibrium between protonated and unprotonated species and, therefore, precludes straightforward determination of the HNO pK_a. Regardless, in 1970, using pulse radiolysis to generate NO^-, Gratzel and co-workers[49] reported a pK_a for HNO of 4.7. This was the generally accepted value until recently. Clearly, a pK_a of 4.7 would predict that the near exclusive species at physiological pH would be the anion. However, studies on the reactivity of HNO at physiological pH were appearing in the literature and were more consistent with chemistry occurring through HNO rather than the anion.[26] This put into question the validity of the reported pK_a of 4.7. Prompted by this paradox, Bartberger and co-workers[53] re-evaluated the HNO pK_a by using quantum mechanical calculations. The first study revised the pK_a upward to a value of approximately 7.2. This report not only revised the pK_a but also indicated that the relevant equilibrium was between singlet HNO and the triplet anion [Eq. (3)] (a conclusion already drawn by Janaway and Brauman for the gas phase[56]). Subsequent experimental work by Shafirovich and Lymar[58] and theoretical re-evaluation by Bartberger and co-workers[57] have now provided a consensus agreement that the pK_a of HNO is approximately 11.4. Thus, it is now established that at physiological pH HNO is the near exclusive species, not NO^-. It should be realized, however, that if $^3NO^-$ can be biologically generated, the slow protonation reaction allows $^3NO^-$ to have a significant lifetime (milliseconds), even though protonation is highly favorable.[59]

One-electron reduction of NO to HNO/NO^- is a possible mechanism for nitroxyl generation in biological systems. Of course, the prevalence of this process in biological systems will be highly dependent on the reduction potential of NO. Experimental determination of the one-electron reduction potential of NO to HNO/NO^- has been problematic because of the existence of catenation reactions between NO^- or HNO and NO [Eqs. (5)–(7) and (8)–(10)].[49,50,61]

$$NO^- + NO \rightarrow N_2O_2^- \quad (k = 1.7 - 3.3 \times 10^9\ M^{-1}\ s^{-1}) \quad (5)$$

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

$$N_3O_3^- \rightarrow N_2O + NO_2^- \quad (k = 87 - 330\ s^{-1}) \quad (7)$$

$$HNO + NO \rightarrow HN_2O_2 \quad (5.8 \times 10^6\ M^{-1}\ s^{-1}) \quad (8)$$

$$HN_2O_2 + NO \rightarrow HN_3O_3 \quad (8 \times 10^6\ M^{-1}\ s^{-1}) \quad (9)$$

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

These reactions can confound experiments involving direct electrochemical reduction of NO, since they are responsible for the irreversibility of this process and lead to other species in solution with distinct electrochemistry. Moreover, NO can bind/adsorb to metal-electrode surfaces, and many reported reduction potential measurements are likely to be of an NO–metal complex rather than NO itself, or to involve multielectron processes. In fact, reduction potentials for NO ranging from 0.4 to −1 V (versus normal hydrogen electrode (NHE) throughout) have been reported. Probably the most reliable, early experimental determinations for the NO reduction potential were by Ehman and Sawyer[62] and Benderski and co-workers[63] who used chroniopotentiometric/controlled-potential coulometric techniques and photoelectrochemical measurements, respectively, on free NO. Both studies yielded reduction-potential values of approximately −0.8 V (these studies do not specifically mention the spin states of the nitrogen oxide species). However, an often-quoted and theoretically-derived reduction potential for the NO/$^3NO^-$ couple is 0.39 V.[54] The huge discrepancy between the theoretical and experimental values for the NO/NO^- couple was disconcerting and remained an enigma until only recently. The theoretically-derived value of 0.39 V was based on two major assumptions; an HNO pK_a of 4.7 and that the relevant equilibrium is between HNO and $^1NO^-$. Since both of these assumptions have recently been determined to be invalid, the calculated NO reduction potential must be in error as well. Indeed, recalculation of the NO/$^3NO^-$ couple by using the revised pK_a and the different equilibrium gives a reduction potential for the NO/$^3NO^-$ couple of approximately −0.8 V.[57] Thus, the difference between experiment and calculation can now be reconciled. Of particular note, since the NO reduction potential and HNO pK_a are mathematically/theoretically linked,[54] unequivocal determination of one value validates the other. Thus, reconciling the experimental and theoretical determinations of the NO reduction potential serve to firmly establish the newly revised HNO pK_a of approximately 11.4. Since protonation of $^3NO^-$ to HNO is highly favorable at physiological pH, there is a positive shift in the potential to approximately −0.5 to −0.6 V as the pH is lowered.[57,58] The negative values for the one-electron reduction potentials for both the NO/$^3NO^-$ and NO,H^+/HNO couples indicate that one-electron reduction of NO by an outer-sphere electron-transfer process might be difficult under biological conditions. However, if the intracellular concentrations of the reductants and oxidants are considered, this process might become biological accessible.[64]

Thus far, the discussion has concentrated on the chemistry of HNO and $^3NO^-$. However, other structural/spin-state congeners of nitroxyl exist. Both a triplet protonated species, ^3NOH,[65,66] and, as mentioned previously, a singlet anionic species, $^1NO^-$, have been the subjects of previous studies. These species are thought to be biologically inaccessible since they are not likely to be generated under typical biological conditions by thermal processes. The relative energetics of these species are depicted in Figure 1. Thus, the biology of nitroxyl is undoubtedly dominated by HNO. However, if generated, $^3NO^-$ can have a significant lifetime prior to protonation and might be able to participate in biological chemistry.

The reaction of nitroxyl with O_2 has become a topic of considerable interest because of its potential biological relevance. It is established that $^3NO^-$ can react with O_2 at near dif-

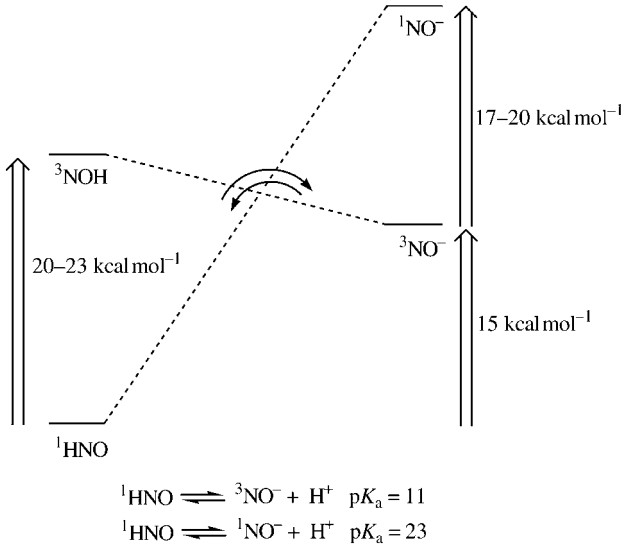

Figure 1. *Relative energies of the various nitroxyl species.*

fusion-controlled rates to generate peroxynitrite, $ONOO^-$ [Eq. (11)].[58,67,68]

$$^3NO^- + {}^3O_2 \rightarrow ONOO^- \quad (2.7 \times 10^9\ M^{-1}\ s^{-1}) \qquad (11)$$

Since the pK_a of HNO is approximately 11.4, equilibrium concentrations of $^3NO^-$ would be vanishingly small at physiological pH and reactions of HNO would predominate. However, as mentioned previously, if $^3NO^-$ can be generated directly, it can have a significant (millisecond) lifetime. A potentially more relevant process in biological systems is the reaction of HNO with O_2. It appears that HNO does indeed react with O_2.[64] However, the mechanism of this reaction and nature of the products are currently two of the most controversial topics in this field. Miranda and co-workers[69,70] find that the reaction of HNO with O_2 gives a potent two-electron oxidant distinct from ^-OONO and/or N_2O_3.

One of the most studied and biologically important reactions of HNO is that with thiols/thiolates. As mentioned earlier, Bartberger and colleagues[53] reported that HNO is extremely "thiolphilic", a claim that is supported by numerous studies describing the reaction of HNO with thiols. In fact, HNO–thiol reactivity can be used to distinguish between the biological chemistry of HNO versus NO.[71] All characterized reactions between a thiol (or thiolate) and HNO initially involve attack of the nucleophilic thiol-sulfur atom on the electrophilic HNO nitrogen atom giving initially an *N*-hydroxysulfenamide [Eq. (12)].[72] The *N*-hydroxysulfenamide has not, as yet, been isolated as it can further react with excess thiol to give the corresponding disulfide and hydroxylamine [Eq. (13)], or, in the absence of large excesses of thiol, it can rearrange to the corresponding sulfinamide [Eq. (14)].[26,73]

$$HNO + RSH \rightarrow RS{-}NHOH \qquad (12)$$

$$RS{-}NHOH + RSH \rightarrow RSSR + NH_2OH \qquad (13)$$

$$RS{-}NHOH \rightarrow [RS^+NH + HO^-] \rightarrow RS^+(OH)NH^- \rightarrow RS(O)NH_2 \qquad (14)$$

Hydrolysis of the sulfinamide can occur, giving a sulfinate and ammonia.[26] Protein or peptide disulfide formation is readily reversible in cells. However, sulfinamide or sulfinate formation is not as readily reversible in cells and might represent an HNO-mediated irreversible protein or peptide modification.

The reactions of HNO with thiols are not the only examples of HNO-mediated oxidation of biologically relevant species. HNO can also oxidize NADPH.[12,74,75] This reaction occurs under anaerobic conditions, precluding NADPH oxidation by HNO/O_2 adducts. The two-electron reduction potential for the ^{1}HNO/$2H^+$,NH_2OH couple at pH 7 is reported to be 0.3 V.[58] The one-electron reduction potentials at pH 7 have been calculated to be 0.1 ± 0.1 and 0.5 ± 0.1 V for the HNO,H^+/$H_2NO^\bullet$ and $H_2NO^\bullet$,H^+/NH_2OH couples, respectively.[76] Thus, reduction of HNO to the dihydronitroxide radical ($H_2NO^\bullet$) and NH_2OH are clearly accessible under biological conditions and, depending on the rates of other competing reactions, might even be expected to be a predominant biological fate.

HNO/$^3NO^-$ can also act as a reductant. Based on the reduction potential for NO (-0.8 V for the NO/$^3NO^-$ couple), $^3NO^-$ is a potent one-electron reductant. For example, nitroxyl reacts with oxidized ferriheme proteins to give ferrous-nitrosyl adducts [Eq. (15), (Hb = hemoglobin)].[77]

$$HNO/NO^- + HbFe^{III} \rightarrow HbFe^{II}{-}NO(+H^+) \qquad (15)$$

Since the H–NO-bond strength is only 48–50 kcal mol^{-1},[53] HNO would also be expected to be a good H-atom donor. H-atom abstraction from HNO by a reactive radical species would quench the reactive radical and generate NO, which is also known to be a proficient radical scavenger. Thus, it might be expected that HNO can be an efficient radical scavenger and/or antioxidant through H-atom donation and generation of NO.

Like NO, HNO is able to form complexes with ferrous heme proteins. For example, reduction of a ferromyoglobin–NO adduct ($MbFe^{II}$–NO) results in the formation of a stable HNO–Fe^{II} adduct ($MbFe^{II}$–HNO).[78] The analogous hemoglobin complex has also been formed by reacting HNO directly with deoxyhemoglobin.[79] Thus, other possible biological targets for HNO are the metal centers of metalloproteins: HNO can reduce oxidized metal centers, possibly followed by complexation of NO to form the metal nitrosyl [Eq. (15)], or directly bind reduced metal centers as HNO. Significantly, the primary "receptor" for NO is the heme protein guanylate cyclase. NO binding to the ferrous heme moiety of the enzyme, giving a ferrous nitrosyl complex, results in an increase in catalytic activity leading to increased levels of cGMP. Although it is reported that HNO cannot replace NO in this regard,[80] these studies were carried out in the presence of high exogenous thiols that might have scavenged HNO. Thus, the effect of HNO on guanylate cyclase activity remains unknown.

This overview describes some of the reactions of HNO that might be biologically relevant. However, in order to truly pre-

dict the likelihood of these reactions occurring in a biological system, the rate constants for these reactions must be known as well as the relative concentrations of the reacting species. Performing kinetic studies on HNO is not trivial since it is a fleeting species, because of self-reactivity [Eq. (4)], and its availability primarily through donor compounds. However, Miranda and co-workers[81] used competition kinetics to determine the relative rates of HNO reactions with several biologically relevant reactants and derived approximate rate constants. Based on the relative rate constants and likely intracellular concentrations of the reactants, they concluded that the most likely biological reaction for HNO is with glutathione (GSH). Significantly, Wink and co-workers[12] reported that exposure of fibroblasts to HNO resulted in a near complete loss of glutathione. The levels of GSH as well as the ratio of the reduced to oxidized species (GSH/GSSG) are primary factors in establishing the redox environment of cells.[82] Therefore, the ability of HNO to affect the status of intracellular GSH might be very important to its ability to alter cell function/fate.

Nitroxyl Toxicity and Toxicological Chemistry

The development of HNO as, for example, a pharmacological treatment for heart failure or as a preconditioning agent for reperfusion toxicity has tremendous potential. However, several studies report HNO toxicity that might negatively impact the utility of HNO as a therapeutic agent. Wink and co-workers[12] were among the first to demonstrate the toxicity of HNO when they found that Angeli's salt (2–5 mM) was cytotoxic to V79 fibroblasts, eliciting GSH depletion and DNA strand breaks. Subsequent work by Ohshima et al.[83] further corroborated the ability of HNO, derived from Angeli's salt, to induce DNA strand breakage in vitro, presumably via the generation of hydroxyl radical (HO$^{\bullet}$). Interestingly, DNA strand breakage by HNO was found to be independent of O_2, indicating that $ONOO^-$ [Eq. (11)] was not involved.

Thus, it is clear that HNO can be cytotoxic at relatively high concentrations (2–5 mM) of the HNO-donor Angeli's salt and, under in vitro conditions, can elicit oxidation chemistry consistent with the generation of HO$^{\bullet}$. Interestingly, HO$^{\bullet}$, spin trapped by using a pyrroline *N*-oxide, was detected in a decomposing solution of Angeli's salt.[84,85] The formation of HO$^{\bullet}$ from HNO was proposed to occur via an azo-type homolytic fission of *cis*-hyponitrous acid [Eq. (16)], a reaction initially proposed almost 40 years ago to explain a small amount of radical chain chemistry associated with the decomposition of hyponitrous acid.[86]

$$2HNO \rightarrow HON{=}NOH(cis) \rightarrow N_2 + 2HO^{\bullet} \qquad (16)$$

Thus, it appears that HNO has the potential to be toxic because of its oxidation chemistry. However, it needs to be understood that mechanisms of oxidant formation that rely on HNO dimerization are second order in HNO and would not be relevant unless physiological HNO levels are high. It has not, as yet, been demonstrated that this oxidation chemistry has any in vivo relevance.

Besides the possible generation of powerful oxidants via *cis*-hyponitrous acid formation, the simultaneous presence of HNO and NO can also lead to the generation of other potent oxidants. Seddon and co-workers[50] reported that the product of the reaction of nitroxyl with NO, hyponitrite radical [Eq. (5) or (8)], was capable of decomposing to generate HO$^{\bullet}$ [Eq. (17)]. Like Equation (16), this reaction was originally proposed by Buchholz and Powell[86] in their attempt to explain the small amount of radical chemistry sometimes seen during the normal decomposition of hyponitrous acid to N_2O [Eq. (4)].

$$N_2O_2^- + H^+ \rightarrow N_2O + HO^{\bullet} \qquad (17)$$

More recently, Poskrebyshev et al.[87] have further investigated this chemistry and reported that the hyponitrite radical itself is a potent oxidant ($E^0(N_2O_2^-/N_2O_2^{2-}) = 0.96$ and $E^0(HN_2O_2,H^+/H_2N_2O_2) = 1.75$ V). However, the physiological/toxicological relevance of this chemistry relies on HNO and NO being generated proximally and simultaneously, something that has not been demonstrated in any in vivo situation.

Summary

In the past few years there has been a significant increase in interest in HNO, undoubtedly prompted by discoveries of its novel and important biological activity. Recent work in this area has led to significant revelations and/or revisions concerning its chemical properties and reactivity. In spite of this research activity, it is still not clear how HNO acts to elicit its unique biology. That is, the chemistry of its biology remains to be established. This review serves only to introduce this species as an important and potentially useful nitrogen oxide and provide an update of some of its interesting and novel chemistry. Our current understanding of the biology and chemistry of nitroxyl can be summarized as follows:

Nitroxyl biology

1. HNO has the unique ability to increase cardiac output and to decrease venous resistance at the same time. These pharmacological attributes make HNO an ideal drug for the treatment of heart failure.
2. Tissue pretreatment with HNO protects against ischemia-reperfusion toxicity.
3. HNO is capable of interacting with thiol proteins or peptides often leading to an inhibition or attenuation of their activity.
4. HNO can interact with metalloproteins, either at the metal center or with the metal ligands.
5. High levels of HNO or simultaneous generation of HNO and NO can lead to toxicity because of the oxidation of biomolecules.

Nitroxyl Chemistry

1. The pK_a of HNO has recently been determined to be 11.4, making HNO, rather than NO^-, the predominant species present at physiological pH.
2. Protonation–deprotonation events of nitroxyl are much slower than typical for other acids/bases. Thus, if formed, the anion can have a significant lifetime.
3. HNO is very thiophilic and the products of a reaction between HNO and a biological thiol can result in either reversible or irreversible modification, depending on the conditions.
4. HNO can act as both an oxidant and reductant and products from either process need to be considered in the overall biology.
5. Endogenous generation of HNO has not been unequivocally demonstrated. Reduction of NO to give nitroxyl is difficult but possible.

Whether all or some of the chemistry discussed above is relevant to the biology of HNO remains to be determined and will likely occupy the efforts of many laboratories for many more years. It will not be at all surprising if other aspects of HNO chemistry are revealed in the future that might also be of biological relevance or will help explain some of the current biological observations. Finally, it is also likely that discoveries of other novel/important aspects of HNO biology await as we are still only in the initial stages of examination of this chemically fascinating species.

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[1] R. F. Furchgott in *Vasodilation: Vascular Smooth Muscle, Peptides, Autonomic Nerves and Endothelium* (Ed. P. M. VanHoutte), Raven Press, New York, **1988**, pp. 401 – 414.
[2] L. J. Ignarro, G. M. Buga, K. S. Wood, R. E. Byrns, G. Chaudhuri, *Proc. Natl. Acad. Sci. USA* **1987**, *84*, 9265 – 9269.
[3] R. M. J. Palmer, A. G. Ferrige, S. Moncada, *Nature* **1987**, *327*, 524 – 526.
[4] *Nitric Oxide Biology and Pathobiology* (Ed. L. J. Ignarro), Academic Press, San Diego, **2000**.
[5] K. M. Miranda, M. G. Espey, D. Jourd'heuil, M. B. Grisham, J. M. Fukuto, M. Feelisch, D. A. Wink in *Nitric Oxide Biology and Pathobiology* (Ed. L. J. Ignarro), Acedemic Press, San Diego, **2000**, pp. 41 – 55.
[6] P. Gross, *Crit. Rev. Toxicol.* **1985**, *14*, 87 – 99.
[7] J. M. Fukuto, K. Chiang, P. Wong, G. Chaudhuri G. , *J. Pharm. Exp. Ther.* **1992**, *263*, 546 – 551.
[8] H. T. Nagasawa, E. G. DeMaster, B. Redfern, F. N. Shirota, D. J. W. Goon, *J. Med. Chem.* **1990**, *33*, 3120 – 3122.
[9] M. J. C. Lee, H. T. Nagasawa, J. A. Elberling, E. G. DeMaster, *J. Med. Chem.* **1992**, *35*, 3648 – 3652.
[10] E. G. DeMaster, B. Redfern, H. T. Nagasawa, *Biochem. Pharmacol.* **1998**, *56*, 2007 – 2015.
[11] N. M. Cook, M. Shinyashiki, M. I. Jackson, F. A. Leal, J. M. Fukuto, *Arch. Biochem. Biophys.* **2003**, *410*, 89 – 95.
[12] D. A. Wink, M. Feelisch, J. M. Fukuto, D. Christodoulou, D. Jourd'heuil, M. Grisham, V. Vodovotz, J. A. Cook, M. Krishna, W. DeGraff, S. Kim, J. Gamson, J. B. Mitchell, *Arch. Biochem. Biophys.* **1998**, *351*, 66 – 74.
[13] B. Halliwell, J. M. C. Gutteridge in *Free Radicals in Biology and Medicine*, 3rd ed, Oxford University Press, Oxford, **1999**, pp. 645 – 661.
[14] X. L. Ma, F. Cao, G. L. Liu, B. L. Lopez, T. A. Christopher, J. M. Fukuto, D. A. Wink, M. Feelisch, *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 14617 – 14622.
[15] R. Takahira, K. Yonemura, Y. Fujise, A. Hishida, *Free Radical Biol. Med.* **2001**, *31*, 809 – 815.
[16] P. Pagliaro, D. Mancardi, R. Rastaldo, C. Penna, D. Gattullo, K. M. Miranda, M. Feelisch, D. A. Wink, D. A. Kass, N. Paolocci, *Free Radical Biol. Med.* **2003**, *34*, 33 – 43.
[17] W.-K. Kim, Y.-B. Choi, P. V. Rayudu, P. Das, W. Asaad, D. R. Arnelle, J. S. Stamler, S. A. Lipton, *Neuron* **1999**, *24*, 461 – 469.
[18] C. A. Colton, M. Gbadegesin, D. A. Wink, K. M. Miranda, M. G. Espey, S. Vicini, *J. Neurochem.* **2001**, *78*, 1126 – 1134.
[19] N. Paolocci, W. F. Saavedra, K. M. Miranda, C. Martignani, T. Isoda, J. M. Hare, M. G. Espey, J. M. Fukuto, M. Feelisch, D. A. Wink, D. A. Kass, *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 10463 – 10468.
[20] N. Paolocci, T. Katori, H. C. Champion, M. E. St. John, K. M. Miranda, J. M. Fukuto, D. A. Wink, D. A. Kass, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 5537 – 5542.
[21] M. Feelisch, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 4978 – 4980.
[22] K. M. Miranda, N. Paolocci, T. Katori, D. D. Thomas, E. Ford, M. D. Bartberger, M. G. Espey, D. A. Kass, J. M. Fukuto, D. A. Wink, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 9197 – 9201.
[23] C. Juaneda, Y. Dumont, R. Quirion, *Trends Pharmacol. Sci.* **2000**, *21*, 432 – 438.
[24] M.-H. Huang, P. R. Knight, J. L. Izzo, *Am. J. Physiol.* **1999**, *276*, R259 – R264.
[25] N. Hogg, *Annu. Rev. Pharmacol. Toxicol.* **2002**, *42*, 585 – 600.
[26] P. S. Y. Wong, J. Hyun, J. M. Fukuto, F. N. Shiroda, E. G. DeMaster, H. T. Nagasawa, *Biochemistry* **1998**, *37*, 5362 – 5371.
[27] D. R. Arnelle, J. S. Stamler, *Arch. Biochem. Biophys.* **1995**, *318*, 279 – 285.
[28] K. N. Houk, B. N. Hietbrink, M. D. Bartberger, P. R. McCarren, B. Y. Choi, R. D. Voyksner, J. S. Stamler, E. J. Toone, *J. Am. Chem. Soc.* **2003**, *125*, 6972 – 6976.
[29] O. W. Griffith, D. J. Stuehr, *Annu. Rev. Physiol.* **1995**, *57*, 707 – 736.
[30] J. Yoo, J. M. Fukuto, *Biochem. Pharmacol.* **1995**, *50*, 1995 – 2000.
[31] J. M. Fukuto, G. C. Wallace, R. Hszieh, G. Chaudhuri, *Biochem. Pharmacol.* **1992**, *43*, 607 – 613.
[32] J. Y. Cho, A. Dutton, T. Miller, K. N. Houk, J. M. Fukuto, *Arch. Biochem. Biophys.* **2003**, *417*, 65 – 76.
[33] M. Hecker, C. Schott, B. Bucher, R. Bussi, J.-C. Stoclet, *Eur. J. Pharmacol.* **1995**, *275*, R1 – R3.
[34] R. Wigand, J. Meyer, R. Busse, M. Hecker, *Ann. Rheum. Dis.* **1997**, *56*, 330 – 332.
[35] G. M. Buga, R. Singh, S. Pervin, N. E. Rogers, D. A. Schmitz, C. P. Jenkinson, C. D. Cederbaum, L. J. Ignarro, *Am. J. Physiol.* **1996**, *271*, H1988 – H1998.
[36] A. J. Hobbs, J. M. Fukuto, L. J. Ignarro, *Proc. Natl. Acad. Sci. USA* **1994**, *91*, 10992 – 10996.
[37] H. H. H. W. Schmidt, H. Hofman, U. Schindler, Z. S. Shutenko, D. D. Cunningham, M. Feelisch, *Proc. Natl. Acad. Sci. USA* **1996**, *93*, 14492 – 14497.
[38] K. M. Rusche, M. M. Spiering, M. A. Marletta, *Biochemistry* **1998**, *37*, 15503 – 15512.
[39] S. Adak, Q. Wang, D. J. Stuehr, *J. Biol. Chem.* **2000**, *275*, 33554 – 33561.
[40] R. B. Clarkson, S. W. Norby, A. Smirnov, S. Boyer, N. Vahidi, R. W. Nims, D. A. Wink, *Biochim. Biophys. Acta* **1995**, *1243*, 496 – 502.
[41] X.-J. Zhao, V. Sampath, W. S. Caughey, *Biochem. Biophys. Res. Commun.* **1995**, *212*, 1054 – 1060.
[42] J. J. Poderoso, M. C. Carreras, F. Schopfer, C. L. Lisdero, N. A. Riobo, C. Guilivi, A. D. Boveris, E. Cadenas, *Free Radical Biol. Med.* **1999**, *26*, 925 – 935.
[43] M. A. Sharpe, C. E. Cooper, *Biochem. J.* **1998**, *332*, 9 – 19.

[44] V. Niketic, S. Stojanovic, A. Nikolic, M. Spasic, A. M. Michelson, *Free Radical Biol. Med.* **1999**, *27*, 992 – 996.
[45] M. Saleem, H. Ohshima, *Biochem. Biophys. Res. Comm.* **2004**, *315*, 455 – 462.
[46] W. H. Koppenol, T. G. Traynham, *Methods Enzymol.* **1996**, *268*, 3 – 6.
[47] A. Angeli, *Gazz. Chim. Ital.* **1903**, *33(II)*, 245.
[48] H. W. Brown, G. C. Pimentel, *J. Chem. Phys.* **1958**, *29*, 883 – 888.
[49] M. Gratzel, S. Taniguchi, A. Henglein, *Ber. Bunsenges. Phys. Chem.* **1970**, *74*, 1003 – 1010.
[50] W. A. Seddon, J. W. Fletcher, F. C. Sopchyshyn, *Can. J. Chem.* **1973**, *51*, 1123 – 1130.
[51] F. T. Bonner, M. N. Hughes, *Comments Inorg. Chem.* **1988**, *7*, 215 – 234, and references therein.
[52] A. S. Dutton, J. M. Fukuto, K. N. Houk, *J. Am. Chem. Soc.* **2004**, *126*, 3795 – 3800.
[53] M. D. Bartberger, J. M. Fukuto, K. N. Houk, *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 2194 – 2198, 2001.
[54] D. M. Stanbury, *Adv. Inorg. Chem.* **1989**, *33*, 69 – 138.
[55] G. A. Janaway, M. Zhong, G. G. Gatev, M. L. Chabinyc, J. I. Brauman, *J. Am. Chem. Soc.* **1997**, *119*, 11 697 – 11 698.
[56] G. A. Janaway, J. I. Brauman, *J. Phys. Chem. A* **2000**, *104*, 1795 – 1798.
[57] M. D. Bartberger, W. Liu, E. Ford, K. M. Miranda, C. Switzer, J. M. Fukuto, P. J. Farmer, D. A. Wink, K. N. Houk, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 10 958 – 10 963.
[58] V. Shafirovich, S. V. Lymar, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 7340 – 7345.
[59] V. Shafirovich, S. V. Lymar, *J. Am. Chem. Soc.* **2003**, *125*, 6547 – 6552.
[60] D. A. Bazylinski, T. C. Hollocher, *Inorg. Chem.* **1985**, *24*, 4285 – 4288.
[61] W. A. Seddon, M. J. Young, *Can. J. Chem.* **1970**, *48*, 393 – 394.
[62] D. L. Ehman, D. T. Sawyer, *J. Electroanal. Chem.* **1968**, *16*, 541 – 549.
[63] V. A. Benderskii, A. G. Krienko, E. A. Ponomarev, *Sov. Electrochem.* **1989**, *25*, 154 – 161.
[64] S. I. Liochev, I. Fridovich *Free Radical Biol. Med.* **2003**, *34*, 1399 – 1404.
[65] J. Peslak Jr., D. S. Klett, C. W. David, *J. Am. Chem. Soc.* **1971**, *93*, 5001 – 5005.
[66] G. A. Gallup, *Inorg. Chem.* **1975**, *14*, 563 – 565.
[67] M. Kirsch, H. de Groot, *J. Biol. Chem.* **2002**, *277*, 13 379 – 13 388.
[68] C. E. Donald, M. N. Hughes, J. M. Thompson, F. T. Bonner, *Inorg. Chem.* **1986**, *25*, 2676 – 2677.
[69] K. M. Miranda, M. G. Espey, K. Yamada, M. Krishna, N. Ludwick, S. Kim, D. Jourd'heuil, M. Grisham, M. Feelisch, J. M. Fukuto, D. A. Wink, *J. Biol. Chem.* **2001**, *276*, 1720 – 1727.
[70] K. M. Miranda, K. Yamada, M. G. Espey D. D. Thomas, W. DeGraff, J. B. Mitchell, M. C. Krishna, C. A. Colton, D. A. Wink, *Arch. Biochem. Biophys.* **2002**, *401*, 134 – 144.
[71] R. Z. Pino, M. Feelisch, *Biochem. Biophys. Res. Commun.* **1994**, *201*, 54 – 62.
[72] M. P. Doyle, S. N. Mahapatro, R. D. Broene, J. K. Guy, *J. Am. Chem. Soc.* **1988**, *110*, 593 – 599.
[73] D. W. Shoeman, H. T. Nagasawa, *Nitric Oxide Biol. Chem.* **1998**, *2*, 66 – 72.
[74] A. Reif, L. Zecca, P. Riederer, M. Feelisch, H. H. H. W. Schmidt, *Free Radical Biol. Med.* **2001**, *30*, 803 – 808.
[75] S. I. Liochev, I. Fridovich, *J. Biol. Chem.* **2001**, *276*, 35 253 – 35 257.
[76] A. S. Dutton, J. M. Fukuto, K. N. Houk, unpublished results.
[77] M. P. Doyle, S. N. Mahapatro, R. D. Broene, J. K. Guy, *J. Am. Chem. Soc.* **1988**, *110*, 593 – 599.
[78] R. Lin, P. J. Farmer, *J. Am. Chem. Soc.* **2000**, *122*, 2393 – 2394.
[79] F. Sulc, C. E. Immoos, D. Pervitsky, P. J. Farmer, *J. Am. Chem. Soc.* **2004**, *126*, 1096 – 1101.
[80] E. A. Dierks, J. N. Burstyn, *Biochem. Pharmacol.* **1996**, *51*, 1593 – 1600.
[81] K. M. Miranda, N. Paolocci, T. Katori, D. D. Thomas, E. Ford, M. D. Bartberger, M. G. Espey, D. A. Kass, J. M. Fukuto, D. A. Wink, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 9197 – 9201.
[82] F. Q. Schafer, G. R. Buettner, *Free Radical Biol. Med.* **2001**, *30*, 1191 – 1212.
[83] H. Ohshima, I. Gilibert, F. Biachini, *Free Radical Biol. Med.* **1999**, *26*, 1305 – 1313.
[84] D. A. Stoyanovsky, R. Clancy, A. I. Cederbaum, *J. Am. Chem. Soc.* **1999**, *121*, 5093 – 5094.
[85] J. Ivanova, G. Salama, R. M. Clancy, N. F. Schor, K. D. Nylander, D. A. Stoyanovsky, *J. Biol. Chem.* **2003**, *278*, 42 761 – 42 768.
[86] J. R. Buchholz, R. E. Powell, *J. Am. Chem. Soc.* **1965**, *87*, 2350 – 2353.
[87] G. A. Poskrebyshev, V. Sharirovich, S. V. Lymar, *J. Am. Chem. Soc.* **2004**, *126*, 891 – 899.

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