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FORUM REVIEW ARTICLE

HNO Signaling Mechanisms

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Abstract

Due to recent discoveries of important and novel biological activity, nitroxyl (HNO) has become a molecule of significant interest. Although it has been used in the past as a treatment for alcoholism, it is currently being touted as a treatment for heart failure. It is becoming increasingly clear that many of the biological actions of HNO can be attributed to its ability to react with specific thiol- and, possibly, heme-proteins. Herein is discussed the chemistry of HNO with likely biological targets. A particular focus is given to targets associated with the pharmacological utility of HNO as a cardiovascular agent and for the treatment of alcoholism. *Antioxid. Redox Signal.* 14, 1649–1657.

Introduction

Although nitroxyl (HNO) has been of interest to chemists since the early 1900s (7, 46, and references therein) recent discoveries of its provocative and potentially profound biological actions have led to a resurgence of interest, particularly in the chemical and biological mechanisms of its activity (*e.g.*, Refs. 34, 54). Of particular importance, from a pharmacological and/or therapeutic perspective, are the effects HNO has on the cardiovascular system. A series of studies indicate that HNO is a unique and near-perfect treatment for heart failure (*e.g.*, Refs. 14, 22, 23, 41, 52, 53, 66). HNO also has the potential to be used to prevent ischemia-reperfusion injury (51) and has been used in the past to treat alcoholism (16). Others have found that HNO has the properties of an endothelium-derived hyperpolarizing factor and/or endothelium-derived relaxing factor (*e.g.*, Ref. 1). Thus, it is becoming increasingly evident that HNO can be developed as a therapeutic agent of significant importance (or is an endogenously generated signaling species). Driven by these recently reported biological effects, as well as others, efforts to elucidate the possible physiological targets and signaling mechanisms of HNO have been substantial. To date, two particular biological moieties have been reported to be primary targets for HNO, thiols, and metals (*i.e.*, thiol- and metallo-proteins). This review will discuss the biologically relevant chemistry of HNO and indicate how/why these interactions may be responsible for the biological signaling of HNO.

General Chemistry of HNO

Before proceeding further, a comment regarding nomenclature is warranted. The term "nitroxyl" as a name for the molecule HNO is deeply embedded in the lexicon of nitrogen oxide chemical biology. This is unfortunate since it is an ambiguous and noninformative term. It is ambiguous since nitroxides (radical species with the general structure $R_2NO\cdot$/$R_2N^{+\cdot}$-O^-) are often referred to as HNOs and it is noninformative since there is nothing in the term that describes the actual HNO structure/bonding. Indeed, the suffix "yl," although fairly ubiquitous, is sometimes used in organic chemistry to indicate a substitution for a hydrogen on an alkane (*i.e.*, a methyl group is formed from substituting a hydrogen on methane) or to indicate a radical species (*i.e.*, removal of hydrogen from the thiol creates a thiyl). Clearly, the suffix "yl" in the term nitroxyl does not conform to this convention and can even lead to a misunderstanding of its actual structure/chemistry. More appropriate terms include nitrosyl hydride or hydrogen oxonitrate for HNO and oxonitrate(1-) for NO^-. It is important for those studying HNO to realize the ambiguity associated with this term and not confuse literature reports that have nothing to do with HNO. Regardless, because most of the current literature (especially reports describing the biological actions and chemical biology of HNO) utilizes the term "nitroxyl," we reluctantly continue to refer to HNO in this way.

As a prelude to discussing the interaction of HNO with likely biological targets, it is worthwhile to first mention some of the salient features of HNO chemistry. The discussion of HNO chemistry herein is brief and concise. More comprehensive treatments of this topic are available (*e.g.*, Refs. 46, 25). Generally speaking, much of the biological chemistry of HNO is a result of its dual reactivity. That is, HNO can react as either an electrophile or nucleophile depending on the reaction conditions/reaction partners. The nitrogen center of HNO is electrophilic (akin to the carbon atom of a carbonyl function)

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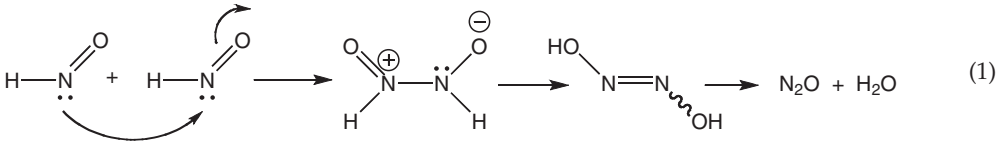
and, therefore, can react with nucleophiles. However, the lone pair of electrons on the nitrogen atom can also be nucleophilic and capable of coordinating metals. This dual reactivity is responsible for a self-dimerization reaction whereby one HNO molecule acts as an electrophile and another acts as a nucleophile, leading to the generation of hyponitrous acid followed by spontaneous decomposition *via* dehydration to form nitrous oxide (N_2O) (Reaction 1) (31).



(1)

The propensity for HNO to react *via* Reaction 1 generally requires studies of HNO to be performed using HNO-donor species (48). Thus, unlike many other nitrogen oxides (NO, NO_2, NO_2^-, NO_3^-, $ONOO^-$, NH_2OH, NH_3, *etc*.) authentic HNO cannot be stored and is typically generated *in situ* for use in biological or chemical studies.

As an electrophile, HNO has the potential to react with a variety of biological nucleophiles. However, it appears that HNO reacts significantly only with soft nucleophiles such as those containing either sulfur or phosphorous (4, 35, 55). The reaction of HNO with hard bases such as water (to form a hydrate) is reported to be thermodynamically unfavorable, likely due to electron repulsion between the numerous proximal lone pairs of electrons on the oxygen and nitrogen atoms (in orbitals of similar size) of the hydrate (4). Thus, in biological systems, thiol proteins appear to be major reaction target for HNO. Moreover, kinetic studies indicate that thiols and phosphines react quickly with HNO (35, 47), further confirming thiols as a likely biological target for HNO. The rate constant for the reaction of HNO with, for example, glutathione (GSH) has been reported to be $2–8\times10^6$ $M^{-1}s^{-1}$ (35, 47). The rate of reaction of the catalytic protein thiolate of papain with HNO has been reported to be significantly greater ($k=2\times10^7$ $M^{-1}s^{-1}$) (69). These rate constants can be compared to other thiol oxidants/electrophiles such as hydrogen peroxide [H_2O_2, $k=0.87\,M^{-1}s^{-1}$, pH 7.4 (70)] and peroxynitrite [$k=1.3\times10^3\,M^{-1}s^{-1}$, pH 7.4 (39)], indicating that HNO is kinetically a much better thiol modifying agent than these other well known small molecule thiol reactive species.

The reaction of HNO with thiols can have at least two possible outcomes depending on the reaction conditions. In a first step, a nucleophilic thiol attacks the electrophilic nitrogen atom of HNO leading to the formation of an N-hydroxysulfenamide (17, 20, 71) (Fig. 1). In the presence of additional thiols (or vicinal thiols), further reaction occurs resulting in the corresponding disulfide and hydroxylamine (Fig. 1, pathway a). In the absence of additional thiols, a rearrangement occurs giving a sulfinamide (Fig. 1, pathway b). The generation of the disulfide product represents a biologically reversible process, whereas the generation of the sulfinamide is much less likely to be easily reversed. Significantly, the generation of the sulfinamide product represents a formal 4-electron oxidation of sulfur performed in a one-to-one stoichiometry between reductant and oxidant. This is unique among most all other biologically relevant thiol oxidants (H_2O_2, ONOOH, NO_2, *etc*.), which are capable of oxidizing a thiol by only 1- or 2-electrons. The thermodynamics of all the reactions in Figure 1 have been examined computationally and every reaction, including the initial formation of the N-hydroxysulfenamide, appears to be exergonic (35).

Although highly speculative at this time, it is also possible that a stable N-hydroxysulfenamide can be formed with select protein thiols. Under purely chemical conditions, N-hydroxysulfenamides are unstable due to the chemistry outlined in Figure 1. However, at the active site of a protein, this intermediate may have increased stability due to interactions with protein residues, sequestration from other reactants in solution, and, therefore, under some circumstances, may have a significant lifetime. Related to this idea is the known stability of several protein sulfenic acids, which under purely chemical conditions are not stable since they will react with thiols (among other things) to give the corresponding disulfide and water. For example, stable sulfenic acids of the cysteine protease papain and the cysteine-based dehydrogenase glyceraldehyde 3-phosphate dehydogenase (GAPDH) have been reported (10, 56). Clearly, to generate a stable N-hydroxysulfenamide in a protein, there cannot be any vicinal thiols that would result in the generation of a disuflide and there needs to be specific stabilization to avoid the rearrangement reaction. Whether any proteins are capable of forming stable N-hydroxysulfenamides is not known. Interestingly, Shen and English (59) have proposed that an intermediate N-hydroxysulfenamide generated in serum albumin may react with a vicinal lysine residue leading to, after oxidation, an internal sulfinamide (Fig. 2). This finding indicates that the N-hydroxysulfenamide formed in serum albumin and possibly other proteins can react with vicinal nucleophiles besides cysteine. Hoffman and coworkers (32) have also proposed a

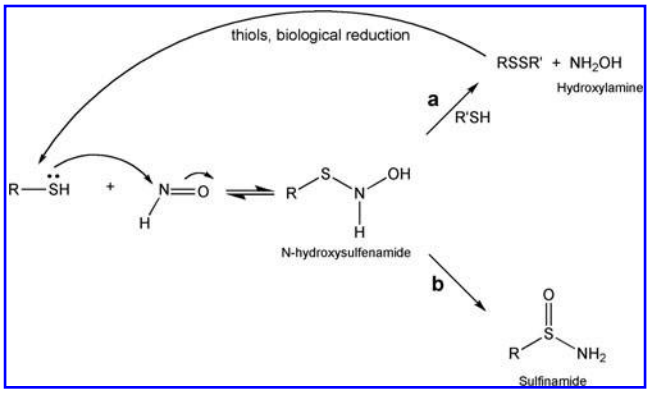


FIG. 1. Pathways for the reaction of HNO with thiols. HNO, nitroxyl.

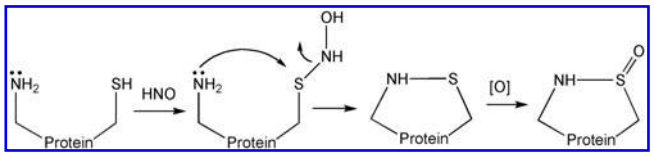


FIG. 2. Possible generation of an internal sulfinamide (29).

similar internal cyclic sulfinamide intermediate in a pathway involving sulfinic acid formation from a sulfinamide.

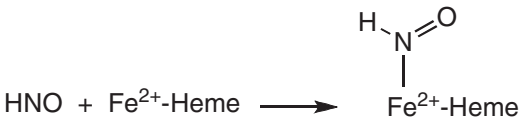
The acid–base chemistry of HNO is a unique and important aspect of its biological chemistry. The pKa of HNO has recently been determined to be ~11.4 (5, 57), a substantial revision of a previously reported value of 4.7 (28). Significantly, the equilibrium partners for the dissociation of a proton from HNO have different electronic ground states (Reaction 2).

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

That is, HNO is a singlet (all electrons are spin paired), whereas NO^- is isoelectronic with O_2 and therefore has a triplet ground state (it has two unpaired electrons residing in degenerate anti-bonding orbitals). What this means is that a spin flip occurs during the deprotonation along with significant nuclear reorganization (58), slowing the process to the point that it is likely to be biologically irrelevant. Thus, generation of either HNO or NO^- in solution will not result in rapid equilibration between the two equilibrium partners and other chemistries associated with either HNO or NO^- will occur long before equilibration. As most donors of HNO are thought to release HNO rather than its conjugate base NO^- (although it is not always clear that this is the case), the biological activity will be due solely to HNO with little, if any, contribution by the conjugate base NO^-. On the other hand, if NO^- (assumed to be the triplet species $^3NO^-$) is generated in a biological solution its protonation is also very slow and other faster reactions are likely to occur before an equilibrium with its conjugate acid, HNO, sets up.

The reduction potential for the $NO/{}^3NO^-$ and $NO,H^+/HNO$ couples is reported to be -0.81 V and -0.55 (*vs.* NHE, pH 7), respectively (57). These negative values indicate that $^3NO^-$ and HNO will be good reductants. Moreover, the H-NO bond dissociation energy is only ~47 kcal/mole (19), indicating that HNO can be a good hydrogen atom donor. Based on these physical constants, it is predicted that HNO can have antioxidant properties akin to ascorbate and/or tocopherols. For the sake of comparison, the reduction potential for the ascorbate·/ascorbate$^-$ couple is 0.28 V (*vs.* NHE, pH 7) and the H–O bond dissociation energy of the phenolic hydrogen of tocopherol is ~78 kcal/mole. Therefore, HNO/NO^- should be very proficient at quenching (reducing) oxidizing radicals.

The nucleophilicity of HNO is manifested in its ability to coordinate metals *via* donation of the nitrogen lone pair to the metal center. However, the overall bonding interactions can also include a prominent π-backbonding component, similar to that found in CO−, NO−, and O_2− metal complexes (21). Of particular possible biological relevance is the reaction of HNO with ferrous heme proteins to form a ferrous–HNO complex (Reaction 3).



(3)

The Farmer lab has synthesized and characterized a series of stable HNO–ferrous heme protein complexes (40, 42, 63, 64). The HNO–ferrous heme complexes of several globins have been reported to be stable for weeks under anaerobic and dark conditions. The fact that these HNO adducts are stable for this period of time indicates that the dissociation rate constant for HNO must be very low since significant dissociation of HNO would lead to spontaneous and irreversible dimerization of the dissociated HNO ligand (Reaction 1) and loss of the complex. The visible absorbance spectra of NO and HNO adducts of ferrous heme proteins (such as hemoglobin or myoglobin) are very similar and often difficult to distinguish. However, they are easily distinguishable *via* NMR since the proton of the HNO-ligated species has a characteristic ^{1}H-NMR peak around 15 ppm (40, 42), which of course is missing in NO adducts. The rate constants for the association of HNO with a variety of ferrous globins have been reported to be $1.2–9\times10^5$ $M^{-1}s^{-1}$ (40). For comparison, the association rate constants for the reaction of NO with similar ferrous heme proteins has been determined to be substantially greater, $1.7–2.4\times10^7$ $M^{-1}s^{-1}$ (49).

One potentially important aspect of biological HNO coordination to a ferrous heme is the fact that this complex is a good one-electron reductant capable of reducing, for example, methyl viologen (42) (Reaction 4). Thus, the binding of HNO to a ferrous heme protein will increase its ability to be oxidized (21), leading to the generation of the corresponding ferrous nitrosyl complex.

$$\mathrm{HNO\text{-}Fe^{2+}\text{-}heme} \rightarrow \mathrm{ON\text{-}Fe^{2+}\text{-}heme} + \mathrm{e}^- + \mathrm{H}^+ \qquad (4)$$

HNO will also react with ferric heme proteins such as myoglobin generating the corresponding ferrous-NO complex in a process referred to as reductive nitrosylation (20) (Reaction 5).

$$\mathrm{HNO} + \mathrm{Fe^{3+}\text{-}Heme} \rightarrow \mathrm{ON\text{-}Fe^{2+}\text{-}Heme} + \mathrm{H}^+ \qquad (5)$$

The rate constant for the reaction of HNO with Fe^{3+}-myoglobin is reported to be 8×10^5 $M^{-1}s^{-1}$ (47), a similar value to what is found for the reaction of HNO with ferrous proteins. Even higher values have been reported for ferric heme model systems, 1×10^7 $M^{-1}s^{-1}$ (3). HNO will also react with ferrous–dioxygen heme complexes, such as oxymyoglobin, leading to the oxidation of iron. Doyle and coworkers propose that this reaction also generates peroxide and nitric oxide (Reaction 6) (20).

$$\begin{aligned}\mathrm{HNO} + \mathrm{O_2\text{-}Fe^{2+}\text{-}Heme\ (myoglobin)} \\ \rightarrow \mathrm{Fe^{3+}\text{-}Heme} + \mathrm{HOO}^- + \mathrm{NO}\end{aligned} \qquad (6)$$

However, Reaction 6 is not fully established and other mechanisms are possible (*e.g.*, Ref. 46). Clearly, HNO has the potential to react with other types of biological metal centers as well. However, the discussion herein will be limited to heme proteins since they appear to be likely targets for HNO,

have been amply studied, and are representative of the types of reactions possible with redox metal species.

The discussions above indicate that the biological chemistry of HNO can be diverse. The lone pair of electrons on the nitrogen atom can be nucleophilic with the potential to react with electrophiles (and metal centers). The nitrogen is also electrophilic and reacts especially well with soft nucleophiles. Finally, HNO can act as a good one-electron reductant *via* H-atom donation with an ability to react with oxidants. The unique acid–base equilibrium of HNO/NO^- indicates that whichever species is initially formed will be the species responsible for the biological activity because formation of the equilibrium partner is too slow to allow its participation in any biochemistry. Figure 3 schematically depicts the most biologically relevant chemical properties of HNO.

Biological Activity of HNO

Numerous effects associated with HNO administration to biological systems have been reported. Among others, HNO has been found to inhibit platelet aggregation (6), exacerbate postischemic myocardial injury (44), elicit a preconditioning effect that is protective against ischemia/reperfusion injury in the myocardium (51), affect NMDA receptor activity (11, 37), and interact with mitochondria and elements of the electron transport chain (2, 8, 60). Many of these and other effects are discussed in more detail in several recent reviews (*e.g.*, Refs. 26, 34, 54). It is not the intent of this review to comprehensively list and discuss all of these activities, but rather the focus herein is to examine the chemical mechanisms by which HNO elicits its actions. Due to its potential pharmacological utility as a cardiovascular agent and its use for the treatment of alcoholism, studies on the activity of HNO on these systems have been the most numerous leading to the greatest degree of mechanistic definition. Therefore, this review will concentrate primarily on the chemistry of HNO at the targets responsible for its currently considered pharmacological applications. However, it seems probable that many, if not all, of the biological effects of HNO can be due to similar interactions at analogous biochemical targets.

Signaling *via* the Interaction of HNO with Thiols and Thiol Proteins

Many of the biological/physiological actions of HNO can be explained as resulting from interactions with specific thiol proteins that control a number of important cellular functions. The first example of HNO acting as a pharmacological agent was as a treatment for alcoholism. The pro-drug cyanamide (H_2N-CN) can be oxidized by the catalase/H_2O_2 system to generate an N-hydroxy intermediate that spontaneously decomposes to HNO and hydrogen cyanide (15, 50). The HNO thus formed can then react with the cysteine thiolate present at the active site of the enzyme aldehyde dehydrogenase, resulting in enzyme inhibition (16, 61) (Fig. 4). Significantly, the treatment of rat hepatocytes with cyanamide at levels that inhibited aldehyde dehydrogenase did not alter intracellular GSH levels (29), indicating that HNO can alter the function of a thiol protein without necessarily altering the thiol redox status of a cell. Moreover, yeast treated with the HNO donor Angeli's salt were found to have significant inhibition of (GAPDH), another cysteine-based dehydrogenase, with little change in either GSH levels or GSH/oxidized GSH ratios (43). These findings provide evidence that HNO can interact with select proteins without significantly changing the redox status of GSH and, presumably, other thiols in the cell. The reasons for this apparent selectivity have not been determined. However, it has been proposed that the structural similarity and analogous chemical properties of HNO and an aldehyde (HCO–) may predict that catalytic entities that promote thiol attack on aldehydes can do the same for HNO (43).

The ability of HNO to modify certain protein thiols with no apparent effect on GSH or oxidized GSH levels can be the result of several possible mechanisms. It is possible that the rate of reaction of HNO with select thiol proteins is significantly faster than with, for example, GSH and selectivity is based on a kinetic preference. The reported rate constant for the reaction of HNO with GSH is quite high [$2–8\times10^6\ M^{-1}s^{-1}$ (47, 35)]. Considering that intracellular levels of GSH are also typically very high (1–10 m*M*) compared to typical thiol protein concentrations (*e.g.*, GAPDH concentration in yeast is only $\sim 2\ \mu M$, 500–5000 times less than GSH), there needs to be a significant difference in the rate constant between a protein and GSH to attain any degree of kinetic specificity. It is difficult to fathom that a rate constant for the reaction of HNO with a thiol protein can be high enough to allow effective competition with the reaction with GSH. Alternatively, if the initial reaction of HNO with a thiol to make the N-hydroxysulfenamide is reversible, there may be thermodynamic control/preference for some thiol adducts over others. Another possibility is that HNO may react with numerous thiols at similar rates and biological reduction/reversal of only select HNO-modified thiols occurs, leaving only certain thiols that remain oxidized. If this were the case, then the ease

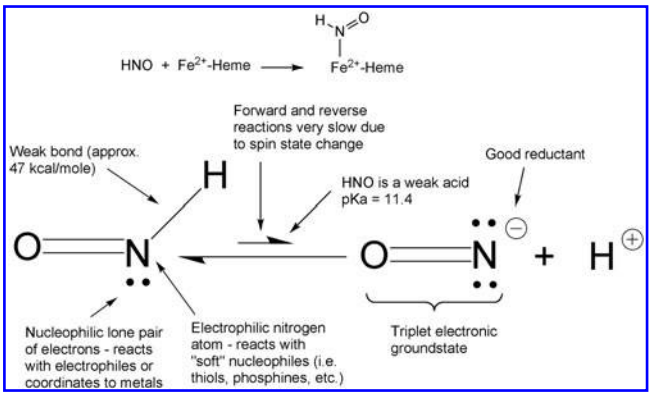


FIG. 3. Biologically relevant chemistry of HNO.

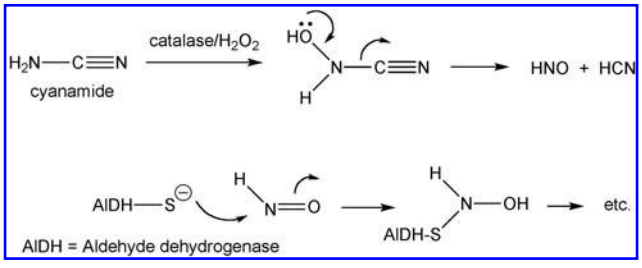


FIG. 4. Bioactivation of cyanamide to HNO and subsequent inhibition of aldehyde dehydrogenase.

of biological reversibility will determine which proteins remain oxidized. Finally, since the reaction of HNO with thiols can result in either reversible or irreversible modification (Fig. 1), thiols that react to give irreversible modifications (*i.e.*, sulfinamide) will eventually be the predominant products since all other HNO-mediated modifications (*i.e.*, disulfide) will be readily reversed by biological reductants. This latter explanation has been used to rationalize the specific inhibition of GAPDH by HNO (43).

Recently, a profound effect of HNO on the failing heart has been described (*vide supra*). Part of the utility of HNO in this regard is the effect HNO has on Ca^{2+} cycling in the myocardium. HNO is able to elicit both inotropy and lusitropy *via* presumed interactions with specific thiol proteins involved in Ca^{2+} release and uptake associated with the sarcoplasmic reticulum (SR) (54). Two seminal reports both indicate that HNO is capable of specifically activating cardiac ryanodine receptors (RyR) leading to an increase in Ca^{2+} release from the SR (9, 66). Significantly, RyRs are known to be regulated *via* the redox activity of critical cysteine thiols (*e.g.*, Ref. 30). For example, the thiol oxidant H_2O_2 is capable of eliciting Ca^{2+} release from the SR (65). Interestingly, the ability for H_2O_2 to cause Ca^{2+} release is enhanced in the presence of GSH or dithiotheitol (DTT), indicating that the mechanism of H_2O_2-mediated activation is more complex than simple direct oxidation of protein thiols by H_2O_2. The idea that HNO activates RyR *via* protein thiol modification is supported by the finding that HNO-mediated Ca^{2+} release from the SR is reversed by the thiol reductant DTT (9, 66). These observations indicate that the reaction of HNO with the critical regulatory cysteines of RyR results in disulfide (or N-hydroxysulfenamide, *vide supra*) rather than sulfinamide products (Fig. 1, pathway a, not pathway b).

The effect of HNO on cardiac function appears to be more involved than simple activation of RyR. Tocchetti and coworkers (66) also showed that the Ca^{2+} uptake pump SERCA2a is also activated by HNO. The mechanism of SERCA2a activation has been attributed to HNO-mediated oxidation of critical cysteine thiols present on its regulatory protein partner phospholamban (PLN). Importantly, it was also shown that thiol targets in the transmembrane domain of PLN were crucial to the actions of HNO since a mutation of cysteine to alanine abolished the effect of HNO compared to wild type (23). As was observed with RyR, the effect of HNO on PLN was reversed with the thiol reductant DTT, implicating HNO-mediated disulfide or N-hydroxysulfenamide (as opposed to sulfinamide) formation in PLN. Interestingly, the presumed regulatory thiols of PLN exist in the hydrophobic transmembrane domain and have been found to be important to the biophysics and regulatory function of PLN (36). Thus, HNO appears to have the ability to partition into hydrophobic compartments and react with membrane cysteines. The net effect of HNO on both RyR and SERCA2a/PLN is to enhance Ca^{2+} cycling resulting in both inotropy (contractile force) and lusitropy (myocardial relaxation).

Along with the effects HNO has on Ca^{2+} cycling from the SR, HNO also has effects directly on the myofilaments (14). That is, HNO sensitizes myofilaments to Ca^{2+}, leading to an increase in contractile force without increasing Mg-ATPase activity. Significantly, HNO-mediated sensitization of the myofilaments was reversed by the addition of DTT, again implicating an HNO–protein thiol interaction that generates a DTT-reversible adduct (*i.e.*, disulfide or N-hydroxysulfenamide as opposed to sulfinamide, Fig. 1). HNO seems to affect only targets associated with Ca^{2+}-cycling since the inhibition of Ca^{2+}-cycling (SR function) resulted in a loss of the positive inotropy associated with the systolic Ca^{2+} transient amplitude (38). Moreover, it was shown that other possible products associated with the use of the HNO-donor Angeli's salt such as nitrite (NO_2^-) and hydroxyl radical ($HO\cdot$) play no role in the effects on Ca^{2+}-cycling.

The remarkable multi-faceted effect of HNO on the heart appears to be the result of specific interactions of HNO on thiol proteins involved in Ca^{2+} cycling and sensitization. In all cases, the reaction of HNO with critical cysteines on these proteins results in oxidation products that are easily converted back to the thiol oxidation state by exogenous thiol reducing agents (*e.g.*, GSH or DTT). This implicates the HNO–thiol reaction product in all cases to be a disulfide since a sulfinamide product would not be reversed by simple thiol reductants. Alternatively, as mentioned previously, there remains the possibility that selective formation of a stable N-hydroxysulfenamide in certain proteins results in an alteration of function and subsequent reduction of the hydroxysulfenamide back to the thiol *via* administration of an exogenous thiol reductant reverses this effect. Regardless, as pointed out by Dai and coworkers (14), it seems improbable that the effects of HNO on the myocardium are a result of a global and nonspecific oxidation process since other indiscriminant oxidants have been shown to elicit decreased force of contraction and Ca^{2+} transients (27).

Other examples of interactions of HNO with thiol proteins have also been described. As might be expected, HNO has been reported to inhibit cysteine proteases, presumably *via* oxidation of the active site cysteine thiolates (67, 68) and, as mentioned above, HNO inhibits the glycolytic enzyme GAPDH. Significantly, Shen and English (59) have confirmed that either sulfinamide or disulfide (Fig. 1) can be formed in a variety of thiol proteins and the microenvironment of the cysteine thiol plays a significant role in determining which reaction product is formed. To date, it is not known whether the interactions of HNO with cysteine proteases or other cysteine proteins are important to its biological activity, although the inhibition of GAPDH has been proposed to play a role in the vascular effects associated with HNO (43).

A recent proteomic study confirms the propensity for HNO to modify thiol proteins *via* two pathways (31). In this study, platelets treated with the HNO (*via* Angeli's salt) resulted in numerous thiol proteins with sulfinic acid modifications (presumably generated from the corresponding sulfinamide). Closer inspection of modified GAPDH isolated from HNO-treated platelets found Cys-152 in the active site and Cys-156 formed a disulfide bond, consistent with previous studies showing inhibition of GAPDH by HNO.

Signaling *via* the Interaction of HNO with Metalloproteins

As described above, HNO has the ability to react with metal centers in proteins. Currently, however, there is little evidence that this type of interaction is relevant in biological (mammalian) signaling. This is not to say that HNO–metal interactions may not be important. Indeed, none of the signaling mechanisms responsible for the known biological actions of HNO are fully established and it is entirely possible (if

not likely) that HNO–metal interactions can participate in HNO biology/pharmacology.

It was reported in the early 1990s that HNO generated from a variety of sources could elicit vasorelaxation and stimulate cGMP production, presumably *via* activation of the enzyme-soluble guanylate cyclase (sGC) (24). Since sGC is a heme protein that is activated by coordination of NO to the regulatory ferrous heme group, this early study alluded to a possible interaction between HNO and a heme protein to elicit a biological response *via* second messenger cGMP generation. Subsequent studies aimed at elucidating the mechanism of vasorelaxation by HNO in resistance vessels found that the HNO–donor Angeli's salt can activate sGC as well as activate voltage-dependent K^+ channels (33). In this study the authors proposed that HNO may have been converted to NO (the firmly established sGC activator) *via in situ* one electron oxidation and the activation of sGC was due to this event. This appeared to be a particularly attractive idea regarding Angeli's salt-mediated sGC activation since a previous study reported that among the simple diatomic redox congeners NO, NO^-(HNO) and NO^+, only NO was capable of activating sGC (18). However, the study claiming the exclusivity of NO in sGC activation was performed in the presence of 10 m*M* dithiothreitol and this level of thiol could have scavenged the HNO before it could interact with the enzyme. More recent work examining the possible interactions and subsequent effects of HNO on purified sGC has led to further confusion regarding the nature of the interaction between HNO and this enzyme. Miller and coworkers found that preparations of purified bovine sGC devoid of exogenous thiols (to prevent possible HNO scavenging by thiols) could be activated by HNO derived from two distinct donors (45). It was proposed that HNO forms a ferrous–HNO complex, leading to enzyme activation, akin to what occurs with NO. However, a similar study also using purified sGC and Angeli's salt as the HNO donor found that HNO does not directly activate sGC (72). However, oxidation of HNO to NO by the enzyme copper–zinc superoxide dismutase does result in enzyme activation. Clearly, more work will be required to reconcile these studies and it remains to be established whether HNO is capable of directly activating sGC or if it is converted to NO, which then activates the enzyme. Significantly, both studies found that HNO does not activate the oxidized ferric heme form of the enzyme (45, 72). This was somewhat surprising since NO activation of sGC is the result of binding to the ferrous enzyme to form the NO–Fe^{2+} species and the reaction of HNO with the Fe^{3+} enzyme should generate the same species (Reaction 4). This finding can be reconciled by considering that the ferric protein is relatively inert to substitution. There is some evidence for this since others have shown that changes in the ferric heme spectrum in sGC requires very high concentrations of azide or cyanide (prototypical Fe^{3+} ligands) (62). It is difficult to perform analogous experiments with HNO since exposure of proteins to high levels of HNO for a significant amount of time is precluded by the dimerization/dehydration reaction (Reaction 1), which prevents the generation of concentrated or long-lasting solutions of HNO. To be sure, the interaction between HNO and sGC is complex as there are multiple regulatory thiols on this enzyme (*e.g.*, Refs. 12, 13) that may also be susceptible to interaction with HNO. Indeed, HNO will inhibit the activity of enzyme devoid of heme or iron, presumably *via* reaction with protein thiols (45). In fact, in the Miller *et al.* study it is proposed that HNO can activate sGC *via* interaction with the ferrous heme and at higher concentrations the activation is blunted *via* HNO-mediated protein thiol oxidation. In this way high levels of HNO will not lead to potentially deleterious hypotension (unlike NO).

As discussed above, a potentially important aspect of HNO–ferrous heme complexes is that it can be easily oxidized to the ferrous–nitrosyl complex with even relatively weak oxidants. Indeed, coordination of HNO to a ferrous–heme may be a mechanism for the facile oxidation of HNO to NO. For example, the ferrous–HNO complex of myoglobin is readily oxidized by methyl viologen (MV^{2+}, $E_{1/2} = -0.44$ V, *vs*. NHE) (42), whereas free HNO is not (57). Thus, it would not be unexpected that generation of an HNO–ferrous heme complex with any protein eventually leads to the ferrous–nitrosyl under biological conditions. Although it is not clear that this chemistry is involved with the HNO-mediated activation of sGC in biological tissues, it certainly is a possibility.

It is very likely that HNO coordination chemistry can occur with many other proteins besides sGC. The paucity of reports in this regard should not be taken as evidence that this is not biologically/pharmacologically relevant. This is especially true since HNO bound to a reduced iron heme will undoubtedly be a fleeting species as it can be readily oxidized to the corresponding NO complex *via* simple one-electron oxidation. Moreover, the reaction of HNO with heme proteins in almost any state (ferrous, ferric, or oxygen bound) can lead to the eventual formation of the ferrous–NO complex, which cannot *a priori* be assumed to be derived from HNO (Fig. 5).

Summary

Clearly, one of the most important and intriguing questions regarding HNO signaling is one of specificity. Taking the myocardium as an example, it seems remarkable that HNO will simultaneously activate RyR and SERCA2a, leading to

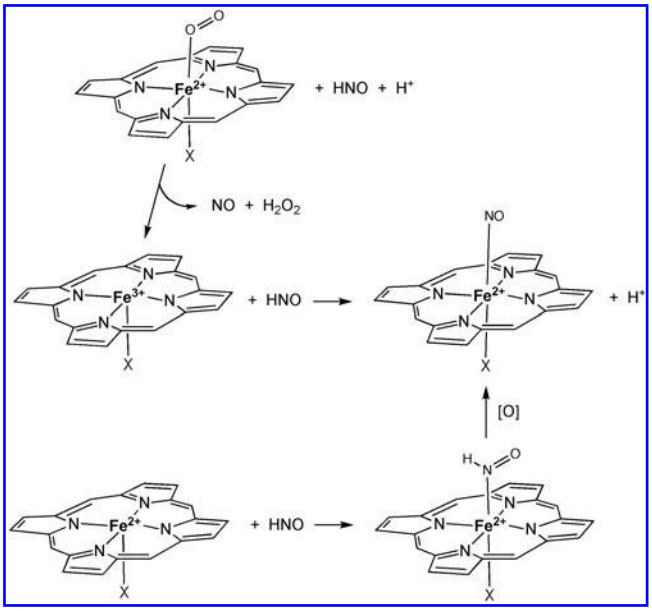


FIG. 5. The reactions of HNO with a variety of iron heme species, ultimately leading to the ferrous–NO complex.

increased Ca^{2+} cycling (giving increased inotropy and lusitropy) as well as sensitize myofilaments to Ca^{2+} without increasing Mg-ATPase activity. Also, all of this occurs without an increase in heart rate or any reported contraindications. Taken altogether, these effects represent a near perfect treatment for heart failure. This appears to be far beyond pharmacological coincidence and raises the question of possible endogenous generation as a response to cardiovascular distress. Clearly, answering this question remains one of the most important goals in this field of research. However, regardless of whether HNO is an endogenously generated species, the question also remains as to how it specifically alters the function of certain proteins without apparently affecting others. This is especially true since the activity of HNO in the heart appears to be the result of interactions with thiol proteins specifically involved in Ca^{2+} signaling. It is important to remember that these proteins exist in a sea of thiols, including millimolar concentrations of GSH. So, how can HNO exhibit selective reactivity with certain thiols/thiol proteins and not with others? Above, we have proposed several possible mechanisms, including kinetic and thermodynamic control, ease of biological reversibility, and reversible *versus* nonreversible modifications. There is also the distinct possibility, though very speculative at this point, that the reaction of HNO with some proteins can result in the formation of a stable N-hydroxysulfenamide and this species confers a special altered activity.

Future work in the area of HNO chemical biology/physiology will undoubtedly focus around several important questions: (i) Is HNO an endogenously generated species participating in fundamental signaling processes? (ii) As either a pharmacological agent or endogenous signaling species, how does HNO achieve any degree of specificity in its actions? These and other questions will likely keep investigators busy for many years to come.

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Abbreviations Used

DTT = dithiothreitol
GAPDH = glyceraldehyde 3-phosphate dehydrogenase
GSH = glutathione
HNO = nitroxyl
H_2O_2 = hydrogen peroxide
PLN = phospholamban
RyR = ryanodine receptors
sGC = soluble guanylate cyclase
SR = sarcoplasmic reticulum

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