

# The Specificity of Nitroxyl Chemistry Is Unique Among Nitrogen Oxides in Biological Systems

Wilmarie Flores-Santana,<sup>1</sup> Debra J. Salmon,<sup>2</sup> Sonia Donzelli,<sup>3-5</sup> Christopher H. Switzer,<sup>1</sup> Debashree Basudhar,<sup>1,2</sup> Lisa Ridnour,<sup>1</sup> Robert Cheng,<sup>1</sup> Sharon A. Glynn,<sup>1</sup> Nazareno Paolocci,<sup>6,7</sup> Jon M. Fukuto,<sup>8</sup> Katrina M. Miranda,<sup>2</sup> and David A. Wink<sup>1</sup>

## Abstract

The importance of nitric oxide in mammalian physiology has been known for nearly 30 years. Similar attention for other nitrogen oxides such as nitroxyl (HNO) has been more recent. While there has been speculation as to the biosynthesis of HNO, its pharmacological benefits have been demonstrated in several pathophysiological settings such as cardiovascular disorders, cancer, and alcoholism. The chemical biology of HNO has been identified as related to, but unique from, that of its redox congener nitric oxide. A summary of these findings as well as a discussion of possible endogenous sources of HNO is presented in this review. *Antioxid. Redox Signal.* 14, 1659–1674.

## Introduction

NITROGEN OXIDES ARE IMPORTANT COMPONENTS of the biochemistry and physiology of many organisms. The actions of these species range from serving as primary sources of ammonia through nitrate/nitrite reductases to regulating vascular tone and neuronal function. Since the seminal discoveries of nitric oxide (NO) as the endothelium-derived relaxing factor (EDRF) (72, 116, 127) and as an antitumor and antipathogen in the immune response (61, 119), the field of NO biology has expanded greatly over the last three decades. Much of the focus has been on the biological effects of NO as well as the interactions of NO with reactive oxygen species (ROS). Since the chemistry of nitrogen oxides is the primary determinant of biological activity (105, 159, 174), an understanding of their mechanisms of action under specific biological conditions is crucial. Due to the high complexity of nitrogen oxide chemistry, producing a comprehensible framework for the chemical biology of nitrogen oxides is an evolving process.

Although nitroxyl (HNO) has long been suspected to be an intermediate in the nitrogen cycle, particularly denitrification, it is a relative latecomer to the class of nitrogen oxides that are important to mammalian biology (106). HNO was

originally a candidate for the EDRF (50), but was largely ignored once NO was identified as this species. However, over the last decade discovery of unique biological properties in the cardiovascular system (128, 129) and in receptor response (56) has led to a significant expansion in HNO-related research. Interestingly, many of the biological effects of HNO are distinct and at times opposite to NO (173). The basis for the unique effects of these nitrogen oxides is distinct targets (112). For example, HNO preferentially reacts with thiols and ferric complexes, whereas NO prefers radicals and ferrous complexes (174). The interesting pharmacological effects of HNO and the relationship to NO suggest the possibility that HNO is a component of redox signaling (44, 112, 130). In this review, the chemistry that makes HNO unique among the nitrogen oxides is described.

## Donors of HNO

Endogenous production of HNO both in bacteria and mammals has been a matter of speculation for decades. Although there are a number of potential routes to HNO biosynthesis, as discussed below, to date data on the effects of HNO on biomolecules, in cultured cells and in mammalian systems, have been acquired with HNO donors. Such species

<sup>1</sup>Radiation Biology Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland.

<sup>2</sup>Department of Chemistry and Biochemistry, University of Arizona, Tucson, Arizona.

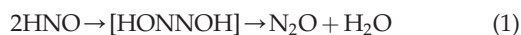
<sup>3</sup>Department of Neurology, <sup>4</sup>Department of Experimental and Clinical Pharmacology and Toxicology, and <sup>5</sup>Cardiovascular Research Center, University Medical Center Hamburg-Eppendorf, Hamburg, Germany.

<sup>6</sup>The Johns Hopkins Medical Institutions, Baltimore, Maryland.

<sup>7</sup>Department of Clinical Medicine, Section of General Pathology, University of Perugia, Italy.

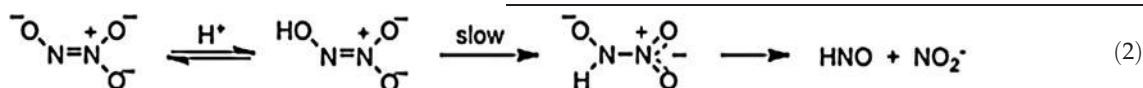
<sup>8</sup>Department of Chemistry, Sonoma State University, Rohnert Park, California.

are required because HNO is metastable due to dimerization to hyponitrous acid followed by irreversible and rapid dehydration [Eq. 1 (84, 89)] [ $8 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$  (145)].

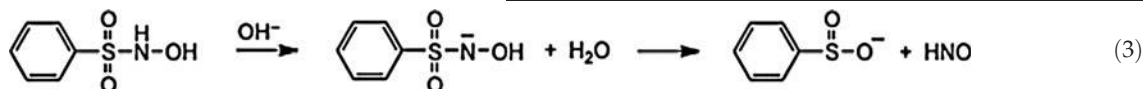


The two most commonly used donors of HNO are Angeli's salt (sodium trioxodinitrate,  $\text{Na}_2\text{N}_2\text{O}_3$ ) and derivatives of sulfohydroxamic acid, particularly Piloty's acid (benzenesulfohydroxamic acid,  $\text{C}_6\text{H}_5\text{SO}_2\text{NHOH}$ ). Several clinically used compounds such as cyanamide and hydroxyurea can also be bioactivated to HNO [for reviews on HNO donors, see (70, 79, 80, 110)].

Angeli's salt decomposes spontaneously to produce HNO and nitrite [Eq. 2 (32, 71)] [ $t_{1/2}$  of 3 min under physiological conditions (100)].



Decomposition of Piloty's acid (131) is base-catalyzed (Eq. 3) (15), which allows for investigation of  $\text{NO}^-$  as well as HNO.



### Fundamental Chemistry of HNO with Respect to NO

The relationship of NO to  $\text{NO}^-$  and  $\text{NO}^+$  is central to the chemical biology of NO since all three species have distinct reaction profiles (45). Early pulse radiolysis studies suggested a  $\text{p}K_a$  for HNO of 4.7 (57), indicating that  $\text{NO}^-$  would be the predominant species under physiological conditions. Based on this  $\text{p}K_a$ , the reduction potential for the  $\text{NO}/\text{NO}^-$  couple was calculated to be 0.39 V [*vs.* normal hydrogen electrode (154)]. Thus, NO would be predicted to be rapidly reduced under biological conditions to  $\text{NO}^-$ . Moreover, the rapid (145) reaction of  $\text{NO}^-$  with  $\text{O}_2$  would form peroxynitrite ( $\text{ONOO}^-$ ), which can lead to modification of biomolecules. A number of inconsistencies with this scenario were evident in the literature, however, such as the more facile reduction of  $\text{O}_2$  compared with NO (40, 171) and the observation of distinct effects of NO and HNO donors (112). These discrepancies led to numerous studies focused on determining the fundamental relationships of NO to  $\text{NO}^-$  and HNO.

In 2002, the reduction potential for NO was determined by a variety of techniques to be  $-0.8 \text{ V}$  (8, 145). From this potential the  $\text{p}K_a$  for HNO was derived to exceed 11, indicating that  $\text{NO}^-$  is actually a minor species at physiological pH. Although the reduction potential for NO is predicted to be increased to  $-0.5 \text{ V}$  at neutral pH (8, 145), outer sphere one-electron reduction of free NO is likely to be nearly inaccessible in mammalian systems. In certain prokaryotes, reduction of NO may be possible (92), which is a plausible reason as to why bacteria and mammals respond differently to NO.

That  $\text{NO}^-$  readily undergoes outer sphere electron transfer was important to experimentally verify the reduction potential for NO. Decomposition of an alkyl sulfohydroxamic acid in the presence of a series of viologens at high pH suggested

that  $\text{NO}^-$  had a potential close to  $-0.7 \text{ V}$  (8). Conversely, at pH 7, the HNO donor Angeli's salt did not reduce methyl viologen, which has a potential of  $-0.44 \text{ V}$ . Thus, either HNO does not readily participate in outer sphere electron transfer or the potential is less negative than that of methyl viologen. Moreover, the rate of the reduction of ferricytochrome *c* by HNO (91, 112) is at least several orders of magnitude slower than that by  $\text{O}_2^-$  (13), suggesting a more positive potential for the  $\text{NO}, \text{H}^+/\text{HNO}$  couple than the  $\text{O}_2/\text{O}_2^-$  couple [ $-0.33 \text{ V}$  (154)]. HNO has been shown to be converted to NO by a variety of other oxidants (106) as well, including ferricyanide (172, 177), Tempol (172), and methylene blue (50).

Assuming that HNO is a poor reductant, oxidation to NO could be considered to occur through the intermediacy of  $\text{NO}^-$ . Given that the ground states of HNO and  $\text{NO}^-$  are singlet and triplet, respectively, the acid-base relationship is

interesting [for detailed discussion, see ref. (106)], and proton transfer is inordinately slow due to spin forbiddenness [at neutral pH, deprotonation of HNO occurs with a  $k_{\text{obs}} \sim 5 \times$

$10^{-3} \text{ s}^{-1}$ , whereas protonation of  $\text{NO}^-$  is 10-fold slower (145)]. A large reorganization energy may also be required. Thermodynamic and kinetic impediments to interconversion of HNO and NO are supported by the many studies that now show distinct effects of HNO and NO donors (106, 112, 125, 173).

### HNO Reactivity

Given the fact that NO is a free radical, its chemistry is unexpectedly limited. In biological systems, NO primarily reacts with ferrous systems, oxygen species, and free radicals (174). Furthermore, NO is neither readily reduced [ $\text{NO}/\text{NO}^-$  potential of  $-0.8 \text{ V}$ , (8, 145)] nor oxidized [ $\text{NO}^+/\text{NO}$  potential of  $1.2 \text{ V}$ , (85)]. Conversely, the reactivity of HNO is quite extensive (Table 1). The reactants are generally oxidants, reductants, nucleophiles, or metalloproteins and their analogs. The reactions are varied, but many lead to the formal oxidation of HNO to NO. Such reactions have often been invoked as the basis for the vasoactive properties of HNO donors. In fact, detection of NO in the presence of oxidants, particularly ferricyanide, has long been used as an indirect indicator of HNO production (110). Two-electron oxidants such as flavin adenine dinucleotide (FAD) can also oxidize HNO to NO (52).

Reduction of HNO has received recent interest. Calculations suggest that both one- and two-electron reduction of HNO is favorable [0.6 and 0.8 V to NHOH and hydroxylamine ( $\text{NH}_2\text{OH}$ ), respectively (39)]. Analysis of two-electron reduction of HNO is complicated by the reaction of HNO with  $\text{NH}_2\text{OH}$  (14, 26). While ascorbate induces simple two-electron reduction (76),  $\text{NH}_2\text{OH}$  was not observed in the presence of NAD(P)H (76, 136). Covalent interactions or  $\text{O}_2$ -dependent

TABLE 1. RATE CONSTANTS FOR REACTION WITH NITROXYL OR NITRIC OXIDE

Reactant	$k$ ( $M^{-1} s^{-1}$ )	
	HNO	NO
HNO	$8 \times 10^6$ (22°C) <sup>a</sup>	$6 \times 10^6$ (23°C) <sup>a</sup>
NO	$6 \times 10^6$ (22°C) <sup>a</sup>	NA
	$8 \times 10^6$ (23°C) <sup>b</sup>	
O <sub>2</sub>	$3 \times 10^3$ (37°C) <sup>c</sup>	$6 \times 10^6$ (23°C) <sup>l</sup>
	$1 \times 10^4$ (23°C) <sup>d</sup>	
NH <sub>2</sub> OH	$4 \times 10^3$ (23°C) <sup>e</sup>	$7 \times 10^{-3}$ (25°C) <sup>m</sup>
NO <sub>2</sub> <sup>-</sup>	$1 \times 10^3$ (23°C) <sup>d</sup>	NR
selenomethionine	$9 \times 10^3$ (23°C) <sup>e</sup>	NR
NADPH	$1 \times 10^4$ (23°C) <sup>e</sup>	NR
NADH	$1 \times 10^4$ (23°C) <sup>e</sup>	NR
Thiosulfate	$2 \times 10^4$ (23°C) <sup>e</sup>	NR
Trolox	$2 \times 10^4$ (23°C) <sup>e</sup>	NR
Tempol	$8 \times 10^4$ (37°C) <sup>c</sup>	NR
Tempo	$6 \times 10^4$ (23°C) <sup>e</sup>	NA
Ascorbate	$1 \times 10^5$ (23°C) <sup>e</sup>	NR
Ferricyanide	$> 10^4$ (23°C) <sup>*</sup>	NR
GSH	$2 \times 10^6$ (37°C) <sup>c</sup>	NR
	$8 \times 10^6$ (23°C) <sup>e</sup>	
N-acetyl-L-cysteine	$5 \times 10^5$ (37°C) <sup>c</sup>	NR
GAPDH	$\sim 10^9$ (37°C) <sup>f</sup>	NR
Triscarboxyethylphosphine	$8 \times 10^6$ (23°C) <sup>e</sup>	NR
Cu,Zn SOD	$1 \times 10^6$ (37°C) <sup>c</sup>	NR
	$9 \times 10^4$ (23°C) <sup>g,†</sup>	
MnSOD	$7 \times 10^5$ (37°C) <sup>c</sup>	NA
Ferricytochrome <i>c</i>	$4 \times 10^4$ (37°C) <sup>c</sup>	$7 \times 10^2$ (20°C) <sup>n</sup>
	$2 \times 10^4$ (23°C) <sup>g,†</sup>	
Ferric Mb	$6-8 \times 10^5$ (37°C) <sup>c,h</sup>	$2 \times 10^5$ (20°C) <sup>n</sup>
Ferrous Mb	$1 \times 10^4$ (37°C) <sup>i</sup>	$2 \times 10^7$ (37°C) <sup>o</sup>
MbO <sub>2</sub>	$1 \times 10^7$ (37°C) <sup>c</sup>	$4 \times 10^7$ (37°C) <sup>p</sup>
Catalase	$3 \times 10^5$ (37°C) <sup>c</sup>	$3 \times 10^7$ (37°C) <sup>n</sup>
HRP	$2 \times 10^6$ (37°C) <sup>c</sup>	$2 \times 10^5$ (20°C) <sup>q</sup>
Fe(III)TPPS	$3 \times 10^5$ (25°C) <sup>j,†</sup>	$5 \times 10^5$ (25°C) <sup>r</sup>
Mn(III)TPPS/Mn(II)(TPP)Cl	$4 \times 10^4$ (25°C) <sup>k</sup>	NA <sup>s</sup>

\*Preliminary data from David A. Wink.

<sup>†</sup>Recalculated using the revised dimerization rate constant of  $8 \times 10^6 M^{-1} s^{-1}$  from (145).

<sup>a</sup>(145), <sup>b</sup>(82), <sup>c</sup>(112) <sup>d</sup>(92), <sup>e</sup>(76), <sup>f</sup>(94), (95), <sup>g</sup>(91), <sup>h</sup>(10), <sup>i</sup>(158), <sup>j</sup>(6), <sup>k</sup>(101), <sup>l</sup>(170), <sup>m</sup>(16), <sup>n</sup>(68), <sup>o</sup>(124), <sup>p</sup>(35), <sup>q</sup>(83), <sup>r</sup>(87), <sup>s</sup>(168).

TPPS, meso-tetra(4-sulfonatophenyl)porphyrinato; NR, no reaction; NA, rate constant not available; HNO, nitroxyl; NO, nitric oxide; NH<sub>2</sub>OH, hydroxylamine; GSH, glutathione; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; SOD, superoxide dismutase; HRP, horseradish peroxidase; Mb, myoglobin.

reactions may influence the products. Since reduction of HNO may influence the pharmacological actions of HNO donors, further analysis of such reactions is required.

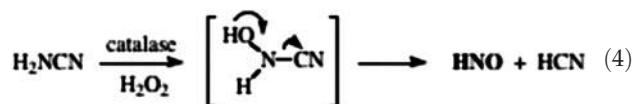
The chemical biology of NO is defined by direct and indirect effects (174). In general, direct interaction of NO with biological targets, particularly ferrous heme or non-heme iron proteins and free radicals, leads to physiological effects or to removal of strong oxidants. Indirect effects arise when NO is oxidized by oxygen species. The resulting nitrogen oxides can lead to oxidative, nitrosative, and nitrative modifications of proteins and DNA, which in turn can have pathological consequences. Given the broader reactivity of HNO (106), such classification may not be as straightforward, but the important direct and indirect reactions of HNO are presented below.

#### Direct reactions

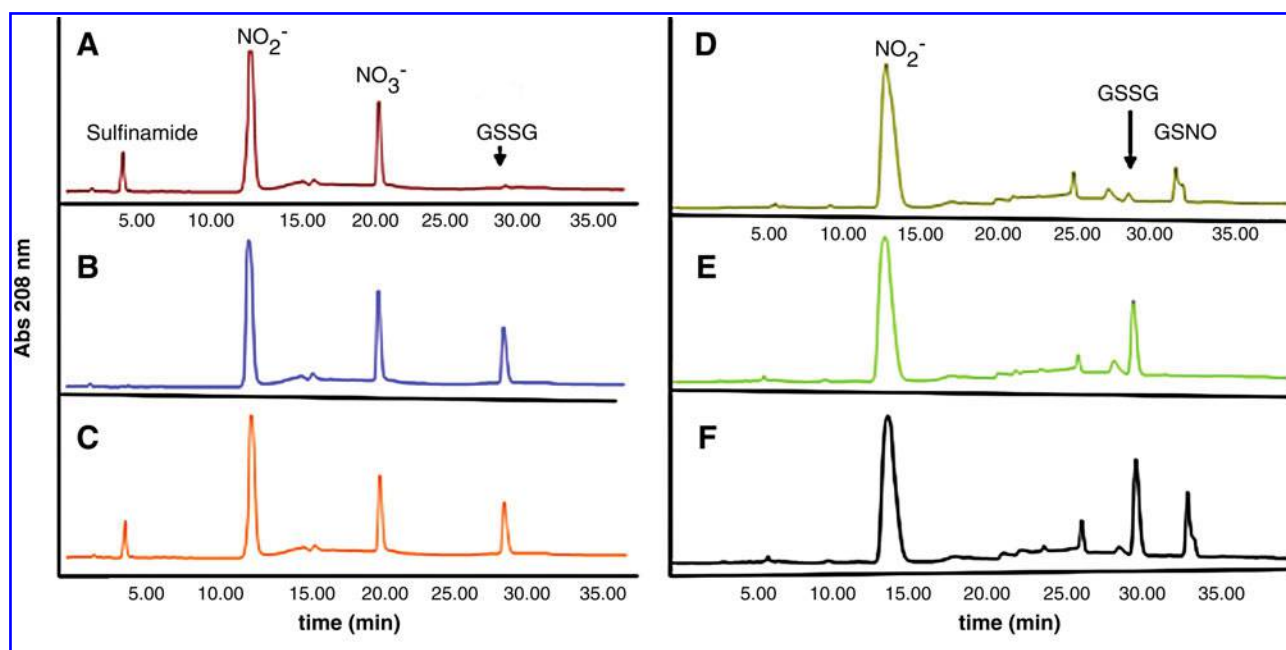
Based on high rate constants relative to the interactions with redox reagents (typically  $\leq 10^5 M^{-1} s^{-1}$ ) and reactant abundance, cellular consumption of HNO is assumed to pri-

marily involve reduction of ferrihemes or oxidation of thiols and oxyhemes.

Thiols. HNO has been demonstrated to function as an electrophile in analogy to formaldehyde (7). Association with thiols is one of the most facile reactions of HNO (Table 1). In contrast, NO does not react directly with thiols (169). The majority of early analysis of HNO was performed by Nagasawa *et al.*, who were interested in the inhibition of aldehyde dehydrogenase with compounds such as cyanamide, which are used clinically to deter alcohol consumption. Metabolism of cyanamide to HNO (Eq. 4) was determined to inhibit a critical thiol on the enzyme (30, 118).



Association of HNO with thiols produces an *N*-hydroxysulfenamide, which is unstable (Eq. 5). In the presence of



**FIG. 1. Indirect high-performance liquid chromatography detection of HNO formation by sulfinamide production.** The reaction was performed in phosphate-buffered saline (pH 7.4) with  $50\ \mu\text{M}$  of the metal chelator diethylene triamine pentaacetic acid (DTPA) for 10 min at  $37^\circ\text{C}$ . All reagents were present at  $100\ \mu\text{M}$ . Samples were filtered prepared and analyzed using the methodology described in (34). Briefly, samples ( $250\ \mu\text{L}$ ) were separated on a Kromasil C-18 column ( $250 \times 4.6\ \text{mm}$ ,  $5\ \mu\text{m}$  particle size, equipped with a guard column; Phenomenex, St. Torrance, CA) by a step gradient from buffer A (pairing reagent  $10\ \text{mM}$  tetrabutylammonium hydroxide,  $10\ \text{mM}$   $\text{KH}_2\text{PO}_4$ , and  $0.25\%$  methanol, pH 7.00) to buffer B ( $2.8\ \text{mM}$  tetrabutylammonium hydroxide,  $100\ \text{mM}$   $\text{KH}_2\text{PO}_4$ , and  $30\%$  methanol, pH 5.50) as follows: 10 min of 100% buffer A, 3 min at up to 80% buffer A, 10 min at up to 70% buffer A, and 12 min at up to 55% buffer A. A flow rate of  $1.2\ \text{ml/min}$ , column temperature of  $18^\circ\text{C}$ , and sample temperature of  $4^\circ\text{C}$  were employed. The wavelength of detection was  $208\ \text{nm}$  except for GSNO, which was  $334\ \text{nm}$ . Ultrapure standards of GSH, GSSG, nitrite, and nitrate were freshly prepared and used to identify eluted peaks by comparison of retention times and absorption spectra. All samples were filtered through a Centricon MW 3000 cutoff filter before high-performance liquid chromatography analysis. (A) Angeli's salt and GSH, (B) Angeli's salt and oxidized glutathione (GSSG), (C) Angeli's salt and a mixture of GSH/GSSG, (D) DEA/NO and GSH, (E) DEA/NO and GSSG, and (F) DEA/NO and a mixture of GSH/GSSG. Sulfinamide formation was observed only in samples containing Angeli's salt and GSH (A, C). DEA/NO reacted with GSH to produce GSNO (D, F). HNO, nitroxyl; GSH, glutathione; GSNO, S-nitrosogluathione; GSSG, glutathione disulfide; DEA/NO, diethylamine NONOate. (To see this illustration in color the reader is referred to the web version of this article at [www.liebertonline.com/ars](http://www.liebertonline.com/ars)).

excess thiol, this intermediate reacts with a second thiol to produce disulfide and  $\text{NH}_2\text{OH}$  [Eq. 6 (37)]. Otherwise, rearrangement generates the sulfinamide [Eq. 7 (152, 175)].

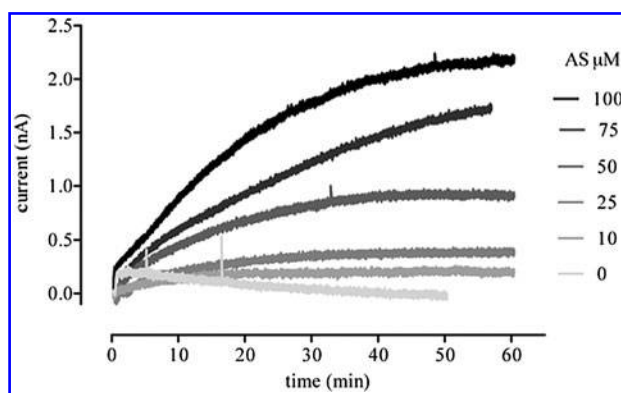


Recent experimental and computational analyses of these interactions have clarified mechanistic details (60, 148, 149). High-performance liquid chromatography detection of sulfinamide has also been offered as an indicator of HNO production (34). Figure 1 shows the specificity of sulfinamide production when glutathione (GSH) is exposed to an equimolar concentration of Angeli's salt compared with the structurally related NO donor diethylamine NONOate. Conversely, production of  $\text{NH}_2\text{OH}$  can be observed electrochemically as  $1\ \text{mM}$  GSH is exposed to increasing concentrations of Angeli's salt (Fig. 2).

Posttranslational modification of thiols by NO has been the subject of intense interest for years. Although NO does not react directly with thiols under biological conditions (169), oxidative products can induce thiol nitrosation (48, 88), which

is reversible upon exposure to dithiothreitol (DTT). Detailed analysis of inhibition of the cysteine proteases cathepsin B (163) and papain (161) by HNO has demonstrated that the modifications by HNO are at least partially irreversible by addition of DTT, which also reduces disulfides. This difference was attributed to selective formation of sulfinamide by HNO. Several pathways exist to convert glutathione disulfide (GSSG) to GSH [*e.g.*, thioredoxin (65), and glutathione reductase (102)], but sulfinamide may represent a terminal modification. Oxidation to the sulfenic acid, which has the same sulfur oxidation state as the sulfinamide, can be amended enzymatically by sulfiredoxin (77), glutaredoxin (103), or peroxiredoxins (176), but a comparable enzyme for sulfinamides has yet to be identified.

A recent mass spectral study examined HNO-induced modifications in human platelet proteins and found that cysteine-containing tryptic peptides were modified to sulfinamides and, to a lesser extent, disulfide linkages with no other long-lived intermediates or side products (63). Storage or sample preparation led to conversion of the sulfinamide to the sulfenic acid. This study identified 10 proteins with stable sulfinamide bonds such as integrin  $\beta 3$  and actin. The highest sensitivity to exogenous HNO was observed with surface receptors. Interestingly, exposure of bovine serum albumin (BSA) to HNO



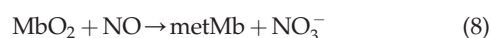
**FIG. 2. Indirect electrochemical detection of HNO formation by hydroxylamine production.** Real-time detection of  $\text{NH}_2\text{OH}$  was performed using an  $\text{H}_2\text{O}_2$  electrode from WPI on an Apollo 4000 series instrument. We found that this electrode is far more sensitive to  $\text{NH}_2\text{OH}$  than to  $\text{H}_2\text{O}_2$  (data not shown).  $\text{NH}_2\text{OH}$  was detected in the presence of  $100 \mu\text{M}$  catalase to eliminate interference from  $\text{H}_2\text{O}_2$ . The reaction conditions were as described in Figure 1 except that  $1 \text{ mM}$  GSH was exposed to varied concentrations of Angeli's salt ( $0$ – $100 \mu\text{M}$ ).  $\text{NH}_2\text{OH}$ , hydroxylamine;  $\text{H}_2\text{O}_2$ , hydrogen peroxide.

results in an as yet unidentified amine cross-link (148), indicating that the initial *N*-hydroxysulfenamide may also undergo nucleophilic attack from another protein residue. Such results suggest that the protein environment is an important determinant in the biological activity of HNO with thiols.

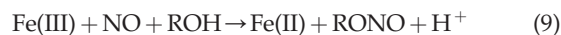
As seen in Table 1, the rate constant for reaction of HNO with GSH is greater than that for *N*-acetyl-L-cysteine. We have estimated a slight increase in the rate constant with BSA ( $8 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ , preliminary data). This trend implies that protein thiols may be more susceptible to attack by HNO than peptides or free amino acids. Furthermore, a number of protein thiols, including aldehyde dehydrogenase (118) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (94, 95), have been observed to be modified by HNO despite high cellular concentrations of GSH. Based on the low cellular concentration of GAPDH [ $2 \mu\text{M}$  in yeast (94)], this could imply a rate constant of  $>10^9 \text{ M}^{-1} \text{ s}^{-1}$ . The basis for such selectivity has been offered to be protein-induced reduction in thiol  $\text{p}K_a$  (34). Given the electrophilic nature of HNO (7), the enhanced nucleophilicity of thiolates is likely to impact the reaction rate over sulfhydryls.

Zinc finger proteins such as poly(ADP-ribose) polymerase (PARP) (153) have also been shown to interact with HNO. In contrast, thioethers such as methione do not react directly with HNO but as discussed below are modified by the product of HNO autoxidation (109). Interestingly, selenomethione, which is a softer nucleophile than methione, does react with HNO ( $10^4 \text{ M}^{-1} \text{ s}^{-1}$ ) (76), implying that selenoproteins may be targets of HNO under certain conditions.

**Metalloproteins.** The primary target of NO is the ferrous heme of soluble guanylyl cyclase (sGC). The nitrosyl complex induces a conformational change in the protein that leads to increased activity and eventually vasodilation. Conversely, reaction of NO with oxyhemoglobin and oxymyoglobin is assumed to be a major consumption pathway for NO (Eq. 8), forming the stable endproduct nitrate and oxidizing the heme.



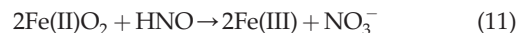
The stability of ferric porphyrin nitrosyl systems is much lower than that of ferrous nitrosyl species (47). However, NO is able to reductively nitrosylate ferric porphyrins upon nucleophilic attack [Eqs. 9, 10 (3, 58, 167)].



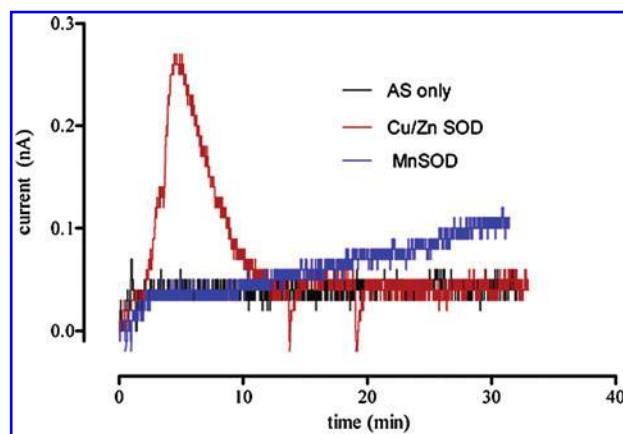
Binding of NO to non-heme systems also has some biological importance [*e.g.*, (18)] that will not be discussed here.

That HNO donors induce vasorelaxation has led to much speculation about whether HNO can interact with sGC in a similar manner to NO. A brief history of the evolution of this question was given above. A complicating factor in the discussion is that very few stable HNO complexes are known, in contrast to the many characterized nitrosyl complexes, which include the formal oxidation states of NO,  $\text{NO}^+$  and  $\text{NO}^-$ . Additionally, sGC is a large protein that includes  $\sim 30$  cysteines, and Fukuto and coworkers (104) have recently suggested that both the heme and at least a subset of the thiols may be important to the effects of HNO on sGC activity. Whether the vasoactivity of HNO donors *in vivo* is directly due to HNO or occurs upon reduction to NO remains unknown. That MbHNO is highly unstable in air suggests that if HNO complexes do form in biological systems, they will typically be short-lived.

As with NO, the reaction of HNO with oxyhemes can be considered to be a consumption pathway. Nitrate and oxidized heme are produced with both HNO and NO with similar rate constants [ $10^7 \text{ M}^{-1} \text{ s}^{-1}$  (35, 38, 112)], but the stoichiometry with respect to the heme is increased for HNO [Eq. 11 (36, 37, 147)].



The mechanism of this reaction has yet to be fully elucidated (106) for discussion.



**FIG. 3. Reactivity of HNO with Cu,Zn SOD and MnSOD.** Real-time measurement of NO produced from Angeli's salt ( $5 \mu\text{M}$ ) and upon the addition of Cu,Zn SOD or MnSOD ( $20 \mu\text{M}$ ) in the presence of GSH ( $100 \mu\text{M}$ ), as in Figure 1 using an NO-specific electrode (ISO-NOP, WPI Apollo 4000 series instrument). In the presence of Cu,Zn SOD, there was rapid conversion of HNO to NO. In contrast, the HNO-MnSOD complex slowly degraded. SOD, superoxide dismutase. (To see this illustration in color the reader is referred to the web version of this article at [www.liebertonline.com/ars](http://www.liebertonline.com/ars)).

Like NO, the association with ferric hemes can lead to reductive nitrosylation, but in a single step [Eq. 12 (11, 37)].



Unlike NO, the association of HNO is generally more favorable with ferric [ $>10^5 \text{ M}^{-1} \text{ s}^{-1}$ , (112)] rather than ferrous [ $<10^4 \text{ M}^{-1} \text{ s}^{-1}$  (86)] hemes. That the ferrous nitrosyl complex can be formed by both NO and HNO illustrates the related nature of these two redox congeners. However, reaction with metMb in air is one of the most common methods to detect HNO (10, 110). Other ferric proteins, including cytochromes and peroxidases, also undergo reductive nitrosylation by HNO (9, 37, 67, 111, 138, 146, 150).

Whether reductive nitrosylation results in free NO depends upon the identity of the metal complex. For example, occupation of the axial sites in ferricytochrome *c* both inhibits the reaction (112) and reduces the stability of the resulting nitrosyl complex (47, 66, 67), such that free NO can be detected (36, 68). Free NO is also produced by the interaction of HNO with Cu,Zn superoxide dismutase (SOD) (90, 117) since Cu(I) does not bind NO due to the  $d^{10}$  configuration metal. Figure 3 shows the rapid increase in detectable signal from an NO-specific electrode when Cu,Zn SOD is incubated with Angeli's salt. Conversely, MnSOD, which forms a stable nitrosyl complex (46), releases NO much more slowly.

Since reductive nitrosylation of a synthetic ferric porphyrin (6) has a similar rate constant to ferric proteins such as metMb (112) [see ref. (106) for full discussion], the rate-limiting step for reductive nitrosylation appears to be addition of HNO to the metal with little structural influence from the protein. For NO, the dissociation of water from ferric porphyrins is the rate-limiting step (68), and this is likely the case for HNO as well. Thus, the stability of the resulting nitrosyl complex determines the profile of NO release and the resulting biological effect of conversion of HNO to NO by interaction with metal complexes.

#### Indirect reactions

As stated above, the direct effects of NO are typically regulatory or protective in nature, whereas oxidized products are typically deleterious. For HNO, posttranslational modification of thiols in particular can be envisioned to inhibit a variety of enzymes, with the impact dependent on function. However, oxidation of HNO induces neurotoxicity and can lead to double-strand DNA breaks.

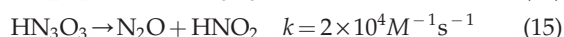
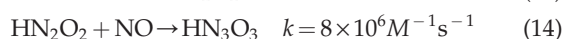
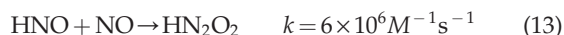
**Reaction of HNO and O<sub>2</sub>.** Decomposition of HNO donors consumes O<sub>2</sub> in aerobic solution (25, 52, 109) without appreciable formation of NO (41, 50, 97, 132, 177). Some caution is therefore required when using a high concentration of HNO donor to evaluate biological properties in closed systems or in containers with low surface areas. Autoxidation of HNO is relatively slow [ $\sim 10^3 \text{ M}^{-1} \text{ s}^{-1}$  (76, 92, 112)] compared with reactions with metal complexes and thiols, suggesting limited importance in the cytoplasm. However, in cell membranes where the concentrations of neutral gases are higher (93) and scavengers such as thiols are less abundant, autoxidation of HNO may become relevant.

Although it is logical to assume that ONOO<sup>-</sup> or peroxynitrous acid is the species generated from the reaction of

HNO with O<sub>2</sub>, there are several important lines of evidence that show distinct differences between synthetic ONOO<sup>-</sup> and HNO donors in aerobic solution (32, 42, 91, 109, 113, 122, 136, 145, 165, 166, 172). Both synthetic ONOO<sup>-</sup> and the HNO/O<sub>2</sub> product readily oxidize dihydrochlorodamine 123 (DHR) by two electrons to rhodamine 123 (109, 172). However, where ONOO<sup>-</sup> readily oxidizes phenols by one electron, this reactivity is not observed with aerobic HNO. Conversely, hydroxylation is more efficient with HNO. Perhaps, most importantly, while ONOO<sup>-</sup> is readily scavenged by CO<sub>2</sub>, the chemistry of aerobic HNO is not altered in carbonate buffer (113). Both ONOO<sup>-</sup> and HNO/O<sub>2</sub> are efficiently quenched by urate and ascorbate (109). Despite experimental and computational efforts (92, 107, 109, 113, 145), the structure of the HNO autoxidation product is currently unknown. The mechanism may involve either direct, spin-forbidden association of O<sub>2</sub> and HNO or nucleophilic addition of HNO to a solvent or target molecule followed by reaction with O<sub>2</sub> with this HNO adduct.

Autoxidation of HNO can be readily distinguished from that of NO by the dyes DHR and 4,5-diaminofluorescein (DAF), which are both converted to fluorescent products much more efficiently by donors of HNO than NO (42, 109). Although NO autoxidation can lead to detrimental modification in proteins and to point mutations in DNA (137, 174), HNO autoxidation can induce DNA double-strand breaks (19, 113, 123, 172), suggesting the potential for higher toxicity.

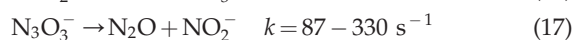
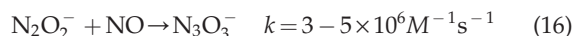
**Reaction of HNO and NO.** Conversion of HNO to NO either by simple oxidation or by reductive nitrosylation can lead to formation of reactive intermediates (Eqs. 13–15) (57, 142, 143, 145).



The coexistence of HNO and NO is certainly conceivable in a cell, although at low concentrations. The rate constant of Eq. 13 (145) is similar to that of HNO dimerization and to reaction with GSH and metalloproteins. Thus, for Eq. 13 to be competitive, biologically the concentrations of efficient scavengers must be low.

The hyponitrous radical is predominantly deprotonated at physiological pH [ $\text{p}K_a$  of 5.5 (96)]. The reduction potential of the  $\text{N}_2\text{O}_2^-/\text{N}_2\text{O}_2^{2-}$  couple has been experimentally determined to be 0.96 V (134), whereas a continuum solvation model has predicted a value of 0.34 V (76). Both potentials support the experimental observation that the NO/HNO reaction produces an oxidant, capable for instance of oxidizing NADH with a rate constant of  $\sim 10^6 \text{ M}^{-1} \text{ s}^{-1}$ . Proton-coupled reduction of  $\text{N}_2\text{O}_2^-$  to  $\text{HN}_2\text{O}_2^-$  ( $\text{p}K_a$ s of  $\text{H}_2\text{N}_2\text{O}_2$  of 7.04, 11.4) is predicted to be more favorable by  $\sim 1 \text{ V}$  (76, 133).

$\text{N}_2\text{O}_2^-$  can also react with a second NO, ultimately resulting in formation of nitrous oxide (N<sub>2</sub>O) and nitrite [Eqs. 16, 17 (57, 96, 142, 143, 145)].



### Pharmacological and Biological Behavior

NO donors are currently in clinical use for treatment of hypertension and angina. In addition to cardiovascular diseases, NO donors are being considered for treatment of a variety of other conditions such as organ transplantation, cancer therapeutics, osteoarthritis, osteoporosis, urinary tract infections, preeclampsia, neurodegenerative diseases, and sickle cell disease. Similarly, significant pharmacological effects of HNO donors have been demonstrated in alcohol metabolism, vasodilation, myocardial contractility, platelet aggregation, neuronal function, angiogenesis, and cancer proliferation. For NO, the most heavily studied effects are in the cardiovascular, immune, and neuronal systems, as illustrated by the names of the three types of NO synthase (endothelial, inducible, and neuronal NOS). Here, the effects of HNO donors on the cardiovascular system, cancer therapy, and neuronal function are described.

#### *The cardiovascular system*

Both NO and HNO are vasoactive in tissue preparations and *in vivo* (50, 52, 53, 117, 164). HNO donors like NO donors induce relaxation in both capacitance and resistance vascular beds in dogs and several rodent species (50, 129). In canine systems, HNO donors behave much as classical nitrovasodilators such as organic nitrates (50, 73, 129). Indeed, HNO relaxes venous capacitance beds as well as arteries and arterioles. In contrast, spontaneous donors of NO have a more prominent action on afterload (*e.g.*, vascular resistance) (129). Thus, both direct and metabolized NO donors such as nitroglycerin are good unloading agents, decreasing both left ventricle dimensions and filling pressures. Consequently, organic nitrates have clinical indications in conditions such as angina, acute myocardial infarction, congestive heart failure (systo- and diastolic dysfunction), and hypertension (1). Since HNO-induced vasodilation is fully preserved in canine failing preparations (128) when compared with controls (129), they may also prove to be useful in treating altered vascular tone in several chronic cardiac diseases. In fact HNO donors have several intriguing features that are unique from clinical NO donors. For instance, initial studies suggest that unlike nitrates (1), HNO donors do not seem to induce tolerance with chronic exposure (74), at least in isolated tissue. Moreover, HNO donors are more amenable for hypertensive crises, or hypertension in general, bringing high blood pressure back to normal values without the risk of severe hypotension such as in the case of certain NO-based agents (4).

Like NO, HNO-induced vasodilation is seemingly a result of activation of a variety of effectors. The primary target for NO is sGC, which converts GTP into cGMP. The initial observations of vasodilation by HNO donors led to speculation about whether HNO could also react with the ferrous heme of sGC. Early studies on partially purified protein suggested that HNO was not able to directly activate sGC (31). However, later demonstrations by Farmer and Sulc (43) that HNO could bind to ferrous myoglobin (Mb) led to further analysis of the interaction of HNO with sGC. Fukuto and coworkers (104) have recently demonstrated that two structurally unrelated HNO donors are able to activate purified sGC. Mayer and coworkers (178) also recently reported that a reductant such as SOD is required for activation by HNO donors. Thus, the question

remains open, and the role of the numerous cysteine residues of sGC may be critical (104).

HNO donors can activate voltage-gated potassium channels ( $K_v$ ) leading to vascular smooth muscle cell hyperpolarization, which is at least in part sGC/cGMP dependent (73, 74). While delineating the mechanistic intricacies of HNO-induced vasorelaxation requires further investigation, the interesting vasodilative profile in combination with studies showing that HNO donors are anti-aggregating agents (12) renders HNO-releasing compounds as attractive alternatives to more traditional nitrovasodilators.

In addition to vasodilation, another striking pharmacological feature of HNO is the ability to enhance myocardial contraction and accelerate relaxation (*i.e.*, positive inotropy and lusitropy, respectively). First described in a canine model under both normal and failing conditions (128, 129), the positive impact of HNO on myocardial function was then mechanistically dissected at the cellular level. Cheong *et al.* (21) showed that HNO donated by Angeli's salt can activate the sarcoplasmic reticulum  $Ca^{2+}$ -ATPase (SERCA2a) pump and ryanodine receptors, which are critical structures in the control of calcium cycling within heart cells. Tocchetti *et al.* then coupled these observations with functional observation in intact myocytes, showing that Angeli's salt increased whole myocyte inotropy/lusitropy in a manner that is cAMP/protein kinase A (PKA) and cGMP/protein kinase G (PKG) independent but sensitive to thiols such as DTT (160). Similar results were obtained in intact rat cardiac muscle in which Angeli's salt increased force development more than the whole calcium transient, also suggesting a prominent impact of HNO directly on the myofilament (27). Again, this effect was offset by pre-treatment or co-infusion of DTT at the peak of force generation. Together, these findings suggest that HNO can target critical cysteine residues in various proteins that are crucial for cardiac electro-contraction coupling.

Froehlich *et al.* have suggested that HNO specifically interacts with cysteine residues present in the transmembrane domain of phospholamban (49). When the inhibitory activation of phospholamban on SERCA2a is relieved, for instance, by PKA-induced phosphorylation of phospholamban (99), SERCA2a activity is enhanced. Thus, despite different mechanisms,  $\beta$ -agonists and HNO donors are able to remove the phospholamban brake on SERCA2a, accelerating the reuptake of calcium from the cytosol into the lumen of the cardiac sarcoplasmic reticulum by SERCA2a (27). That the positive inotropy and lusitropy induced by HNO is not diminished in models of congestive heart failure renders HNO donors as an attractive new avenue to provide inotropic support (at least in the short term) to failing hearts in which inotropic agents such as  $\beta$ -agonists not only lose their efficacy, but are also contraindicated in most cases (115).

Finally, in addition to enhancing cardiac function, HNO donors are protective against myocardial structural and functional disarrangement following global ischemia and reperfusion. NO donors are well known to confer early and late protection against this type of myocardial insult, and have been shown to afford beneficial effects even when given directly at reperfusion (98). This is not the case, however, of HNO donated by Angeli's salt, which is only beneficial when administered before ischemia in a pre-condition type of fashion (126). When Angeli's salt is infused directly at reperfusion, it is both structurally and functionally detrimental.

While this apparent adverse effect may reflect a dose issue, it also points to the intriguing possibility that in ischemia/reperfusion injury, HNO in analogy to its effects on inotropy/lusitropy, may require redox intact switches to provide myocardial protection. Such availability may be greatly compromised in the heart at reperfusion where reintroduction of  $O_2$  leads to uncontrolled formation of ROS. Under such conditions modification of critical or protective thiols by HNO may be an aggravating factor (21). Additional studies are needed to precisely delineate whether, when, and how HNO donors are beneficial in countermanding myocardial damage during ischemia/reperfusion. However, evidence showing that HNO donors are powerful preconditioning agents is another potential factor for fully deploying HNO donating agents as cardiovascular therapeutics.

### Cancer therapy

In addition to cardiovascular effects, HNO donors have shown promise as anti-cancer agents. Angeli's salt is cytotoxic to V79 cells and neurons only at millimolar concentrations (172), which is typical for the diazeniumdiolate class of nitrogen oxide donors. In cell culture or with purified DNA, Angeli's salt can induce DNA double-strand breaks (19, 113, 123, 172). It should be noted that DNA damage is induced with millimolar concentrations of donor, whereas the cardiovascular effects require micromolar doses. The oxygen dependence of such damage suggests that the autoxidation product of HNO is the active species. Whether infusion of HNO donors into animals results in DNA cleavage has yet to be determined. However, autoxidation of HNO is assumed to be a minor pathway as discussed above.

Irreversible thiol modification of GAPDH by HNO affects glycolysis (94, 95). Since most solid tumors must survive in a hypoxic environment, glycolysis is the major energy pathway. Thus, GAPDH and other critical thiol enzymes that facilitate cancer progression may be targets by HNO-donating anti-cancer agents. Consistent with this idea, HNO has been shown to inhibit breast (120) and neuroblastoma (155) cancer proliferation in mouse xenografts as well as in culture. Reduced blood vessel density with the tumors was accompanied by reduced levels of circulating vascular endothelial growth factor and in total hypoxia-inducible factor  $1\alpha$  protein (120). As a result, an increase in apoptosis and the apoptotic factor caspase-9 were observed.

In addition to the direct effects of HNO on cancer cells, HNO donors may be useful adjuvant agents to cancer chemotherapy. HNO has been shown to inhibit PARP in a breast cancer cell line (153). As an important component of the DNA repair machinery, PARP is a major target in the design of anti-cancer agents. Also, since a number of chemotherapies and radiation therapy are based on inducing DNA damage in cancer cells, inhibition of PARP by HNO donors may increase the efficacy of these treatments.

### Neurotoxicity

Although HNO donors show substantial promise in the treatment of alcoholism, cardiovascular conditions and cancer, the toxicological effects, particularly as a result of thiol reactivity or autoxidation, remain to be fully elucidated. As stated above, DNA strand breakage in particular needs to be assessed in whole organisms. Administration of  $10\ \mu\text{mol}$

Angeli's salt into rats has induced neurotoxicity. For instance, injection into cerebral spinal fluid led to motor neuron injury, although sensory neurons were not affected. Similarly,  $400\ \text{nmol}$  of Angeli's salt reduced striatal dopamine 7 after treatment (162). Since GSH appears to play important roles in neuroprotection and neurotoxicity (114), direct injection of an HNO donor into the cerebrospinal fluid may critically alter GSH levels.

Similarly, modification of thiols on receptors may impact neurotoxicity. For instance, HNO has been shown to modify a thiol on *N*-methyl-D-aspartate (NMDA) receptors (78), which are pivotal in the regulation of neuronal communication and synaptic function in the central nervous system. Influx of calcium through NMDA receptors is both glutamate and voltage dependent. Overstimulation of the NMDA receptor, for example, during ischemia/reperfusion injury, leads to excitotoxicity (23). The impact of HNO on NMDA channel function has been shown to be oxygen dependent (25), with augmentation observed under aerobic conditions, whereas attenuation occurred under hypoxia. GSH may also play a role in excitotoxicity *via* NMDA receptor activation (114). Thus, HNO may protect against neuronal damage, for instance, during stroke, similarly to the above-described protective effect of HNO on myocardial damage during infarct. However, a recent examination of the effects on infarct size in mouse brain of Angeli's salt infusion before cerebral artery occlusion suggests that HNO is detrimental due to oxidative damage (22). It is difficult to correlate doses and animal size between mice, rats, rabbits, and dogs, but further analysis of the potential neurotoxicity of HNO donors is required.

### Pathways for Formation of Endogenous HNO

NO is primarily biosynthesized by NOSs (157). There is growing interest in the reduction of nitrite as a secondary source of NO (28). Although significant nitrite can be provided dietarily, endogenous nitrite largely originates from NO. Thus, the  $\text{NO}/\text{NO}_2^-$  cycle is often assumed to be a stor-

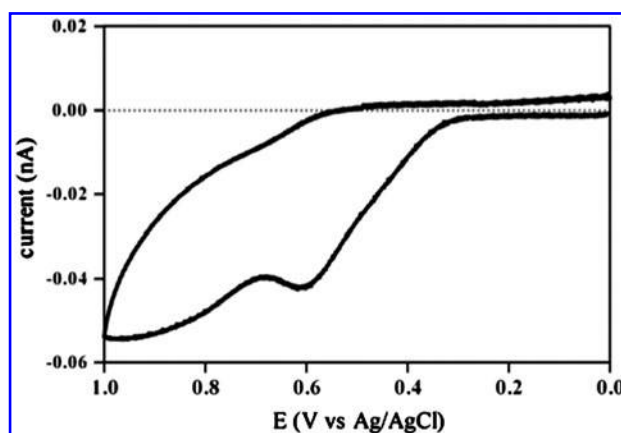
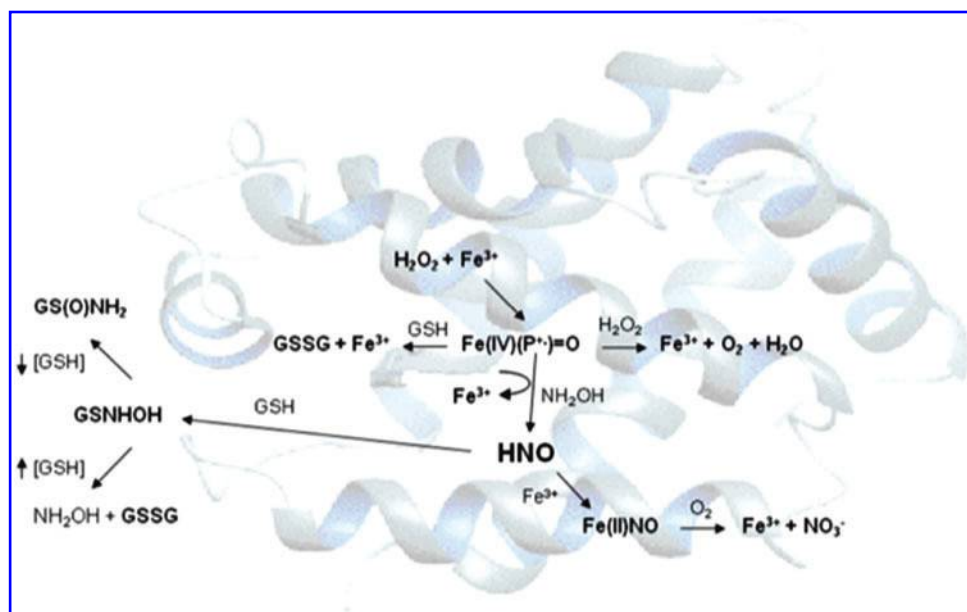


FIG. 4. Cyclic Voltammetry measurements of suberoylanilidehydroxamic acid. The voltammogram was obtained with an EG Potentiostat/Galvanostat Model 273A from AMETEK Princeton Applied Research (Oak Ridge, TN). Measurements were performed on  $2\ \text{mM}$  suberoylanilidehydroxamic acid under aerobic conditions at room temperature in acetonitrile ( $2\ \text{mM}$ ) using the platinum auxiliary electrode and  $\text{Ag}/\text{AgCl}$  (saturated  $\text{KCl}$ ) reference electrode.

**FIG. 5. Peroxidation of  $\text{NH}_2\text{OH}$  by heme proteins [adapted from (33)].**  $\text{NO}_3^-$ , nitrate,  $\text{GSNHOH}$ , glutathione *N*-hydroxysulfenamide;  $\text{GS(O)NH}_2$ , sulfenamide. (To see this illustration in color the reader is referred to the web version of this article at [www.liebertonline.com/ars](http://www.liebertonline.com/ars)).



age mechanism to extend the temporal response to NO production. Conversely, despite the significant pharmacological effects of HNO donors, endogenous production of HNO has yet to be demonstrated. However, the diversity of these pharmacological effects suggests at least the potential for biosynthesis of HNO. Recently, Kemp-Harper and coworkers proposed that HNO is an endothelium-derived relaxing and hyperpolarizing factor in rodent resistance arteries, which supports this hypothesis (4). The metastability and higher reactivity of HNO compared with NO severely complicates definitively detecting HNO in biological systems. Sulfenamide formation (Eq. 7) in particular is of significant interest in the pursuit of selective detection of HNO (34, 63, 148). To date, there are three primary mechanisms proposed for HNO generation: decomposition of *S*-nitrosothiols (RSNO), oxidation of *L*-arginine by NOS under certain conditions, and oxidation of  $\text{NH}_2\text{OH}$ .

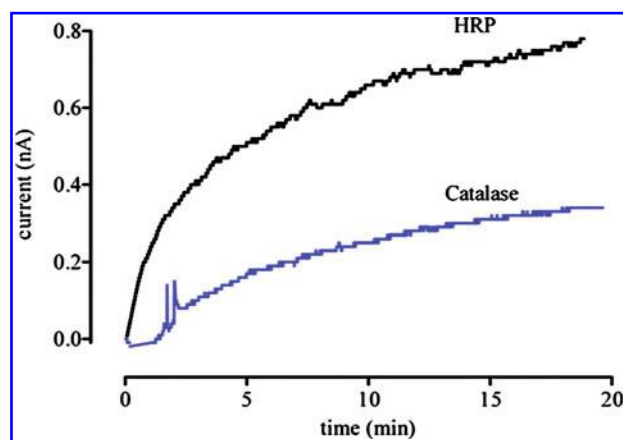
Formation of HNO from RSNO occurs through nucleophilic attack by a sulfhydryl leading to the release of HNO and concurrent formation of the corresponding disulfide [Eq. 18 (5, 64, 121, 141)].



Low-molecular-weight RSNO such as *S*-nitrosogluathione (GSNO) as well as protein RSNOs that do not contain juxtaposed thiols can be long lived. For example, decomposition of GSNO is on the order of hours, whereas the half-lives of *S*-nitrosated DTT or dihydrolipoic acid are  $\sim 9$  min and  $< 20$  s, respectively (5). Furthermore, significant HNO has been trapped only with dithiols. Under cellular conditions, a dithiol can metabolize low-molecular-weight RSNOs through a transnitrosation reaction, which may provide a means to both produce HNO and modulate protein function by disulfide formation. The cellular dithiols thioredoxin and dihydrolipoic acid have recently been shown to catabolize protein RSNOs (144, 156). These species thus may be involved in the regulation of cellular RSNO and in protein function.

RSNOs are commonly detected by the biotin-switch assay (17). This method involves reduction of RSNOs by ascorbate followed by biotin labeling. Interestingly, the reduction of RSNOs by ascorbate leads to significant production of HNO, which can impede detection (81).

Another alternative for HNO generation is during NOS catalysis (2, 4, 50, 55, 62, 135, 140). Detection of  $\text{N}_2\text{O}$  and  $\text{NH}_2\text{OH}$  and dependence of NO production on  $\text{Cu,Zn SOD}$  (62, 75, 140) all suggest that HNO could be an intermediate in NOS metabolism, although this has yet to be conclusively established. Again, detection of HNO is critical to elucidate this pathway.



**FIG. 6. Reactivity of HNO with HRP and catalase.** Detection of NO produced from the aerobic reaction of  $\text{NH}_2\text{OH}$  ( $500 \mu\text{M}$ ) and  $\text{H}_2\text{O}_2$  ( $50 \mu\text{M}$ , added last to initiate the reaction) in the presence of HRP ( $100 \mu\text{M}$ ) or catalase ( $100 \mu\text{M}$ ) as in Figure 1 using an NO-specific electrode (ISO-NOP, WPI Apollo 4000 series instrument). The two profiles show the higher production of NO from HRP ( $500 \text{ nM}$  at 20 min) compared with catalase ( $250 \text{ nM}$  at 20 min). HRP, horseradish peroxidase. (To see this illustration in color the reader is referred to the web version of this article at [www.liebertonline.com/ars](http://www.liebertonline.com/ars)).

The NOS cofactor tetrahydrobiopterin (BH4) has also been indicated to be critical for NO formation (24, 135, 138, 140). That the oxidation state of the iron nitrosyl complex produced during NOS turnover was BH4 dependent (2) suggested that the function of BH4 is to enable NOS to generate NO instead of HNO. Oxidation of NOS metabolites may also generate HNO. *N*<sup>ω</sup>-hydroxy-L-arginine (NOHA), which can be uncoupled from NOS at high levels (59, 138), can be oxidized to release HNO by a variety of oxidants (54, 55). Coupled NOHA (135, 138) can also produce HNO under low BH4 conditions (2, 135, 138, 140). Again, such reactivity has yet to be shown *in vivo*, but condition-dependent products are intriguing, for example, in the control of channel function (25).

NOHA is a derivative of NH<sub>2</sub>OH, which is two electrons more reduced than HNO. NH<sub>2</sub>OH is also a product of the reaction of HNO with thiols (Eq. 6). Thus, oxidation of NH<sub>2</sub>OH can be considered to be a recycling mechanism for HNO as nitrite reduction is for NO. NH<sub>2</sub>OH has been shown to induce vasodilation, indicating that it is metabolized to NO or HNO (29). The two-electron reduction of HNO to NH<sub>2</sub>OH has a potential of 0.3 V at pH 7 (145). Successive one-electron reduction of HNO to NH<sub>2</sub>OH *via* NHOH is also favorable at pH 7 [0.1 and 0.5 V, respectively (51)]. NOHA and other hydroxamic acids such as suberoylanilide hydroxamic acid (SAHA, an anticancer agent *via* inhibition of histone deacetylase) and hydroxyurea (clinically used for treatment of sickle cell crisis) can be oxidized to mixtures of NO and N<sub>2</sub>O (54, 55, 69, 139). Cyclic voltammetry of NH<sub>2</sub>OH shows a single two-electron process (data not shown), whereas NOHA and SAHA are oxidized in two one-electron steps (Fig. 4). Similarly, hydroxyurea has been shown to be oxidized by one-electron to the nitroxide radical (69).

Both NH<sub>2</sub>OH and hydroxyurea can be oxidized by peroxidases (33, 69) [Fig. 5 for NH<sub>2</sub>OH; a related two step process is suggested for hydroxyurea (69)]. Production of HNO is accompanied by reduction of high-valent iron-oxo intermediates to the ferric state, suggesting that reductive nitrosylation (Eq. 12) may occur. Whether HNO will be capable of escaping the heme pocket seems to be protein dependent. A survey of heme proteins (33) showed that proteins such as Mb and myeloperoxidase, which have proximal histidine residues, can generate free HNO, as indicated by formation of sulfinamide. A lower affinity of the ferric heme for HNO may also correlate to a lower affinity for NO upon reductive nitrosylation. Figure 6 indicates that horseradish peroxidase (HRP), which produces a higher level of sulfinamide than catalase, also produces a higher amount of NO as measured by NO-specific electrode. The yield of NO is quite low, however, indicating that this is a minor pathway compared with release of HNO or reductive nitrosylation.

Oxidation of NOHA (33) by HRP did not lead to detection of HNO, indicating some specificity for hydroxamic acid identity in this reaction. HNO has been assumed to be an intermediate of SAHA oxidation by a peroxidase mechanism (139). Addition of HRP/H<sub>2</sub>O<sub>2</sub>/NH<sub>2</sub>OH to cells loaded with DAF resulted in an increase in fluorescence (33), indicating that HNO produced by this reaction can migrate into cells (42). Interestingly, HNO pharmacology has been shown to be effective with cardiovascular conditions in which inflammation will produce H<sub>2</sub>O<sub>2</sub>.

Another potential source of HNO is from the metal-catalyzed dismutation of NO, for example by MnSOD (46). Production of NH<sub>2</sub>OH and GSNO in the presence of GSH

suggests generation of both HNO and NO<sup>+</sup> upon sustained exposure of MnSOD to NO. This reactivity has been suggested to represent a mechanism to protect cells from the deleterious effects associated with overproduction of NO. Furthermore, the interrelated nature of HNO and NO and the impact of biomolecules on fundamental reactivity are highlighted by such a reaction.

## Conclusion

As with other nitrogen oxides of biological interest, the chemical biology of HNO must be analyzed in the context of other biomolecules as well as derivative products. That is, the biological effects associated with HNO can be the result of either direct interactions with specific targets (*e.g.*, thiols or metalloproteins) or indirect effects associated with reaction for example with O<sub>2</sub> and NO. As redox congeners, NO and HNO have similar targets but produce different products through distinct mechanisms (*e.g.*, direct modification of thiols by HNO *vs.* nitrosation of thiols upon NO autoxidation). Additionally, HNO and NO can produce identical products from different targets or by unique mechanisms (*e.g.*, interaction with heme systems to produce ferrous nitrosyl complexes).

At this juncture, protein thiols should be considered to be the primary target of endogenous HNO. Inhibition of enzymes containing critical thiols by HNO can affect alcohol metabolism (30, 118) as well as DNA repair, apoptosis, and glycolysis (95, 120, 153, 155), which affect tumor progression. The cardiovascular effects of HNO donors are driven by thiol-modifications, particularly of calcium channels such as the ryanodine receptor and SERCA2a calcium pump (20, 160). Modification of calcium channels such as the NMDA receptor may also attenuate neurotoxicity (25). However, the neurotoxicity observed in rats upon infusion of low levels of Angeli's salt has been attributed to thiol modification (162). Clearly, the effects of broad-spectrum modification of thiols by HNO donors need to be assessed in detail.

Although stable association of HNO with metals is assumed to be limited in organisms, reductive nitrosylation may be significant pharmacologically. Release of NO upon reductive nitrosylation may explain some of the similarities observed for NO and HNO donors, such as vasodilation. Conversely, formation of a reasonably stable ferrous nitrosyl complex from a ferric resting state may lead to HNO-specific inhibition of enzymes such as cytochrome P450s (108), peroxidases (9), and the mitochondrial respiration complexes I and II (151).

Given the slow reaction of HNO with O<sub>2</sub> relative to GSH, metalloproteins, and other scavengers, autoxidation of HNO is expected to be minimal in whole organisms, in contrast to exposure to purified biomolecules or in cultured cells, where antioxidant systems can be readily overwhelmed. For instance, although Angeli's salt increased lipid peroxidation in brain homogenates at concentrations below 100 μM, such modifications were not observed upon intranigral infusion of rats with up to 400 nmol of Angeli's salt (162). A recent study in mice showing that HNO can exacerbate oxidative stress (22) emphasizes the need for further study of the potential toxicological impacts of HNO administration. This also includes the potential for oxidative modifications when HNO and NO are produced proximally. Such studies are necessary in order to fully realize the potential of HNO donors as pharmacological agents.

### Acknowledgments

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Address correspondence to:  
Dr. David A. Wink  
Radiation Biology Branch  
National Cancer Institute  
National Institutes of Health  
Building 10, Room B3-B35  
Bethesda, MD 20892

E-mail: wink@mail.nih.gov

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#### Abbreviations Used

BH4 = tetrahydrobiopterin
BSA = bovine serum albumin
DAF = 4,5-diaminofluorescein
DEA/NO = diethylamine NONOate
DHR = dihydrorhodamine 123
DTT = dithiothreitol
EDRF = endothelium-derived relaxing factor
GAPDH = glyceraldehyde 3-phosphate dehydrogenase
GSH = glutathione
GSNHOH = glutathione N-hydroxysulfenamide
GSNO = S-nitrosogluathione
GS(O)NH <sub>2</sub> = sulfonamide
GSSG = glutathione disulfide
HNO = nitroxyl
H <sub>2</sub> O <sub>2</sub> = hydrogen peroxide
HRP = horseradish peroxidase
Mb = myoglobin
N <sub>2</sub> O = nitrous oxide
NH <sub>2</sub> OH = hydroxylamine
NMDA = N-methyl-D-aspartic acid
NO = nitric oxide
NO <sub>3</sub> <sup>-</sup> = nitrate
NOHA = N <sup>G</sup> -hydroxy-L-arginine
NOS = nitric oxide synthase
ONOO <sup>E</sup> = peroxynitrite
PARP = poly(ADP-ribose) polymerase
PKA = protein kinase A
PKG = protein kinase G
ROS = reactive oxygen species
RSNO = S-nitrosothiol
SAHA = suberoylanilidehydroxamic acid
SERCA2A = sarcoplasmic reticulum Ca <sup>2+</sup> -ATPase
sGC = soluble guanylyl cyclase
SOD = superoxide dismutase
TPPS = meso-tetra(4-sulfonatophenyl) porphyrinato

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