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The Orthogonal Properties of the Redox Siblings Nitroxyl (HNO) and Nitric Oxide (NO) in the Cardiovascular System: A Novel Redox Paradigm.

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Abstract

Endogenous formation of nitric oxide (NO) and related nitrogen oxides in the vascular system is critical to regulation of multiple physiological functions. An imbalance in the production or availability of these species can result in progression of disease. Nitrogen oxide research in the cardiovascular system has primarily focused on the effects of NO and higher oxidation products. However, nitroxyl (HNO), the one electron reduced product of NO, has recently been shown to have unique and potentially beneficial pharmacological properties. HNO and NO often induce discrete biological responses, providing an interesting redox system. This review discusses the emerging aspects of HNO chemistry and attempts to provide a framework for the distinct effects of NO and HNO *in vivo*.

Keywords: nitroxyl, nitric oxide, cGMP, CGRP, Angeli's salt

Introduction

The surprising discovery in the mid 1980s that vascular tone is modulated by the interaction of endogenous nitric oxide (NO) with soluble guanylyl cyclase (sGC) (60, 93) has stimulated a substantial number of studies attempting to elucidate the role of NO in physiology, particularly in the cardiovascular system. To date, NO has been shown to regulate numerous processes including vascular tone, platelet function, leukocyte recruitment, mitochondrial respiration and cardiac function (7, 23, 42, 58, 92).

The most important determinant of the biological activity of NO is the cellular redox environment. Although NO is a free radical, it is remarkably unreactive toward most biomolecules and primary interacts with other free radicals or with metal complexes such as heme proteins. The redox environment can both modulate these direct reactions and activate NO through generation of reactive nitrogen oxide species (RNOS) that are capable of modifying a wider range of biomolecules than NO itself through oxidative and nitrosative mechanisms (138).

Superoxide (O_2^-) has been shown to attenuate vascular relaxation mediated by NO (37, 45, 61), suggesting that reactive oxygen species (ROS) and NO regulate function in discrete ways. Since these initial observations, the literature addressing the chemistry associated with ROS and NO has been substantial. Early on, autoxidation of NO was proposed to have deleterious consequences through formation of RNOS that could nitrosate, oxidize or nitrate macromolecules such as proteins and DNA (55, 141). These modifications were predicted to exacerbate pathophysiological conditions. However, later kinetic determinations demonstrated that the low concentrations of NO found under *in vivo* conditions limits the extent to which NO undergoes autoxidation (134).

Conversely, the interaction of NO with O_2^- does not have the kinetic constraints of NO autoxidation. This reaction has been proposed to not simply result in scavenging of NO but to convert it to the deleterious RNOS peroxynitrite ($ONOO^-$). This intermediate can both oxidize and nitrate macromolecules (12, 109) and has been suggested to increase oxidative stress resulting in tissue injury (11). However, further evaluation of the chemistry elicited by the NO/O_2^- reaction showed that high oxidative yields were limited to specific ratios of the two radicals (84, 108, 125).

Biosynthesis of NO is now known to not enhance oxidative stress but rather establishes an antioxidant environment (137), protecting cells from oxidative damage by abating lipid peroxidation, DNA strand cleavage and process involved in peroxide-mediated cytotoxicity (41, 56, 99, 136). Vascular homeostasis is regulated by a critical balance between oxidative species and NO with NO shielding against damage to macromolecules by ROS, and ROS in turn restricting the effects of NO.

For example, shear stress in endothelial cells leads to a burst of ROS from NADPH oxidase, which activates a variety of signal transduction pathways including MAP kinase and NFκB (21, 91). This ultimately results in expression of leukocyte adhesion molecules such as MCP-1 (22, 143). Biosynthesis of NO during shear stress down regulates these signal cascades by scavenging ROS whereas consumption of NO by ROS impairs NO-mediated pathways, for instance vasodilation via stimulation of sGC or down regulation of NFκB activity (91).

Complete abatement of both the ROS and NO pathways would in general require the presence of nearly equimolar concentrations of both reactants. Under conditions of excess NO, the oxidative chemistry that leads both directly and indirectly to cellular

injury through modifications of critical biomolecules or activation of certain signal transduction mechanisms will be diminished. However, regulation of cellular metabolism by NO, such as enhanced blood flow and prevention of leukocyte adhesion and neutrophil proliferation during shear stress (72), may still be significant. These interactions between NO and ROS provide a substantially more subtle means to maintain homeostasis than macromolecular interactions, since this binary system functions on the millisecond rather than minute or hour timescale.

Evaluation of the biological properties of NO has primarily focused on species with higher valance states of nitrogen than NO, such as NO_2, N_2O_3 and $ONOO^-$. Reduced valence species such as nitroxyl (HNO/NO^-; nitrosyl hydride/nitroxyl anion), the one-electron reduction product of NO, have been largely ignored. Nitroxyl was initially a candidate for the endothelial-derived relaxing factor (EDRF) (35), however, when NO was clearly established as the EDRF (32, 59, 60), enthusiasm for investigation of nitroxyl waned. Interest in the biological properties of nitroxyl was revived when NO synthase (NOS) was shown to produce nitroxyl rather than NO under certain conditions, particularly at low substrate or cofactor concentrations (1, 54, 110, 113, 115). Nitroxyl may also be formed through other biochemical pathways including decomposition of *S*-nitrosothiols and oxidation of the decoupled intermediate of NOS catalysis, N^G-hydroxy-L-arginine (NOHA) or of hydroxyurea by peroxidase/catalase-like reactions (4, 35, 67, 113, 132).

The availability of NO donor compounds has been invaluable to the elucidation of the biological properties of NO (126). The rate of NO production by NOS is cell-dependent, and NO donors with controlled decomposition rates have been used

extensively to simulate NO biosynthesis (79). At present, Angeli's salt ($Na_2N_2O_3$; sodium trioxodinitrate), which was originally synthesized in the late 1800s (3), is the only compound available that spontaneously releases HNO under physiological conditions (31). Sulfohydroxamic acid derivatives, such as Piloty's acid, also spontaneously release nitroxyl, but only under basic conditions and are subject to rapid oxidation yielding NO rather than HNO (31, 36, 145).

The half-life of HNO release from decomposition of Angeli's salt is 2.5 min under physiological conditions (79).

$$N_2O_3^{2-} + H^+ \rightarrow HNO + NO_2^- \quad (1)$$

The diethylamine/NO adduct, DEA/NO (sodium salt), releases NO with well established, nearly identical kinetics to Angeli's salt (79),

$$Et_2NN(O)NO^- + H^+ \rightarrow Et_2NH + 2NO \quad (2)$$

allowing direct comparison of the biological properties of NO and HNO. For instance, the cytotoxicity of Angeli's salt, assessed by clonogenic assay (2 logs of kill at 2 mM), is several orders of magnitude greater than that of other RNOS and is comparable to alkylhydroperoxides (135). DEA/NO is not appreciably toxic at a similar concentration (1 mM since decomposition of DEA/NO releases 2 NO; Eq. 2). Further, while DEA/NO protects against oxidative stress, Angeli's salt (0.1 mM) increases the toxicity of ROS such as H_2O_2 and O_2^- (133), suggesting that HNO formation *in vivo* could have deleterious consequences.

The cytotoxicity of Angeli's salt is abated under hypoxic conditions (135), indicating that the toxic species is a product of the interaction of HNO with O_2. The resulting oxidant cleaves purified and cellular DNA (89, 98), while HNO itself inhibits

DNA repair protein activity (120). The oxidative properties of the HNO/O_2 product are similar to synthetic $ONOO^-$, however, the overall chemical profiles are sufficiently distinct to suggest that the reactive intermediate is not $ONOO^-$ (86, 89). For instance, the radical chemistry of $ONOO^-$ such as oxidation of phenols does not appear to be a component of HNO/O_2 chemistry. An important difference between these reactions is that while the flux of NO relative to O_2^- is critical for the oxidation or nitrosation chemistry of $ONOO^-$ (84, 125), reaction of HNO with O_2 results in oxidation at any ratio (89). Although the reactant stoichiometry is 1:1 (86), the structure of the oxidant derived from the HNO/O_2 interaction remains to be determined.

Many studies have utilized alternate NO donors such as sodium nitroprusside (SNP), nitrates, for instance nitroglycerine (NTG), or nitrosothiols, allowing indirect comparisons of the pharmacological properties of NO and HNO in a number of different systems. These *in vitro*, *in vivo* and *ex vivo* analyses have revealed that NO and HNO in general elicit distinct responses (for example (30, 38, 78, 105, 135)), which are highly dependent upon experimental conditions.

NO and HNO in myocardial ischemia/reperfusion and preconditioning.

Ischemia/reperfusion. There is long-standing debate as to whether NO plays a beneficial or detrimental role in ischemia/reperfusion (I/R) injury. The ambiguity is in part a result of extrapolation of *in vivo* pathogenic conditions from *in vitro* toxicological experiments. A retrospective analysis of 92 studies evaluating the modulatory effects of NO in the severity of I/R injury in non-preconditioned myocardium showed beneficial effects of exogenous or endogenous NO in the majority of the contributions (67%; (14)).

In the early 1990s NO donors were determined to decrease myocardial necrosis and reperfusion-induced endothelial dysfunction (121). Similar observations were made concomitantly in the gut mesentery (71). Protective effects of NO were also later demonstrated during brain and liver ischemia (74, 80). In the ischemic heart, NO can provide protection through several mechanisms including inhibition of platelet aggregation (83) and neutrophil activity and adhesion (72) in a cGMP-dependent manner.

The effect of NO, either through exposure to NO donors or L-arginine, is proposed to be dependent upon the stage of I/R with maximal protection against myocardial injury occurring with drug administered either immediately prior to or during onset of reperfusion (14). Furthermore, infarct size and post-ischemic myocardial functional recovery are worse in endothelial NOS knockouts compared to wild-type mice (46, 63, 124). In addition, endothelial NOS deficient hearts demonstrate a transient (<1 h) heightened contractile response in the early periods of reperfusion. In this setting, bolus administration of NO donors prior to the ischemic period prevents the early hypercontractile response during reperfusion while significantly reducing myocardial damage (65).

Protection is due to the antioxidant properties of NO, which not only safeguards against chemical insult from ROS (or RNOS) (138, 140) but also exerts other beneficial effects. For example, NO is a powerful vasodilator and may improve blood flow during reperfusion (80). NO also inhibits inositol-1,4,5-triphosphate signaling, thereby reducing calcium overload (90), and mediates protein kinase C translocation at reperfusion, thus protecting contractile function in isolated rat heart (144). Although the reaction of NO with O_2^- produces $ONOO^-$ (12, 109), which is considered to be cytotoxic (131), NO

donors confer vascular protection against exogenously applied $ONOO^-$ (130) via secondary reactions (44). This multiple protective functionality renders NO an ideal substance to protect against I/R-induced tissue injury.

In striking contrast, HNO from Angeli's salt dramatically increased infarct area, tissue injury and myocardial creatine kinase release in the same cardiac I/R model while aggravating myocardial performance, as suggested by elevated left ventricular end-diastolic pressure (LV dP/dt_{max}) (78). These observations reinforced the perception originating from the initial cytotoxicity studies that HNO enhances oxidative stress while NO is an antioxidant. Although it would be attractive to invoke a mechanism that directly relates I/R injury to cell death, it is worth noting that the Angeli's salt concentration (~1 μM) used in the *in vivo* model was three orders of magnitude lower than that used in the cytotoxicity studies (2 mM; (135)). This suggests that the mechanism of tissue damage is due to modification of a physiological response rather than to massive cell death. In the cardiac I/R model, Angeli's salt increased neutrophil infiltration into the infarcted area by approximately threefold, indicating that HNO enhances the induction of adhesion proteins in endothelial cells (78). Thus, HNO at relatively low concentrations *in vivo* appears to modulate leukocyte trafficking at reperfusion in an opposite manner to NO, in agreement with previous *in vitro* observations demonstrating that Angeli's salt enhances human neutrophil migration under both aerobic and anaerobic conditions (127).

Preconditioning. There is no doubt that pharmacological tools able to improve myocardial function during and after I/R as well as to prevent the incidence of arrhythmias and/or to reduce the extent of the necrotic mass in the reperfused

myocardium are of immense clinical relevance. At present, however, clinically available drugs do not fully mitigate the consequences of myocardial I/R injury. However, hearts exposed to brief, sublethal ischemic insults are more resistant against subsequent, prolonged ischemia (68, 95). This phenomenon of preconditioning (PC) was originally described as an immediate adaptation to brief coronary occlusion (ischemic PC), but was later recognized to be a biphasic phenomenon. Ischemic PC consists of an early phase of protection, which is typically manifested within a few minutes of the initial ischemic episode and lasts 2-3 h, and a late phase, which is characterized by a slower onset (12-24 h) and longer duration (3-4 d) (14). The main difference in terms of functional outcome is that the early phase confers protection only against myocardial infarction whereas the late phase is also effective against myocardial stunning.

NO, RNOS and ROS have been extensively investigated both as triggers and modulators of PC. The role of NO is now fully recognized in PC, and its effects are well defined, particularly in the late phase (25). Recently, a comparative study with Angeli's salt and DEA/NO determined that equimolar HNO (1 μM) appeared to be a more effective preconditioning agent than NO (101). In fact, postischemic (2 h) contractility was similarly improved with ischemic PC or pre-exposure to Angeli's salt, as opposed to control or DEA/NO-treated hearts. Infarct size and LDH release were also significantly reduced in IPC and Angeli's salt groups, whereas DEA/NO was less effective in limiting necrosis. Moreover, the preconditioning features of Angeli's salt appeared to be specific to HNO signaling since the HNO scavenger, *N*-acetyl-L-cysteine (NAC; 4 mM) completely reversed the beneficial effects of Angeli's salt. Thus, exposure to HNO

during reperfusion increases myocardial damage but imparts protection if present prior to reperfusion.

The precise mechanism by which Angeli's salt provides myocardial protection, including if HNO *per se* or its oxidative product is responsible for preconditioning, remain to be elucidated as does whether the isolated heart model extrapolates to *in vivo* conditions and to a more sustained response (i.e. late PC). Nevertheless, these studies suggest that the physiological properties of NO and HNO are orthogonal (i.e. of the same origin but not overlapping) and that the biological response to either species is highly condition dependent.

***In vivo* effects of NO and HNO on normal and failing myocardial contractility.**

NO donors. The modulatory role of NO and its donors on myocardial contractility (inotropy) is still controversial since conflicting results showing positive, negative or neutral inotropic effects for NO have been presented (5). This variability may result from the donor compounds utilized, the amounts of NO generated (129), the redox status of the myocardium (18, 103), the target tissue (e.g. atrial versus ventricular cells) (20) and/or the concurrence of stimuli deriving from the activation of the immune or the autonomic nervous systems (6, 48). Moreover, the majority of these studies were performed *in vitro*, precluding assessment of the effects of NO (or its donors) in a more integrative context.

On the other hand, *in vivo* studies, including those performed in humans, often rely only upon load-dependent parameters, such as changes in LV dP/dt_{max}, which vary greatly with alterations in heart rate, loading (e.g. modifications in preload and afterload)

or myocardial perfusion conditions (e.g. changes in coronary flow). This approach may sometimes contribute to drawing incorrect conclusions, for example, attributing direct primary effects on the myocardium to NO and its related species that in reality are secondary to changes in arterial resistance (afterload), venous capacitance and return (preload) or to coronary perfusion (102). Despite these potential limitations, the effects of NO donors have been explored extensively in humans as well as in experimental preparations under both normal and disease conditions.

In healthy humans, Paulus and colleagues reported that intracoronary infusion of SNP significantly dropped estimated LV end-systolic elastance (Ees), which is a load-independent index of myocardial contractility, while LV dP/dt_{max} remained unchanged (107). Similar results were obtained with substance P, which causes endothelial release of NO, in patients affected by dilated cardiomyopathy (53) or following cardiac transplantation (10). In contrast, use of NOS inhibitors revealed a small baseline inotropic effect in the normal human heart that was not apparent in heart failure patients (24). This observation is in agreement with other studies using L-NMMA as an inhibitor of endogenous NO formation (49, 50). However, whether the effect is positive or negative, both exogenous and endogenous NO appears to have a rather small effect on basal non-stimulated myocardial contractility both in normal and failing hearts.

Unlike the inotropic effects, other cardiovascular features of NO seem to be firmly established and less controversial. For instance, both endogenously produced or exogenously administered NO favorably impact diastolic dysfunction. In particular, intracoronary infusion of substance P induced LV hastening effects, which were accompanied by decreased LV dP/dt_{max}. These effects where even more pronounced in

dilated non-ischemic cardiomyopathy subjects (53). Moreover, NO-induced relaxation in these patients was potentiated by pretreatment with the β-agonist dobutamine (10). These finding are in agreement with a later demonstration that NO production declines in a canine heart failure (HF) model after 4 weeks of pacing. This decrease was accompanied by a significant increase in LV dP/dt_{max} and a reduction in LV stroke work (112). Thus, it appears that NO is beneficial to congestive heart failure patients and particularly for subjects whose cardiac output is largely dependent upon the LV Frank-Starling response (106).

On the other hand, NO donors or nitrates are first-line drugs for acute or chronic treatment of several cardiac diseases conditions. Nitrates and similar agents are used to reduce elevated filling pressures and unload failing hearts by reducing pre- and afterload, thereby enhancing cardiac output (34). However, there is a large body of evidence suggesting that NO and nitrates can themselves blunt adrenergic signaling. This has been indirectly supported by the observation of enhanced dobutamine or isoproterenol-stimulated function in control animals and in humans following NOS inhibition (47, 49, 51). The negative impact of NO on the β-adrenergic response appears to be enhanced in failing myocardium. This effect has been attributed to several factors including altered inducible NOS activity (19), down regulated cGMP catabolism (116) and enhanced oxidant stress (114, 122). β-adrenergic stimulation further increases NO release (64) and can amplify its depressant modulation. Importantly, this negative synergy seems exacerbated in failing myocardium, and this phenomenon has been ascribed to altered inducible NOS. However, the outcome of this interaction might also be a dose-dependent effect of NO, since low doses appear to enhance β-adrenergic stimulation (see (118)).

HNO donors. Recently, the cardiovascular properties of Angeli's salt were examined in a conscious canine model (104, 105). Analysis of pressure-volume (PVA) relationships provided a load-independent approach to dissect the primary effects of NO and HNO on myocardial contractility from changes inherent to loading conditions (e.g. alterations in pre- and after-load). Administration of Angeli's salt to normal chronically instrumented dogs, at a similar dose to the I/R study by Ma *et al*. (78), led to rapid enhancement of left ventricular contractility (positive inotropy) with concomitant lowering of cardiac preload and diastolic pressure (venodilation) without altering arterial resistance (105)). In contrast an equidilatative dose (-14-16% in end-systolic pressure) of DEA/NO and NTG triggered vasodilatation on both the arterial and venous side of the circulation, which is typically accompanied by an increase in heart rate while lacking a significant inotropic response.

A comparable increase in inotropy was observed following administration of Angeli's salt to failing canine preparations obtained after rapid pacing (116) (Figure 1). The reduced pressure through venous dilation and the increased inotropy are similar to the effects obtained with NTG and β-agonists, respectively, in subjects suffering from heart failure. However, inotropy was enhanced additively with coinfusion of Angeli's salt and the β-agonist dobutamine, in stark contrast to the NO donors DEA/NO and NTG, which had a negative or zero impact on dobutamine response, respectively (104). Thus, the unique ability of Angeli's salt to increase myocardial performance without altering heart rate may have therapeutic potential for treatment of cardiovascular diseases that are associated with cardiac depression and elevated venous filling pressures, including congestive heart failure.

The inotropic effect of Angeli's salt was further examined and linked to release of calcitonin gene related peptide (CGRP). In fact, administration of anti-CGRP to normal conscious dogs resulted in abatement of the inotropic effect induced by Angeli's salt (105). Further, infusion of Angeli's salt into normal and failing dogs resulted in elevated plasma levels of CGRP, whereas neither DEA/NO nor NTG had an appreciable effect on basal levels (104). Conversely, plasma cGMP was increased by infusion of DEA/NO or NTG, presumably through activation of sGC, but was unaffected by Angeli's salt. Whether these changes in vasculature CGRP and cGMP levels reflect myocardial level alterations remains to be established.

These results suggest the existence of two mutually exclusive response pathways that involve stimulated release of discrete signaling agents by HNO and NO. Nonadrenergic/noncholinergic (NANC) neurons contain CGRP, which is released upon stimulation by calcium. We propose that HNO mediates release of CGRP, which is responsible, at least in part, for the inotropic effects of Angeli's salt and other HNO donors.

CGRP *per se* has positive inotropic activity involving augmented calcium release (57) and is effective in failing human hearts (39). However, there are dissimilarities between HNO and CGRP signaling. Angeli's salt was equally effective in the presence or absence of β-receptor blockade, yet CGRP-positive inotropy is thought to be coupled to protein kinase A stimulation via increased cAMP (57), and thus, would likely be blunted in heart failure. Hence, it is possible to speculate that NANC peptides are involved in HNO signaling or, alternatively, that there may be other direct effects of

HNO on cardiomyocytes that influence contractility or enhance sensitivity to CGRP signaling.

Chemical Properties of HNO Revisited

The discrete modulation by HNO and NO donors is surprising since NO^- and NO differ by a single electron much like O_2^- and O_2. From this perspective, interconversion between the NO^-/NO couple would be anticipated to be facile since there are a number of biological agents that can interact with either redox sibling through outer-sphere electron transfer (36, 54, 76, 94, 115, 119). However, the orthogonal effects of Angeli's salt and DEA/NO in the cardiovascular system, the I/R injury model and the cytotoxicity studies (101, 104, 105, 135) imply that this causal redox chemistry does not occur *in vivo*.

The pK_a for deprotonation of HNO was originally reported as 4.7 (43), indicating that NO^- was the predominant species at biological pH. Recently, the acid-base equilibria of nitroxyl have been reevaluated (8), and the pK_a for HNO is suggested to exceed 11 (9, 117). Therefore, HNO is now implicated as not only a significant, but likely the *exclusive* species present in the acid/base equilibrium of HNO/NO^- in biological systems. This is an important distinction since the chemistry of the protonated and unprotonated forms of nitroxyl varies substantially. The chemistry of the acid is primarily electrophilic in nature while the conjugate base is principally involved in redox chemistry by outer-sphere electron transfer (e.g. simple electron transfer with the electron in essence jumping from the oxidant to the reductant without covalent association of the reactants).

The reduction potential of NO was recently determined to be lower than -0.7 V versus normal hydrogen electrode (NHE) (9, 117). This potential lies at the high end of

the biological redox scale for eukaryotic cells, indicating that direct reduction of NO to NO^- by simple electron transfer is unlikely to occur *in vivo*. Rather, reduction mechanisms in mammalian biology will reduce O_2 to O_2^- due to a substantially more positive reduction potential (-0.33 V versus NHE) and higher concentration. However, the reverse reaction, oxidation of NO^- to NO, with a potential of higher than +0.7 V, should be quite facile (9). This suggests that infusion of Angeli's salt should increase cGMP in plasma. Yet, as discussed above, plasma cGMP is unaffected by Angeli's salt (104).

The explanation originates from the pK_a of HNO. Decomposition of Angeli's salt (Eq. 1) at physiological pH produces HNO rather then NO^-. The high pK_a and the necessity of a spin flip (from ^{1}HNO to $^3NO^-$) severely limits deprotonation such that NO^- would be expected to have little or no role in the biological chemistry of HNO (88).

Reduction of ferricytochrome *c* by Angeli's salt ($k \sim 10^4$ M^{-1} s^{-1}; (88)) is approximately two orders of magnitude slower than by O_2^- ($k = 4 \times 10^6$ M^{-1} s^{-1}; (13)). Assuming these reactions proceed through outer-sphere electron transfer, this suggests that the oxidation potential of HNO is considerably less than that of O_2^- (+0.33 V versus NHE). Thus, unlike NO^-, HNO is a relatively poor reductant (9), which will thus participate *in vivo* in other faster reactions than outer-sphere electron transfer (88). The redox potentials for NO and HNO indicate that the chemistry of these two species will be entirely discrete (Figure 2). Thus, elevated CGRP levels may be a specific marker for HNO *in vivo*.

Recently, two concurrent studies determined for the first time the approximate rate constants for HNO with common biomolecules. Liochev and Fridovich (75)

evaluated the relative rate constants for reaction of HNO with ferricytochrome *c*, Cu,Zn SOD, O_2 and GSH by competitive studies. Similar techniques were utilized by Miranda *et al*. (88) to ascertain the relatively reactivity of HNO with an expanded number of biomolecules including metmyoglobin (metMb) and peroxidases. In anaerobic aqueous solution, HNO will dimerize to form N_2O after dehydration.

$$2HNO \rightarrow N_2O + H_2O \quad (3)$$

The rate constant for dimerization has been measured as 8×10^6 M^{-1} s^{-1} (117), unlocking a quantitative determination of the rate constants for HNO chemistry from the relative reactivates. The derived rate constants are presented in Table I and exhibit considerable similarities between the two studies (75, 88).

Kinetic evaluation can provide insight into the independent mechanisms of HNO and NO. For instance, the activity of sGC, which has a ferrous resting state (17), is enhanced by NO and unaffected by HNO (26). NO generally associates quite rapidly with both ferric and ferrous hemes (e.g., k = 2×10^5 and 2×10^7 M^{-1} s^{-1} for metMb and Mb, respectively; (73, 100))

$$Fe(III) + NO \rightarrow Fe(III)NO \quad (4)$$

$$Fe(II) + NO \rightarrow Fe(II)NO \quad (5)$$

In contrast, the extra electron in HNO results in reductive nitrosylation of the ferric center (87), and reaction with a ferrous complex would be expected to be transitory.

$$Fe(III) + HNO \rightarrow Fe(II)NO + H^+ \quad (6)$$

The general tendency for ferrous-nitrosyl complexes to exhibit substantially higher stability than the corresponding ferric species is well established (e.g., K = 10^3 and 10^{11} for metMb and Mb, respectively; (73, 100)), although exceptions exist (for review

see (33)). The relative reversibility of ferric-nitrosyl complexes suggests that biological response is likely a result only of ferrous-nitrosyl formation for most iron proteins. Thus, the differential physiological effects of NO and HNO may be in part a result of respective complexation with ferrous (Eq. 5) or ferric (Eq. 6) proteins to generate the same stable ferrous-nitrosyl product.

The derived rate constants in Table I can now provide additional insight into kinetic viability, which is dependent upon both the rate constant, which is a function of the molecular target, and the concentrations of reactants. For instance, the cytoplasm is rich in GSH (1-10 mM; (82)), which reacts with HNO with a high rate constant ((88); Table 1). Conversely, NO only reacts with thiols after conversion to an RNOS (139). Thus, direct stimulation of sGC by HNO will be inhibited by the kinetic restraints imposed by the relative rate constants for and the relative concentrations of GSH and sGC. These kinetic parameters for NO, however, cause reaction with sGC to be quite favorable under the same conditions (17). Thus, molecular targeting at the protein level, by valence state in this case, and the location in the cell dictate reactivity such that elevated cGMP production is only consequent to NO exposure.

Compartmentalization of molecular targets is an important factor in unraveling the basis for the orthogonal behavior of NO and HNO in the cardiovascular system and perhaps elsewhere. Although the rate constants for reaction of HNO with GSH and Cu,Zn SOD are similar (Table I), the relative concentrations of both species in the cytosol (1-10 mM GSH, (82); 10 μM Cu,Zn SOD, (69, 96)) indicate that HNO will preferentially react with GSH. Thus, although interconversion of HNO and NO does occur *in situ*, by for example oxidation of HNO by purified Cu,Zn SOD

$$Cu(II) + HNO \rightarrow Cu(I) + NO + H^+ \quad (7)$$

it can be argued that these reactions are likely to be of little relevance in the cytoplasm and other cellular compartments rich in GSH and other biological redox-active biomolecules due to kinetic constraints.

As stated above, ferrous-nitrosyl complexes commonly exhibit relatively high stability under biological conditions. For example the half life of nitrosylated hemoglobin in red blood cells is approximately 30 min (85). Cyt *c* is an exception to this generality, and free NO is released from the heme (29, 76)

$$Fe(III) + HNO \rightarrow Fe(II) + NO + H^+ \quad (8)$$

However, the low rate constant for reaction of HNO with ferricyt *c* (10^4 M^{-1} s^{-1}; (75, 88) likely precludes significant oxidation of HNO to NO by this protein *in vivo*.

One the other hand, reductive nitrosylation of metMb (Eq. 6) may have significant kinetic relevance (Table 1). However, the ferrous-nitrosyl complex in the globins decays in the aerobic environment of the cell to nitrate rather than free NO (2)

$$Fe(II)NO + O_2 \rightarrow Fe(III) + NO_3^- \quad (9)$$

This reaction is similar to that of NO by oxyHb or oxyMb (27)

$$Fe(II)O_2 + NO \rightarrow Fe(III) + NO_3^- \quad (10)$$

which is considered to be a major pathway for consumption of NO. HNO also reacts with oxyMb although with different stoichiometry and an as yet undetermined nitrogen product (28, 29)

$$2Fe(II)O_2 + HNO \rightarrow 2Fe(III) \quad (11)$$

Thus, HNO diffusion is also likely to be significantly controlled by oxygen-binding proteins such as Hb and Mb. Production of an identical end-product following reaction

with NO or HNO would also conveniently require only a single mechanism to return to the ferrous state. Whether reaction with oxyHb or oxyMb completely inactivates HNO, as it does for NO, will depend upon the uncharacterized nitrogen product. However, the concentration of HNO itself will be highly controlled both by Eq. 11 and reaction with GSH as well as other scavengers.

Recent studies indicate that unlike NO donors, HNO induces preferential venous dilation (105). Baroreflex blockers balance arterial and venous relaxation by HNO, suggesting that the observed selectivity is due to enhancement of the compensatory neuronal response by HNO (104). The observed dilation of blood vessels, as previously shown (62), indicates either that mechanisms exist in the cardiovascular system to convert HNO to NO or that HNO mediates dilation through a separate mechanism.

Early reports indicated that Angeli's salt induces relaxation of isolated vasculature tissue (36). Later, HNO was found to be reduced to NO by free metals found in common buffers and media (36, 76, 94), and the prior results were assumed to be a function of this reaction. However, recent studies have shown that induced relaxation by Angeli's salt in isolated tissue models is not affected by addition of the NO scavenger PTIO (a nitroxide that converts NO to NO_2 via O atom transfer) (62). Therefore, casual metal-catalyzed conversion of HNO to NO does not entirely account for the observed effects. The sensitivity of potassium channels in resistance arteries to Angeli's salt was also not affected by PTIO, unlike the response to NO donors. Furthermore, a small fraction of relaxation resulted from stimulation of sGC. Since HNO does not stimulate sGC (26), intercellular conversion to NO was concluded to occur.

In these cases, selective reduction of HNO may take place near the activation site by specific catalytic conversion rather than random redox reactions. Covalent addition of HNO to transition metal complexes such as in peroxidases, metHb or metMb will result in reductive nitrosylation (Eq. 6). Release of NO from the resulting ferrous-nitrosyl complex, which will be highly dependent upon the protein, will have then resulted from inner-sphere electron transfer to HNO. A similar mechanism may be important to the oxidation of L-arginine to NO by NOS. Stuehr *et al*. have shown the HNO is produced by NOS under low cofactor conditions (1). In solution, HNO may back react to convert the ferric heme to ferrous nitrosyl, which could then eliminate NO and return NOS to the ferrous resting state.

These studies suggest that the reactions of HNO that may be generally restricted in the cell may be kinetically viable in specific cellular regions or when HNO is produced directly adjacent to a particular target. For instance, although the reaction of HNO with O_2 proceeds with a relatively slow rate constant, this reaction may become significant in cell membranes, in which nitrogen oxides and O_2 have enhanced solubilities (77) while the concentration of GSH is substantially lower compared to the cytosol. In fact, *in vitro* studies with the fluorophore diaminofluorescein (DAF) have suggested that HNO chemistry primarily occurs in cell membranes (30).

The differential physiological properties of HNO and NO are ultimately a result of the unique molecular targets for each species (Figure 3). Iron complexes, for example, are nitrosylated by NO and reductively nitrosylated by HNO (Eqs. 5 and 6). Thus, NO will favor ferrous iron while HNO will preferentially react with ferric iron in accordance with the extra electron in HNO. Further, NO will not react directly with thiols and must

first be activated to an RNOS such as the NO^+ donor N_2O_3. Conversely, HNO has a high affinity for thiols. Metal complexation may account for the rapid, reversible physiological effects while thiol oxidation/modification might be irreversible, requiring enzymatic regeneration within the cell. Other biological motifs unique to HNO have yet to be identified.

***In vitro* effects of NO and HNO on calcium channel function.**

Exocytosis of CGRP from NANC neurons is regulated by calcium influx through voltage gated channels (70, 97). HNO may stimulate neuropeptide release by interacting directly with the channel, perhaps through binding to a metal or thiol. This is an attractive proposition since the chemical modification would occur in the membrane where HNO scavengers such as GSH are low. The effects of HNO and NO donors on the NMDA calcium channel have been examined (38, 66, 128), and the observed responses have revealed several important aspects to the relationship of NO and HNO.

Under aerobic conditions, long term exposure to high micromolar and low millimolar NO donors attenuated glutamate-stimulated calcium influx (128), possibly through *S*-nitrosation of a thiol residue. The high concentration of NO required suggests that this mechanism would only be mediated by stimulated inducible NOS. Interestingly, short term, pulsed, aerobic infusion of NO donors potentiated calcium influx, and this effect was enhanced under hypoxia (38). Substitution of the NO donor with Angeli's salt produced similar augmentation aerobically, however, channel response was attenuated by HNO under hypoxia.

These condition-dependent responses to nitrogen oxides can be envisioned to be vital to cell physiology. Under normal conditions, low levels of NO would promote calcium influx, thus regulating normal metabolism. Conversely, high fluxes of NO from activated leukocytes would signal channel closure, potentially reducing damage from the immune response. Peroxides stimulate a similar response, indicating that both ROS and NO redox chemistry can protect the neuron from immunological or pathological conditions.

Tissues initially experiencing hypoxia often produce a burst of NO from endothelial NOS in an attempt to reestablish normal blood flow by vasodilation (80). Whether this burst is sufficient for calcium channel closure is unknown. However, if the ischemic event continues, the cofactors and substrates for NOS will diminish. Under these conditions *in vitro*, neuronal NOS converts to an HNO synthase (1). This alteration could be critical for cell survival since NO under hypoxia enhances calcium influx, which upon reperfusion would aggravate deleterious processes. Channel closure by HNO would instead be protective toward these pathways. Return of O_2 during reperfusion would initially open the channel while enhanced cofactor concentration would reestablish NO synthesis, again promoting normal function.

Since neuronal NOS may be attached to the NMDA receptor in certain neurons (15), the differential response to NO and HNO observed *in vitro* offers intriguing possibilities for regulation of neuronal responses by NOS under varied conditions. HNO appears to modulate channel function in parallel with O_2 concentration. Thus, HNO may be able to reinforce the initial signal in an O_2-dependent manner as described above and thus provide a protective response to regional fluctuations in blood flow. Additionally,

the pharmacological properties of HNO donors may originate from modification of calcium channels.

The responses of the NMDA channel to nitrogen oxide exposure are rapid and readily reversible (38), thus indicating interaction with a channel-associated metal rather than covalent modification of channel proteins. The hypoxia data suggest a reduced metal such as a ferrous iron. It is therefore likely that the distinct effects of NO and HNO again are a function of differential reactivity with the target.

Production of HNO *in vivo*.

The pharmacological and toxicological properties of HNO are slowly being elucidated. However, whether HNO is an endogenous mediator is still questionable. The properties of HNO described above provide circumstantial evidence for a role as an endogenous agent in the cardiovascular system. The unique biological signatures of HNO and NO, which are often opposing, renders these redox siblings ideal for control of a variety of physiological processes. Since casual redox conversion between these species is kinetically improbable, specific changes in redox states of proteins containing critical metal or thiol sites provide an intriguing scenario for regulation.

The primary candidate as an endogenous source of HNO is NO synthase. Production of HNO by NOS has been both speculated and demonstrated under specific *in vitro* conditions (1, 54, 110, 113, 115). The major shortcoming to this mechanism is escape of HNO from the protein pocket if the resultant valence state of the heme is ferric, unless rapid electron transfer from the cofactors reduces the iron prior to complexation of

HNO. If this highly regulated enzyme does in fact produce HNO, it is likely that the molecular target would have to be proximal to the enzyme due to consumptive pathways.

Another attractive possibility for HNO production is oxidation of the decoupled intermediate of NOS catalysis, N^{G}-hydroxy-L-arginine (NOHA). This molecule can constitute as much as 50% of the metabolism of NOS (16), and in this respect is likely an underappreciated metabolite, possessing its own unique properties. NOHA is an antioxidant and modulates metabolism and transport of L-arginine (16, 52). Catalyzed oxidation of NOHA by peroxidases has been proposed to produce HNO (110). Hydroxyurea can be similarly oxidized to HNO (67), possibly providing a novel class of HNO donors.

Decomposition of *S*-nitrosothiols may also provide a mechanism for *in vivo* generation of HNO (4). *S*-Nitrosothiols have been proposed as intermediates in the biology of NO (81, 123), although the physiological roles and concentrations are a matter of current debate (40, 111). The reaction of *S*-nitrosothiols with excess reduced thiol produces HNO and disulfide (142).

$$RSNO + RSH \rightarrow HNO + RSSR \qquad (12)$$

This reaction is an intriguing mechanism to convert NO chemistry to HNO chemistry but would require limited thiol concentration to avoid scavenging of HNO. Further, the tight regulation of NOS suggests that casual production of nitrogen oxides, perhaps including HNO, is both unlikely and undesirable.

Summary

Although nitroxyl production by NOS *in vivo* remains speculative, the potential therapeutic avenues for HNO donors are intriguing. As with NO, location and concentration will ultimately determine the biological effects of HNO and whether the response will be advantageous or deleterious. The chemistry of HNO is more diverse than that of NO allowing both a wider array of modifications and tighter control through multiple consumption pathways, such that diffusion will be restricted to an even greater extent than for NO. Further, the differential behavior of the redox siblings HNO and NO is to a great extent a function of their specific chemistry toward distinct molecular targets, providing discrete regulatory mechanisms under a variety of conditions.

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Table I. Derived Rate Constants for Reaction of HNO with Biomolecules.

	k ($M^{-1}s^{-1}$)	
biomolecule	Miranda *et al*. (37°C) (88)	Liochev/Fridovich (23°C) (75)
ferricyt *c*	4×10^4	2×10^4 [a]
O_2	3×10^3	1×10^4
Cu,Zn SOD	1×10^6	8×10^4 [a]
MnSOD	7×10^5	ND
GSH	2×10^6	$>>1 \times 10^5$
NAC	5×10^5	ND
catalase	3×10^5	ND
metMb	8×10^5	ND
oxyMb	1×10^7	ND
HRP	2×10^6	ND
Tempol	8×10^4	ND

[a]also in reference (76)
ND = not determined

Figure 1. Cardiovascular effects of Angeli's salt, DEA/NO and NTG in congestive heart failure. Ees, end-systolic elastance; D-edd, preload-normalized maximal *d*P/*dt*; Pes, end-systolic pressure; RT, total resistance; EDD, end-diastolic dimension. * P<0.005 versus baseline; ** P<0.01 versus. baseline; *** P<0.05 versus. baseline; † P<0.005 between groups; † † P<0.05 between groups.

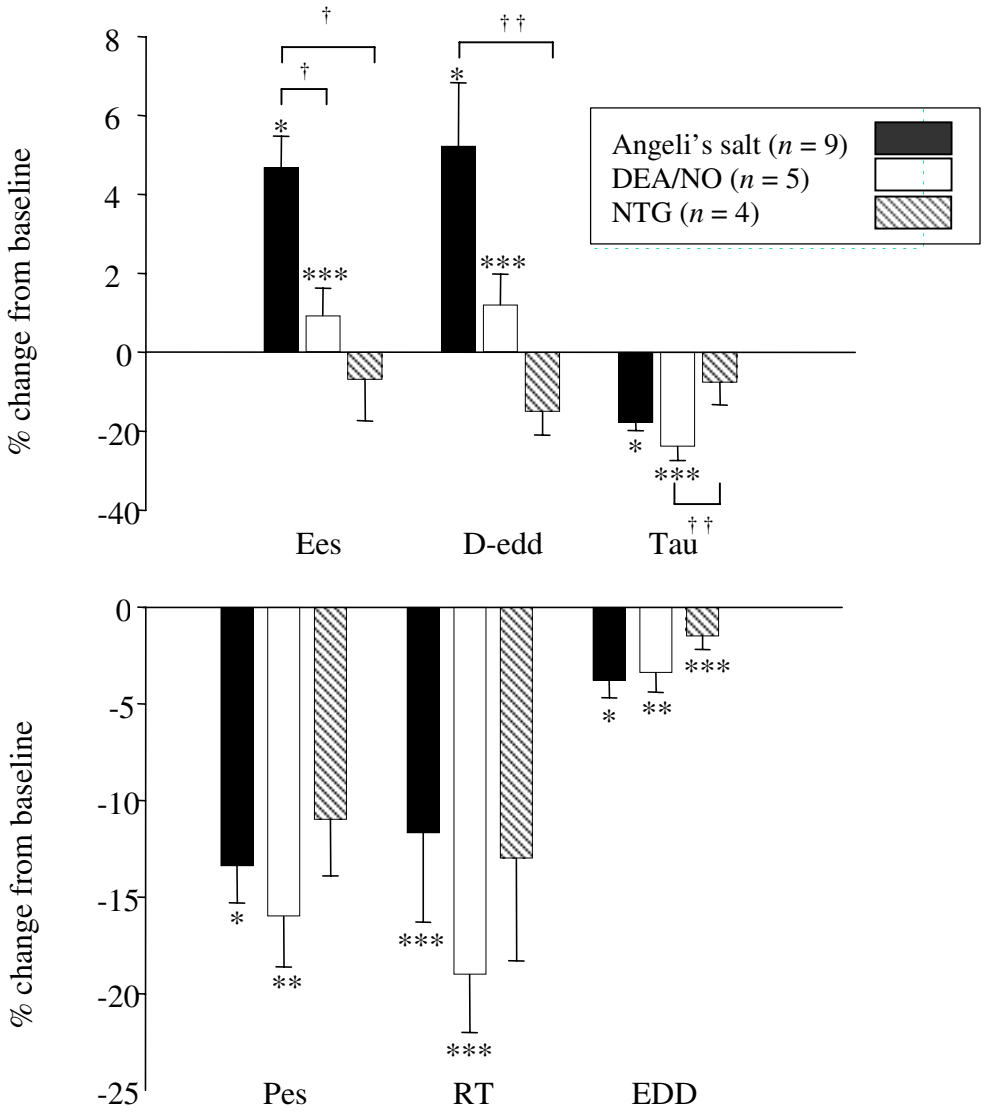

Figure 2. Relative Redox Potentials of NO and HNO/NO^- under Physiological Conditions. This diagram is a qualitative description of the calculated redox potentials for NO, HNO and NO^- and illustrates that the orthogonality observed in biological systems can be explained by the high barrier to either NO reduction or HNO oxidation through the intermediacy of NO^-. However, interconversion between NO and HNO can by achieved through specific metal-catalyzed inner-sphere electron transfer.

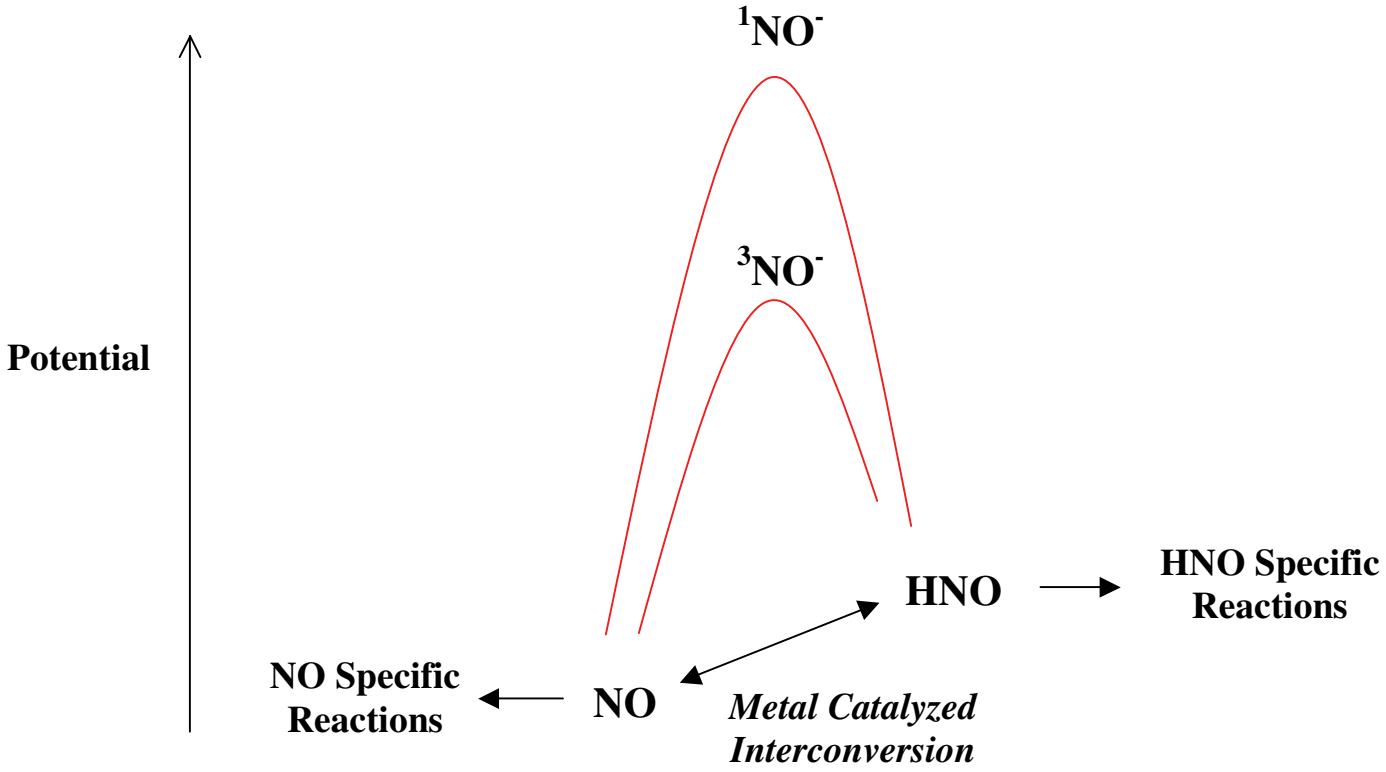

Figure 3. Differentiation of HNO and NO Chemistry by Cellular Compartmentalization. This diagram illustrates the likely biological targets of HNO and NO from a kinetic viewpoint. The orthogonal responses observed with HNO and NO are suggested to result from both the non-facile interconversion (red arrows) and the differential reactivity toward thiols and metal centers. Further, the reactivity of HNO with different biomolecules indicates that specific cellular compartments will foster either scavenging or activation/deactivation reactions. For instance, ferrous sGC, which is surrounded by large concentrations of GSH is expected to only be activated by NO. Modification of critical biomolecules by HNO is likely to only occur in membranes or other regions devoid of common scavengers such as GSH.