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Kinetic feasibility of nitroxyl reduction by physiological reductants and biological implications

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Abstract

Nitroxyl (HNO), the one-electron reduced and protonated congener of nitric oxide (NO), is a chemically unique species with potentially important biological activity. Although HNO-based pharmaceuticals are currently being considered for the treatment of chronic heart failure or stroke/transplant-derived ischemia, the chemical events leading to therapeutic responses are not established. The interaction of HNO with oxidants results in the well-documented conversion to NO, but HNO is expected to be readily reduced as well. Recent thermodynamic calculations predict that reduction of HNO is biologically accessible. Herein, kinetic analysis suggests that the reactions of HNO with several mechanistically distinct reductants are also biologically feasible. Product analysis verified that the reductants had in fact been oxidized and that in several instances HNO had been converted to hydroxylamine. Moreover, a theoretical analysis suggests that in the reaction of HNO with thiol reductants, the pathway producing sulfinamide is significantly more favorable than that leading to disulfide. Additionally, simultaneous production of HNO and NO yielded a biphasic oxidative capacity.

Keywords

Nitroxyl; Kinetics; Nitric oxide; Reduction; Nitrogen oxides; Thiols; Free radicals

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In recent years, nitroxyl (HNO) has been demonstrated to be a chemically unique species with potentially important pharmacological activity [1,2]. Unlike many other biologically relevant nitrogen oxides (e.g., nitric oxide (NO), nitrogen dioxide (NO₂), nitrite (NO₂⁻)), for which an abundance of biochemical data has been amassed, the physiologically relevant chemistry of HNO has been largely obfuscated by its metastability. The propensity of HNO to dimerize and then dehydrate to produce nitrous oxide (N₂O) [3,4] precludes storage and necessitates the use of donor compounds. Angeli's salt (sodium trioxodinitrate, Na₂N₂O₃) [5] has been the primary HNO donor for chemical and pharmacological studies. Temposil (citrated calcium carbimide), which is metabolically activated to release HNO, has been used for over half a century as a behavioral therapeutic for chronic alcoholism [6] and works via an HNO-mediated modification of the catalytic cysteine residue of aldehyde dehydrogenase [7].

Other potential therapeutic uses for HNO have been reported more recently. For example, HNO from Angeli's salt enhances cardiac contractility and output in a chronic heart failure model [8]. Additionally, HNO promotes smooth muscle relaxation and vasodilation [9,10] and decreases lobar arterial pressure without changing left atrial pressure [11]. Diverse cellular processes such as ryanodine receptor activation [12], transcription factor modification [13], cyclooxygenase activation [14], heme oxygenase induction [15], voltage gated potassium channel activation [16], protein thiol modification [17], increased arginine uptake [18], cAMP fluctuation [1], and calcitonin gene-related peptide release [19] can be moderated by HNO. Interestingly, HNO seems to specifically affect calcium signaling in myocardial tissue by interacting with a variety of functionally distinct thiol proteins ([2] and references therein). However, the biochemistry responsible for many of these effects of HNO is not well understood.

Toxicological outcomes have also been associated with exogenous application, and potentially endogenous production, of HNO. In this regard, HNO may mediate the inflammatory effects of ischemia–reperfusion by enhancing dexamethasone-inhibitable [20] neutrophil invasion [21]. Additionally, the etiology of amyotrophic lateral sclerosis has been suggested to involve the pro-oxidative chemistry associated with aberrant HNO production [22].

The biological targets of HNO have been reported to include protein thiols [17], transition metals [23], iron–sulfur clusters [24], and DNA [25,26]. Reaction with oxidants results in conversion of HNO to NO [27]. HNO is also recognized as an electrophile [28], which suggests that reduction may also occur. Recent thermodynamic calculations predict that reduction of HNO is accessible under biological conditions [29,30], but the kinetic proclivity toward reduction has not been elucidated.

Notably, compounds that can be considered structurally similar to HNO are readily reduced in diverse chemical and physiological settings. For example, oxoammonium cations (R₂NO⁺) could serve to mechanistically model two-electron transfer to HNO (albeit oxoammonium cations will be much more oxidizing than HNO). Two-electron transfer to R₂NO⁺ from NADH proceeds with a 1:1 stoichiometry [31]. Oxoammonium electrophiles are also able to oxidize alcohols under relatively mild conditions [32], which may correspond to the reaction

of HNO with ascorbate. There is also precedent in the reduction of *S*-nitrosothiols (RSNOs) by NADH and ascorbate. The stoichiometry for reduction of *S*-nitrosoglutathione by Hantzsch dihydropyridines is 1:1, with concurrent production of one equivalent of oxidized pyridine [33]. Enzymatic reduction of RSNOs by dihydropyridine-dependent dehydrogenase activity is also well known [34]. Additionally, ascorbate reduces RSNOs in a metal-independent manner [35].

Considering that the cytosolic environment of cells is reducing in nature, reductive metabolism of HNO may be facile and thus important to its pharmacology and ultimate fate. As a first step in addressing this hypothesis, the reduction of HNO by several mechanistically distinct reductants has been examined. The findings of this study indicate that HNO reduction is a likely metabolic fate and confirm the idea that thiols and thiol proteins are likely targets for HNO activity. Moreover, generation of both the disulfide and the sulfinamide products from this interaction is energetically accessible, although sulfinamide formation is more favored than disulfide formation, with the latter being driven by the availability of other reactive thiols. As disulfide is readily reversible *in vivo*, whereas sulfinamide is thought not to be, this may have potential implications for the pharmacology of HNO and the reversibility of its effects.

Experimental procedures

Chemicals and solutions

Reduced nicotinamide adenine dinucleotide (NADH), oxidized nicotinamide adenine dinucleotide (NAD⁺), glutathione, and glutathione disulfide were purchased from Sigma (St. Louis, MO, USA). L-Ascorbic acid was purchased from Fischer Scientific, and L-dehydroascorbic acid was from Sigma. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), hydroxylamine hydrochloride, and isobutyl nitrate were purchased from Aldrich (Milwaukee, WI, USA). All other chemicals were obtained from commercial suppliers and were of the highest purity available. Rigorous exclusion of metals was performed through the use of deionized water, treatment with Chelex resin, and inclusion of diethylenetriamine pentaacetate (DTPA) chelator. Use of glass containers was avoided or, when required, glassware was demetallated by holding full volumes of stirred 1% nitric acid over night.

Angeli's salt synthesis

Angeli's salt (AS) was used as a source of HNO and synthesized according to a method modified from that of Smith and Hein [3] but utilizing a process whereby sodium hydroxide was replaced by sodium methoxide equivalents dissolved in methanol. The reagents employed were hydroxylamine hydrochloride and isobutyl nitrate, the ratio of NH₂OH·HCl:RONO₂:MeONa was 3:1:5, and the primary contaminants were nitrite and hydroxylamine, totaling 3% of the solid AS preparation. Stock solutions of Angeli's salt were made up in at least fourfold excess sodium hydroxide immediately before use and kept on ice. All stocks were from the same batch of Angeli's salt and purity was determined by the absorbance at 248 nm (extinction coefficient 8100 M⁻¹ cm⁻¹ [5]) of an Angeli's salt

preparation in 0.1 N NaOH before assays, periodically throughout the experiments, and to ensure that complete decomposition of prodrug had occurred in the product analysis studies.

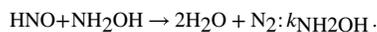
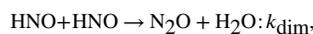
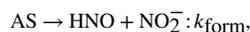
Kinetics of reduction

Direct reaction—Solutions of reductants were made up in distilled water. Reaction was initiated by the addition of Angeli's salt. Typically, 100–1000× AS in 0.1 M NaOH was rapidly mixed into water containing 0.1 M potassium phosphate buffer (pH 7.4) and the reductant at final concentration. Reactions were carried out in triplicate or greater unless otherwise noted, under either aerobic or anaerobic (argon-saturated) conditions at room temperature for 1–2 min (at 23°C and pH 7.45 the $t_{1/2}$ of AS decomposition was 17 min ($6.33 \times 10^{-4} \text{ s}^{-1}$ as determined here)). Loss of chromophore was linear over this period, and initial rates were derived from these data.

Competition reaction—Competition kinetics were determined as in the direct reaction studies, but the competitor (10–100× stocks in water) was added to the reaction mixture immediately before Angeli's salt addition.

Mathematical modeling—The loss of reductant chromophore in these kinetic runs was quantified, and the data were analyzed with the freely available kinetic software DYNFIT [36]. The kinetic constants used were from the literature and are shown in Scheme 1. Shown below is an example of a reaction mechanism and the differential equations utilized to determine the second-order rate constant for the reaction of HNO with NH_2OH .

The reaction mechanism is as follows:



The differential equations used are:

$$d[\text{AS}]/dt = -k_{\text{form}}[\text{AS}],$$

$$d[\text{HNO}]/dt = +k_{\text{form}}[\text{AS}] - k_{\text{dim}}[\text{HNO}][\text{HNO}] - k_{\text{dim}}[\text{HNO}][\text{HNO}] - k_{\text{NADH}}[\text{HNO}][\text{NADH}] - k_{\text{NH}_2\text{OH}}[\text{HNO}][\text{NH}_2\text{OH}]$$

$$d[\text{NO}_2^-]/dt = +k_{\text{form}}[\text{AS}],$$

$$d[\text{N}_2\text{O}]/dt = +k_{\text{dim}}[\text{HNO}][\text{HNO}]$$

$$d[\text{H}_2\text{O}]/dt = +k_{\text{dim}}[\text{HNO}][\text{HNO}] + k_{\text{NH}_2\text{OH}}[\text{HNO}][\text{NH}_2\text{OH}],$$

$$d[\text{NADH}]/dt = -k_{\text{NADH}}[\text{HNO}][\text{NADH}],$$

$$d[\text{NH}_2\text{OH}]/dt = +k_{\text{NADH}}[\text{HNO}][\text{NADH}] - k_{\text{NH}_2\text{OH}}[\text{HNO}][\text{NH}_2\text{OH}].$$

$$d[\text{NAD}]/dt = +k_{\text{NADH}}[\text{HNO}][\text{NADH}],$$

$$d[\text{N}_2]/dt = +k_{\text{NH}_2\text{OH}}[\text{HNO}][\text{NH}_2\text{OH}].$$

In addition to DYNAFIT, the kinetic reactions were also modeled using MATLAB version 7.01 SP1. In general, the reactions under consideration, the known rate constants, and a crude estimate of the unknown rate constants were determined. The programs then set up a series of simultaneous differential equation solvers to derive predicted rate constants based on actual initial rates of experimental data. A subsequent comparison of the fit between model-predicted data and the experimental data using the newly developed rate constant for NAD (P)H determines whether the user-defined reactions were necessary and sufficient to describe the system under study. All variables were nondimensionalized with respect to the initial concentration of each species or with the parent compound of each species if the initial concentration was zero. The set of differential equations was solved using the stiff ordinary differential equation solver “ode15s” with the absolute error tolerance set to 10^{-12} and the relative error tolerance set to 10^{-6} . Optimization of unknown reaction coefficients was achieved using the “fminsearch” function, which performs a multidimensional unconstrained nonlinear minimization based on the Nelder–Mead method. The objective function was defined as the sum of the square error of the theoretical prediction from the experimentally derived values for the formation of a selected species normalized to the experimental values. Various initial conditions were tested to ensure the results were not due to trapping within a local minima.

NADH and NAD⁺ analysis

NADH was determined by monitoring the absorbance at 340 nm ($\lambda = 6.2 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$). The formation of NAD⁺ was monitored using a fluorometric assay published previously

[37]. Fluorescence was measured using a Perkin–Elmer LS50B spectrofluorometer (excitation at 365 nm; emission at 460 nm), against an NAD⁺ standard curve. Reactions were typically performed in 400 μ l of 50mM Chelex-treated sodium phosphate buffer, pH 7.45, containing 10 μ M DTPA. After complete decomposition of AS (greater than five half-lives), the NADH remaining and NAD⁺ produced were measured from the same incubation.

Ascorbate and dehydroascorbate analysis

Ascorbate was determined by monitoring the absorbance at 265 or 285 nm ($\epsilon_{265} = 1.4 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$, $\epsilon_{285} = 5.44 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$, determined in this study). Dehydro-1-ascorbate produced from the reaction of ascorbate with Angeli's salt was assayed as the hemiketal methanol adduct, absorbing at 346 nm, according to Badrakhan et al. [38]. After complete decomposition of AS (greater than five half-lives), the ascorbate remaining and dehydroascorbate produced were measured from parallel incubations with methanol. The reactions were carried out in 400 μ l of 50 mM Chelex-treated sodium phosphate buffer, pH 7.45, containing 10 μ M DTPA.

Hydroxylamine (NH₂OH) analysis

The formation of NH₂OH from the reactions of HNO with the various reductants was determined using the indoxine test [39]. In summary, a 0.4-ml sample was mixed with an equal volume of 8-hydroxyquinoline (1% w/v in absolute ethanol) and Na₂CO₃ (10% w/v in absolute ethanol). The solution was reacted at 95°C for 5 min. Upon cooling to room temperature the absorbance at 710 nm was determined using a Shimadzu UV-2501PC spectrophotometer. Occasionally the assay was carried out in microplate format with heating via thermocycler (Perkin–Elmer) and spectrophotometry performed on a microplate reader (Emax; Molecular Devices). Quantitation was accomplished using a standard curve generated from known concentrations of authentic NH₂OH•HCl (Sigma).

Theoretical calculations

All structures were initially optimized using the B3LYP density functional theory method with a 6–311+G(d) basis set implemented in the Gaussian 03 program [40–42]. Gas phase energies were computed for all structures using the high-accuracy CBS-QB3 multicomponent method, which generally gives average errors of ± 1 kcal/mol compared to experimentally measured data for the G3 data set [43]. Aqueous solvation energies were calculated as single points on the B3LYP/6–311+G(d) gas phase-optimized geometries using B3LYP/6–31+G(d) in the SM6 continuum solvation model. The SM6 model has been demonstrated to give aqueous solvation free energies with an average error of ~ 0.5 kcal/mol for a limited test set of neutral solutes [44]. Aqueous solvation energies were applied to the CBS-QB3 gas phase energies to obtain free energies in aqueous solution.

Results

Reaction rate constants

Prior reports indicate that HNO may be subject to reduction under biological conditions [45]. In an attempt to assess the feasibility of this process, HNO derived from the donor Angeli's salt was exposed to a variety of common biologically relevant reductants. To obtain

the order of the reaction with respect to reductant, it is desirable to maintain the reactions under excess HNO. Given the necessity to produce HNO in situ and the relatively slow decay of the principal HNO donor Angeli's salt, such conditions are difficult to achieve. The consumption of HNO by dimerization (Scheme 1) further complicates achieving a high steady-state concentration of HNO. Therefore, the reactions seem to be nearly zero order in reductant for much of the examined concentration range (Fig. 1A; NADH or ascorbate as reductant). To examine the dependence of reaction rate on Angeli's salt and HNO concentration, constant NADH or ascorbate (100–500 μM) was reacted with varying Angeli's salt (0.1–2000 μM) for 1–2 min, whereas the rate of loss of reductant was monitored spectrophotometrically (Fig. 1B).

Correlating the effect of HNO treatment with Angeli's salt concentration is often not informative; ideally the effect would be plotted versus the concentration of HNO ($[\text{HNO}]$). This is especially crucial in the determination of reaction rate constants. To address this, others have predicted HNO concentrations based upon steady-state assumptions of nitroxyl, governed by donor decomposition (v_f), dimerization to N_2O (v_d), and the reaction under study (v_r) (Eqs. (1) and (2), where RH_2 is any reductant) [12,46–48]. Cheong et al. [12] used the steady-state equation, $v_f = v_d + v_r$, to plot ryanodine receptor activation versus $[\text{HNO}]$. By including another component in the steady state, v_r , Liochev and Fridovich [49] predict $[\text{HNO}]$ more accurately (Eq. (3)). They observed rates of product formation (or loss of reactant) and determined rate constants for reaction with, for example, superoxide dismutase. Utilizing a fitting parameter from plots of Angeli's salt against binding velocity Bari et al. [46] and Marti et al. [50] have published rate constants for the reaction of HNO with metalloporphyrins. Using end-product analysis instead, Miranda et al. [1] determined k_r after complete decomposition of nitroxyl donor (Eq. (4)):

$$v_f = v_d(\text{or } v_d + v_r), \quad (1)$$

$$k_f[\text{AS}] = k_d[\text{HNO}]^2(\text{or } k_d[\text{HNO}]^2 + k_r[\text{HNO}][\text{RH}_2]), \quad (2)$$

$$[\text{HNO}] = (k_f[\text{AS}]/k_d)^{1/2}(\text{or } ((k_f[\text{AS}] - v_r)/k_d)^{1/2}), \quad (3)$$

$$k_r = 1/[\text{RH}_2] \times \left(k_f[\text{AS}]/((k_f[\text{AS}] - v_r)/k_d)^{1/2} - k_d((k_f[\text{AS}] - v_r)/k_d)^{1/2} \right). \quad (4)$$

These general strategies provide insight into the potency and stoichiometry of HNO reactions, but the equations are not readily solvable in the presence of multiple species capable of reaction with HNO. A further limitation of these equations is their lack of a time-dependent component.

Because it is clear that neither Angeli's salt concentrations nor simple calculations of HNO concentration will enable determination of second-order rate constants for the reaction of nitroxyl with a scavenger, we quantified the loss of reductant via kinetic analysis using the software programs DYNAFIT and MATLAB according to Scheme 1, in which the donor

(AS) produces HNO and multiple paths lead to its consumption (for example O₂, NO, reductant, nitrite). We determined the value of k_{NADH} for the HNO/NADH reaction to be $1.1 (\pm 0.2) \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$. When we used our reactant concentrations and observed the velocities of reaction to calculate k_{NADH} with the equations of Liochev and Fridovich [48] or Bari et al. [46], the resultant values ranged from 0.9×10^4 to $2.2 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$. The reaction of HNO with ascorbate was difficult to monitor directly as the UV absorbance of Angeli's salt overlaps with that of ascorbate, necessitating the use of off-maximum ascorbate absorbances for quantitation. Additionally, we noted that authentic dehydroascorbate, a product of the HNO/ascorbate reaction, produces an absorbance at 273 nm over time in solution, blunting our observed loss of ascorbate. Because many biomolecules absorb minimally in the visible region, NADH was used as an indicator in the assessment of the propensity of other reductants to reduce HNO via a competition assay. The degree to which common reductants competed with the oxidation of NADH by HNO is shown in Fig. 1C. The values predicted by DYNAFIT, based on Scheme 1, are superimposed as solid lines on the observed data points. The fit is reasonable, providing validation of the analysis model utilized in the competition assay. Control experiments verified that addition of competing reductants did not affect the NADH concentration or reverse of the HNO-induced bleaching of NADH. Table 1 shows the rate constants proposed for the reaction of nitroxyl with glutathione, thiosulfate, tris-carboxyethylphosphine, the water-soluble vitamin E analog trolox, ascorbic acid, and hydroxylamine. Trolox was nearly insoluble above low-millimolar concentrations, leading to a deviation from predicted values at higher concentrations. Interestingly, selenomethionine reacted with a rate constant of around $1 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ but methionine was unreactive up to its limit of solubility (not shown). The selenide is therefore much more reactive than its sulfide analog. From Table 1, it is clear that nitroxyl is capable of reaction with a diverse array of mechanistically distinct reductants, with second-order reaction rate constants that span several orders of magnitude.

Enhanced oxidative capacity of HNO/NO mixtures

As HNO can be readily oxidized to NO, it is possible that HNO and NO can be present simultaneously in systems examining HNO. Because HNO and NO react with each other to form a putative oxidant (*vide infra*), there is the need to determine whether these reaction products can be oxidizing. To assess the oxidative potential of the product of the reaction of HNO with NO in our system, the NO donor DEA/NO [51] was titrated with constant Angeli's salt and NADH. DEA/NO decays with a half-life similar to that of Angeli's salt but produces two equivalents of NO. At concentrations up to 600 μM , DEA/NO alone resulted in no oxidation of NADH. In the presence of Angeli's salt, the rate of NADH oxidation was dependent on the concentration of DEA/NO in a biphasic manner (Fig. 1D).

The association of HNO and NO produces the hyponitrous radical (HN₂O₂) [52], which at pH 7.4 is predominantly deprotonated as the hyponitrite radical anion (N₂O₂⁻). Poskrebyshev et al. [53] reported an experimental value for the potential of the N₂O₂⁻/N₂O₂²⁻ couple of +0.96 V, indicating that N₂O₂⁻ is a reasonably strong oxidant. In contrast, a markedly different value for this couple of -0.4 V has been reported by us based on theoretical analysis with CBS-QB3 level theory and (C)PCM solvation mode [54]. This negative potential suggested that HNO/NO mixtures should actually be relatively reducing,

an idea that has been challenged recently [55]. Because the reaction of HNO with NO clearly produces an oxidant and thermodynamic calculations involving solvation of a dianion are known to be prone to significant error, we have reinvestigated the potential of the $\text{N}_2\text{O}_2^-/\text{N}_2\text{O}_2^{2-}$ couple herein. The SM6 continuum solvation model was utilized as it is better able predict thermodynamic parameters than the (C)PCM model when a dianion is present in the reaction [56]. The recalculated potential of the $\text{N}_2\text{O}_2^-/\text{N}_2\text{O}_2^{2-}$ couple based on the SM6 model is 0.34 ± 0.1 V. Additionally, as the $\text{p}K_{\text{a}1}$ and $\text{p}K_{\text{a}2}$ of hyponitrous acid are 7.05 and 11.4, respectively, a predominate product of the HNO/NO product reduction reaction under physiological conditions will be the monoprotonated hyponitrous anion. Thus, we have calculated the potential of the more relevant proton-coupled reduction of N_2O_2^- to HN_2O_2 as a significantly more favorable 1.0 ± 0.1 V, consistent with recent arguments [55].

Reduction products

To qualitatively validate the proposed reaction scheme, the products of oxidation of NADH and ascorbate (Figs. 2A and B, respectively) and of HNO reduction (Fig. 2C) were analyzed. The HNO-dependent loss of NADH or ascorbate monitored spectroscopically was accompanied by an increase in NAD^+ or dehydroascorbate, albeit nonquantitatively. Reduction of HNO to NH_2OH is inherently not quantitative owing to the reaction of HNO with NH_2OH [57] and the interference of excess reductants in the 8-hydroxyquinoline/indoxine assay [58] (shown for comparison is the effect elicited by a strong reductant, dithiothreitol). Plots of NH_2OH formed versus increasing reductant or Angeli's salt were therefore invariably bell shaped (higher concentrations not shown). We currently do not have an explanation for the apparent lack of NH_2OH production in the HNO/NAD(P)H reaction (Fig. 2C). NAD(P)H interfered to a lesser extent with the indoxine assay, yet the reaction with HNO produced disproportionately less NH_2OH than might be expected from the relative magnitude of the rate constant. This lack of NH_2OH production in the reaction of HNO with NADH has been previously reported by Reif and coworkers [59]. To address the possibility that NH_2OH could be an intermediate that is further reduced (to NH_3 , for example), the reaction between NH_2OH and all reductants was examined. In all cases, the direct reaction of NH_2OH with reductants did not lead to reductant oxidation (data not shown), indicating that NH_2OH is not further reduced under the conditions of our experiments.

Reaction of HNO with NAD(P)H

NADH/NADPH—The ratio of reduced to oxidized dihydropyridine nucleotides in mammalian systems differs for the NADH and NADPH pools [60]. Therefore we sought to ascertain whether the 2' phosphate plays a role in the relative reactivity of NADPH versus NADH, as has been reported for reaction with ferric iron [61]. To this end, the rate of reaction of NADPH with HNO was examined. As this rate constant ($1.3 (\pm 0.4) \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$) did not differ significantly from that of NADH, the reaction of nitroxyl with dihydropyridine nucleotides can be generally signified as $\text{HNO} + \text{NAD(P)H} \rightarrow \text{products}$.

Reaction with oxygen

Monoprotonated nitroxyl reacts with dioxygen with a published bimolecular rate constant of $k_{O_2} < 10^5 \text{ M}^{-1} \text{ s}^{-1}$ [29] or $k_{O_2} \sim 10^3 \text{ M}^{-1} \text{ s}^{-1}$ [1]. Although the reaction is thermodynamically favorable [62] the kinetic relevance of this reaction under biological conditions is questionable, as it has a significant energy of activation. The identity of the product of HNO autoxidation has been a matter of debate. The resulting oxidant has been argued to be either peroxynitrite (ONOO^-) [63] or an as yet unidentified and distinct species [64]. The reaction of HNO with O_2 would provide an additional pathway for HNO loss (Scheme 1), and its inclusion in the kinetic analysis scheme slightly improves the fits of modeling software predictions to experimental data; however, the rates of reaction of HNO with NADH in the presence and absence of dioxygen were not significantly different over the short time periods employed (<2 min). The rate constant under anaerobic conditions was calculated as $0.9 (\pm 0.3) \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$, versus $1.1 (\pm 0.2) \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ in the presence of oxygen. Inclusion of ONOO^- in the model as a downstream oxidant of NADH ($k_r = 4 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ [65]) worsened the fits of experimental data, suggesting that ONOO^- is not consuming NADH in this system. Furthermore, the rate constant ($0.9 \pm 0.3 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$) for NADH consumption was not significantly altered in the presence of bicarbonate (20 or 50 mM, pH 7.4–7.6), which rapidly scavenges ONOO^- [66]. Therefore, although autoxidation of HNO is a viable consumption pathway, we concur with Reif and coworkers [59] that the resulting oxidant leading to the loss of NAD(P)H is not ONOO^- . Thus, it is not likely that any of the biological activity of HNO is due to formation of ONOO^- . This is further supported by recent work indicating that the cardiovascular actions of HNO and ONOO^- are distinct [67].

Hydroxyl radical formation

HNO has been suggested to produce hydroxyl radical activity either via a secondary decomposition pathway of the hyponitrous acid formed upon HNO dimerization [68] or by elimination from the hyponitrous radical formed from the reaction of HNO with trace NO [63]. To examine whether NAD(P)H oxidation was mediated through $\bullet\text{OH}$ -type chemistry, the impact of mechanistically distinct $\bullet\text{OH}$ scavengers was examined. Because neither methionine nor salicylate, at concentrations up to 10 mM, inhibited NADH oxidation (data not shown), it is concluded that significant $\bullet\text{OH}$ is not produced in this system. This is consistent with the findings of Buchholz and Powell [68], who reported that a radical mechanism is promoted only in the presence of metal ion or nitrous acid.

Thermodynamic favorability of the reaction of HNO with thiols

After the initial association of HNO with a thiol, the resulting *N*-hydroxysulfenamide (RSNHOH) is short-lived and can either react with an additional thiol equivalent to form the disulfide and NH_2OH [45,69] or isomerize to a sulfinamide [45]. Together, these pathways account for the modifications of thiol-dependent proteins by HNO, which can be either reversible or irreversible by dithiothreitol [17].

The relative changes in free energy for these reactions of HNO with a simple thiol have been calculated (Fig. 3). The formation of *N*-hydroxysulfenamide was found to be thermodynamically favorable (G_{aq} of $-11.5 \pm 1 \text{ kcal/mol}$). Additionally, both the

subsequent reaction with thiol (to give the disulfide and hydroxylamine) and the rearrangement to sulfinamide were calculated to be exergonic (G_{aq} of -10.6 and -35.7 ± 1 kcal/mol, respectively).

Discussion

In this study we demonstrate that HNO can react with several biological reductants with rate constants that would predict potential biological relevance. The reductant we utilized as a primary tool of investigation was NADH. Wink and coworkers first examined the reactivity of Angeli's salt with NADPH [26] and indicated the presence of both aerobic and anaerobic pathways. Reif et al. [59] further concluded that nitroxyl was the actual oxidant of NADPH and that hydroxylamine was not likely to be one of the reduced products of HNO. Liochev and Fridovich have examined the NADPH/HNO reaction in some detail [49], including reaction rates and inhibition by nitroxyl scavengers. These studies provide conflicting estimates for the stoichiometry of the HNO/NAD(P)H reaction and, although reaction rates have been examined, no second-order rate constant was proposed. We have determined this rate constant to be on the order of $10^4 \text{ M}^{-1} \text{ s}^{-1}$ and also demonstrate as a corollary that the stoichiometry for the reaction is 1:1. In addition, our experimental data support the findings of Reif et al. [59] that HNO is the proximal oxidant, not peroxynitrite, and that hydroxylamine is apparently not formed as a product in this reaction.

The rate constants in Table 1 enable considerations of the relative contribution that these reductive pathways might make to HNO metabolism as a whole. A similar assessment, based on final product analysis after complete decomposition of HNO donor rather than initial rates of reaction, was performed by Miranda et al. for GSH, cysteine, and a short series of oxidants [1]. The rate constant for the reaction of HNO with GSH determined here is within fivefold of the previously published value ($6 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$). We are therefore in agreement that among the common biological reductants, GSH is likely to be one of the predominant targets of HNO, based on the calculated rate constant and relatively high intracellular GSH concentrations.

The case of vitamin E (tocopherol) is more ambivalent. Studies here with the water-soluble analog trolox do not accurately account for the localization of tocopherol in lipid membranes [70]. Similar to NO and O₂ [71], HNO is expected to partition into the membrane. The rate of the reaction of HNO with tocopherol in cells may thus be significantly higher than that reported here for trolox, which may define a lower limit for tocopherol reactivity.

It is possible that HNO and NO may be found in proximity to one another in chemical and biological settings; however, the relative amounts will differ greatly depending on formation conditions and the cellular environment [27]. As these two nitrogen oxide congeners are observed to have orthogonal biological properties [72], differing ratios of HNO to NO may be expected to stimulate markedly varying biochemical responses. Indeed, it has been previously noted that NO has a biphasic effect on product yields in reactions of HNO with benzoic acid or NADH [63]. Similarly, varying NO:HNO ratios produced opposing effects on the activity of a copper-thiolate-dependent transcription factor, Ace-1 [13]. Additionally,

Miranda and coworkers recently invoked an HNO/NO-dependent reaction sequence to explain the scavenging effects of superoxide dismutase [1].

The reaction of HNO with NO initially produces HN_2O_2 , which at pH 7.4 largely exists as the deprotonated species, N_2O_2^- . This anion can either react with an additional NO to produce N_3O_3^- , which is a relatively unreactive species, or carry out chemistry of its own. To account for the observed increase in oxidative capacity exhibited by combinations of HNO with low amounts of NO, Kirsch et al. [63] have proposed elimination of $\bullet\text{OH}$ from HN_2O_2 . However, at physiological pH the ratio of HN_2O_2 to N_2O_2^- will not exceed 1/100 [53] and will perhaps be as low as 1/10,000 [73], thus limiting the pathway for $\bullet\text{OH}$ release from HN_2O_2 (N_2O_2^- is not likely to eliminate the oxyl radical anion, $\text{O}^{\bullet-}$) [55]. Additionally, although elimination of $\bullet\text{OH}$ from HN_2O_2 is thermodynamically favorable (G_{aq} of -28.1 kcal/mol [63]), Poskrebyshev has shown that both HN_2O_2 and N_2O_2^- are kinetically stable to self-decomposition and are more likely to participate in redox reactions [53]. We thus propose that at lower NO concentrations, HNO reacts with NO to form N_2O_2^- , which instead of subsequently producing OH, directly oxidizes NADH with a rate constant greater than that of HNO. We are in agreement with Kirsch and de Groot [63] regarding the inhibition of oxidation at higher ratios of NO to HNO, namely that the hyponitrite radical is itself trapped by additional NO to form N_3O_3^- , which is not reactive in this system. DYNAFIT predictions based upon data like that shown in Fig. 1B (inset) led to a calculated rate constant of $\sim 1 \times 10^6 \text{M}^{-1} \text{s}^{-1}$ for the reaction of N_2O_2^- with NADH. Although there is significant error ($\pm 30\%$) associated with this prediction, the proposed reaction sequence generally describes the changes in the rate of NADH oxidation after concomitant exposure to HNO and NO.

The biological relevance of N_2O_2^- -mediated oxidation relies on the likelihood that it can be generated from simultaneous and localized NO and HNO production. Although it is not established that HNO is generated endogenously, its possible pharmacological utility (vide supra) makes its interaction with endogenously generated NO a distinct possibility. Clearly, the presence of HNO and NO together will divert both from their respective biological targets (i.e., soluble guanylate cyclase for NO and thiol proteins for HNO) to form an oxidant that can react with cellular reductants. Clearly, this will be an important factor in evaluating the pharmacology/toxicology of HNO donors as drugs.

The kinetic and thermodynamic data presented here support the growing consensus that thiols are a primary target of HNO in vivo. Of the two known reactions of HNO with thiols (Fig. 3), sulfinamide formation was calculated to be thermodynamically more favorable than disulfide production. This may have significant implications for the biological effects of HNO. However, it is likely the reaction pathway chosen will be highly dependent on the proximity of other reactive thiols, allowing disulfide formation to be competitive with the thermodynamically favorable sulfinamide pathway. Regardless, sulfinamide formation from glutathione and proteins after treatment with an HNO donor has been recently shown [74,75]. It is difficult to speculate on the biological relevance of the initial adduct of the HNO and RSH reaction, as few published data address RSNHOH directly. However, both the *N*-hydroxysulfenamide and the sulfinamide products are intriguing as protein thiol modifications. For example, a putative *N*-hydroxysulfenamide form of glyceraldehyde-3-

phosphate dehydrogenase (GAPDH) was shown to have inhibited dehydrogenase activity but increased acylphosphatase activity [76]. The acylphosphatase activity of GAPDH has been linked to a futile cycle that increases glycolytic flux but decreases energy yield by hydrolyzing the product of its own dehydrogenase reaction [77]. We, and others, have seen that HNO is indeed a potent inhibitor of GAPDH dehydrogenase activity in vitro [78] and in yeast cells [79], but it remains to be seen whether HNO can induce acylphosphatase activity and futile cycling.

Of note, yeast cells obligatorily dependent upon glycolysis were more susceptible to HNO toxicity than respiring cells [79]. Additionally, HNO has been shown to be more toxic to cancer cell lines, which are strongly dependent upon glycolysis, than to non-cancer cell types [80]. This may have important implications for treatments in which the mechanistic basis for therapy rests upon the modification of glycolysis.

Rearrangement of the N-hydroxysulfenamide to the corresponding sulfinamide may be relevant in the peroxiredoxin H_2O_2 scavenging system. Sulfinamides are relatively stable at pH 7.4 [81], but could potentially be subject to hydrolysis in acidic subcellular compartments or by cellular amidases and esterases to form the corresponding sulfinic acids. The discovery of a sulfinate reductase [82], which acts upon peroxiredoxin sulfinate, led to the hypothesis that this “overoxidation” acts as a molecular floodgate allowing peroxide-dependent signal transduction to occur [83]. Whereas the reaction of thiol with H_2O_2 to form a sulfinate is second order in H_2O_2 , the reaction of HNO with RSH is first order in oxidant. To the best of our knowledge, HNO is the only species that can form the sulfinic oxidation state ($RS(O)NH_2$, RSO_2H) via reaction of a thiol with only *one* equivalent of oxidant. Thus, HNO is a potentially valuable tool in the investigation of signal transduction. It is also interesting to note that HNO may potentially alter the redox state of the NADPH/NADP⁺ couple either by direct oxidation of NADPH or by oxidizing cellular thiols, leading to indirect NADPH oxidation as cellular glutathione and thioredoxin reductases reduce the disulfides so formed.

In summary, with the intracellular environment being generally reducing in nature, reduction of HNO might be a facile, if not the primary, metabolic event. The significance of these findings lies in the possibility that the conversion of HNO to a reduced species may account in part for the observed pharmacological effects of HNO donors. Clearly, it will be important for future work in this area to include the possibility of reduced species in the overall biology of HNO. Significantly, NH_2OH , one of the most likely reduction products, has recently been shown to be capable of being oxidized by peroxidases to HNO [84], allowing for a possible recycling mechanism involving reducing species (e.g., thiols), H_2O_2 , and HNO/ NH_2OH .

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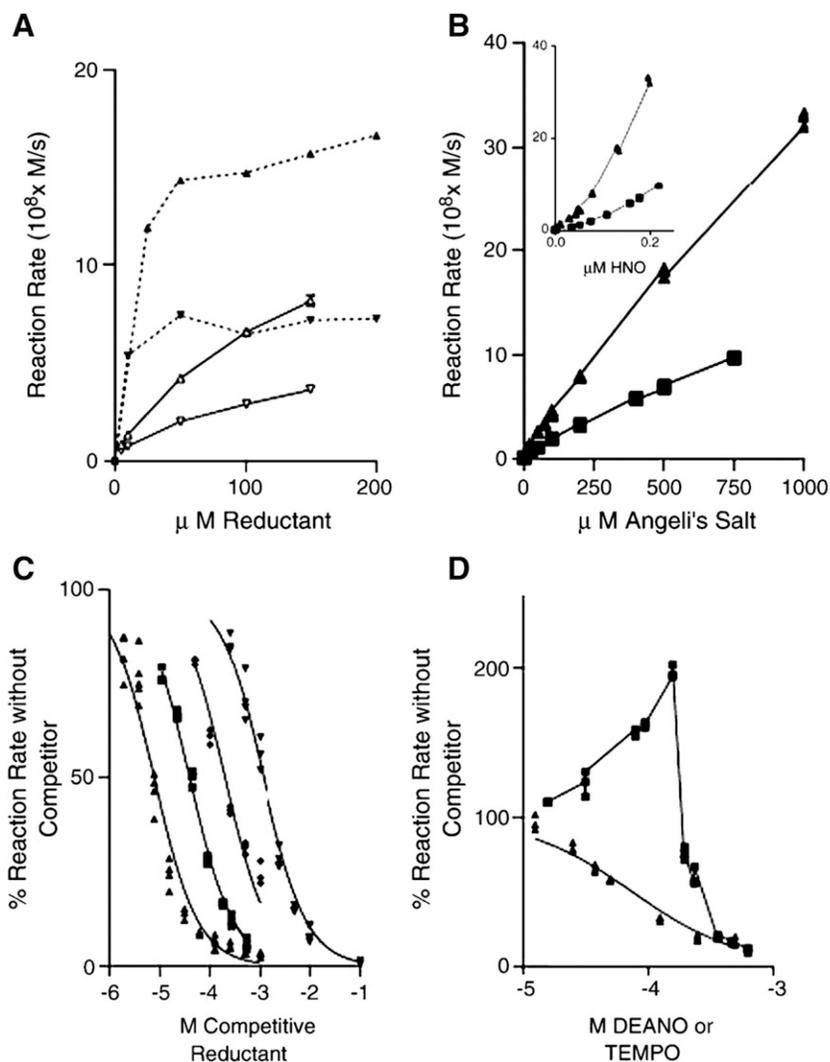


Fig. 1. Direct and competition reactions of HNO with reductants. A. Plot of [reductant] versus rate of reductant oxidation. (\blacktriangledown) 200 μM Angeli's salt and (\blacktriangle) 500 μM Angeli's salt: filled symbols, NADH (average of duplicates; open symbols, ascorbate ($n=3$ B. Plot of [donor] (Angeli's salt, 1–1000 μM) versus rate of reductant oxidation. NADH (\blacktriangle , 85 μM), ascorbate (\blacksquare , 100 μM). B inset. Plot of [HNO] versus rate of reductant oxidation. NADH (\blacktriangle , 85 μM), ascorbate (\blacksquare , 100 μM). [HNO] was derived from the method of Liochev and Fridovich [49]. C. Competition assay. Angeli's salt (500 μM) and NADH (85 μM) were reacted together in the presence of increasing concentrations of competing reductant, and the loss of NADH was monitored as in A. Glutathione (\blacksquare), ascorbate (\blacktriangledown), trolox (\bullet), and hydroxylamine (\blacklozenge) data are shown, with the values predicted by DYNAFIT shown as a solid line superimposed on the data. D. Anomalous behaviour of NO in the competition assay. Conditions were as in C, but with nitric oxide donor, DEANO (\blacksquare), as the competitor. A typical response, exhibited by TEMPO (\blacktriangledown) is shown for comparison.

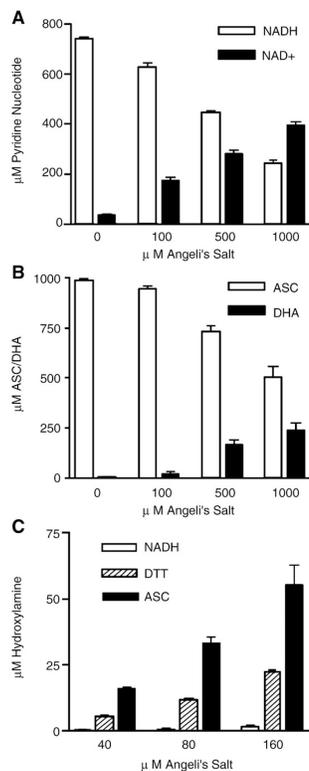


Fig. 2. Product formation from the reaction of HNO with reductant. (A) 800 μ M NADH or (B) 1000 μ M ascorbate was reacted for 90 min in 50 mM potassium phosphate with the indicated concentrations of Angeli's salt before assay for remaining reductant and oxidized products. (C) Hydroxylamine was assayed using the indoxine test after reaction under conditions as in (A) and (B). ASC, ascorbate; DHA, dehydroascorbate; DTT, dithiothreitol.

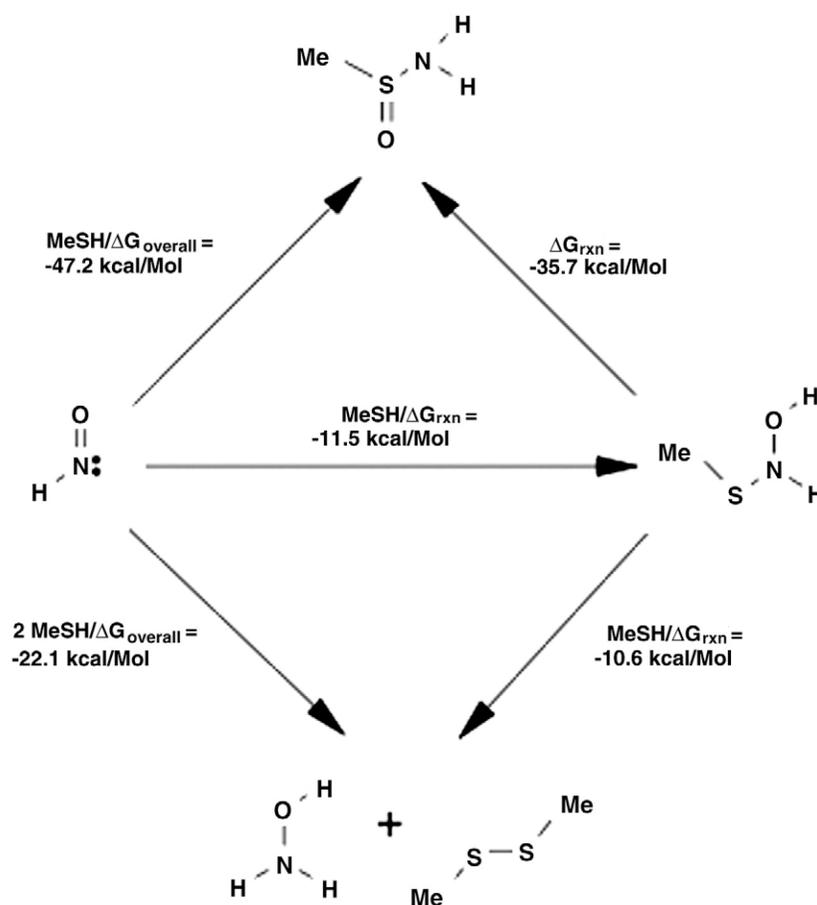
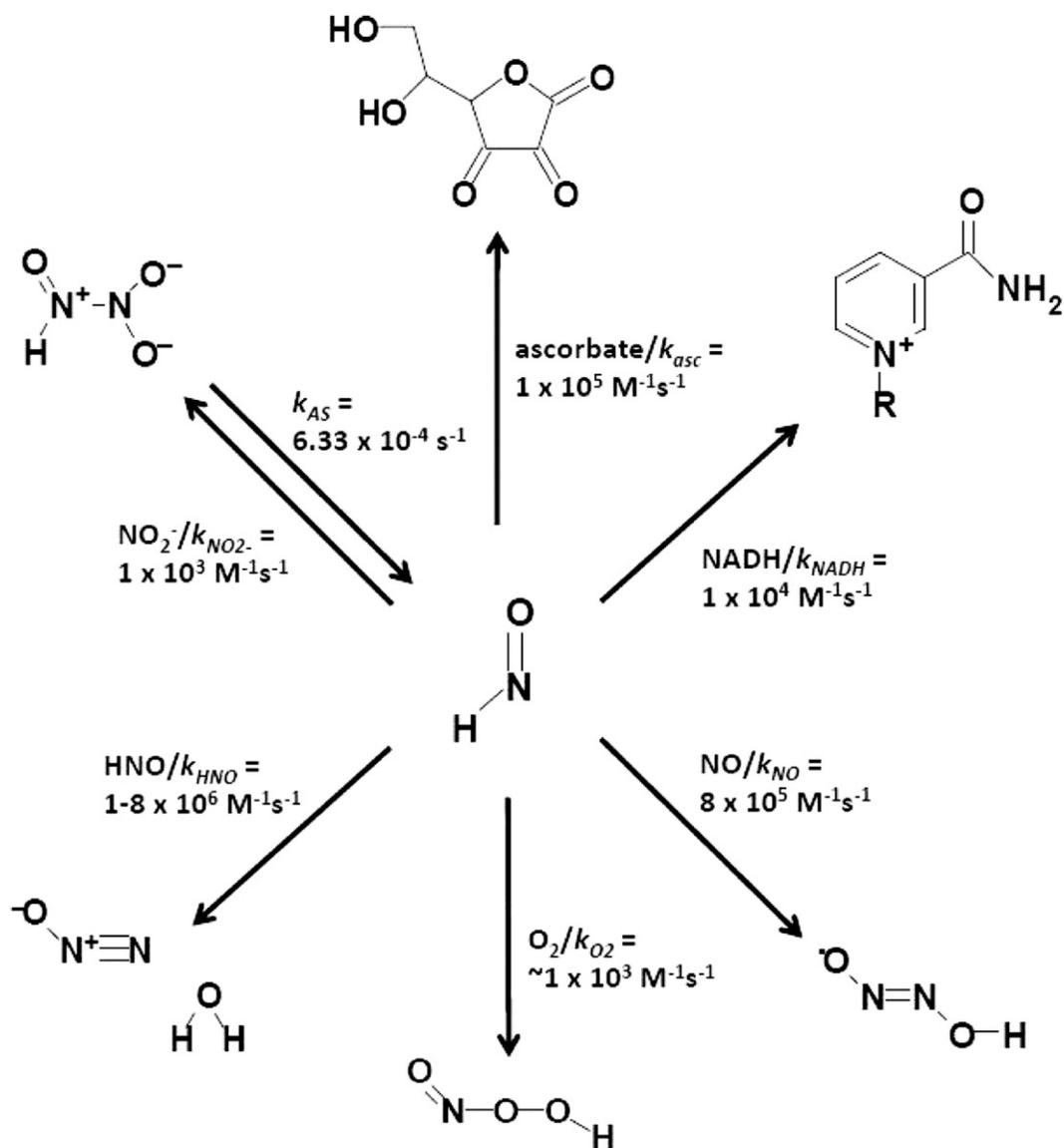


Fig. 3. Thermodynamic scheme describing reactions of HNO with thiol (B3LYP/6-31G(d) method with solvation via SM6 model).

**Scheme 1.**

Kinetic scheme describing the combined processes under consideration. This was used to process initial rate data through DYNAFIT for direct and competition reactions. The coreactant leading to product is shown adjacent the reaction arrow, followed by the published rate constant. References: k_{AS} , determined here; $k_{\text{NO}_2^-}$ [48]; k_{HNO} [29,85]; k_{NO} [65]; k_{O_2} [29,48].

Table 1

Rate constants for the reaction of HNO with reductants

Reductant	$K_{\text{red}} \times 10^{-5} \text{ M}^{-1} \text{ s}^{-1}$	% Error	$k_{\text{red}}/k_{\text{NADH}}^1$
Hydroxylamine	0.04	7.4	0.31
Selenomethionine	0.09	12	0.67
NAD(P)H	0.11	9.1	1
Thiosulfate	0.21	8.4	1.58
Trolox	0.2	14	1.72
TEMPO	0.63	13	4.38
Ascorbate	1.1	9.8	6.1
Glutathione	76	9.5	42.80
Tris-carboxyethylphosphine	84	21	53.42

Conditions were as for Fig. 1C, and values were derived using DYNAFIT and MATLAB via fits of data to the kinetic scheme described in Scheme 1.