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Direct and Nitroxyl (HNO)-Mediated Reactions of Acyloxy Nitroso Compounds with the Thiol-Containing Proteins Glyceraldehyde 3- Phosphate Dehydrogenase and Alkyl Hydroperoxide Reductase Subunit C

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Abstract

Nitroxyl (HNO) reacts with thiols and this reactivity requires the use of donors with 1-nitrosocyclohexyl acetate, pivalate and trifluoroacetate forming a new group. These acyloxy nitroso compounds inhibit glyceraldehyde 3-phosphate dehydrogenase (GAPDH) by forming a reduction reversible active site disulfide and a reduction irreversible sulfinic acid or sulfinamide modification at Cys 244. Addition of these acyloxy nitroso compounds to AhpC C165S yields a sulfinic acid and sulfinamide modification. A potential mechanism for these transformations includes nucleophilic addition of the protein thiol to a nitroso compound to yield an N-hydroxysulfenamide, which reacts with thiol to give disulfide or rearranges to sulfinamides. Known HNO donors produce the un-substituted protein sulfinamide as the major product while the acetate and pivalate give substituted sulfinamides that hydrolyze to sulfinic acids. These results suggest that nitroso compounds form a general class of thiol-modifying compounds allowing their further exploration.

Introduction

Nitroxyl (HNO) is a triatomic compound related to the well-known signaling agent nitric oxide (NO) through one-electron reduction and protonation.^{1–3} HNO demonstrates distinct chemistry and biology compared to NO and these differences combined with HNO's potential as a new congestive heart failure (CHF) treatment fuel studies to better understand the pharmacology and biochemistry of HNO.^{1, 3–11} Chemically, HNO acts as a potent electrophile, rapidly reacting with itself to ultimately yield nitrous oxide¹² and thiols to produce N-hydroxysulfenamide adducts (RSNHOH) that react with additional thiol to yield disulfides (RSSR), a reversible modification via reduction, and hydroxylamine (Scheme 1).^{13, 14} In the absence of thiol, this intermediate dehydrates to a reactive sulfenium species, which adds water to yield sulfinamide (via tautomerization, Scheme 1).^{1, 15–18} These thiol reactions form the basis of many of the observed biological effects of HNO including the actions of HNO on cardiac tissue (increased inotropy and calcium sensitivity) that appear based on HNO's interaction with key thiols in phospholamban,¹⁹ the sarcoplasmic reticulum (SR) calcium pump,^{9, 20} the ryanodine receptor and cardiac myofilaments.^{20–23} HNO,

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generated from AS, inhibits glyceraldehyde-3-phosphate dehydrogenase (GAPDH) through interaction with the active site thiol and this activity forms the basis of the use of HNO donors as potential anti-cancer agents.^{24–26} HNO mediates the action of cyanamide, a drug used to treat alcoholism through modification of the active site thiol of aldehyde dehydrogenase (ALDH) blocking normal ethanol metabolism.^{27, 28} HNO inhibition of GAPDH and ALDH consists of a reversible (upon treatment with a reducing agent) and irreversible component with the reversible portion attributed to disulfide formation and the irreversible being ascribed to protein sulfinamide generation.^{24, 25, 27}

Given HNO's high reactivity with itself and thiols, introduction of HNO to biological systems relies upon kinetically-defined chemical HNO donors.^{29, 30} Currently, Angeli's salt (AS, $\text{Na}_2\text{N}_2\text{O}_3$) remains the most common HNO source and decomposes to HNO and nitrite at pH 7 with a rate constant of $5 \times 10^{-3} \text{ s}^{-1}$ at 37 °C.^{31, 32} Other compounds including Piloty's acid (PhSO_2NHOH) and cyanamide (H_2NCN) generate HNO under specific conditions and numerous new, structurally diverse HNO donors have been described.^{33–39} Endogenous HNO production remains uncertain, although several pathways for biological production have been suggested.^{18, 40–44} Acyloxy nitroso compounds (**1–3**) generate HNO through ester hydrolysis to yield an unstable intermediate that decomposes to HNO and the corresponding ketone (Scheme 2).^{45, 46} These C-nitroso compounds form a class of HNO donors that vary in their stability and rate of HNO release depending on the ester group structure. 1-Nitrosocyclohexyl acetate (**1**) acts as a slow ($t_{1/2} = 800 \text{ min}$) HNO donor while 1-nitrosocyclohexyl pivalate (**2**) does not release HNO under neutral conditions at an appreciable rate ($t_{1/2} = 2268 \text{ min}$).⁴⁶ 1-Nitrosocyclohexyl trifluoroacetate (**3**) releases HNO at a rate too rapid to measure using standard methods and here we provide stopped-flow measurements for the hydrolytic decomposition (and HNO release) of **3**.⁴⁶ Similar to AS, the HNO donor utilized in most studies of HNO's biological actions, acyloxy nitroso compounds activate soluble guanylate cyclase,⁴⁷ relax pre-constricted rat aortic rings and enhance myocardial contractility by increasing calcium cycling and sensitizing myofilament responsiveness to calcium.^{46, 48} Enhanced myocardial contractility directly results from specific acyloxy nitroso compound-mediated thiol modification of the myofilament proteins actin, tropomyosin and the myosin light and heavy chains.⁴⁸ Acyloxy nitroso compounds block leukemia inhibitory factor (LIF) signaling in endothelial cells and cardiac myocytes through oxidative modification of protein thiols.⁴⁹

Acyloxy nitroso compounds also structurally resemble C-nitroso compounds, such as nitrosobenzene (PhNO), which cannot hydrolyze to HNO but directly react with thiols as electrophiles to give various products including disulfides and sulfinamides.⁵⁰ Similar to HNO (derived from AS), PhNO inhibits ALDH, possibly by forming an N-phenyl sulfinamide derivative (Scheme 1).⁵¹ Small molecule thiols also directly react with acyloxy nitroso compounds (Scheme 2).⁴⁶ Thiol addition competes with hydrolytic HNO formation and choice of reaction pathway primarily depends on the structure of the acyloxy nitroso compound and the reaction conditions. While the chemistry and involvement of protein thiols in the observed biological activity of acyloxy nitroso compounds suggests HNO-mediated activity, the ability of acyloxy nitroso compounds to directly react with thiols provides a potential non-HNO-mediated pathway for these species to elicit their effects. This paper examines the reactions of the acyloxy nitroso compounds (**1–3**) with the thiol-containing proteins, GAPDH and mutant alkyl hydroperoxidase reductase subunit (AhpC C165S). The results, in comparison to AS, indicate that acyloxy nitroso compounds modify thiol-containing proteins in a similar fashion to HNO but through two pathways: HNO release (similar to AS) and direct reactions of the thiol and acyloxy nitroso compound.

Results

Stopped-Flow Determination of the Rate of Decomposition of **3**

Previous studies define the decomposition kinetics of **1** and **2**, and trifluoroacetate (**3**) decomposes under neutral buffered conditions so rapidly that normal UV-vis spectroscopy cannot measure a rate of decomposition.⁴⁶ Stopped-flow UV-vis spectroscopy experiments were used to determine the rate constant ($k = 5.74 \pm 1.40 \text{ s}^{-1}$) and half-life ($t_{1/2} = 121 \text{ ms}$) of **3** in buffer by global fitting of the spectra from 553–783 nm (Supporting Information). These results indicate that **3** releases HNO over 1000 times faster than AS identifying it as an extremely fast non-nitrite generating HNO donor.

In vitro inhibition of GAPDH by **1–3**, PhNO and AS

GAPDH contains 4 cysteine residues (Cys149, Cys153, Cys244, and Cys281; EC=1.2.1.12) and possesses a sequence motif that facilitates disulfide formation between the active site cysteines (Cys 149 and 153). AS-derived HNO inhibits GAPDH activity and consists of a reversible (upon treatment with a reducing agent) and irreversible component of inhibition making GAPDH an excellent model to evaluate and compare new HNO donors.^{24–26} Assays monitoring the change of NADH absorbance at 340 nm reveal that incubation of rabbit muscle GAPDH with acyloxy nitroso compounds **1–3** or AS results in the inhibition of enzyme activity in a concentration-dependent manner (Figure 1). Based on this data, apparent IC_{50} values for **1–3** of 0.2, 0.15 and $0.67 \mu\text{M}$, respectively could be calculated and compared to AS ($IC_{50} = 0.4 \mu\text{M}$, Supporting Information). Nitrosobenzene also inhibited rabbit muscle GAPDH with an apparent calculated IC_{50} of $0.23 \mu\text{M}$ (Supporting Information). Solvent or decomposed solutions of **1** did not show any GAPDH inhibition. Incubation of GAPDH with GSNO or DEANO (an NO donor) results in inhibition of enzyme activity as previously reported (Supporting Information).²⁴

To test the reversibility of GAPDH inhibition by reducing agents, GAPDH was incubated with AS or **1–3** (0.1, 1, 10, 100 or $1000 \mu\text{M}$) for 60 min followed by incubation with DTT (10 mM). Addition of DTT to the incubation mixture following AS or **1–3** treatment results in partial restoration of GAPDH activity (Figure 1). For example, AS ($1 \mu\text{M}$) treatment of GAPDH decreases enzyme activity to 23% of control and incubation with DTT restores activity to 51 % of control (Figure 1). The acyloxy nitroso compounds show similar trends and incubation of GAPDH with **1–3** ($1 \mu\text{M}$) decreases enzyme activity to 5, 76 and 42% and DTT incubation restores activity to 12, 88 and 66% of control (Figure 1). In general, acyloxy nitroso compounds behave similarly to AS in these assays, which do not quantify the number or identify the modified thiols, by inhibiting GAPDH with both a reversible and irreversible component. While these results may be expected for an HNO donor, ^{24, 25} the ability of **2**, an extremely slow HNO donor, to inhibit GAPDH in a relatively similar fashion to AS and **3**, rapid HNO donors, strongly suggests the possibility of direct thiol reactions (Figure 1).

Structural Modifications of GAPDH by **1–3** and AS

Preliminary gel electrophoretic analysis of the reactions between GAPDH with AS and **1–3** does not reveal higher molecular weight disulfides but changes appear in the intensity and position of the band assigned to GAPDH (approximately 35 kDa, Supporting Information). Low resolution peptide mass mapping of GAPDH using matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry reveals modification of Cys 149, 153 and 244 of GAPDH upon incubation with AS and **1–3**. Previous work shows that incubation of GAPDH with AS yields a disulfide (Cys 149 – Cys 153) and sulfenamides at Cys 244 and 281.¹⁵ Incubation of with **1** only shows peaks with $m/z = 1699.05$ and 1701.07 for tryptic peptide I143-K159 (Supporting Information). MALDI-TOF MS spectra show a

peak with $m/z = 1499.8$ for un-modified V232–R245 peptide but GAPDH incubation with AS, **1** or **2** shows the appearance of a peak with a $m/z = 1532.0$ ($M+32$), which may represent the oxidative conversion of the thiol group (Cys 244) to a sulfinic acid (RSO_2H) or sulfinamide (R-S(O)NH_2 , $M + 1$ peak, Supporting Information). During this initial analysis, modifications at Cys 281 were not determined. Again, acyloxy nitroso compounds behave similarly to AS by yielding a disulfide between Cys 149 and 153 and an oxidized derivative at Cys 244.¹⁵

High Resolution Mass Spectrometric Determination of the Structural Modifications of GAPDH by **1–3** and AS

High resolution LC-MS experiments provide further molecular insight into the identity of the oxidative thiol modifications formed during the reaction of GAPDH and AS and the acyloxy nitroso compounds (**1–3**). Such MS experiments on untreated GAPDH followed by trypsin digest and liquid chromatography (LC) show a peak with a $m/z = 853.4349$, which corresponds to the two unreacted thiols (Cys 149 and 153) in the GAPDH tryptic peptide I143–K159 (Table 1). Incubation of GAPDH with either ^{14}N or ^{15}N AS (10 eq.) gave a major peak with a $m/z = 852.4269$ and 852.4277 , respectively, which corresponds to the formation of a disulfide (Cys 149 – Cys 153) within the I143–K159 peptide (Table 1 and Supporting Information Section, which contains detailed MS data for each incubation). Similar high resolution LC/MS examination of the I143–K159 peptide of GAPDH incubated with **1–3** or PhNO reveals only disulfide formation and no other significant peaks within this peptide. Treatment of GAPDH with *o*-bromo Piloty's acid, a mechanistically alternative HNO donor,⁵² only generates the same Cys 149 – Cys 153 disulfide (Table 1). Incubation of GAPDH with GSNO, an S-nitrosothiol or DEANO, an NO donor, followed by similar analysis only yields the internal disulfide of the I143–K159 peptide (Table 1).

Similar high resolution LC/MS measurements reveal more diverse structural modification at Cys 244 of the GAPDH peptide V232–R245 after incubation with AS and **1–3** followed by trypsin digestion (Table 2). Incubation of GAPDH with ^{14}N AS (10 eq.) gave a major peak (based on ion count) with $m/z = 765.9014$ and a minor peak with a $m/z = 766.3929$, which corresponds to a peptide sulfinamide and sulfinic acid, respectively (Table 2). Similar experiments using ^{15}N AS gave a major and minor product with identical retention times but with increased mass of 1 amu for the proposed sulfinamide product indicating ^{15}N incorporation (Table 2). Incubation of GAPDH with the acyloxy nitroso compounds **1** and **2** yields only the sulfinic acid of Cys 244 of the V232–R245 peptide but reaction of GAPDH with **3** or *o*-bromo Piloty's acid gives a mixture of sulfinamide (major) and sulfinic acid (minor), similar to AS treatment.¹⁵ Incubation of GAPDH with nitrosobenzene yields the N-phenylsulfinamide derivative (along with sulfinic acid) of this peptide (Table 2). Treatment of GAPDH with GSNO and DEANO shows the formation of the S-nitrosated peptide (Table 2). These results confirm that the reaction of HNO derived from AS, **3** or *o*-bromo Piloty's acid (relatively rapid HNO donors) with GAPDH yields a sulfinamide at Cys 244, which differs from the observed modification of NO donors. Acyloxy nitroso compounds **1** and **2**, slow HNO donors, generate a sulfinic acid at this residue suggesting different reactivity.

The size and the presence of multiple charges in the tryptic peptide of GAPDH containing Cys 281 (G269–K306, MW = 3986.85) prevents the straightforward separation and analysis of this peptide. Mass spectrometric data extraction from a reaction mixture containing this peptide shows that treatment of GAPDH with AS (10 eq.) gave a peak with $m/z = 1355.2894$ while treatment with **1** yields a peak with $m/z = 1355.6229$, which correspond to the Cys 281 sulfinamide and sulfinic acid, respectively. These results show that different products arise from the faster HNO donors compared to **1** and **2** and also reveal that the

acyloxy nitroso compounds capably react with all four GAPDH cysteine residues under these conditions.

Structural Modifications of AhpC C165S by 1–3 and AS

The mutant alkyl hydroperoxidase reductase subunit (AhpC, C165S) contains a single cysteine residue (Cys46), the peroxidatic center of the wild-type protein and has been used extensively in studies elucidating the formation and reactivity of protein sulfenic acids (RSOH).^{53, 54} To date, the reaction of HNO with AhpC C165S has not been examined and incubation of protein with AS and **1** followed by gel electrophoresis shows a major band migrating at the same distance as the parent protein (~20kDa) and a dose-dependent band of approximately 40 kDa, which likely corresponds to an AhpC disulfide dimer as treatment with a reducing agent results in the disappearance of this band (Supporting Information). Low resolution MALDI-TOF mass spectrometry of Asp-N digested unreacted AhpC peptide D41–G51 reveals a peak with a m/z = 1228.55 and N-ethyl maleimide (NEM) treated protein gives a peak of $M + 125$ indicating alkylation of a single thiol (Supporting Information). Incubation of AhpC, AS or **1–3** followed by Asp-N digestion and MALDI-TOF mass spectrometry consistently yields a peak with a m/z = 1260.85 ($M + 32$), which was assigned as either the peptide sulfinic acid (RSO₂H) or sulfinamide (RS(O)NH₂, $M + 1$) modification (Supporting Information). Given that AhpC C165S contains a single cysteine residue and cannot form an internal disulfide, the formation of these oxidized thiol derivatives appears consistent with the GAPDH results.

High Resolution Mass Spectrometric Determination of the Structural Modifications of AhpC by 1–3 and AS

High resolution LC-MS experiments provide more detailed insight into the identity of the oxidative thiol modifications formed during the reaction of AhpC and AS and acyloxy nitroso compounds (**1–3**). Such MS experiments on untreated AhpC followed by Asp-N digestion and liquid chromatography show a peak with a m/z = 1228.5537, which corresponds to unreacted thiol in the AhpC peptide D41–G51 (Table 3). Incubation of AhpC with ¹⁴N AS (10 eq.) gave a major peak (based on ion count) with m/z = 1259.5598 and a minor peak with a m/z = 1260.5444, which corresponds to a peptide sulfinamide and sulfinic acid, respectively (Table 3). Similar experiments using ¹⁵N AS gave a major and minor product with identical retention times but with an increased mass of 1 amu for the proposed sulfinamide product indicating ¹⁵N incorporation (Table 3). Incubation of AhpC with **1** and **2** yields only the sulfinic acid of Cys 46 of the D41–G51 peptide (m/z = 1260.5444) but reaction of AhpC (like GAPDH) with **3** or α -bromo Piloty's acid gives a mixture of sulfinamide and sulfinic acid at Cys 46 (Table 3). Incubation of AhpC with nitrosobenzene only yields sulfinic acid (m/z = 1260.5444) and no N-phenylsulfinamide as shown by high resolution LC/MS (Table 3). Treatment of AhpC with GSNO and DEANO followed by the same analysis reveals the presence of the S-nitrosated D41–G51 peptide with m/z = 1257.5238 (Table 3). These results show in a second protein that reaction of HNO derived from AS, **3** or α -bromo Piloty's acid (relatively rapid HNO donors) yields a sulfinamide modification while reaction with slow acyloxy nitroso compounds (**1** and **2**) generates a sulfinic acid.

Discussion and Conclusions

Nitroxyl (HNO) remains a relatively lesser studied nitrogen oxide that avidly reacts with thiols as an electrophile and these reactions underlie many of HNO's observed biology.^{1–3} The chemical and biological properties of HNO focus attention on its potential development as a congestive heart failure therapy.^{8, 10} The high reactivity of HNO with itself requires the use of donors to further study HNO's chemistry and biology and Angeli's salt (AS) provides

HNO for nearly all of these studies.^{29, 30} Scheme 2 gives a framework for understanding HNO's reactions with thiols and a growing body of work illustrates the reactions of HNO (generated from AS) with thiol-containing proteins. The reaction of tubulin with AS generates more inter-chain disulfides than peroxynitrite or other reactive nitrogen species, demonstrating the potent reactivity of HNO with thiols.⁵⁵ Exposure of phospholamban, a regulatory SERCA protein, to HNO also forms an internal disulfide bond.¹⁹ Treatment of GAPDH with HNO gives an active site disulfide (Cys 149 – 153) and sulfinamides at Cys 244 and 281.¹⁵ Treatment of human calbindin and bovine serum albumin with AS also yields sulfinamides.¹⁵ Incubation of platelets with AS yields ten modified thiol-containing proteins, including GAPDH, in a dose-dependent fashion.⁵⁶ Mass spectrometric analysis of the cysteine-containing tryptic digests of GAPDH reveals sulfinamide and sulfinic acid (RSO₂H) formation, which may arise through sulfinamide hydrolysis (Scheme 2).⁵⁶ AS treatment of cysteine-containing peptides show sulfinamide, but not sulfinic acid formation.⁵⁷ Exposure of these peptide-based sulfinamides to excess DTT results in thiol, indicating sulfinamide formation may be reversible.⁵⁷

Acyloxy nitroso compounds form an alternative group of HNO donors that release HNO without the co-production of nitrite during their hydrolytic decomposition.^{45, 46, 58} The rate of ester group hydrolysis of **1–3** dictates their rate of HNO release and these compounds bear structural resemblance to *C*-nitroso compounds, like PhNO, and react with small thiols to give disulfides and oximes rather than releasing HNO.⁴⁶ Acyloxy nitroso compounds demonstrate a biological profile similar to AS but recent work reveals differences between these compounds and AS. Compound **1** enhances myocardial contractility more potently than AS by sensitizing myofilament responsiveness to calcium, but **2** has no effect on this system, suggesting a direct role for HNO.⁴⁸ New work shows **2**, which does not release HNO blocks STAT3 activation similar to AS, indicating **2** likely elicits these effects through direct reactions.⁴⁹ The differing biological properties of these compounds compared to AS coupled with their ability to directly react with thiols warrant further study of their reaction with thiol-containing proteins to understand their biology and define their therapeutic potential.

Acyloxy nitroso compounds (**1–3**) behave similarly to AS by inhibiting GAPDH in a dose-dependent fashion, with comparable potency and both a reversible (upon DTT addition) and irreversible component (Figure 1).^{24, 25} Nitrosobenzene, which does not release HNO, also inhibits GAPDH, which shows the electrophilic nitroso group is capable for inhibiting this enzyme. The extreme difference in the HNO release rates of **1–3** and PhNO coupled with their ability to inhibit GAPDH strongly suggests that direct reactions with protein thiols contribute to enzyme inhibition as **1**, **2** and PhNO should not produce HNO under these conditions and time scale. AS and **1–3** inhibit GAPDH at much lower concentrations than GSNO and DEA/NO, whose inhibition is completely DTT reversible, suggesting these HNO and *C*-nitroso compounds inhibit the enzyme in a different fashion.

Mass spectrometric studies provide more specific details regarding the reactions of acyloxy nitroso compounds and thiol-containing proteins. Low and high resolution MS show that the reactions of GAPDH with **1–3**, PhNO, GSNO and DEA/NO yields the disulfide (between Cys 149 and 153), as previously shown for AS.¹⁵ Disulfide formation represents a DTT reversible protein modification and likely accounts for the restoration of activity upon reduction. High resolution MS shows that only the rapid HNO donors AS and **3** generate a sulfinamide modification (major) and some sulfinic acid at Cys 244, but the slower acyloxy nitroso HNO donors (**1–2**) only form sulfinic acids. Sulfinic acid formation could occur via sulfinamide hydrolysis (Scheme 2), but the identification of sulfinamides in these reactions, confirmed by isotopic labeling, reveals their hydrolytic stability and strongly suggests direct sulfinic acid formation. These four-electron oxidized modifications, which form distant from

the active site cysteine residues (Cys 149 and 153), may contribute to the irreversible portion of GAPDH inhibition by altering the overall protein structure or aggregation state, as suggested for AS. Examination of these reactions mixtures show that **1–3** also modify Cys 281 of GAPDH revealing that all four cysteine residues react but neither high nor low resolution MS provides evidence of a direct adduct of **1–3** and the protein. Experiments with AhpC C165S show a similar pattern with AS and **3** yielding mixtures of sulfinamide and sulfinic acid modified protein and **1** and **2** giving only sulfinic acids. The addition of PhNO, a *C*-nitroso compound, to GAPDH generates a stable N-phenyl sulfinamide adduct at Cys 244 but similar experiments with AhpC C165S only gives sulfinic acid.

These results organize acyloxy nitroso compounds into those that rapidly release HNO and those that act as electrophilic *C*-nitroso compounds directly reacting with protein thiols. Compounds that rapidly release HNO (such as **3** and AS) form protein sulfinamides as the predominant product and those that slowly release or do not release HNO (**1–2** or PhNO) primarily yield sulfinic acids and N-substituted sulfinamides. To further test whether HNO donors yield sulfinamides, incubation of *o*-bromo Piloty's acid, a rapid yet structurally and mechanistically distinct HNO donor,⁵² with gives the Cys 244 sulfinamide as the major modification, supporting the idea that sulfinamide formation represents a unique protein marker for HNO. Sulfinamide identification at a specific residue in a specific protein (Cys 244 of GAPDH for example) may provide a new avenue of HNO detection strategy. The reaction of GAPDH and AhpC C165S with GSNO (an S-nitrosothiol) or DEA/NO (an NO donor) do not yield the sulfinamide but rather the S-nitrosothiol showing unique formation of sulfinamides with HNO donors. These results indicate that acyloxy nitroso compounds that slowly release HNO still yield protein thiol modifications similar to HNO. Compounds **1** and **2** both convert the active site thiols Cys 149 and 153 of GAPDH to a disulfide and result in a four electron oxidation of Cys 244 and 281 to a sulfinic acid. Such observations identify these compounds as "organic nitroxyls" capable of effecting similar biological chemistry to HNO without releasing HNO. Such compounds may provide answers in terms of forwarding the discovery and development of new HNO donors as the organic portion can be easily synthetically manipulated in terms of size, water-solubility, stereochemistry and possess diverse HNO release rate compared to AS.

Scheme 2 provides an accepted general mechanism for the reaction of HNO (regardless of the source) with protein thiols that leads to the observed products. Scheme 2 also accounts for the formation of reaction products of *C*-nitroso compounds with thiols. The formation of minor amounts of sulfinic acid in the reactions of HNO donors or PhNO with GAPDH and AhpC C165S may arise from sulfinamide hydrolysis or through other undefined reactions of the sulfenium ion intermediate (Scheme 2). Similar reactions of aromatic *C*-nitroso compounds with glutathione and thiol-containing proteins including human serum albumin yield thiol oxidized products including N-aryl sulfinamides.^{50, 59} Scheme 3 depicts a potential explanation for the exclusive formation of sulfinic acids from the reaction of **1** and **2** with thiol containing proteins. Acyloxy nitroso compounds behave like other electrophilic *C*-nitroso compounds and undergo nucleophilic thiol addition to yield an N-hydroxysulfenamide intermediate. Further addition of thiol to the N-hydroxysulfenamide yields the disulfide and an α -substituted hydroxylamine that decomposes to the oxime (Scheme 3). In the absence of a second thiol, these intermediates dehydrate to a sulfenium ion (similar to Scheme 2) and the presence of the acyl group permits internal trapping by the acyloxy nitroso compound to form an intermediate that collapses to an α -N-substituted sulfinamide (Scheme 3). Such a pathway would not be available to HNO or PhNO and the exclusive identification of sulfinic acids from these incubations suggest rapid hydrolysis of these structurally unusual sulfinamides (Scheme 3).

Nitroxyl possesses a distinct chemistry and biology compared to NO and these differences combined with HNO's potential as a new congestive heart failure treatment have generated interest in new HNO donors as potential therapeutics. Much of HNO's biological activity appears mediated through reactions with thiol-containing proteins and this work examines reactions of three kinetically distinct acyloxy nitroso compounds (**1–3**) with GAPDH and AhpC C165S. Nitroso compounds (X-N=O, whether X = H, (nitroxyl) or X = carbon, (C-nitroso, acyloxy nitroso)) react with thiol containing proteins to give various products depending on the rate of HNO release and the identity of X. Nucleophilic addition of the protein thiol to a nitroso compound yields an N-hydroxysulfenamide that reacts with a thiol to give a disulfide as observed for the GAPDH active site. In the absence of thiol, this intermediate loses water to yield a sulfenium ion that rehydrates to form sulfinamides that may hydrolyze to sulfinic acids. For GAPDH and AhpC C165S, the known HNO donors AS, **3** and α -bromo Piloty's acid produce the un-substituted protein sulfinamide as the major product but **1**, **2** and PhNO (that either release HNO slowly or do not release HNO) yield sulfinic acids or N-aryl sulfinamides.⁴⁶ Nitroso compounds form a general class of thiol-modifying compounds ranging from HNO to PhNO to the acyloxy nitroso compounds that show different reactivity that can be exploited for biochemical, pharmacological and clinical benefit.

Experimental

General

Sodium triiodidinitrate (Angeli's Salt, AS),⁶⁰ ¹⁵N AS,⁶⁰ S-nitrosoglutathione (GSNO),⁶¹ α -bromo Piloty's Acid,⁶² 1-nitrosocyclohexyl acetate,⁴⁵ 1-nitrosocyclohexyl pivalate,⁴⁶ and 1-nitrosocyclohexyl trifluoroacetate⁴⁶ were prepared as previously described with the purity of the acyloxy nitroso compounds being determined by elemental analysis (> 95%). Diethylammonium-1- (N,N-diethylamino) diazen-1-ium- 1,2-diolate (DEANO) was purchased from Cayman Chemicals. Glyceraldehyde-3- phosphate dehydrogenase (GAPDH, EC 1.2.1.12) from rabbit muscle was obtained as a lyophilized powder (1KU protein) from Sigma (St.Louis, MO). Glyceraldehyde-3- phosphate (G-3-P) and -nicotinamide adenine dinucleotide (NAD⁺) were purchased from Sigma. All other chemicals and materials were purchased from Sigma (St. Louis, MO) or Fisher (Pittsburgh, PA) and were of the highest quality available. Mutant alkyl hydroperoxide reductase subunit C C165S (AhpC C165S) was obtained from Professor Leslie Poole (Wake Forest University). UV-Vis spectrometry was performed on a Cary 100 Bio UV-Vis spectrophotometer (Varian, Walnut Creek, CA). Data were typically obtained from experiments run in triplicate where each value represents the mean $\pm$ SE (standard error). IC₅₀ values were obtained by fitting concentration-dependent percent of control data to a non-linear curve using a sigmoidal curve with a standard slope algorithm (GraphPad Prism).

Stopped-flow UV-vis spectroscopic kinetic decomposition of **3**^{63, 64}

A solution of **3**(1mM) in dimethyl formamide (DMF) was loaded into one syringe of a BioLogic SFM- 300stopped flow apparatus with a 2mm cuvette (Knoxville, TN), coupled to an Olis RSM 1000 spectrophotometer (Bogart, GA), while phosphate buffered saline (PBS, 10 mM, pH = 7.4) was loaded into another syringe. The syringes and internal mixing pathways of the apparatus were deoxygenated prior to and during the experiment with argon to ensure that hydrolysis of **3** did not occur prior to mixing the solutions. The flow rate of **3** was 40 μ L/ms, while the flow rate for the buffer was 120 μ L/ms, and the final concentration upon mixing of **3** was 250 μ M. Absorbance measurements were taken at 1 ms intervals over the range of 553–783 nm, and kinetics were determined using SPECFIT Global Analysis software.

In vitro inhibition of GAPDH by 1–3, AS and NB

Lyophilized GAPDH (rabbit muscle, Sigma) was dissolved in sodium phosphate buffer (PB, 0.1 M, pH 7.4) to give a solution with a final concentration of 100 U/mL (approximately 30 μ M). Stock solutions of NAD⁺ (10 mM) and glyceraldehyde-3-phosphate (G-3-P, 10 mM) were also prepared in PB. Stock solutions of AS, DEANO and GSNO were prepared in cold 0.01 M NaOH and solutions of 1–3 and NB were prepared in DMF and kept on ice until used. Incubations of GAPDH (4 μ L) with AS, 1–3 or NB (0.1, 0.5, 1, 5, 10, 100 μ M) were done in PB (0.1 M, pH 7.4, total volume of 700 μ L) at 25°C. After 60 min, the reaction mixtures were assayed for residual enzyme activity in a septum sealed cuvette by adding NAD⁺ (100 μ L) and G-3-P (100 μ L). The activity was determined spectrophotometrically by following the increase in concentration of NADH (monitored at 340 nm) over time.

To determine whether GAPDH inhibition could be reversed by thiols, experiments were carried out by first incubating the enzyme with the inhibitor (AS, DEANO, GSNO, 1–3 or NB) as described followed by DTT (100 mM) incubation for 60 min. The reaction mixture was analyzed for residual enzyme activity as before. All incubations were carried out at 25 °C. All reactions were monitored at one minute intervals over 30 min at room temperature in a 1 cm cuvette on a Cary Bio 100 spectrophotometer.

Tryptic Digestion of GAPDH

Untreated and modified GAPDH was digested at a GAPDH monomer to trypsin ratio of 20:1 (w/w) at 37 °C for 12–16 h in 50 mM Tris-HCl buffer. Digestion was quenched by freezing at –20 °C.

Reaction of AS and 1–3 with AhpC C165S

AhpC C165S was pre-incubated with DTT (20 μ L, 1M in H₂O) for 30 min. DTT was removed by Bio-Gel P6 spin columns and protein concentration was assessed using UV-vis spectroscopy ($\epsilon_{280}=23400 \text{ M}^{-1} \text{ cm}^{-1}$). An aliquot of protein (50 μ M) was incubated with AS or 1 (1, 5, 10 and 50 equivalents) in Tris Buffer (0.1 M, pH 7.6, total volume, 200 μ L) at room temperature for 60 min. Samples were applied to Bio-Gel P6 spin columns to remove small molecules and exchange protein into ammonium bicarbonate buffer (25 mM, pH 7.75).

Asp-N Digestion of AhpC C146S

Untreated and modified AhpC C146S was digested at a protein monomer to Asp-N ratio of 20:1 (w/w) at 37 °C for 12–16 h in 50 mM Tris-HCl buffer. Digestion was quenched by freezing at –20 °C.

MALDI-TOF Mass Spectrometric Analyses of GAPDH and AhpC C146S

Following enzymatic digestion, GAPDH and AhpC samples were mixed in a 1:1 ratio with matrix (0.05 g dihydroxy benzoic acid in 50% acetonitrile and 0.3% trifluoroacetic acid, 1 mL) and spotted onto a MALDI target plate. The tryptic peptides from GAPDH containing cysteinyl residues examined were: I143-K159 (IVSNASCTTNCLAPLAK) and V232–R245 (VPTPNVSVVDLTCTCR). AhpC C146S peptides containing a cysteinyl residue: D41–G51 (DFTFVCPTELG). Data Analysis was performed on a Bruker Daltonics MALDI–TOF mass spectrometer.

High Resolution Liquid Chromatography-Mass Spectrometry

Liquid chromatography-mass spectrometry experiments were performed on a Thermo Fisher Orbitrap LTQ XL high-resolution mass spectrometer. Separations were achieved using a Thermo Hypersil GOLD column (50 x 2.1 mm, 1.9 μ m) at 25 °C with solvent A = Optima

grade 0.1% formic acid in water, solvent B = Optima grade 0.1% formic acid in methanol, and solvent C Optima grade acetonitrile with a flow rate of 250 μ L/min. The column was conditioned using a gradient of 100% solvent C to 95% solvent B and 5% solvent A over 5 minutes, and held for 5 minutes. The gradient for separation of GAPDH peptides was : 70% solvent A (30% solvent B) ramped to 5% solvent A (95% solvent B) over 25 minutes, immediately ramped to 95% solvent A (5% solvent B) and held for 5 minutes, for a total run time of 30 minutes. The gradient for separation of AhpCC146S peptides was: 80% solvent A (20% solvent B) ramped to 5% solvent A (95% solvent B) over 55 minutes, immediately ramped to 95% solvent A (5% solvent B) and held for 5 minutes, for a total run time of 60 minutes. All analyses were performed with 10 μ M solutions of digested protein (GAPDH or AhpC) prepared in Optima grade reagents of 1:1 2% formic acid in water:methanol. The mass spectrometer was operated in positive ion with the following conditions: Sheath gas flow rate 29 arb, spray voltage 4.0kV, capillary temperature 325°C, Capillary voltage 40.0 V, Tube Lens voltage (95.0 V) and resolution at 60000.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations Used

HNO	nitroxyl
NO	nitric oxide
CHF	congestive heart failure
AS	Angeli's Salt
GSNO	glutathione S-nitrosothiol
SR	sarcoplasmic reticulum
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
ALDH	aldehyde dehydrogenase
sGC	soluble guanylatecyclase
AhpC	alkyl hydroperoxidase
DEANO	diethylammonium-1- (N,N-diethylamino) diazen-1-ium-1,2-diolate
G-3-P	glyceraldehyde-3-phosphate
NAD⁺	-nicotinamide adenine dinucleotide
AhpC C165S	mutant alkyl hydroperoxide reductase subunit C C165S
DHB	dihydroxybenzoic acid
DMF	dimethyl formamide
PBS	phosphate buffered saline
DTT	dithiothreitol
SDS-PAGE	sodiumpolyacrylamide gel electrophoresis

NBD-Cl	4-chloro-7-nitrobenzofurazan
DMSO	dimethyl sulfoxide
NEM	N-ethylmaleimide

References

1. Fukuto JM, Switzer CH, Miranda KM, Wink DA. Nitroxyl (HNO): Chemistry, biochemistry, and pharmacology. *Annual Review of Pharmacology and Toxicology*. 2005; 45:335–355.
2. Miranda KM. The chemistry of nitroxyl (HNO) and implications in biology. *Coordination Chemistry Reviews*. 2005; 249:433–455.
3. Kemp-Harper BK. Nitroxyl (HNO): A Novel Redox Signaling Molecule. *Antioxidants & Redox Signaling*. 2011; 14:1609–1613. [PubMed: 21299468]
4. Switzer CH, Flores-Santana W, Mancardi D, Donzelli S, Basudhar D, Ridnour LA, Miranda KM, Fukuto JM, Paolocci N, Wink DA. The emergence of nitroxyl (HNO) as a pharmacological agent. *Biochimica et Biophysica Acta (BBA) - Bioenergetics*. 2009; 1787:835–840.
5. Fukuto JM, Bartberger MD, Dutton AS, Paolocci N, Wink DA, Houk KN. The physiological chemistry and biological activity of nitroxyl (HNO): The neglected, misunderstood, and enigmatic nitrogen oxide. *Chemical Research in Toxicology*. 2005; 18:790–801. [PubMed: 15892572]
6. Irvine JC, Ritchie RH, Favalaro JL, Andrews KL, Widdop RE, Kemp-Harper BK. Nitroxyl (HNO): the Cinderella of the nitric oxide story. *Trends in Pharmacological Sciences*. 2008; 29:601–608. [PubMed: 18835046]
7. Feelisch M. Nitroxyl gets to the heart of the matter. *Proceedings of the National Academy of Sciences of the United States of America*. 2003; 100:4978–4980. [PubMed: 12704227]
8. Paolocci N, Saavedra WF, Miranda KM, Martignani C, Isoda T, Hare JM, Espey MG, Fukuto JM, Feelisch M, Wink DA, Kass DA. Nitroxyl anion exerts redox-sensitive positive cardiac inotropy in vivo by calcitonin gene-related peptide signaling. *Proceedings of the National Academy of Sciences of the United States of America*. 2001; 98:10463–10468. [PubMed: 11517312]
9. Tocchetti CG, Stanley BA, Murray CI, Sivakumaran V, Donzelli S, Mancardi D, Pagliaro P, Gao WD, van Eyk J, Kass DA, Wink DA, Paolocci N. Playing with Cardiac "Redox Switches": The "HNO Way" to Modulate Cardiac Function. *Antioxidants & Redox Signaling*. 2011; 14:1687–1698. [PubMed: 21235349]
10. Paolocci N, Katori T, Champion HC, St John ME, Miranda KM, Fukuto JM, Wink DA, Kass DA. Positive inotropic and lusitropic effects of HNO/NO⁻ in failing hearts: Independence from beta-adrenergic signaling. *Proceedings of the National Academy of Sciences of the United States of America*. 2003; 100:5537–5542. [PubMed: 12704230]
11. Paolocci N, Jackson MI, Lopez BE, Miranda K, Tocchetti CG, Wink DA, Hobbs AJ, Fukuto JM. The pharmacology of nitroxyl (HNO) and its therapeutic potential: Not just the janus face of NO. *Pharmacology & Therapeutics*. 2007; 113:442–458. [PubMed: 1722913]
12. Bonner FT, Hughes MN. The Aqueous Solution Chemistry of Nitrogen in Low Positive Oxidation States. *Comments in Inorganic Chemistry*. 1988; 7:215–234.
13. Doyle MP, Mahapatro SN, Broene RD, Guy JK. Oxidation and Reduction of Hemoproteins by Trioxodinitrate(Ii) - the Role of Nitrosyl Hydride and Nitrite. *Journal of the American Chemical Society*. 1988; 110:593–599.
14. Fukuto JM, Carrington SJ, Tantillo DJ, Harrison JG, Ignarro LJ, Freeman BA, Chen A, Wink DA. Small Molecule Signaling Agents: The Integrated Chemistry and Biochemistry of Nitrogen Oxides, Oxides of Carbon, Dioxygen, Hydrogen Sulfide, and Their Derived Species. *Chemical Research in Toxicology*. 2012; 25:769–793. [PubMed: 22263838]
15. Shen B, English AM. Mass spectrometric analysis of nitroxyl-mediated protein modification: Comparison of products formed with free and protein-based cysteines. *Biochemistry*. 2005; 44:14030–14044. [PubMed: 16229492]
16. Shoeman DW, Shirota FN, DeMaster EG, Nagasawa HT. Reaction of nitroxyl, an aldehyde dehydrogenase inhibitor, with N-acetyl-L-cysteine. *Alcohol, United States*. 2000; 20:55–59.

17. Donzelli S, Espey MG, Thomas DD, Mancardi D, Tocchetti CG, Ridnour LA, Paolocci N, King SB, Miranda KM, Lazzarino G, Fukuto JM, Wink DA. Discriminating formation of HNO from other reactive nitrogen oxide species. *Free Radical Biology and Medicine*. 2006; 40:1056–1066. [PubMed: 16540401]
18. Wong PSY, Hyun J, Fukuto JM, Shirota FN, DeMaster EG, Shoeman DW, Nagasawa HT. Reaction between S-Nitrosothiols and Thiols: Generation of Nitroxyl (HNO) and Subsequent Chemistry. *Biochemistry*. 1998; 37:5362–5371. [PubMed: 9548918]
19. Froehlich JP, Mahaney JE, Keceli G, Pavlos CM, Goldstein R, Redwood AJ, Sumbilla C, Lee DI, Tocchetti CG, Kass DA, Paolocci N, Toscano JP. Phospholamban Thiols Play a Central Role in Activation of the Cardiac Muscle Sarcoplasmic Reticulum Calcium Pump by Nitroxyl. *Biochemistry*. 2008; 47:13150–13152. [PubMed: 19053265]
20. Tocchetti CG, Wang W, Froehlich JP, Huke S, Aon MA, Wilson GM, Di Benedetto G, O'Rourke B, Gao WD, Wink DA, Toscano JP, Zaccolo M, Bers DM, Valdivia HH, Cheng H, Kass DA, Paolocci N. Nitroxyl improves cellular heart function by directly enhancing cardiac sarcoplasmic reticulum Ca²⁺ cycling. *Circ Res United States*. 2007; 100:96–104.
21. Dai TY, Tian Y, Tocchetti CG, Katori T, Murphy AM, Kass DA, Paolocci N, Gao WD. Nitroxyl increases force development in rat cardiac muscle. *Journal of Physiology-London*. 2007; 580:951–960.
22. Stoyanovsky D, Murphy T, Anno PR, Kim YM, Salama G. Nitric oxide activates skeletal and cardiac ryanodine receptors. *Cell Calcium*. 1997; 21:19–29. [PubMed: 9056074]
23. Cheong E, Tumbev V, Abramson J, Salama G, Stoyanovsky DA. Nitroxyl triggers Ca²⁺ release from skeletal and cardiac sarcoplasmic reticulum by oxidizing ryanodine receptors. *Cell Calcium*. 2005; 37:87–96. [PubMed: 15541467]
24. Lopez BE, Wink DA, Fukuto JM. The inhibition of glyceraldehyde-3- phosphate dehydrogenase by nitroxyl (HNO). *Archives of Biochemistry and Biophysics*. 2007; 465:430–436. [PubMed: 17678614]
25. Lopez BE, Rodriguez CE, Pribadi M, Cook NM, Shinyashiki M, Fukuto JM. Inhibition of yeast glycolysis by nitroxyl (HNO): A mechanism of HNO toxicity and implications to HNO biology. *Archives of Biochemistry and Biophysics*. 2005; 442:140–148. [PubMed: 16139238]
26. Norris AJ, Sartippour MR, Lu M, Park T, Rao JY, Jackson MI, Fukuto JM, Brooks MN. Nitroxyl inhibits breast tumor growth and angiogenesis. *International Journal of Cancer*. 2008; 122:1905–1910.
27. DeMaster EG, Redfern B, Nagasawa HT. Mechanisms of inhibition of aldehyde dehydrogenase by nitroxyl, the active metabolite of the alcohol deterrent agent cyanamide. *Biochemical Pharmacology*. 1998; 55:2007–2015. [PubMed: 9714321]
28. Nagasawa HT, Kawle SP, Elberling JA, Demaster EG, Fukuto JM. Prodrugs of Nitroxyl as Potential Aldehyde Dehydrogenase Inhibitors Vis-a-Vis Vascular Smooth-Muscle Relaxants. *Journal of Medicinal Chemistry*. 1995; 38:1865–1871. [PubMed: 7783118]
29. DuMond JF, King SB. The Chemistry of Nitroxyl-Releasing Compounds. *Antioxidants & Redox Signaling*. 2011; 14:1637–1648. [PubMed: 21235345]
30. Miranda KM, Nagasawa HT, Toscano JP. Donors of HNO. *Current Topics in Medicinal Chemistry*. 2005; 5:647–664.
31. Hughes MN, Cammack R. Synthesis, chemistry, and applications of nitroxyl ion releasers sodium trioxodinitrate or Angeli's salt and Piloty's acid. *Methods Enzymol*. 1999; 301:279–287. [PubMed: 9919577]
32. Maragos CM, Morley D, Wink DA, Dunams TM, Saavedra JE, Hoffman A, Bove AA, Isaac L, Hrabie JA, Keefer LK. Complexes of .NO with nucleophiles as agents for the controlled biological release of nitric oxide. Vasorelaxant effects. *Journal of Medicinal Chemistry*. 1991; 34:3242–3247. [PubMed: 1956043]
33. Andrei D, Salmon DJ, Donzelli S, Wahab A, Klose JR, Citro ML, Saavedra JE, Wink DA, Miranda KM, Keefer LK. Dual Mechanisms of HNO Generation by a Nitroxyl Prodrug of the Diazeniumdiolate (NONOate) Class. *Journal of the American Chemical Society*. 2010:16526–16532. [PubMed: 21033665]

34. Huang ZJ, Velazquez C, Abdellatif K, Chowdhury M, Jain S, Reisz J, DuMond J, King SB, Knaus E. Acyclic triaryl olefins possessing a sulfohydroxamic acid pharmacophore: synthesis, nitric oxide/nitroxyl release, cyclooxygenase inhibition, and anti-inflammatory studies. *Organic & Biomolecular Chemistry*. 2010; 8:4124–4130. [PubMed: 20664853]
35. Huang ZJ, Velazquez CA, Abdellatif KRA, Chowdhury MA, Reisz JA, DuMond JF, King SB, Knaus EE. Ethanesulfohydroxamic Acid Ester Prodrugs of Nonsteroidal Anti-inflammatory Drugs (NSAIDs): Synthesis, Nitric oxide and Nitroxyl Release, Cyclooxygenase Inhibition, Anti-inflammatory, and Ulcerogenicity Index Studies. *Journal of Medicinal Chemistry*. 2011; 54:1356–1364. [PubMed: 21280601]
36. Guthrie DA, Kim NY, Siegler MA, Moore CD, Toscano JP. Development of N-Substituted Hydroxylamines as Efficient Nitroxyl (HNO) Donors. *Journal of the American Chemical Society*. 2012; 134:1962–1965. [PubMed: 22233148]
37. Sutton AD, Williamson M, Weismiller H, Toscano JP. Optimization of HNO production from N, O-bis-acylated hydroxylamine derivatives. *Org Lett*. 2012; 14:472–475. [PubMed: 22196028]
38. Nakagawa H. Photocontrollable nitric oxide (NO) and nitroxyl (HNO) donors and their release mechanisms. *Nitric Oxide*. 2011; 25:195–200. [PubMed: 21193058]
39. Nakagawa H. Controlled release of HNO from chemical donors for biological applications. *J Inorg Biochem*. 2013; 118:187–190. [PubMed: 23140899]
40. Filipovic MR, Miljkovic J, Allgauer A, Chaurio R, Shubina T, Herrmann M, Ivanovic-Burmazovic I. Biochemical insight into physiological effects of H₂S: reaction with peroxynitrite and formation of a new nitric oxide donor, sulfinyl nitrite. *Biochemical Journal*. 2012; 441:609–621. [PubMed: 21950347]
41. Wei CC, Wang ZQ, Hemann C, Hille R, Stuehr DJ. A tetrahydrobiopterin radical forms and then becomes reduced during N-omega-hydroxyarginine oxidation by nitric-oxide synthase. *Journal of Biological Chemistry*. 2003; 278:46668–46673. [PubMed: 14504282]
42. Cline MR, Chavez TA, Toscano JP. Oxidation of N-hydroxy-L-arginine by hypochlorous acid to form nitroxyl (HNO). *J Inorg Biochem*. 2013; 118:148–154. [PubMed: 23102772]
43. Arnelles DR, Stamler JS. NO⁺, NO and NO⁻ Donation by S-Nitrosothiols: Implications for Regulation of Physiological Functions by S-Nitrosylation and Acceleration of Disulfide Formation. *Archives of Biochemistry and Biophysics*. 1995; 318:279–285. [PubMed: 7733655]
44. Kirsch M, Buscher AM, Aker S, Schulz R, de Groot H. New insights into the S-nitrosothiol-ascorbate reaction. The formation of nitroxyl. *Org Biomol Chem*. 2009; 7:1954–1962. [PubMed: 19590793]
45. Sha X, Isbell TS, Patel RP, Day CS, King SB. Hydrolysis of acyloxy nitroso compounds yields nitroxyl (HNO). *Journal of the American Chemical Society*. 2006; 128:9687–9692. [PubMed: 16866522]
46. Shoman ME, DuMond JF, Isbell TS, Crawford JH, Brandon A, Honovar J, Vitturi DA, White CR, Patel RP, King SB. Acyloxy Nitroso Compounds as Nitroxyl (HNO) Donors: Kinetics, Reactions with Thiols, and Vasodilation Properties. *Journal of Medicinal Chemistry*. 2011; 54:1059–1070. [PubMed: 21247168]
47. Miller TW, Cherney MM, Lee AJ, Francoleon NE, Farmer PJ, King SB, Hobbs AJ, Miranda KM, Burstyn JN, Fukuto JM. The Effects of Nitroxyl (HNO) on Soluble Guanylate Cyclase Activity INTERACTIONS AT FERROUS HEME AND CYSTEINE THIOLS. *Journal of Biological Chemistry*. 2009; 284:21788–21796. [PubMed: 19531488]
48. Gao WD, Murray CI, Tian Y, Zhong X, Dumond JF, Shen X, Stanley BA, Foster DB, Wink DA, King SB, Van Eyk JE, Paolocci N. Nitroxyl-mediated disulfide bond formation between cardiac myofilament cysteines enhances contractile function. *Circ Res, United States*. 2012; 111:1002–1011.
49. Zgheib C, Kurdi M, Zouein FA, Gunter BW, Stanley BA, Zgheib J, Romero DG, King SB, Paolocci N, Booz GW. Acyloxy nitroso compounds inhibit LIF signaling in endothelial cells and cardiac myocytes: evidence that STAT3 signaling is redox-sensitive. *PLoS One, United States*. 2012; 7:e43313.

50. Callan HE, Jenkins RE, Maggs JL, Lavergne SN, Clarke SE, Naisbitt DJ, Park BK. Multiple adduction reactions of nitroso sulfamethoxazole with cysteinyl residues. *Chem Res Toxicol*. 2009; 22:937–948. [PubMed: 19358516]
51. Nagasawa HT, Yost Y, Elberling JA, Shiota FN, Demaster EG. Nitroxyl Analogs as Inhibitors of Aldehyde Dehydrogenase - C-Nitroso Compounds. *Biochemical Pharmacology*. 1993; 45:2129–2134. [PubMed: 8512594]
52. Cline MR, Tu C, Silverman DN, Toscano JP. Detection of nitroxyl (HNO) by membrane inlet mass spectrometry. *Free Radic Biol Med*. 2011; 50:1274–1279. [PubMed: 21349325]
53. Ellis HR, Poole LB. Roles for the Two Cysteine Residues of AhpC in Catalysis of Peroxide Reduction by Alkyl Hydroperoxide Reductase from *Salmonella typhimurium*†. *Biochemistry*. 1997; 36:13349–13356. [PubMed: 9341227]
54. Poole LB. Bacterial defenses against oxidants: mechanistic features of cysteine-based peroxidases and their flavoprotein reductases. *Arch Biochem Biophys*. 2005; 433:240–254. [PubMed: 15581580]
55. Landino LM, Koumas MT, Mason CE, Alston JA. Modification of tubulin cysteines by nitric oxide and nitroxyl donors alters. *Chem Res Toxicol*. 2007; 20:1693–1700. [PubMed: 17907787]
56. Hoffman MD, Walsh GM, Rogalski JC, Kast J. Identification of Nitroxyl-induced Modifications in Human Platelet Proteins Using a Novel Mass Spectrometric Detection Method. *Molecular & Cellular Proteomics*. 2009; 8:887–903. [PubMed: 19119137]
57. Keceli G, Toscano JP. Reactivity of nitroxyl-derived sulfinamides. *Biochemistry*. 2012; 51:4206–4216. [PubMed: 22571165]
58. DuMond JF, Wright MW, King SB. Water soluble acyloxy nitroso compounds: HNO release and reactions with heme and thiol containing proteins. *Journal of Inorganic Biochemistry*. 2013; 118:140–147. [PubMed: 23083700]
59. Gallemann D, Greif A, Eyer P, Dasenbrock J, Wimmer E, Sonnenbichler J, Sonnenbichler I, Schafer W, Buhrow I. Formation of 4,4-dialkoxycyclohexa-2,5- dienone N-(thiol-S-yl)imine during. *Chem Res Toxicol*. 1998; 11:1423–1433. [PubMed: 9860483]
60. King SB, Nagasawa HT. Chemical approaches toward generation of nitroxyl. *Methods in Enzymology: Nitric Oxide*, Pt C. 1999; 301:211–220.
61. Hart TW. Some observations concerning the S-nitroso and S-phenylsulphonyl derivatives of L-cysteine and glutathione. *Tetrahedron Letters*. 1985; 26:2013–2016.
62. Toscano, JP.; Brookfield, FA.; Cohen, AD.; Courtney, SM.; Frost, LM.; Kalish, VJ. Preparation of N-hydroxysulfonamide derivatives as nitroxyl (HNO) donors. WO 2007109175. 2007.
63. Azarov I, Huang KT, Basu S, Gladwin MT, Hogg N, Kim-Shapiro DB. Nitric Oxide Scavenging by Red Blood Cells as a Function of Hematocrit and Oxygenation. *Journal of Biological Chemistry*. 2005; 280:39024–39032. [PubMed: 16186121]
64. Donadee C, Raat NJ, Kanas T, Tejero J, Lee JS, Kelley EE, Zhao X, Liu C, Reynolds H, Azarov I, Frizzell S, Meyer EM, Donnenberg AD, Qu L, Triulzi D, Kim-Shapiro DB, Gladwin MT. Nitric oxide scavenging by red blood cell microparticles and cell-free hemoglobin as a mechanism for the red cell storage lesion. In. *Circulation*, United States. 2011; 124:465–476.

Figure 1. GAPDH activity inhibition and restoration as described in Scheme 1. GAPDH was pre-incubated with either AS or **1–3**, followed by subsequent incubation with DTT (100 mM) or Millipore water: A) AS, B) **1**, C) **2**, D) **3**.

Scheme 1.

Reactions of HNO and C-nitroso compounds with thiols.

Scheme 2.

HNO formation from and thiol reactions of acyloxy nitroso compounds(1–3).

Scheme 3.
Proposed Direct Reaction of Acyloxy Nitroso Compounds with Protein Thiols

Table 1

LC-MS Analysis of the Reaction of *C*-Nitroso Compounds and HNO Donors and GAPDH tryptic peptide I143–K159.

Reagent	Retention Time (min)	m/z	Products
-	9.18	853.4349 [M+2H] ⁺ 2	Unmodified cysteine
¹⁴ N AS (10 eq.)	7.11	852.4269 [M(RSSR)+2H] ⁺ 2	Internal disulfide; RSSR
¹⁵ N AS (10 eq.)	7.10	852.4277 [M(RSSR)+2H] ⁺ 2	Internal disulfide; RSSR
1 (acetate) (10 eq.)	7.60	852.4277 [M(RSSR)+2H] ⁺ 2	Internal disulfide; RSSR
2 (pivalate) (10 eq.)	7.59	852.4268 [M(RSSR)+2H] ⁺ 2	Internal disulfide; RSSR

Reagent	Retention Time (min)	m/z	Products
3 (trifluoroacetate) (10 eq.)	7.09	852.4268 [M(RSSR)+2H] ⁺²	Internal disulfide; RSSR
Nitrosobenzene	7.30	852.4260 [M(RSSR)+2H] ⁺²	Internal disulfide; RSSR
DEANO	7.32	852.4260 [M(RSSR)+2H] ⁺²	Internal disulfide; RSSR
GSNO	7.31	852.4261 [M(RSSR)+2H] ⁺²	Internal disulfide; RSSR
<i>o</i> -Bromo-Piloty's Acid	7.41	852.4260 [M(RSSR)+2H] ⁺²	Internal disulfide; RSSR

Table 2

LC-MS Analysis of the Reaction of *C*-Nitroso Compounds and HNO Donors and GAPDH tryptic peptide V232–R245.

Reagent	Retention Time (min)	m/z	Products
-	10.82	750.3999 [M+2H] ⁺ 2	Unmodified cysteine
¹⁴ N AS (10 eq.)	9.58	765.9014 [M(SONH ₂)+2H] ⁺ 2	Sulfinamide
	10.04	766.3929 [M(SO ₂ H)+2H] ⁺ 2	Sulfinic acid
¹⁵ N AS (10 eq.)	9.54	766.3995 [M(SONH ₂)+2H] ⁺ 2	Sulfinamide
	10.02	766.3929 [M(SO ₂ H)+2H] ⁺ 2	Sulfinic acid
1 (acetate) (10 eq.)	10.54	766.3929 [M(SO ₂ H)+2H] ⁺ 2	Sulfinic acid

Reagent	Retention Time (min)	m/z	Products
2 (pivalate) (10 eq.)	10.57	766.3928 [M(SO ₂ H)+2H] ⁺ ²	Sulfinic acid
3 (trifluoroacetate) (10 eq.)	9.47	765.9014 [M(SONH ₂)+2H] ⁺ ²	Sulfinamide,
	10.00	766.3928 [M(SO ₂ H)+2H] ⁺ ²	Sulfinic acid
Nitrosobenzene	14.46	803.9100 [M(C ₆ H ₅ NO)2H] ⁺ ²	N-phenyl sulfinamide (equal amounts)
	10.16	766.3928 [M(SO ₂ H)+2H] ⁺ ²	Sulfinic acid
DEANO	11.99	764.8943 [M(SNO)+2H] ⁺ ²	Nitrosylated product
GSNO	11.94	764.8943 [M(SNO)+2H] ⁺ ²	Nitrosylated product

Reagent	Retention Time (min)	m/z	Products
<i>o</i> -Bromo Piloty's Acid	9.79	765.9025 [M(SONH ₂)+2H] ⁺ ²	Sulfinamide
	10.32	766.3929 [M(SO ₂ H)+2H] ⁺ ²	Sulfinic acid

Table 3

LC-MS Analysis of the Reaction of *C*-Nitroso Compounds and HNO Donors and C165S AhpC Asp-N peptide D41–G51.

Reagent	Retention Time (min)	m/z	Products
-	30.11	1228.5537 [M+H] ⁺	Unmodified cysteine
¹⁴ N AS (10 eq.)	27.20	1259.5598 [M(SONH ₂)+H] ⁺	Sulfinamide
	29.52	1260.5444 [M(SO ₂ H)+H] ⁺	Sulfinic acid
¹⁵ N AS (10 eq.)	27.36	1260.5577 [M(SONH ₂)+H] ⁺	Sulfinamide
	29.59	1260.5444 [M(SO ₂ H)+H] ⁺	Sulfinic acid
1 (acetate) 10 eq.	29.44	1260.5444 [M(SO ₂ H)+H] ⁺	Sulfinic acid

Reagent	Retention Time (min)	m/z	Products
2 (pivalate) 10 eq.	29.50	1260.5444 [M(SO ₂ H)+H] ⁺	Sulfinic acid
3 (trifluoroacetate) 10 eq.	29.99	1259.5598 [M(SONH ₂)+H] ⁺	Sulfinic acid
	29.20	1260.5444 [M(SO ₂ H)+H] ⁺	Sulfinamide
Nitrosobenzene	29.17	1260.5444 [M(SO ₂ H)+H] ⁺	Sulfinic acid
DEANO	32.54	1257.5238 [M(SNO)+H] ⁺	Nitrosylated Product
GSNO	33.46	1257.5238 [M(SNO)+H] ⁺	Nitrosylated Product
α-Bromo Piloty's Acid	27.45	1259.5529 [M(SONH ₂)+H] ⁺	Sulfinamide

Reagent	Retention Time (min)	m/z	Products
	29.51	1260.5444 [M(SO ₂ H)+H] ⁺	Sulfinic acid