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Glutathione Sulfinamide Serves as a Selective, Endogenous Biomarker for Nitroxyl Following Exposure to Therapeutic Levels of Donors

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Abstract

Nitroxyl (HNO) donors exhibit promising pharmacological characteristics for treatment of cardiovascular disorders, cancer and alcoholism. However, whether HNO also serves as an endogenous signaling agent is currently unknown, largely due to the inability to selectively and sensitively detect HNO in a cellular environment. Although a number of methods to detect HNO have been developed recently, sensitivity and selectivity against other nitrogen oxides or biological reductants remain problematic. To improve selectivity, the electrophilic nature of HNO has been harnessed to generate modifications of thiols and phosphines that are unique to HNO, especially compared to nitric oxide (NO). Given high bioavailability, glutathione (GSH) is expected to be a major target of HNO. As a result, the putative selective product glutathione sulfinamide (GS(O)NH₂) may serve as a high yield biomarker of HNO production. In this work, the formation of GS(O)NH₂ following exposure to HNO donors was investigated. Fluorescent labeling followed by separation and detection using capillary zone electrophoresis with laser-induced fluorescence allowed quantitation of GS(O)NH₂ with nanomolar sensitivity, even in the presence of GSH and derivatives. Formation of GS(O)NH₂ was found to occur exclusively upon exposure of GSH to HNO donors, thus confirming selectively. GS(O)NH₂ was detected in the lysate of cells treated with low micromolar concentrations of HNO donors, verifying that this marker has sufficient stability to server as a biomarker of HNO. Additionally, the concentration-dependent formation of GS(O)NH₂ in cells treated with an HNO donor suggests that the concentration of GS(O)NH₂ can be correlated to intracellular levels of HNO.

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Conflict of Interest Disclosure

The authors declare no competing financial interest.

Supporting Information

Fluorescent excitation and emission spectra for NDA/CN⁻ labeled GSH and GS(O)NH₂ (Figure S1), calibration curves for NDA/CN⁻ labeled GSH and GS(O)NH₂ analyzed by CZE-LIF detection (Figure S2), and electropherograms illustrating selectivity of GSH against other nitrogen oxides and potential interferents (Figure S3; 10 eqs) and indicating the resolution between and GS(O)NH₂ and GSNO (Figure S4) are included in the Supporting Information. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Keywords

Angeli's salt; NONOates; glutathione; glutathione sulfinamide; naphthalene-2; 3-dicarboxaldehyde

Introduction

Nitroxyl (HNO) donors are used clinically as deterrents for alcohol use [1, 2] and also exhibit anti-cancer properties [3], protect tissue against reperfusion injury [4], and show promise for the treatment of heart failure [5]. The pharmacology of HNO is generally associated with modification of thiols and heme proteins [6]. Exposure to HNO has been shown to modify the cysteine residues of a number of proteins, both of purified samples and in vitro, with the effect of modification dependent on protein function. Examples include the DNA repair protein poly(ADP-ribose) polymerase (PARP) [7], the cysteine proteases papain [8] and cathepsin B [9, 10], the glycolytic protein glyceraldehyde 3-phosphate dehydrogenase (GAPDH) [11] and aldehyde dehydrogenase (ALDH), which is critical to alcohol metabolism [12]. HNO also regulates vasorelaxation [13] and alters calcium channel function [14] through binding of HNO to a ferric or thiol site.

Although the pharmacology of HNO has been investigated in considerable detail over the last decade, whether HNO functions as an endogenous signaling agent is currently a matter of speculation (see reference [15]). Biochemical studies suggest that HNO may be biosynthesized from oxidation of *N*-hydroxy-L-arginine [16–20], reaction of *S*-nitrosothiols with thiols [21, 22], or heme mediated peroxidation of hydroxylamine [23]. However, determination of the physiological relevance of HNO has been hindered by a lack of HNO-specific detection methods compatible with cell samples.

Selective and sensitive detection of HNO in biological systems is nontrivial. The short lifetime of HNO under physiological conditions due to irreversible dimerization complicates detection (eq 1; [24, 25] $8 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$, pH 7.4, 22°C) [26].

Additionally, the reactive nature of HNO with a variety of biological targets depletes the concentration of free and measurable HNO in cells [15]. Thus, detection of HNO requires high selectivity for HNO over other nitrogen oxides, in particular nitric oxide (NO), which is produced endogenously [27–29] in the range of pico- to micromolar concentrations [30, 31].

A number of assays exist for detection of HNO, but those based on fluorescence detection are most suitable for use in cultured cells or tissues due to high sensitivity. HNO has been indirectly detected through its autoxidation product, which induces a fluorogenic response from dihydrorhodamine and diaminofluorescein derivatives [32, 33]; however, these reagents respond to NO as well as HNO. Fluorescent probes based on the reduction of Cu(II) to Cu(I) by HNO have also been used to detect HNO in cells [34]. Although more selective against NO, these complexes are subject to interference from biological reducing

agents such as glutathione (GSH) and ascorbate [35]. Very recently, an adduct between rhodol and triphenylphosphine was introduced as a bioorthogonal trapping agent for HNO [36]. Although the aza-ylide product is specific to HNO [37], observation of fluorescence requires an intramolecular rearrangement and hydrolysis to release rhodol [36]. The initial analysis suggests a specificity to HNO in cells that is comparable to that of 4,5-diaminofluorescein diacetate [33]. Although each of these assays has been utilized for fluorescent imaging of HNO in cells, they rely on the purchase or synthesis and introduction of an exogenous probe, and quantitation of HNO has yet to be demonstrated.

In this work, we sought to identify a selective, endogenous intracellular biomarker for HNO that could be subsequently detected in a quantitative manner in biological systems. To overcome issues with selectivity, we focused on the reactivity of HNO with GSH. This reaction produces glutathione sulfenamide (GS(O)NH₂), which is proposed to be a unique marker of HNO, and oxidized glutathione (GSSG) (Scheme 1) [21, 38]. Moreover, the reaction between HNO and GSH is facile ($2 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$, pH 7.4, 37 °C) [6] in comparison to other biomolecules, and the concentration of GSH in cells (0.5–10 mM) [39] is higher than many other cellular components. Together, these parameters suggest that significant reactivity may occur between HNO and GSH in cells [15]. Both GSH and derivative species such as GS(O)NH₂ that contain a primary amine are amenable to fluorescent derivatization, thus enhancing analytical sensitivity. Furthermore, because GSH is biologically available, detection of HNO does not require the addition of an exogenous probe, which may enable endogenous detection.

Materials and Methods

Chemicals

Unless otherwise noted, chemicals were purchased from Sigma-Aldrich and used without further purification. Angeli's salt (Na₂N₂O₃, sodium trioxodinitrate), DEA/NO (Na[Et₂NN-(O)NO], sodium (Z)-1-(N,N-diethylamino) diazen-1-ium-1,2-diolate), IPA/NO (Na[(CH₃)₂CHNH(N(O)NO)], sodium 1-(N-isopropylamino)diazene-1-ium-1,2-diolate), AcOM-IPA/NO (O²-(acetoxymethyl) 1-(isopropylamino)diazene-1-ium-1,2-diolate), and AcOM-DEA/NO (O²-(acetoxymethyl) 1-(diethylamino) diazen-1-ium-1,2-diolate) were synthesized as previously described [40–44]. Stock solutions (>10 mM) of Angeli's salt, DEA/NO and IPA/NO were prepared in 10 mM NaOH while those of AcOM-IPA/NO and AcOM-DEA/NO were prepared in ethanol. UV absorption spectroscopy was used to determine the concentrations with extinction coefficients as follows: 7,600 M⁻¹ cm⁻¹ for Angeli's salt, 8,800 M⁻¹ cm⁻¹ for IPA/NO, and 6,700 M⁻¹ cm⁻¹ for DEA/NO at 250 nm in 10 mM NaOH and 8,700 M⁻¹ cm⁻¹ for AcOM-IPA/NO and 7,600 M⁻¹ cm⁻¹ for AcOM-DEA/NO at 240 nm in ethanol. These donors were stored at -20 °C for several weeks and then on ice while in use. GS(O)NH₂ was synthesized as previously described [45]. Stock solutions of peroxynitrite (ONOO⁻) were prepared as previously reported [32, 46] and diluted in 10 mM NaOH to yield a concentration of 5 mM.

The assay buffer included the metal chelator diethylenetriaminepentaacetic acid (DTPA, 50 μM) in calcium- and magnesium-free Dulbecco's phosphate-buffered saline (PBS, pH 7.4) to reduce oxidation of HNO to NO [47]. Borate buffer (20 mM) was made with boric acid

(Spectrum) and adjusted to pH 9.2 with NaOH. To generate CZE-LIF run buffer, borate buffer was filtered using a 0.2 μm filter (Millipore), photobleached for at least 30 min using a 100 W mercury arc lamp with an infrared-water filter, and deaerated for at least 15 min by sparging with helium. Water was obtained from a Barnstead EasyPure UV/UF purification system.

Stock solutions of GSH (>10 mM) were prepared daily in assay buffer and stored at 4 $^{\circ}\text{C}$. Stock solutions of naphthalene-2,3-dicarboxaldehyde (NDA; BioChemika) were made in DMSO (4 mM), stored at 4 $^{\circ}\text{C}$ protected from light and used for several weeks. Tris(2-carboxyethyl)phosphine (TCEP; Alfa Aesar; 50 mM), *N*-ethylmaleimide (NEM; 50 mM), and potassium cyanide (KCN; 80 mM) were made daily in H_2O and stored at 4 $^{\circ}\text{C}$ during the course of the experiment. Fluorescein stock solutions (>1 mM) were prepared weekly in DMF (EMD Millipore) and stored protected from light at 4 $^{\circ}\text{C}$. Hydrogen peroxide (H_2O_2), nitrite (NO_2^-), nitrate (NO_3^-), *S*-nitrosoglutathione (GSNO), and ascorbic acid stock solutions (50 mM) were prepared daily in assay buffer and stored at 4 $^{\circ}\text{C}$ for the duration of data collection.

Instrumentation

UV-visible absorption spectroscopy was performed with a Hewlett-Packard 8453 diode-array spectrophotometer. Fluorescence measurements were made on a Thermo Spectronic Aminco Bowman Series 2 Luminescence Spectrometer using 457 nm as the excitation wavelength, 480 or 490 nm as the emission wavelength, a band-pass of 4 nm, and a detector voltage of 600 V.

For capillary zone electrophoresis with laser-induced fluorescence detection (CZE-LIF), a 47 cm long capillary (Polymicro Technologies, Phoenix, AZ, USA) with 25 μm inner diameter (ID) and 37 cm to the detection window (<1 cm) was rinsed consecutively for 5 min each with 0.1 M HCl, nanopure H_2O , 0.1 M NaOH, nanopure H_2O and run buffer. Hydrodynamic injections were performed by raising one end of the capillary to a height of 10 cm for 5 s, which corresponded to 10 nL injection volume. For detection, the 457 nm line of an Ar^+ laser (Innova 70C, Coherent, Santa Clara, CA, USA) with a beam power of 25 mW was focused into the center of the separation capillary. The fluorescence signal was collected using a microscope objective (10 $\times$, 0.25 N.A., Newport, Irvine, CA, USA) and passed through a 480 nm longpass filter (E480LP, Chroma Technology, Bellow Falls, VT, USA). The signal was then detected by a photomultiplier tube (H957, Hamamatsu Photonics, Bridgewater, NJ, USA), current from which was amplified and filtered using a current amplifier (Model 428, Keithley Instruments, Cleveland, OH, USA) with a filter rise time of 100 ms and a gain of 10^6 V/A. The signals were then collected by an A/D converter board using software written in LabVIEW. Electric fields were applied using a high voltage power supply (CZE-1000, Spellman High Voltage Corporation, Hauppauge, NY, USA) with potential of 25 kV. The separation parameters for CZE were as follows: 20 mM borate buffer of pH 9.2 (CZE run buffer); $E = 426$ V/cm, $L_d = 37$ cm, ID = 25 μm ; $t_{inj} = 5$ s (10 nL), $\lambda_{ex} = 457$ nm.

Labeling of GSH and GS(O)NH₂

Solutions of GSH (50 μ M; 1 mL) were treated with 50 mM NEM (10 μ L) for 15 min at room temperature to specifically block the free thiol on GSH [48]. To label the primary amines [49] of GSH-NEM, GS(O)NH₂ or GSSG (50 μ M; 1 mL), 20 mM borate buffer (pH 9.2, 800 μ L), 4 mM NDA (120 μ L), and 80 mM KCN (8 μ L) were added, and the samples were kept in the dark at 4 °C for 15, 30 or 60 min before fluorescence measurements were collected for labeling time and stability analysis.

To determine separation efficiency and sensitivity, solutions of GSH and GS(O)NH₂ (50 μ M; 1 mL) were labeled as described above for 30 min before diluting to final concentrations of 35, 100, 175 or 350 nM with CZE run buffer that contained fluorescein (5 nM) as the internal standard. To validate quantitative analysis, GSH samples (50 or 500 μ M; 500 μ L) were treated with Angeli's salt (10, 20, 30, 100, 200 or 300 μ M) and incubated at 37 °C for 1 h to allow complete donor decomposition. After incubation, the original 500 μ M GSH samples were diluted ten-fold in assay buffer to ensure complete labeling (30 min) before being processed for CZE-LIF measurements as described above. Furthermore, to assess GSSG concentrations, the samples were treated with TCEP (5 μ L of a 50 mM stock for 15 min at room temperature) to reduce GSSG to GSH and then blocked with NEM before fluorogenic labeling.

For selectivity experiments, GSH (50 μ M; 500 μ L) was treated with Angeli's salt, H₂O₂, NO₂⁻, NO₃⁻, GSNO, ascorbic acid or DEA/NO (50 or 500 μ M) for 1 h at 37 °C to allow complete donor decomposition. After incubation, the samples were processed as described above.

Detection of GS(O)NH₂ in cell lysate

Human breast cancer cells (MDA-MB-231; American Type Culture Collection, Manassas, VA) were grown in RPMI medium supplemented with 10% heat inactivated fetal bovine serum and 1% penicillin-streptomycin (50 U/mL; Life Technologies, Inc., Grand Island, NY). The cells were maintained at 37 °C in an atmosphere with 95% room air and 5% CO₂, and the media was changed twice weekly. In a 35 mm culture plate, cells were grown to a density of 1×10^6 cells/plate. Experiments were performed in triplicate sets of whole cells spiked with a known concentration of GS(O)NH₂. Single cell suspensions were obtained by trypsinization (0.05% trypsin-EDTA Life Technologies, Inc., Grand Island, NY) and were counted with a bright line hemocytometer (Sigma-Aldrich). Cells were then diluted to a density of 6×10^5 cells/mL, transferred to a 1.5 mL tube, and centrifuged to remove the supernatant. The cells were resuspended in 400 μ L PBS buffer that contained GS(O)NH₂ (0.5, 1, 5, 10, 25, or 50 μ M). The cells were then lysed using five freeze/thaw cycles. The samples were collected and filtered through a Centricon MW 3000 cutoff filter. GSH and GS(O)NH₂ in the cell lysate were processed as described above.

Treatment of MDA-MB-231 with HNO donors

Cells were maintained and suspended as described above. Cells were plated in a 35 mm culture plate and allowed to incubate in cell culture media for 4 h. At this point, the cell culture media was replaced by PBS (1 mL) followed by treatment with 10 μ L of 10 mM

donor, 10 mM NaOH or ethanol (vehicle). The cells were then incubated for 5 h to allow complete decay of the donors. After incubation, cells were washed with 0.05% trypsin-EDTA, transferred to a 1.5 mL tube, and centrifuged to remove the supernatant. The cells were resuspended in 400 μ L PBS buffer and processed as described above.

Results and Discussion

Here, we describe the generation and detection of GS(O)NH₂ in cultured cells as an HNO-selective biomarker. We predicted that reaction of HNO with endogenous GSH and subsequent fluorescent labeling and detection of GS(O)NH₂ would provide the necessary sensitivity and selectivity to monitor HNO in cultured cells treated with therapeutic doses of HNO donors. Combined with CZE-LIF analysis, this approach provides fast separation, high resolution and sensitivity and requires small sample sizes to facilitate monitoring of HNO exposure in cultured cells.

Labeling time and stability of GSH and GS(O)NH₂

Primary amines can be selectively labeled with NDA in the presence of cyanide as the nucleophile to produce fluorescent adducts with good stability and high fluorescent quantum yields [49–51]. This modification is shown for GS(O)NH₂ in eq 2. Due to the close proximity of the amine and thiol, GSH can also be labeled with NDA alone to produce a fluorescent adduct with more red-shifted excitation and emission wavelengths than NDA/CN[−] adducts [52–55]. Such unique excitation and emission spectra are undesirable for LIF detection due to the potential for significant differences in sensitivity for the free versus modified thiols [49, 53]. Thus, the thiol of GSH was first blocked with NEM to form GS-NEM [56], before labeling of the amine with NDA/CN[−] (eq 3) [49].

(2)

(3)

The spectra of NDA/CN[−] labeled GS(O)NH₂ and GSH showed excitation maxima at 421 and 449 nm and emission maxima at 488 nm (Figure S1), which is consistent with prior

studies of NDA/CN⁻ fluorescent adducts [49–51]. The excitation and emission intensities for NDA/CN⁻ labeled GSSG were markedly lower than those of either GS(O)NH₂ or GSH. This is presumably due to quenching of fluorescence from labeling of two amines in close proximity [57, 58], which has been previously observed upon labeling of lysine [51]. Therefore, to detect changes in GSSG concentration, GSSG was reduced first to GSH with TCEP before blocking with NEM and fluorogenic labeling, as previously described [45].

Although, NDA/CN⁻ labeled adducts are reportedly rather stable compared to other fluorogenic species [49], they are nonetheless known to be sensitive to hydrolysis [59]. Thus, we optimized the labeling time for maximum fluorescence and collected fluorescence data for an extended period of time to analyze the stability of the labeled samples. The labeling reactions were complete for both GSH and GS(O)NH₂ in 30 min, and the samples were stable for at least an additional 30 min (Figure 1). These values are similar to those obtained by Roach and Harmony for analysis of amino acids labeled with NDA/CN⁻ [49].

Separation efficiency, sensitivity and selectivity

Selective detection of GS(O)NH₂ will require resolution from its precursor GSH and other intracellular interferences. CZE facilitates high resolution separation of amino acids and related compounds [60, 61]. The NDA/CN⁻-labeled GSH and GS(O)NH₂ adducts are both dianionic at pH 9.2, and are similar in size. Blocking the thiol of GSH with NEM not only provides common excitation and emission wavelengths after NDA/CN⁻ labeling but also increases the size difference compared to GS(O)NH₂, which should further enhance resolution.

GSH appears as a split peak in the electropherogram (Figure 2), presumably due to the chiral carbon on NEM resulting in diastereomers during the thiol blocking step [62]. The migration times for the split peaks of GSH were 136 ± 1 and 137 ± 1 s. GS(O)NH₂ migrates more slowly through the capillary (142 ± 1 s) due to a larger negative electrophoretic mobility. Fluorescein is also dianionic at pH 9.2, but due to its smaller size, it has the largest negative electrophoretic mobility, resulting in a migration time of 155 ± 1 s.

Importantly, GSH and GS(O)NH₂ or GS(O)NH₂ and fluorescein were separated with greater than baseline resolution ($R_s \gg 1.5$). This facilitates quantitative detection of both GSH and GS(O)NH₂, provided that both analytes are at concentrations greater than the limit of detection and that no other analyte peaks migrate in the same time window when analyzing cell samples. In addition to migration time differences, quantitative differences in the ratio of GSH to GS(O)NH₂ can be observed in Figure 2. The split peaks for GSH are 20 and 10% less, respectively, than the peak resulting from GS(O)NH₂, when normalized to the fluorescein internal standard peak. Furthermore, the separation of all three analytes is complete in less than 3 min (Figure 2), and analysis of samples in triplicate requires less than 10 min. Thus, the fluorescently labeled samples are stable for longer than the duration of the experiment (Figure 1).

To determine the linear dynamic range and analytical sensitivity of the newly developed assay, calibration curves were generated using mixtures of labeled GSH and GS(O)NH₂, using peak areas rather than the peak heights, since GSH migration results in a split peak.

Using the standard deviation of the baseline between GS(O)NH₂ and fluorescein as the noise, limits of detection for GSH and GS(O)NH₂ were determined to be 0.9 ± 0.1 and 1.4 ± 0.1 nM, respectively. Additionally, calibration curves were linear between 35–350 nM for both analytes (Figure S2). These limits are similar to those reported for amino acids labeled with NDA/CN[−] and analyzed with CZE-LIF [63].

Analysis of the chemical and biological properties of HNO requires use of donor compounds due to the metastability of HNO (eq 1). Since Angeli's salt is a well-studied and characterized HNO donor that decomposes to form HNO and nitrite under physiological conditions [64–69] (eq 4; $t_{1/2}$ 2.5 min, pH 4.0–8.0, 37 °C) [41], it was chosen as the standard HNO donor to evaluate the quantitative ability of this assay.

We have previously used Angeli's salt to evaluate the performance of a related fluorescence assay that employed a plate reader for high throughput analysis of HNO from donors [45]. Briefly, HNO released from Angeli's salt was trapped by GSH, followed by labeling unreacted GSH with NDA, and subsequent quantitation of HNO by fluorescence difference. This assay was used to quantify HNO from primary amine diazeniumdiolates (NONOates), such as IPA/NO. Furthermore, the yield of GS(O)NH₂ and GSSG formed from the reaction of HNO with GSH was confirmed to be dependent on the initial concentration of GSH (Scheme 1; see reference [45] for complete discussion). In particular, when the concentrations of GSH and Angeli's salt are less than 50 μM, GS(O)NH₂ is the exclusive product. Higher concentrations of GSH result in a mixture of both GS(O)NH₂ and GSSG (Scheme 1).

Here, when 50 μM GSH was treated with various concentrations of Angeli's salt, GS(O)NH₂ was again the only product detected by CZE-LIF. Moreover, the trapping of HNO by 50 μM GSH was quantitative as indicated by the changes in GS(O)NH₂ concentration (eq 5; Table 1).

$$\% \text{ HNO trapped} = ([\text{GS(O)NH}_2]) / [\text{Angeli's salt}] \times 100 \quad (5)$$

Treatment of 500 μM GSH with various concentrations of Angeli's salt resulted in a mixture of GSSG and GS(O)NH₂ with GS(O)NH₂ as the major product. The concentration of HNO released from Angeli's salt was quantitative as determined by the sum of GSSG and GS(O)NH₂ concentrations (eqs 6 and 7; Table 1).

$$[\text{HNO}] = [\text{GS(O)NH}_2] + [\text{GSSG}] \quad (6)$$

$$\% \text{ HNO trapped} = ([\text{HNO}]) / [\text{Angeli's salt}] \times 100 \quad (7)$$

Although these results are consistent with our previous analysis [45], a higher degree of reliability is suggested here since GS(O)NH₂ itself is detected rather than a decrease in

fluorescence intensity. Additionally, the CZE-LIF method requires much smaller sample volumes (10 nL) than our high throughput assay (200 μ L), while also providing enhanced sensitivity. Note that since GSH may be oxidized to GSSG by many cellular pathways, the assumption in eq 6 cannot be applied to cellular analyses.

High selectivity against other biomolecules, in particular NO, is a critical feature of an HNO biomarker. Our approach is predicated on the assumption that GS(O)NH₂ serves as a selective biomarker of HNO. Prior analysis indicates that the reaction of NO with GSH does not produce GS(O)NH₂ [21, 38]. Here, the reactivity of GSH was evaluated with a variety of nitrogen oxides (NO₂⁻, NO₃⁻, and ONOO⁻), NO donors (DEA/NO and GSNO; note that the identity of the nitrogen oxide released from GSNO is environment-dependent, this compound is included here given the prevalence of use), oxidants (H₂O₂) and reductants (ascorbic acid). GS(O)NH₂ was not detected in the presence of any potentially interfering compounds when using 1 (Figure 3) or 10 equivalents (Figure S3) of these compounds. Although GSNO, which is a product of exposure of GSH to NO in aerobic environments, elutes in close proximity to GS(O)NH₂, the resolution and migration time reproducibility are sufficient to distinguish these species (Figure S4). In summary, this analysis demonstrates that our CZE-LIF method provides the necessary sensitivity and selectivity to attempt to detect HNO in cultured cells.

Detection of GS(O)NH₂ in cultured cells

CZE-LIF yields better than baseline resolution between GS(O)NH₂ and GSH. However, many cellular components may be labeled with NDA/CN⁻, so it is imperative that GS(O)NH₂ be well-resolved from these potential interferents. This was evaluated by spiking whole cells with GS(O)NH₂. After labeling cell lysate with NDA/CN⁻, control samples did not exhibit an observable peak for GS(O)NH₂ in contrast to cell samples that were treated with GS(O)NH₂ (Figure 4). Both GSH and GS(O)NH₂ are well-resolved from other cellular components, allowing quantitation in the cell lysate.

The percent recovery of a GS(O)NH₂ aliquot during sample processing was $102 \pm 1\%$, indicating that there was no sample loss during workup and labeling. However, the percent recovery of GS(O)NH₂ from cells spiked with 50 μ M GS(O)NH₂ was $62 \pm 1\%$ (Table 2). This loss of GS(O)NH₂ will decrease the sensitivity of the method for detecting HNO in biological samples, but nonetheless demonstrates the feasibility of using GS(O)NH₂ as a marker for HNO.

The stability and reactivity of sulfinamides (RS(O)NH₂) has not been studied in detail. The suggestion has been made that sulfinamides may be hydrolyzed to the respective sulfinic acid (RSO₂H) or react further with excess thiols to form thiosulfinates (RS(O)SR) [21]. Given the complexity of cell lysate, it will be difficult to determine all the sources of loss and metabolism. Nonetheless, the practical limit of detection for GS(O)NH₂ can be estimated by spiking cells with decreasing concentrations of GS(O)NH₂, until the GS(O)NH₂ peak is no longer discernible from the noise. Such an assessment suggests a practical limit of detection near 1 μ M GS(O)NH₂ (Table 2), which is well below the concentration of HNO donor (10–200 μ M) that leads to therapeutic responses [2, 4, 5, 11, 34, 36]. For example, 10 μ M Angeli's salt effectively inhibited yeast ALDH in cells [2].

Angeli's salt and other ionic NONOates are cytotoxic at low millimolar concentrations in part due to reactions with thiols [70]. In fact, that depletion of GSH enhanced cytotoxicity was the first indicator that GSH is a major target of HNO in vivo. Given the concentration dependence on response, we sought to examine the effect of exposure of HNO donors on modification of GSH in cells.

As described earlier, Angeli's salt is the most common donor of HNO. DEA/NO, a related secondary amine NONOate, is commonly used to compare the selectivity of an assay for HNO over NO because it is a pure NO donor with respect to HNO⁵⁹ ($t_{1/2}$ 2.1 min, pH 7.4, 37 °C) [41]. IPA/NO, a primary amine NONOate, decomposes to form both HNO and NO due to competing pH-dependent decomposition mechanisms ($t_{1/2}$ 7 min, pH 7.4, 37 °C) [42] and has been used for evaluating concurrent release of both HNO and NO. Additionally, IPA/NO has proven amenable to alkylation to produce a neutral species, AcOM-IPA/NO, that releases HNO by either a hydrolytic or enzymatic pathway [43]. Under physiological conditions, the half-life of AcOM-IPA/NO is 41 min in the absence of esterases. Andrei *et al.* found that in comparison to IPA/NO, AcOM-IPA/NO caused an increase in pharmacological responses of the heart, suggesting greater efficacy of this compound [43]. Similarly, AcOM-DEA/NO has been synthesized [44], and only 2% decay occurred after 16 h in phosphate buffer at pH 7.4 and 37 °C, showing the high stability of this donor. In the presence of esterases, both AcOM-DEA/NO and AcOM-IPA/NO have half-lives similar to their ionic precursors. In this study, GS(O)NH₂ formation in cultured cells was evaluated following treatment with these donors.

Cells treated with Angeli's salt or IPA/NO (100 μM) did not show observable formation of GS(O)NH₂ (data not shown), presumably due to rapid decay of the ionic donors outside the cell and consumption of HNO through dimerization or other pathways. Longer-lived donors are likely to reduce loss of HNO to dimerization. Furthermore, esterified derivatives such as AcOM-IPA/NO are more likely to cross the cell membrane before decomposing, and thus to improve delivery of HNO [71]. Treatment of cells with AcOM-IPA/NO (100 μM), did in fact result in a significant peak for GS(O)NH₂ (Figure 5). Additionally, the GS(O)NH₂ analyte peak was well-resolved from other cellular components allowing quantitation of GS(O)NH₂ in the cell lysate sample. We also confirmed that the NO donors, DEA/NO and AcOM-DEA/NO or vehicle (10 mM NaOH) did not produce observable peaks for GS(O)NH₂ (data not shown).

The production of GS(O)NH₂ upon exposure of cultured cells to AcOM-IPA/NO increased in a concentration-dependent manner (Table 3). The response was not linear, and levels of GS(O)NH₂ were quite low compared to initial HNO donor concentration. The non-linear response is presumably due to the second order nature of HNO dimerization, and perhaps also to formation of GSSG. The low yields of GS(O)NH₂ also suggest consumption pathways for HNO other than formation of GS(O)NH₂. However, the concentration-dependence suggests for the first time that the concentration of a selective biomarker can be correlated to intracellular concentrations of HNO. Furthermore, the sensitivity of the assay is demonstrated, suggesting that endogenous formation may be detectable.

Conclusions

A new strategy for detection of HNO based on quantification of GS(O)NH₂, a highly selective biomarker, using CZE-LIF was designed. Key advantages of this approach are selectivity for HNO, particularly with respect to NO, fast separation and high resolution and analytical sensitivity. Furthermore, GS(O)NH₂ has been demonstrated to be both a specific marker of HNO production and stable enough in a cellular environment to be useful for detection of HNO. The intracellular concentration of GS(O)NH₂ increased when treated with higher concentrations of HNO donors, suggesting that GS(O)NH₂ levels can be correlated to the concentration of HNO in cells. The quantitative nature of this method will allow assessment of HNO release from new donor compounds, which is critical for continued development of the pharmacology of HNO. Moreover, this assay may allow simultaneous detection of HNO and analysis of the profile of thiol modification from prodrugs such as cyanamide. Finally, both the biosynthetic routes and the physiological responses to HNO remain to be resolved, and this method is suggested to provide a valuable investigative tool to address these questions. There is currently no evidence that human breast cancer cells biosynthesize HNO. The next step is to investigate suggested pathways and environments for HNO biosynthesis.

Supplementary Material

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Acknowledgments

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Abbreviations

AcOM-IPA/NO	Acetoxymethyl protected isopropylamine NONOate
AcOM-DEA/NO	acetoxymethyl protected diethylamine NONOate
AIDH	aldehyde dehydrogenase
Na₂N₂O₃	Angeli's salt
CZE	capillary zone electrophoresis
NONOate	diazoniumdiolate
DEA/NO	diethylamine NONOate
DTPA	diethylenetriaminepentaacetic acid
GSH	glutathione
GS-NEM	glutathione <i>N</i> -ethylmaleimide adduct
GS(O)NH₂	glutathione sulfinamide
GAPDH	glyceraldehyde 3-phosphate dehydrogenase

H₂O₂	hydrogen peroxide
ID	inner diameter
IPA/NO	isopropylamine NONOate
LIF	laser-induced fluorescence
NDA	naphthalene-2,3-dicarboxaldehyde
NO	nitric oxide
NO₃⁻	nitrate
NO₂⁻	nitrite
HNO	nitroxyl
NEM	<i>N</i> -ethylmaleimide
GSSG	oxidized glutathione
ONOO⁻	peroxynitrite
PBS	phosphate-buffered saline
PARP	poly(ADP-ribose) polymerase
GSNO	<i>S</i> -nitrosoglutathione
RSO₂H	sulfinic acid
RS(O)SR	thiosulfinate
TCEP	tris(2-carboxyethyl)phosphine

References

1. Shoeman DW, Shiota FN, DeMaster EG, Nagasawa HT. Reaction of nitroxyl, an aldehyde dehydrogenase inhibitor, with N-acetyl-L-cysteine. *Alcohol*. 2000; 20:55–59. [PubMed: 10680717]
2. DeMaster EG, Redfern B, Nagasawa HT. Mechanisms of inhibition of aldehyde dehydrogenase by nitroxyl, the active metabolite of the alcohol deterrent agent cyanamide. *Biochem Pharmacol*. 1998; 55:2007–2015. [PubMed: 9714321]
3. Norris AJ, Sartippour MR, Lu M, Park T, Rao JY, Jackson MI, Fukuto JM, Brooks MN. Nitroxyl inhibits breast tumor growth and angiogenesis. *Int J Cancer*. 2008; 122:1905–1910. [PubMed: 18076071]
4. Pagliaro P, Mancardi D, Rastaldo R, Penna C, Gattullo D, Miranda KM, Feelisch M, Wink DA, Kass DA, Paolocci N. Nitroxyl affords thiol-sensitive myocardial protective effects akin to early preconditioning. *Free Radic Biol Med*. 2003; 34:33–43. [PubMed: 12498977]
5. Paolocci N, Katori T, Champion HC, StJohn ME, Miranda KM, Fukuto JM, Wink DA, Kass DA. Positive inotropic and lusitropic effects of HNO/NO⁻ in failing hearts: independence from β-adrenergic signaling. *Proc Natl Acad Sci USA*. 2003; 100:5537–5542. [PubMed: 12704230]
6. Miranda KM, Paolocci N, Katori T, Thomas DD, Ford E, Bartberger MD, Espey MG, Kass DA, Feelisch M, Fukuto JM, Wink DA. A biochemical rationale for the discrete behavior of nitroxyl and nitric oxide in the cardiovascular system. *Proc Natl Acad Sci USA*. 2003; 100:9196–9201. [PubMed: 12865500]
7. Sidorkina O, Espey MG, Miranda KM, Wink DA, Laval J. Inhibition of poly(ADP-ribose) polymerase (PARP) by nitric oxide and reactive nitrogen oxide species. *Free Radic Biol Med*. 2003; 35:1431–1438. [PubMed: 14642390]

8. Väänänen AJ, Kankuri E, Rauhala P. Nitric oxide-related species-induced protein oxidation: reversible, irreversible, and protective effects on enzyme function of papain. *Free Radic Biol Med.* 2005; 38:1102–1111. [PubMed: 15780768]
9. Väänänen AJ, Salmenperä P, Hukkanen M, Rauhala P, Kankuri E. Cathepsin B is a differentiation-resistant target for nitroxyl (HNO) in THP-1 monocyte/macrophages. *Free Radic Biol Med.* 2006; 41:120–131. [PubMed: 16781460]
10. Väänänen AJ, Salmenperä P, Hukkanen M, Miranda KM, Harjula A, Rauhala P, Kankuri E. Persistent susceptibility of cathepsin B to irreversible inhibition by nitroxyl (HNO) in the presence of endogenous nitric oxide. *Free Radic Biol Med.* 2008; 45:749–755. [PubMed: 18572022]
11. Lopez BE, Rodriguez CE, Pribadi M, Cook NM, Shinyashik M, Fukuto JM. Inhibition of yeast glycolysis by nitroxyl (HNO): mechanism of HNO toxicity and implications to HNO biology. *Arch Biochem Biophys.* 2005; 442:140–148. [PubMed: 16139238]
12. Nagasawa HT, DeMaster EG, Redfern B, Shiota FN, Goon DJ. Evidence for nitroxyl in the catalase-mediated bioactivation of the alcohol deterrent agent cyanamide. *J Med Chem.* 1990; 33:3120–3122. [PubMed: 2258896]
13. Miller TW, Cherney MM, Lee AJ, Francoleon NE, Farmer PJ, King SB, Hobbs AJ, Miranda KM, NBJ, Fukuto JM. The effects of nitroxyl (HNO) on soluble guanylate cyclase activity: interactions at ferrous heme and cysteine thiols. *J Biol Chem.* 2009; 284:21788–21796. [PubMed: 19531488]
14. Colton CA, Gbadegesin M, Wink DA, Miranda KM, Espey MG, Vicini S. Nitroxyl anion regulation of the NMDA receptor. *J Neurochem.* 2001; 78:1126–1134. [PubMed: 11553686]
15. Miranda KM. The chemistry of nitroxyl (HNO) and implications in biology. *Coord Chem Rev.* 2005; 249:433–455.
16. Fukuto JM, Wallace GC, Hszieh R, Chaudhuri G. Chemical oxidation of N-hydroxyguanidine compounds: release of nitric oxide, nitroxyl and possible relationship to the mechanism of biological nitric oxide generation. *Biochem Pharmacol.* 1992; 43:607–613. [PubMed: 1540216]
17. Fukuto JM, Stuehr DJ, Feldman PL, Bova MP, Wong P. Peracid oxidation of an N-hydroxyguanidine compound - a chemical model for the oxidation of N-omega-hydroxy-L-arginine by nitric oxide synthase. *J Med Chem.* 1993; 36:2666–2670. [PubMed: 7692046]
18. Yoo J, Fukuto JM. Oxidation of N-hydroxyguanidine by nitric oxide the possible generation of vasoactive species. *Biochem Pharmacol.* 1995; 50:1995–2000. [PubMed: 8849325]
19. Miranda KM, Nims RW, Thomas DD, Espey MG, Citrin D, Bartberger MD, Paolucci N, Fukuto JM, Feelisch M, Wink DA. Comparison of the reactivity of nitric oxide, nitroxyl with heme proteins, A chemical discussion of the differential biological effects of these redox related products of NOS. *J Inorg Biochem.* 2003; 93:52–60. [PubMed: 12538052]
20. 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]
21. Wong PSY, Hyun J, Fukuto JM, Shiota 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]
22. Arnelle 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. *Arch Biochem Biophys.* 1995; 318:279–285. [PubMed: 7733655]
23. Donzelli S, Espey MG, Flores-Santana W, Switzer CH, Yeh GC, Huang J, Stuehr DJ, King SB, Miranda KM, Wink DA. Generation of nitroxyl by heme protein-mediated peroxidation of hydroxylamine but not N-hydroxy-L-arginine. *Free Radic Biol Med.* 2008; 45:578–584. [PubMed: 18503778]
24. Kohout FC, Lampe FW. On the role of the nitroxyl molecule in the reaction of hydrogen atoms with nitric oxide. *J Am Chem Soc.* 1965; 87:5795–5796.
25. Lin MC, He Y, Melius CF. Theoretical interpretation of the kinetics and mechanisms of the HNO + HNO and HNO + 2NO reactions with a unified model. *Int J Chem Kinet.* 1992; 24:489–516.
26. Shafirovich V, Lyman SV. Nitroxyl and its anion in aqueous solutions: spin states, protic equilibria, and reactivities toward oxygen and nitric oxide. *Proc Natl Acad Sci USA.* 2002; 99:7340–7345. [PubMed: 12032284]

27. Ignarro LJ, Buga GM, Wood KS, Byrns RE, Chaudhuri G. Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proc Natl Acad Sci USA*. 1987; 84:9265–9269. [PubMed: 2827174]
28. Ignarro LJ. Biosynthesis and metabolism of endothelium-derived nitric oxide. *Annu Rev Pharmacol Toxicol*. 1990; 30:535–536. [PubMed: 2188578]
29. Palmer RMJ, Ferrige AG, Moncada S. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature*. 1987; 327:524–526. [PubMed: 3495737]
30. Wink DA, Mitchell JB. Chemical biology of nitric oxide: insights into regulatory, cytotoxic, and cytoprotective mechanisms of nitric oxide. *Free Radic Biol Med*. 1998; 25:434–456. [PubMed: 9741580]
31. Sato M, Nakajima T, Goto M, Umezawa Y. Cell-based indicator to visualize picomolar dynamics of nitric oxide release from living cells. *Anal Chem*. 2006; 78:8175–8182. [PubMed: 17165805]
32. Miranda KM, Espey MG, Yamada K, Krishna M, Ludwick N, Kim S, Jourdain D, Grisham MB, Feelisch M, Fukuto JM, Wink DA. Unique oxidative mechanisms for the reactive nitrogen oxide species, nitroxyl anion. *J Biol Chem*. 2001; 276:1720–1727. [PubMed: 11042174]
33. Espey MG, Miranda KM, Thomas DD, Wink DA. Ingress and reactive chemistry of nitroxyl-derived species within human cells. *Free Radic Biol Med*. 2002; 33:827–834. [PubMed: 12208370]
34. Rosenthal J, Lippard SJ. Direct detection of nitroxyl in aqueous solution using a tripodal copper(II) BODIPY complex. *J Am Chem Soc*. 2010; 132:5536–5537. [PubMed: 20355724]
35. Royzen M, Wilson JJ, Lippard SJ. Physical and structural properties of [Cu(BOT1)Cl]Cl, a fluorescent imaging probe for HNO. *J Inorg Biochem*. 2013; 118:162–170. [PubMed: 23102502]
36. Kawai K, Ieda N, Aizawa K, Suzuki T, Miyata N, Nakagawa H. A reductant-resistant and metal-free fluorescent probe for nitroxyl applicable to living cells. *J Am Chem Soc*. 2013; 135:12690–12696. [PubMed: 23865676]
37. Reisz JA, Zink CN, King SB. Rapid and selective nitroxyl (HNO) trapping by phosphines: kinetics and new aqueous ligations for HNO detection and quantitation. *J Am Chem Soc*. 2011; 133:11675–11685. [PubMed: 21699183]
38. 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 Radic Biol Med*. 2006; 40:1056–1066. [PubMed: 16540401]
39. Forman HJ, Zhang H, Rinna A. Glutathione: overview of its protective roles, measurement, and biosynthesis. *Mol Aspects Med*. 2009; 30:1–12. [PubMed: 18796312]
40. Smith PAS, Hein GE. The alleged role of nitroxyl in certain reactions of aldehydes and alkyl halides. *J Am Chem Soc*. 1960; 82:5731–5740.
41. 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. *J Med Chem*. 1991; 34:3242–3247. [PubMed: 1956043]
42. Salmon DJ, Torres de Holding CL, Thomas L, Peterson KV, Goodman GP, Saavedra JE, Srinivasan A, Davies KM, Keefer LK, Miranda KM. HNO and NO release from a primary amine-based diazeniumdiolate as a function of pH. *Inorg Chem*. 2011; 50:3262–3270. [PubMed: 21405089]
43. 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. *J Am Chem Soc*. 2010; 132:16526–16532. [PubMed: 21033665]
44. Saavedra JE, Shami PJ, Wang LY, Davies KM, Booth MN, Citro ML, Keefer LK. Esterase-sensitive nitric oxide donors of the diazeniumdiolate family: in vitro antileukemic activity. *J Med Chem*. 2000; 43:261–269. [PubMed: 10649981]
45. Johnson GM, Chozinski TJ, Salmon DJ, Moghaddam AD, Chen HC, Miranda KM. Quantitative detection of nitroxyl upon trapping with glutathione and labeling with a specific fluorogenic reagent. *Free Radic Biol Med*. 2013; 63:476–484. [PubMed: 23685286]

46. Beckman JS, Beckman TW, Chen J, Marshall PH, Freeman BA. Apparent hydroxyl radical production by peroxynitrites: implications for endothelial injury from nitric oxide and superoxide. *Proc Natl Acad Sci USA*. 1990; 87:1620–1624. [PubMed: 2154753]
47. Lopez BE, Shinyashiki M, Han TH, Fukuto JM. Antioxidant actions of nitroxyl (HNO). *Free Radic Biol Med*. 2007; 42:482–491. [PubMed: 17275680]
48. Mojica ER, Kim S, Aga DS. Formation of N-ethylmaleimide (NEM)-glutathione conjugate and N-ethylmaleamic acid revealed by mass spectral characterization of intracellular and extracellular microbial metabolites of NEM. *Appl Environ Microbiol*. 2008; 74:323–326. [PubMed: 17981949]
49. Roach MC, Harmony MD. Determination of amino acids at subfemtomole levels by high-performance liquid chromatography with laser-induced fluorescence detection. *Anal Chem*. 1987; 59:411–415. [PubMed: 3565761]
50. Carlson RG, Srinivasachar K, Givens RS, Matuszewski BK. New derivatizing agents for amino acids, peptides I, Facile synthesis of N- substituted 1- cyanobenz[f] isoindoles and their spectroscopic properties. *J Org Chem*. 1986; 51:3978–3983.
51. De Montigny P, Stobaugh JF, Givens RS, Carlson RG, Srinivasachar K, Sternson LA, Higuchi T. Naphthalene- 2, 3- dicarboxyaldehyde/cyanide ion: a rationally designed fluorogenic reagent for primary amines. *Anal Chem*. 1987; 59:1096–1101.
52. Camera E, Picardo M. Analytical methods to investigate glutathione and related compounds in biological and pathological processes. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2002; 781:181–206.
53. Parmentier C, Wellman M, Nicolas A, Siest G, Leroy P. Simultaneous measurement of reactive oxygen species and reduced glutathione using capillary electrophoresis and laser-induced fluorescence detection in cultured cell lines. *Electrophoresis*. 1999; 20:2938–2944. [PubMed: 10546831]
54. Orwar O, Fishman HA, Ziv NE, Scheller RH, Zare RN. Use of 2,3-naphthalenedicarboxaldehyde derivatization for single-cell analysis of glutathione by capillary electrophoresis and histochemical localization by fluorescence microscopy. *Anal Chem*. 1995; 67:4261–4268. [PubMed: 8633771]
55. Leroy P, Nicolas A, Wellmann M, Michelet F, Oster T, Siest G. Evaluation of o-phthalaldehyde as a bifunctional fluorogenic post-column reagent for glutathione in LC. *Chromatographia*. 1993; 36:130–134.
56. Gregory JDJ. The stability of N-ethylmaleimide and its reaction with sulfhydryl groups. *J Am Chem Soc*. 1955; 77:3922–3923.
57. Johansson MK, Cook RM. Intramolecular dimers: a new design strategy for fluorescence-quenched probes. *Chemistry & Biology*. 2003; 9:3466–3471.
58. Marmé N, Knemeyer JP, Sauer M, Wolfrum J. Inter- and intramolecular fluorescence quenching of organic dyes by tryptophan. *Bioconjug Chem*. 2003; 14:1133–1139. [PubMed: 14624626]
59. Manica DP, Lapos JA, Jones AD, Ewing AG. Analysis of the stability of amino acids derivatized with naphthalene-2,3-dicarboxaldehyde using high-performance liquid chromatography and mass spectrometry. *Anal Biochem*. 2003; 322:68–78. [PubMed: 14705782]
60. Lada MW, Vickroy TW, Kennedy RT. High temporal resolution monitoring of glutamate and aspartate in vivo using microdialysis on-line with capillary electrophoresis with laser-induced fluorescence detection. *Anal Chem*. 1997; 69:4560–4565. [PubMed: 9375517]
61. Lada MW, Kennedy RT. In vivo monitoring of glutathione and cysteine in rat caudate nucleus using microdialysis on-line with capillary zone electrophoresis-laser induced fluorescence detection. *J Neurosci Methods*. 1997; 72:153–1159. [PubMed: 9133579]
62. Smyth DG, Blumenfeld OO, Konigsberg W. Reactions of N-ethylmaleimide with peptides and amino acids. *Biochem J*. 1964; 91:589–595. [PubMed: 5840721]
63. Hernandez L, Escalona J, Verdeguez P, Guzman NA. In vivo monitoring of brain glutamate by microdialysis coupled to capillary electrophoresis and laser induced fluorescence detection. *J Liq Chromatogr*. 1993; 16:2149–2160.
64. Angeli A. Sopra la nitroidrossilammina. *Gazz Chim Ital*. 1896; 26:17–25.
65. Bonner FT, Ravid B. Thermal decomposition of oxyhyponitrite (sodium trioxodinitrate(II)) in aqueous solution. *Inorg Chem*. 1975; 14:558–563.

66. Hughes MN, Wimbledon PE. The chemisry of trioxodinitratesPart I. Decompostion of sodium trioxodinitrate (Angeli's salt) in aqueous solution. *J Chem Soc Dalton Trans.* 1976:703–707.
67. Hendrickson DN, Jolly WL. The structure of the oxyhyponitrite ion. *Inorg Chem.* 1969; 8:693–694.
68. Hughes MN, Wimbledon PE. The chemistry of trioxodinitratesPart 2, The effect of added nitrite on the stability of sodium trioxodinitrate in aqueous solution. *J Chem Soc Dalton Trans.* 1977:1650–1653.
69. Donald CE, Hughes MN, Thompson JM, Bonner FT. Photolysis of the nitrogen-nitrogen double bond in trioxodinitrate: reaction between triplet oxonitrate(1-) and molecular oxygen to form peroxonitrite. *Inorg Chem.* 1986; 25:2676–2677.
70. Wink DA, Feelisch M, Fukuto J, Christodoulou D, Jourdeuil D, Grisham MB, Vodovotz Y, Cook JA, Krishna M, DeGraff WG, Kim S, Gamson J, Mitchell JB. The cytotoxicity of nitroxyl: possible implications for the pathophysiological role of NO. *Arch Biochem Biophys.* 1998; 351:66–74. [PubMed: 9501920]
71. Basudhar D, Bharadwaj G, Cheng RY, Jain S, Shi S, Ridnour LA, Caceres VM, Spadari-Bratfisch RC, Paolucci N, Velázquez-Martínez CA, Wink DA, Miranda KM. Synthesis and comparison of nitroxyl and nitric oxide releasing diazeniumdiolate-based aspirin derivatives. *J Med Chem.* 2013; 56:7804–7820. [PubMed: 24102516]

Highlights

- The products of association of HNO with glutathione were examined
- Glutathione sulfinamide is a major product and a specific biomarker of HNO
- A method to quantify glutathione sulfinamide with nanomolar sensitivity is reported
- Glutathione sulfinamide was detected in cell lysate after exposure to an HNO donor

Figure 1.

Fluorescence intensity at 490 nm vs. time for NDA/CN⁻-labeled GSH (red) or GS(O)NH₂ (blue) (50 μ M in assay buffer, pH 7.4). Error bars are displayed as mean $\pm$ SD ($n = 3$) but are smaller than the symbol size.

Figure 2. Electropherogram of NDA/CN⁻-labeled GSH-NEM (175 nM), GS(O)NH₂ (175 nM), and fluorescein (5 nM; *). The inset provides an expanded view of peaks eluting from 130–145 s.

Figure 3.

Electropherograms of NDA/CN⁻-labeled products following treatment of GSH (50 μM) with 1 eq of (A) Angeli's salt, (B) DEA/NO, (C) GSNO, (D) ONOO⁻, (E) H₂O₂, (F) NO₂⁻, (G) NO₃⁻, (H) ascorbic acid or (I) assay buffer with fluorescein (5 nM; *).

Figure 4.

Electropherograms of NDA/CN⁻-labeled (A) cell lysate, (B) cell lysate of cells spiked with 50 μM GS(O)NH₂ or (C) a standard mixture of GSH and GS(O)NH₂ (175 nM) with fluorescein (5 nM; *). In B, the peak for GS(O)NH₂ corresponded to 62% recovery of the spiked sample.

Figure 5.

Electropherograms of (A) cell lysate, (B) cell lysate of cells treated with 100 μ M AcOM-IPA/NO or (C) a standard mixture of GSH and GS(O)NH₂ (175 nM) with fluorescein (5 nM; *).

Scheme 1.

Reaction of HNO with GSH to produce the potential biomarker GS(O)NH₂ (pathway *a*) or the common oxidation product GSSG (pathway *b*) [21]. Pathway *b* becomes progressively competitive as the concentration of GSH increases, such that a mixture of GS(O)NH₂ and GSSG is produced.

Table 1

Percent HNO trapping efficiencies determined from GSH (50 or 500 μM) treated with Angeli's salt (pH 7.4 and 37 $^{\circ}\text{C}$).

[GSH], μM	[Angeli's salt], μM	[GS(O)NH ₂], μM	[GSSG], μM	% trapping efficiency
50	0	ND	ND	–
	11	11 ± 1	ND	102 ± 5
	22	22 ± 1	ND	99 ± 1
	31	31 ± 1	ND	100 ± 2
500	0	ND	ND	–
	90	64 ± 1	22 ± 1	96 ± 1
	180	97 ± 1	76 ± 1	96 ± 1
	270	160 ± 1	96 ± 1	95 ± 1

ND, not detected; $n = 3$

Table 2

Percent recovery of GS(O)NH₂ in the lysate of cells (6×10^5 cells/mL) exposed to known concentrations of GS(O)NH₂ in 400 μ L of PBS buffer.

[GS(O)NH ₂], μ M	Recovery (%)
0	ND
0.5	ND
1	17 ± 3
5	18 ± 2
10	41 ± 1
25	49 ± 1
50	62 ± 1

ND, not detected; $n = 3$

Table 3

Concentration of GS(O)NH₂ in cell lysate of cells treated with AcOM-IPA/NO ($n = 3$).

[AcOM-IPA/NO], μM	[GS(O)NH ₂], nM
25	8 ± 2
	26 ± 2
100	49 ± 3