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Original Contribution

Glutathione sulfinamide serves as a selective, endogenous biomarker for nitroxyl after 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 because of 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 ($GS(O)NH_2$) may serve as a high-yield biomarker of HNO production. In this work, the formation of $GS(O)NH_2$ after exposure to HNO donors was investigated. Fluorescent labeling followed by separation and detection using capillary zone electrophoresis with laser-induced fluorescence allowed quantitation of $GS(O)NH_2$ with nanomolar sensitivity, even in the presence of GSH and derivatives. Formation of $GS(O)NH_2$ was found to occur exclusively upon exposure of GSH to HNO donors, thus confirming selectivity. $GS(O)NH_2$ was detected in the lysate of cells treated with low-micromolar concentrations of HNO donors, verifying that this species has sufficient stability to server as a biomarker of HNO. Additionally, the concentration-dependent formation of $GS(O)NH_2$ in cells treated with an HNO donor suggests that the concentration of $GS(O)NH_2$ can be correlated to intracellular levels of HNO.

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 [7], the cysteine proteases papain [8] and cathepsin B [9,10], the glycolytic protein glyceraldehyde-3-phosphate dehydrogenase [11], and aldehyde dehydrogenase (AlDH)[1], which is critical to alcohol metabolism [12]. HNO also regulates vasorelaxation [13] and alters calcium channel function [14] through binding of HNO to an iron or thiol site.

Although the pharmacology of HNO has been investigated in considerable detail over the past decade, whether HNO functions as an endogenous signaling agent is currently a matter of speculation (see Ref. [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 owing to irreversible dimerization complicates detection (Eq. (1) [24,25]; $8 \times 10^6\ M^{-1}\ s^{-1}$, pH 7.4, 22 °C [26]):

$$2HNO \rightarrow [HONNOH] \rightarrow N_2O + H_2O. \quad (1)$$

Abbreviations: AcOM-IPA/NO, acetoxymethyl-protected isopropylamine NONOate; AcOM-DEA/NO, acetoxymethyl-protected diethylamine NONOate; AlDH, aldehyde dehydrogenase; $Na_2N_2O_3$, Angeli's salt; CZE, capillary zone electrophoresis; NONOate, diazeniumdiolate; DEA/NO, diethylamine NONOate; GSH, glutathione; GS–NEM, glutathione *N*-ethylmaleimide adduct; $GS(O)NH_2$, glutathione sulfinamide; IPA/NO, isopropylamine NONOate; LIF, laser-induced fluorescence; NDA, naphthalene-2,3-dicarboxaldehyde; NEM, *N*-ethylmaleimide; GSSG, oxidized glutathione/glutathione disulfide; PBS, phosphate-buffered saline; GSNO, *S*-nitrosoglutathione; TCEP, tris(2-carboxyethyl)phosphine.

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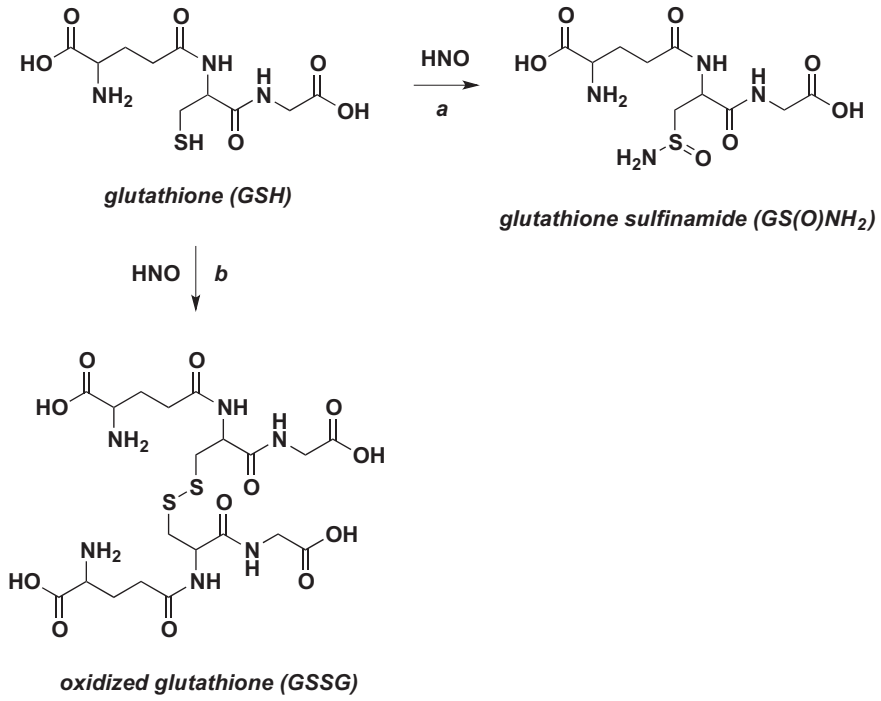

Scheme 1. Reaction of HNO with GSH to produce the potential biomarker $GS(O)NH_2$ (pathway *a*) or the common oxidation product GSSG (pathway *b*) [21]. Pathway *b* becomes progressively competitive as the concentration of GSH increases, such that a mixture of $GS(O)NH_2$ and GSSG is produced.

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 owing to their 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 fluorescence 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 sulfinamide ($GS(O)NH_2$), which is proposed to be a unique marker of HNO, and oxidized glutathione/glutathione disulfide (GSSG) (Scheme 1) [21,38]. Moreover, the reaction between HNO and GSH is facile ($2 \times 10^6\ M^{-1}\ s^{-1}$, 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 those of 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 $GS(O)NH_2$ 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 ($Na_2N_2O_3$, sodium trioxodinitrate), DEA/NO ($Na[Et_2NN(O)NO]$, sodium (*Z*)-1-(*N*,*N*-diethylamino)diazen-1-ium-1,2-diolate), IPA/NO ($Na[(CH_3)_2CHNH(N(O)NO)]$, sodium 1-(*N*-isopropylamino)diazen-1-ium-1,2-diolate), AcOM-IPA/NO (O^2-(acetoxymethyl) 1-(isopropylamino)diazen-1-ium-1,2-diolate), and AcOM-DEA/NO (O^2-(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, whereas 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: $7600\ M^{-1}\ cm^{-1}$ for Angeli's salt, $8800\ M^{-1}\ cm^{-1}$ for IPA/NO, and $6700\ M^{-1}\ cm^{-1}$ for DEA/NO at 250 nm in 10 mM NaOH and $8700\ M^{-1}\ cm^{-1}$ for AcOM-IPA/NO and $7600\ M^{-1}\ cm^{-1}$ 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. $GS(O)NH_2$ 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 (50 μM) in calcium- and magnesium-free Dulbecco's phosphate-buffered saline (PBS; pH 7.4) to reduce oxidation of HNO to