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Original Contribution

Generation of nitroxyl by heme protein-mediated peroxidation of hydroxylamine but not *N*-hydroxy-L-arginine

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ABSTRACT

The chemical reactivity, toxicology, and pharmacological responses to nitroxyl (HNO) are often distinctly different from those of nitric oxide (NO). The discovery that HNO donors may have pharmacological utility for treatment of cardiovascular disorders such as heart failure and ischemia reperfusion has led to increased speculation of potential endogenous pathways for HNO biosynthesis. Here, the ability of heme proteins to utilize H_2O_2 to oxidize hydroxylamine (NH_2OH) or *N*-hydroxy-L-arginine (NOHA) to HNO was examined. Formation of HNO was evaluated with a recently developed selective assay in which the reaction products in the presence of reduced glutathione (GSH) were quantified by HPLC. Release of HNO from the heme pocket was indicated by formation of sulfinamide ($GS(O)NH_2$), while the yields of nitrite and nitrate signified the degree of intramolecular recombination of HNO with the heme. Formation of $GS(O)NH_2$ was observed upon oxidation of NH_2OH, whereas NOHA, the primary intermediate in oxidation of L-arginine by NO synthase, was apparently resistant to oxidation by the heme proteins utilized. In the presence of NH_2OH, the highest yields of $GS(O)NH_2$ were observed with proteins in which the heme was coordinated to a histidine (horseradish peroxidase, lactoperoxidase, myeloperoxidase, myoglobin, and hemoglobin) in contrast to a tyrosine (catalase) or cysteine (cytochrome P450). That peroxidation of NH_2OH by horseradish peroxidase produced free HNO, which was able to affect intracellular targets, was verified by conversion of 4,5-diaminofluorescein to the corresponding fluorophore within intact cells.

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Introduction

Nitroxyl (HNO) has been shown to exhibit unique biological and pharmacological properties compared to those of other nitrogen oxides. In the cardiovascular system, HNO donors increase cardiac function (e.g., inotropy and lusitropy) in an additive and independent manner to β-adrenergic signaling [1,2]. While HNO can induce early preconditioning-like effects that protect heart tissue against ischemia–reperfusion injury [3], the outcome is dependent on the timing of administration [4]. In addition, HNO regulates calcium channels, in both the cardiovascular and nervous systems [5–10]. These responses to HNO are often discrete from those of its redox cousin nitric oxide (NO) [11,12]. Such findings have led recently to extensive investigation of the chemical, biochemical, and pharmacological properties of HNO, particularly in comparison to NO (reviewed in [12,13]). Moreover, the potential for positive cardiovascular impacts has intensified interest both in the production of HNO donors as pharmacological agents [14] and in the elucidation of biosynthetic routes to HNO production.

HNO donors have been used clinically since the 1950s [15]. For instance, HNO is the active metabolite of the alcohol-sensitizing drug cyanamide (NH_2CN) [16], which is used for pharmacotherapy of alcohol abuse. Additionally, hydroxyurea, which is a chemotherapeutic used to reduce the complications of sickle cell disease, was found to be oxidatively degraded to HNO [17,18]. Although endogenous production of HNO has yet to be demonstrated in vivo, numerous in vitro assays have indicated the existence of several potential biosynthetic mechanisms. For instance HNO can be produced by the enzyme NO

Abbreviations: DAF, 4,5-diaminofluorescein diacetate; DTPA, diethylenetriaminepentaacetic acid; HRP, horseradish peroxidase; HNO, nitroxyl; LPO, lactoperoxidase; Hb, hemoglobin; Mb, myoglobin; NO, nitric oxide; NOS, NO synthase; NH_2OH, hydroxylamine; NOHA, *N*-hydroxy-L-arginine; RSNO, *S*-nitrosothiols.

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synthase (NOS) under conditions where cofactor concentrations are limited [19–24] or by association of an *S*-nitrosothiol and a thiol [25–28]. Furthermore, formation of HNO during the enzymatic oxidation of cyanamide and hydroxyurea suggests that endogenous reduced nitrogen species may be similarly converted to HNO.

Many heme proteins function as peroxidases in the catalyzed oxidation of a wide range of substrates. Despite the protective and functional utility of peroxidases, an increase in peroxidase activity has been implicated in the pathology of a number of diseases [29,30]. Here, we examined the ability of heme proteins to produce HNO from peroxidation of hydroxylamine (NH_2OH) or *N*-hydroxy-L-arginine (NOHA), which are both produced during catalytic turnover of NOS [20,31]. Hydroxylamine and derivatives such as NOHA and hydroxyurea are logical substrates for formation of HNO by peroxidation since their nitrogens are two electrons more reduced than that in HNO. Although in vivo concentrations of NH_2OH and NOHA are not well established, the pharmokinetics of hydroxyurea are better understood. At a standard 20 mg/kg dose of hydroxyurea, maximum serum levels can exceed 100 mM [32]. Although such levels are expected only from exogenous sources, the demonstrated ability to accumulate hydroxylamines suggests the possibility that this class of compound may function as endogenous precursors of HNO.

Production of HNO from oxidation of NH_2OH or NOHA was evaluated with a recently developed, selective assay in which formation of sulfinamide ($GS(O)NH_2$) from the association of HNO with reduced glutathione (GSH) was quantified by HPLC [33]. The reaction of HNO with GSH was further investigated in real time by both chemiluminescence and fluorometric assays.

Experimental procedures

Materials

Hydroxylamine hydrochloride, reduced and oxidized (GSSG) glutathione, sodium nitrite (NO_2^-), sodium nitrate (NO_3^-), diethylenetriaminepentaacetic acid (DTPA), horseradish peroxidase (HRP), horse heart myoglobin (metMb), human hemoglobin (metHb), hemin, catalase, and lactoperoxidase (LPO) were obtained from Sigma-Aldrich. 4,5-Diaminofluorescein diacetate (DAF) was purchased from Calbiochem (San Diego, CA). Human leukocytes myeloperoxidase (MPO) [34] was purchased from Alexis while human cytochrome P450 (P450) was acquired from Oxford Biomedical Research. All proteins were used without further purification, and stock solutions were prepared fresh daily at 100× in Milli-Q filtered water unless otherwise specified. NOHA was a generous gift from Prof. Jon Fukuto (UCLA School of Medicine). 1-[2-(Carboxylato)pyrrolidin-1-yl]diazem-1-ium-1,2-diolate (PROLI/NO) was a generous gift from Drs. Joseph Saavedra and Larry Keefer (Laboratory of Comparative Carcinogenesis, National Cancer Institute at Frederick). Sulfinamide was synthesized as previously described [33,35]. Stock solutions of NO were prepared by sparging argon-deaerated 100 mM phosphate buffer (pH 7.4) with hydroxide scrubbed NO (>99%; Matheson, Montgomeryville, PA).

Heme protein- and hemin-mediated peroxidation of NH_2OH or NOHA

Enzymes, metMb, metHb, or hemin (5 μM) were dissolved in sodium phosphate buffer (10 mM, pH 7.4) containing the metal chelator DTPA (50 μM), GSH (100 μM) and either NH_2OH or NOHA (500 μM). This level of substrate was assumed to be biologically feasible given the high millimolar concentrations of hydroxyurea that can be established [32]. Furthermore, these conditions were chosen to optimize evaluation of the reaction given the concentration of GSH (100 μM) required for the assay. The reaction was initiated by the addition of H_2O_2 (100 μM) and was allowed to proceed at 37°C for 10 min. The reaction was terminated by removal of protein by centrifugal filtration (14,000 rpm for 30 min at 4°C, Microcon 3-kDa centrifugal filter device, Millipore). The deproteinized ultrafiltrate was injected directly onto a C-18 HPLC column to characterize the reaction products as previously described [33].

Fluorescence analysis

Human breast carcinoma (MCF-7) cells were cultured as attached cells to 80% confluence in T-75 flasks (Nalge Nunc International, Rochester, NY) containing RPMI 1640 medium at 37°C in a humidified incubator with 5% CO_2 in air. A suspension of cells (10^6) was incubated with DAF (5 μM) at 37°C for 15 min. Cells were rinsed with PBS three times by a cycle of suspension and centrifugation. Fluorescence signal from cell suspensions in phosphate buffer (10 mM, pH 7.4) containing DTPA (50 μM) were analyzed on a Perkin-Elmer LS50B fluorometer using an excitation at 495 and emission at 515 nm with 5.0 mm slit widths in a 2 mL reaction volume held at 37°C while stirring with a water-jacketed cuvette holder [36].

Chemiluminescence analysis

Production of NO is commonly quantified from the chemiluminescence signal resulting from reaction of NO with ozone [37]. Recently, a commercially available chemiluminescence system (Sievers NO Analyzer, Ionics, Boulder, CO) was shown to also detect HNO in a GSH-dependent manner [38]. Therefore, production of HNO during peroxidation of NH_2OH or NOHA was examined by performing the reaction in the presence or absence of 100 μM GSH in the helium-purged reaction vessel of the analyzer.

Gas chromatographic and electron paramagnetic resonance analysis

Peroxidation of NH_2OH (100 mM) by HRP (500 μM) was initiated by addition of H_2O_2 (50 mM) to the deaerated solution in phosphate buffer (50 mM, pH 7.4, 1.0 mL) contained in a septum-stoppered flask. After 2 h at room temperature, the yield of nitrous oxide (N_2O), as a marker of free HNO, was analyzed by injecting an aliquot of the reaction headspace (250 μL) onto a 6890 Hewlett Packard gas chromatograph equipped with a thermal conductivity detector and a 6 ft × 1/8 in Porapak Q column at an operating oven temperature of 50°C (injector and detector 150°C) with a flow rate of 16.67 mL/min (He carrier gas). The retention time of N_2O of 2.78 min was identical to authentic N_2O (Aldrich), and the yield was calculated from an N_2O standard curve. An aliquot (300 μL) of the reaction solution was also transferred to an EPR tube and frozen in liquid nitrogen. EPR spectra were recorded on a Bruker ER200D spectrometer using 8.5 mW microwave power, 5.0 G modulation amplitude, and 9.32 GHz microwave frequency.

Table 1
Micromolar product formation from heme-mediated peroxidation of NH_2OH in the presence of GSH

	$GS(O)NH_2$	GSH	GSSG	NO_2^-	NO_3^-
HRP	20.8±1.3	51±2	4.5±0.4	ND	3±0.3
metMb	29.4±1.5	23.6±2.4	11.5±2.3	6.8±0.7	5.2±1.3
metHb	14.6±1.8	39.5±6.4	6.9±1.1	5.6±0.6	3.5±1.1
LPO	7.6±0.5	72±11	9.4±0.9	0.5±0.1	5.0±0.5
MPO	43±5	ND	7.0±0.7	ND	ND
Catalase	2±1	49±11	17±2	ND	13±2
Hemin	7.3±1.3	46.0±1.6	11.0±1.3	4.3±0.2	6.0±1.2

Heme protein or hemin (5 μM) was incubated in 10 mM phosphate buffer (pH 7.4) containing DTPA (50 μM), GSH (100 μM), and NH_2OH (500 μM). The reaction was initiated by the addition of H_2O_2 (100 μM), and following a 10-min incubation at 37°C was terminated by centrifugal filtration. The deproteinized ultrafiltrate was analyzed by HPLC or spectrophotometrically. Each product concentration (μM) is reported as the mean±SE of at least three experiments with the exception of MPO. The balance of the products is assumed to be oxidized sulfur compounds (e.g., sulfenic acid), which are not detectable by the methods utilized. ND, not detectable.