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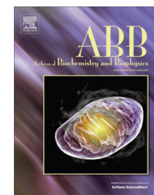

The effects of nitroxyl (HNO) on H_2O_2 metabolism and possible mechanisms of HNO signaling

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ABSTRACT

Nitroxyl (HNO) possesses unique and potentially important biological/physiological activity that is currently mechanistically ill-defined. Previous work has shown that the likely biological targets for HNO are thiol proteins, oxidized metalloproteins (i.e. ferric heme proteins) and, most likely, selenoproteins. Interestingly, these are the same classes of proteins that interact with H_2O_2. In fact, these classes of proteins not only react with H_2O_2, and thus potentially responsible for the signaling actions of H_2O_2, but are also responsible for the degradation of H_2O_2. Therefore, it is not unreasonable to speculate that HNO can affect H_2O_2 degradation by interacting with H_2O_2-degrading proteins possibly leading to an increase in H_2O_2-mediated signaling. Moreover, considering the commonality between HNO and H_2O_2 biological targets, it also seems likely that HNO-mediated signaling can also be due to reactivity at otherwise H_2O_2-reactive sites. Herein, it is found that HNO does indeed inhibit H_2O_2 degradation via inhibition of H_2O_2-metaboilizing proteins. Also, it is found that in a system known to be regulated by H_2O_2 (T cell activation), HNO behaves similarly to H_2O_2, indicating that HNO- and H_2O_2-signaling may be similar and/or intimately related.

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Introduction

Nitroxyl (HNO)[2] is one of many nitrogen oxide species with important and potentially useful biological activity. Akin to its redox congener nitric oxide (NO), HNO has significant and unique effects on the cardiovascular system that portends its development as a potential therapeutic species (for example, [1]). However, unlike NO, the mechanisms by which HNO elicits its effects on the cardiovascular system are only beginning to be understood (for example, [2–5]). Based on previous reports indicating the propensity for HNO to react with thiols [6,7], it has been proposed that primary targets for HNO in biological systems are thiol proteins (for example, [8]). That is, HNO can react with specific thiol proteins leading to reversible and/or irreversible thiol modification [9] with consequent effects on protein activity and physiological function. Significantly, HNO has been reported to be one of the fastest thiol modifying species among all biologically (or pharmacologically) relevant species [10,11], consistent with the idea that HNO biological activity can be due to reaction with thiol proteins.

The biologically relevant chemistry of HNO has been examined and a clear picture of its likely biochemical targets has been described (for example, [12,13]). As mentioned immediately above, HNO reacts readily with thiols/thiol proteins. Surprisingly, HNO does not react readily with water (to form a hydrated, gem-diol species akin to formaldehyde). The selectivity of HNO to react with sulfur nucleophiles as opposed to oxygen nucleophiles is likely due to a lack of electron-pair repulsion in the sulfur adduct compared to that in the oxygen adduct [7]. Consistent with this idea, phosphines (like sulfur, phosphorous is a 3rd row element) are also very reactive towards HNO [11,14]. Based on this reasoning, it is expected that HNO will have an especially high affinity for selenoproteins since the selenium atom in selenocysteine can be much more nucleophilic than the corresponding sulfur atom in cysteine [15] and electron-pair repulsion in the seleno product will be considerably less than that of even the sulfur adduct. HNO is also known to readily react with ferric hemes to form typically stable ferrous nitrosyl complexes (**Reaction 1**) [6].

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[2] *Abbreviations used:* GPx, glutathione peroxidase; GSR, glutathione reductase; AS, Angeli's salt; CAT, catalase; DTPA, diethylenetriaminepentaacetic acid; PMA, phorbol myristic acetate; DCFDA, dichlorofluorescein diacetate; DTT, dithiothreitol; ERK, extracellular signal-regulated kinase; FDP, fluorescein diphosphate; HRP, horseradish peroxidase; AR, amplex red.

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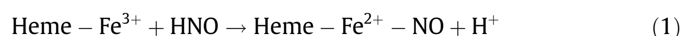

$$\text{Heme} - Fe^{3+} + HNO \rightarrow \text{Heme} - Fe^{2+} - NO + H^{+} \quad (1)$$

Thus, the reactivity profile of HNO predicts that it can modify and possibly inhibit proteins with highly reactive (nucleophilic) cysteines, nucleophilic selenocysteines and ferric heme proteins.

Interestingly, hydrogen peroxide (H_2O_2) is known to react with the same biological groups/motifs and proteins containing these functionalities are supposed targets for H_2O_2 reactivity. The role of H_2O_2 in various signaling pathways has been well-established and is thought to be mediated primarily by the oxidation of specific thiols that are activated to react with electrophiles such as H_2O_2 (for example, [16]). Therefore, proteins that regulate the metabolism of H_2O_2 can have potential effects on many signaling pathways (for example, [17]). The proteins primarily responsible for the degradation of H_2O_2 are peroxiredoxins (a family of activated cysteine proteins), glutathione peroxidase (GPx, a selenoprotein) and catalase (a ferric heme protein) (for example, [18]). Therefore, it may be expected that HNO is capable of inhibiting H_2O_2 metabolism via selective modification of these proteins directly. Taken altogether, it is clearly possible that the biological actions of HNO and H_2O_2 can be intimately intertwined. That is, HNO has the potential to elicit biological activity by increasing H_2O_2 levels in cells (via inhibition of H_2O_2 degrading enzymes) and also directly by interacting with otherwise H_2O_2-reactive signaling proteins. Herein are described studies of the effects of HNO and H_2O_2 on purified proteins and in cells. Specifically, we compare of the actions of H_2O_2 and HNO on well-characterized signaling systems within established macrophage and T cell lines.

Experimental methods

Reagents

Angeli's salt (AS) was synthesized according to a previously published procedure [19]. Concentrated stock solutions of AS were made up in 10 mM NaOH and used immediately. In all cases, the pH of solutions was checked after addition of aliquots of the stock AS solutions to assure that the basic solution did not alter the experimental pH.

Treatment of purified enzymes and activity assay

Prior to use, glutathione peroxidase (GPx, Sigma, St. Louis, MO) and glutathione reductase (GSR, Sigma, St. Louis, MO) enzymes purchased in a lyophilized form were dissolved and dialyzed against 50 mM potassium phosphate that had been pretreated with Chelex to remove any thiols present in the commercial product. Dialysis was carried out under anaerobic conditions and was preceded by treatment with 1 mM tris-carboxyethyl phosphine for 30 min (Research Products International, Mount Prospect, Il) to assure that the proteins were fully reduced. After dialysis, enzymes were diluted anaerobically to concentrations 100-fold greater than that used for activity assays. Aerobic treatments were carried out at 37 °C in a water bath for 20 min, while anaerobic treatments were performed at 37 °C in a temperature controlled glycerol bath inside a glove box (PLAS Labs, Lansing, MI) with ambient equilibrium oxygen content present at 0.03 mg/L or less as determined using an oxygen meter in the glove box (WTW 197i, College Station, TX, USA). All activity assays were carried out aerobically, and in 1 mL cuvettes, according to the following references: catalase (CAT) [20], GPx [21], GSR [22]. Control experiments demonstrated that neither thermally decomposed AS nor sodium nitrite, the primary aqueous decomposition co-product of AS (besides HNO), inhibited purified or cellular enzymes at levels where AS did produce inhibition. This indicates that HNO and not an ancillary byproduct of AS decomposition was responsible for enzyme inhibition. To ascertain the reversibility of CAT inhibition, the treated protein was washed on a 30,000 dalton M.W. cut-off centrifugal spin column (Centricon, Millipore, Billerica, MA, USA) twice with Chelex treated 50 mM potassium phosphate.

Cell culture and treatment

RAW264.7 cells were obtained from ATCC (Manassas, VA, USA) and cultured in DMEM with 20 mM HEPES (HyClone, Logan UT, USA) supplemented with 10% FBS (HyClone, Logan UT, USA), penicillin, streptomycin, glutamine and 0.1 μM selenium (sodium selenite) and utilized until passage 18. It has been demonstrated that common cell culture conditions do not supply sufficient selenium for endogenous GPx activity; specific activity of GPx increased approximately 7-fold after addition of selenite to culture maintenance [23]. Cells were maintained at 37 °C and 5% CO_2 until ~95% confluent before passaging and seeding into 6-well plates (for assays involving lysates or supernatants, 3×10^6 cells/well) or 96 well plates (for assays involving whole cells, $2–10 \times 10^4$ cells/well). For experiments involving the Amplex Red (AR)/Horseradish peroxidase (HRP) assay, phenol red and FBS free media was employed. Cells were treated with AS in full media or, when noted, in serum free media or PBS containing 20 mM HEPES buffer. Treatments were 20 min in length, as the HNO donor AS has a half life of ~2.5 min at 37 °C and there is no additional benefit to be derived from longer treatments.

Jurkat T cells and Lck-deficient T cells (JCaM1) were grown in RPMI 1640 (Cellgro) supplemented with 10% FBS (JRH Biosciences), penicillin, streptomycin, and glutamine.

Lysate preparation and cellular enzyme assays with RAW cells

For cell pellet preparation, cell monolayers were washed and scraped into PBS before pelleting and freezing in liquid nitrogen, and stored at −80 °C until analysis. Lysates were obtained by adding a detergent based hypotonic lysis buffer containing chelators and phosphatase inhibitors (75 mM potassium phosphate (pH 7.45), 0.5% NP-40, 0.1% TritonX-100, 1% glycerol, 1 mM EGTA, 1 mM DTPA, 5 mM potassium fluoride), thawing the cells on ice, disrupting via pipetting and clearing the lysates by centrifugation at 14,000g for 10 min. Analysis of cell lysates was performed in duplicate, at two different protein concentrations: aliquots used in enzyme assays contained approximately 30 μg of protein (~10 μL under our lysis conditions). The lysates were analyzed for enzymatic activity according to the following microplate format assays.

CAT

Catalase was assayed according to a previously published method adapted to microplate format [24]. Aliquots of cell lysate (~10 μL) were added to 27 μL of 50 mM potassium phosphate buffer, pH 7.45. A further 37 μL of 50% methanol in the same buffer was added and the plate mixed on an orbital shaker. The reaction was initiated with the addition of 10 μL 0.3% hydrogen peroxide, mixed and was allowed to proceed for 20 min at room temperature with shaking. Subsequently 18.5 μL of 7.8 M NaOH was added and mixed thoroughly. Next, 37 μL of 35 mM Purpald in 0.48 M HCl was added and allowed to incubate for 20 min at room temperature with shaking. After incubation, 18.5 μL of 65 mM KIO_4 in 0.48 M NaOH was added to further color development and the plate mixed. Absorbance at 590 nm was measured with a microplate spectrophotometer (Emax, Molecular Devices). Results were normalized to protein concentrations in cell lysates.

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