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Original Contribution

Mechanism and regulation of peroxidase-catalyzed nitric oxide consumption in physiological fluids: Critical protective actions of ascorbate and thiocyanate

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ABSTRACT

Catalytic consumption of nitric oxide (NO) by myeloperoxidase and related peroxidases is implicated as playing a key role in impairing NO bioavailability during inflammatory conditions. However, there are major gaps in our understanding of how peroxidases consume NO in physiological fluids, in which multiple reactive enzyme substrates and antioxidants are present. Notably, ascorbate has been proposed to enhance myeloperoxidase-catalyzed NO consumption by forming NO-consuming substrate radicals. However, we show that in complex biological fluids ascorbate instead plays a critical role in inhibiting NO consumption by myeloperoxidase and related peroxidases (lactoperoxidase, horseradish peroxidase) by acting as a competitive substrate for protein-bound redox intermediates and by efficiently scavenging peroxidase-derived radicals (e.g., urate radicals), yielding ascorbyl radicals that fail to consume NO. These data identify a novel mechanistic basis for how ascorbate preserves NO bioavailability during inflammation. We show that NO consumption by myeloperoxidase Compound I is significant in substrate-rich fluids and is resistant to competitive inhibition by ascorbate. However, thiocyanate effectively inhibits this process and yields hypothiocyanite at the expense of NO consumption. Hypothiocyanite can in turn form NO-consuming radicals, but thiols (albumin, glutathione) readily prevent this. Conversely, where ascorbate is absent, glutathione enhances NO consumption by urate radicals via pathways that yield *S*-nitrosoglutathione. Theoretical kinetic analyses provide detailed insights into the mechanisms by which ascorbate and thiocyanate exert their protective actions. We conclude that the local depletion of ascorbate and thiocyanate in inflammatory microenvironments (e.g., due to increased metabolism or dysregulated transport) will impair NO bioavailability by exacerbating peroxidase-catalyzed NO consumption.

Nitric oxide (NO) exerts diverse functions in animals (e.g., by signaling for vasodilation and bronchodilation and by inhibiting platelet aggregation) and in plants (e.g., by mediating host defense). Increasing evidence indicates that heme proteins play dominant roles in regulating NO bioavailability and signaling [1–4]. For example, in mammals, the neutrophil-derived heme enzyme myeloperoxidase (MPO)[1] is released into extracellular fluids in high levels during inflammatory processes and is implicated as a key mediator of impaired NO bioavailability in the vascular compartment and in the lung lumen because of its ability to utilize hydrogen peroxide (H_2O_2) as a cosubstrate to catalytically consume NO [5–10]. A range of other heme proteins (e.g., lactoperoxidase (LPO), myoglobin, hemoglobin, and indoleamine 2,3-dioxygenase) may also utilize H_2O_2 to consume NO via peroxidase-dependent reactions [5,11–13], although the nature of these reactions and their potential biological relevance have, to date, received less attention. In the light of evidence that MPO-dependent decreases in NO bioavailability play an important contributory role in the pathophysiology of inflammatory disorders (e.g., cardiovascular disease, cystic fibrosis), there is particular interest in understanding how MPO consumes NO in physiological fluids and how this process may be controlled [10,14].

An initial study reported that, in the absence of competing substrates, NO can be directly oxidized by MPO via reactions with the redox intermediates MPO Compound I and MPO Compound II [5].

Abbreviations: Asc$^{\bullet}$, ascorbyl radical; GSH, glutathione; GS$^{\bullet}$, glutathione thiyl radical; HRP, horseradish peroxidase; LPO, lactoperoxidase; MPO, myeloperoxidase; GSNO, *S*-nitrosoglutathione; urate–NO, nitrosourate; Tyr$^{\bullet}$, tyrosyl radical; urate$^{\bullet}$, urate radical

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More recent studies have shown that MPO-catalyzed NO consumption can be strongly influenced by the availability of peroxidase substrates in extracellular fluids, namely ascorbate, urate, and tyrosine; however, their effects are incompletely understood. Thus, it has been shown that ascorbate, urate, and tyrosine, when added individually, can increase the rate of NO consumption by MPO in physiological buffer. These increased rates are attributed to the oxidation of these substrates into NO-consuming radicals rather than enhanced NO consumption by MPO redox intermediates [6,15]. Extrapolating from these observations, it has been proposed that ascorbate, urate, and tyrosine will all serve a "pro-oxidant" function in vivo during inflammatory conditions and exacerbate MPO-dependent decreases in NO bioavailability by acting as sources of NO-consuming radicals [6,7,10,14,15]. However, it has yet to be examined whether ascorbate is indeed capable of stimulating MPO-catalyzed NO consumption in substrate-rich biological fluids such as human plasma. Significantly, this proposed pro-oxidant function of ascorbate is difficult to reconcile with a considerable body of evidence showing that ascorbate consistently preserves NO bioavailability during inflammatory conditions in which MPO is implicated as a key mediator of impaired NO bioavailability, e.g., in humans with cardiovascular disease [1,6,9,16]. No previous study has examined the possibility that ascorbate might instead inhibit peroxidase-catalyzed NO consumption under physiological conditions.

Thiocyanate (SCN^-) is a major physiological substrate for MPO and other mammalian peroxidases such as LPO and is present in micromolar concentrations in extracellular fluids, i.e., it is present at 10–100 μM in plasma and is further enriched to ca. 500 μM in lung lining fluid owing to the action of active transport systems [17]. There is considerable interest in whether SCN^- serves to prevent or to promote deleterious changes in tissue function caused by MPO during inflammatory conditions [17–20]. Previous studies identify that SCN^- can be oxidized by MPO Compound I in the presence of NO [21] and that peroxidase-catalyzed SCN^- oxidation can yield highly oxidizing free radicals [22–24]. These findings indicate that SCN^- has the potential to modulate the rate at which MPO and related peroxidases consume NO (i.e., by competitively inhibiting NO consumption by Compound I or by forming NO-consuming radicals); however, this has yet to be investigated.

Finally, the free thiol residues of albumin and glutathione (GSH) efficiently scavenge peroxidase-derived oxidants and radicals and are therefore likely to alter the availability of NO-consuming radicals generated during peroxidase turnover. However, the effects of thiols on peroxidase-catalyzed NO consumption are also unknown.

In this study we have applied a combination of experimental and theoretical approaches to resolve how peroxidase-catalyzed NO consumption is regulated in complex biological fluids. We establish that urate, thiols, ascorbate, and SCN^- all play important and previously undescribed roles in modulating the rate of NO consumption by MPO and related peroxidases under physiological conditions, with ascorbate and SCN^- exerting critical protective effects.

Experimental procedures

Materials

Peroxidase enzymes were obtained from Merck (MPO, from human neutrophils), Worthington (bovine LPO), and Sigma (horseradish peroxidase (HRP)). Superoxide dismutase (Cu/Zn SOD, from bovine erythrocytes) was from Sigma. H_2O_2 was purchased from Merck. The NO donor NOC-9 ($t_{1/2}$ 2.7 min at 22 °C) was purchased from Santa Cruz Biotechnology. Unless indicated otherwise, all other materials were purchased from Sigma–Aldrich and were of the highest purity available. The concentration of H_2O_2 was routinely determined spectrophotometrically (H_2O_2 ε_{240} = 43.6 M^{-1} cm^{-1}). All solutions were prepared using water filtered through a four-stage MilliQ system and Chelex-treated phosphate buffer (100 mM, pH 7.4). Working solutions of urate were prepared by dissolving urate (10 mM) in 40 mM sodium hydroxide and immediately diluting in phosphate buffer.

Isolation and fractionation of human plasma

Plasma was prepared from fresh heparinized blood donated by healthy consenting adult volunteers by centrifugation, immediately frozen, and stored at −80 °C. Plasma stocks were thawed immediately before use and used within 1 h. For removal of non-protein-bound low-molecular-weight species, plasma (1 ml) was eluted from PD10 size-exclusion columns with phosphate buffer (100 mM, pH 7.4) and the protein-containing fractions (2 ml) were pooled.

Electrochemical measurement of NO

Under "standard" conditions, the NO donor NOC-9 (prepared as a stock solution in 0.01 M sodium hydroxide) was added to 0.1 M phosphate buffer (pH 7.4, 22 °C, air-saturated) with rapid mixing to achieve the desired concentrations of NO at the time point at which reactions were initiated. H_2O_2 (10 μM) and heme proteins (10 nM MPO; 20 nM LPO; 200 nM HRP) were added sequentially (1.5 and 2 min after addition of NOC-9) in the presence and absence of other reaction components. NO was measured continuously with an NO-specific electrode (ISO-NOP) interfaced to an Apollo 4000 free radical analyzer (World Precision Instruments). For experiments examining the effects of O_2 depletion, NO measurements were made in a sealed reaction chamber (NOCHM-4) and small volumes of air-saturated stock solutions of the NO donor, H_2O_2, and MPO (2% of the final reaction volume in total, final O_2 concentration of ca. 4 μM) were delivered to N_2-purged solutions in the sealed chamber via microsyringes, with H_2O_2 and MPO being added 1.5 and 3 min after addition of NOC-9. The rate of NO consumption was measured 10 s after the addition of heme proteins, relative to control treatments in which the addition of heme proteins was omitted. In reactions with GSH, nitrosothiol formation was assessed by Cu^{2+}-stimulated NO release (2 mM $CuSO_4$) [25,26].

Electrochemical measurement of H_2O_2

The NO donor NOC-9 (0.5–10 μM) was added to 0.1 M phosphate buffer with rapid mixing (as above). H_2O_2 (25 μM) and MPO (10 nM MPO) were added sequentially (30 and 120 s after addition of NOC-9) in the presence and absence of other reaction components. H_2O_2 was measured continuously with a H_2O_2-specific electrode (ISO-HPO-2) interfaced to an Apollo 4000 free radical analyzer (World Precision Instruments). The rate of H_2O_2 consumption was measured 10 s after the addition of MPO, relative to control treatments in which the addition of MPO was omitted.

Measurement of nitrite

NOC-9-derived NO (ca. 10 μM NOC-9) was exposed to MPO and H_2O_2 under conditions identical to those employed in equivalent NO consumption experiments (i.e., 10 nM MPO and 10 μM H_2O_2, with catalysis initiated 2 min after the addition of the NO donor). Reactions (200 μl) were performed under aerobic conditions in sealed 1.5-ml tubes (to minimize the loss of NO into the