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Original Contribution

Myeloperoxidase acts as a source of free iron during steady-state catalysis by a feedback inhibitory pathway

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ABSTRACT

Myeloperoxidase (MPO) is a heme-containing enzyme that generates hypochlorous acid (HOCl) from chloride (Cl^-) and hydrogen peroxide (H_2O_2). It is implicated in the pathology of several chronic inflammatory conditions such as cardiovascular and pulmonary diseases and cancer. Recently we have shown that HOCl can destroy the heme prosthetic group of hemoproteins. Here, we investigated whether the HOCl formed during steady-state catalysis is able to destroy the MPO heme moiety and thereby function as a major source of free iron. UV–visible spectra and H_2O_2-specific electrode measurements recorded during steady-state HOCl synthesis by MPO showed that the degree of MPO heme destruction increased after multiple additions of H_2O_2 (10 μM), precluding the enzyme from functioning at maximum activity (80–90% inhibition). MPO heme destruction occurred only in the presence of Cl^-. Stopped-flow measurements revealed that the HOCl-mediated MPO heme destruction was complex and occurred through transient ferric species whose formation and decay kinetics indicated it participates in heme destruction along with subsequent free iron release. MPO heme depletion was confirmed by the buildup of free iron utilizing the ferrozine assay. Hypochlorous acid, once generated, first equilibrates in the solution as a whole before binding to the heme iron and initiating heme destruction. Eliminating HOCl from the MPO milieu by scavenging HOCl, destabilizing the MPO–Compound I–Cl complex that could be formed during catalysis, and/or inhibiting MPO catalytic activity partially or completely protects MPO from HOCl insults. Collectively, this study elucidates the bidirectional relationship between MPO and HOCl, which highlights the potential role of MPO as a source of free iron.

Myeloperoxidase (MPO) is a heme protein found in azurophilic granules of neutrophils and monocytes [1–3]. Myeloperoxidase uses hydrogen peroxide (H_2O_2) to catalyze the two-electron oxidation of chloride (Cl^-) to generate hypochlorous acid (HOCl) [3,4]. The catalytic cycle of MPO is depicted in Fig. 1. Myeloperoxidase in its ferric form, MPO–Fe(III), reacts with H_2O_2 to form a ferryl π cation radical, Compound I (MPO–Fe(IV)=$O^{\bullet+\pi}$) [3–5]. Compound I, in the presence of Cl^-, is converted back to MPO–Fe(III) with the concomitant two-electron oxidation of Cl^- to HOCl. Alternatively, in the absence of Cl^-, Compound I is converted back to MPO–Fe(III) through a two-step one-electron ($1e^-$) oxidation pathway involving organic or inorganic one-electron substrates such as melatonin and nitric oxide [6,7]. Compound II can execute only $1e^-$ oxidation reactions. Thus, formation of HOCl is not possible with this intermediate. Compound II is the catalytically inactive form of the enzyme and the long-lived intermediate in the cycle. In the presence of an excess of H_2O_2, Compound II is readily converted to Compound III (MPO–Fe(II)–O_2) [3–5]. Alternatively, superoxide or molecular oxygen interact with the MPO–Fe(III) and MPO–Fe(II) heme iron to generate Compound III [8,9]. Formation of Fe(II)–O_2 complexes through these routes is reversible, is relatively fast, and occurs via a one- or multiple-step mechanism [8,9]. The presence of superoxide dismutase completely inhibited Compound III formation, but the presence of catalase had no significant effect on this process [9].

Hypochlorous acid is a potent oxidant that functions as a powerful antimicrobial agent and is produced by phagocytic cells including neutrophils [1–3]. However, sustained high levels of HOCl have been implicated in the etiology of several pathological conditions, including cardiovascular diseases, diabetes mellitus, pulmonary fibrosis, and neurodegenerative conditions, as well as certain forms of cancer [1,10–12]. Under many pathological

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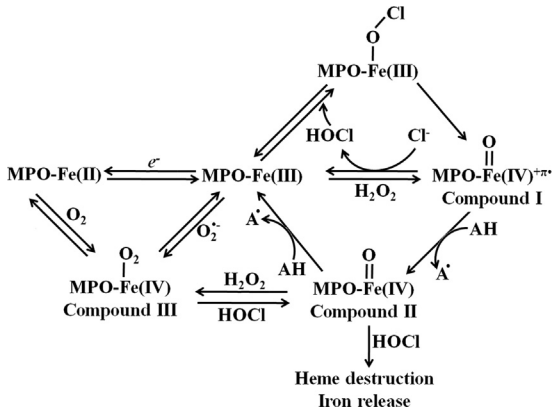

Fig. 1. Working kinetic model for HOCl-mediated modulation of MPO.

conditions, e.g., atherosclerosis, pulmonary fibrosis, endometriosis, and cancer, in which MPO is elevated, there have been reports of significant free iron accumulation [12–17]. In light of these data, there is considerable interest in finding the specific source and mechanism of generation of free iron. Our recent studies with purified hemoglobin in a cell-free system, and with isolated human red blood cells, elucidated the mechanistic link between high MPO/HOCl and elevated free iron [18–20]. Previous studies by Floris and Wever [21] and Furtmuller et al. [22] have shown that MPO–Fe(III) reacts with exogenous HOCl to generate Compound II through the formation of Compound I. It has also been shown that MPO Compound III reacts with HOCl generating Compound II [22]. Several groups have suggested the formation of an Fe–OCl complex upon mixing HOCl with hemoproteins (e.g., catalase, lactoperoxidase, MPO, and hemoglobin) [18–20,23,24] (Fig. 1). Our results showed that HOCl can oxidatively destroy the heme moiety of hemoproteins such as hemoglobin and lactoperoxidase, through a multistep mechanism, which is initiated by the oxidation of the heme iron to a ferryl form (akin to Compound I and Compound II) and then subsequent oxidative cleavage of the carbon methyne bridge of the tetrapyrrole moiety leading to heme fragmentation and release of free iron [18–20]. Additionally, HOCl also mediates the destruction of other metal-ion derivatives of tetrapyrrole macrocyclic rings, such as cyanocobalamin, the most common supplemental form of vitamin B_{12}, generating free cobalt and cyanogen chloride [25]. The toxicity of free transition metals is attributed to their capacity to generate highly reactive secondary free radicals such as the hydroxyl radical ($^{\bullet}OH$) through the Fenton reaction [26–28]. In addition, free iron could lead to increased bacterial growth and worsen risk for infection [29].

In this work, we investigated whether self-generated HOCl displays a feedback regulation of MPO catalytic activity through a mechanism that involves MPO heme destruction and subsequent free iron release. Our results show that HOCl downregulates MPO activity through a dual mechanism: (1) the transient formation of a MPO–Fe(III)–OCl complex, an inactive form of the enzyme, and (2) heme destruction and free iron release through the formation of Compound I and II. Collectively, this study may provide a direct mechanistic link between the elevated MPO and free iron observed in the biological systems under conditions of deficient HOCl scavengers.

Materials and methods

Materials

All the materials used were of the highest grade purity and used without further purification. Sodium hypochlorite (NaOCl), ammonium acetate (CH_3COONH_3), ferrozine, melatonin, L-methionine, taurine, and ascorbic acid were obtained from Sigma–Aldrich (St. Louis, MO, USA).

MPO purification

Myeloperoxidase was initially purified from detergent extracts of human leukocytes by sequential lectin affinity and gel-filtration chromatography [30–32]. Trace levels of contaminating eosinophil peroxidase were then removed by passage over a sulfopropyl Sephadex column [31]. Purity of isolated MPO was established by demonstrating a Reinheitzal value of >0.85 (A_{430}/A_{280}), SDS–PAGE analysis with Coomassie blue staining, and gel tetramethylbenzidine peroxidase staining to confirm no contaminating eosinophil peroxidase activity. Enzyme concentration was determined spectrophotometrically utilizing extinction coefficients of 89,000 M^{-1} cm^{-1}/heme of MPO [33]. The concentration of the MPO dimer was calculated as half the indicated concentration of heme-like chromophore [34].

H_2O_2-selective electrode measurements

Hydrogen peroxide consumption was measured using an H_2O_2-selective electrode (Apollo 4000 free radical analyzer; World Precision Instruments, Sarasota, FL, USA). Experiments were performed at 25 °C by immersing the electrode in 3 ml of 0.2 M sodium phosphate buffer, pH 7.4. H_2O_2 (10–20 μM) was added to a continuously stirred buffer solution containing fixed (40 nM, final) or various concentrations of MPO (1.25–40 nM, final) and/or Cl^- (100 mM) during which the change of H_2O_2 concentration was continuously monitored.

Absorbance measurements

The absorbance spectra were recorded using a Cary 100 Bio UV–visible spectrophotometer, at 25 °C, pH 7.0. Experiments were performed in 1 ml phosphate buffer solution supplemented with fixed amounts of MPO (1.0 μM) and Cl^- (100 mM) and increasing concentrations of H_2O_2 (0–200 μM), in the absence and presence of sodium thiocyanate (NaSCN), sodium nitrite ($NaNO_2$), or sodium bromide (NaBr) (100 μM). To study the effect of HOCl on MPO heme destruction, similar experiments were repeated in 1 ml phosphate buffer solution supplemented with a fixed amount of MPO (1.0 μM) and increasing concentrations of HOCl (0–200 μM). After 10-min incubation for reaction completion, methionine (fivefold the final HOCl concentration) was added to eliminate excess HOCl and absorbance changes were recorded from 300 to 700 nm.

Rapid kinetic measurements

The kinetic measurements of HOCl-mediated MPO heme destruction were performed using a dual-syringe stopped-flow instrument (Hi-Tech Ltd., Model SF-61). Measurements were carried out under an aerobic atmosphere at 10 °C after rapid mixing of equal volumes of a buffer solution containing a fixed amount of MPO (2.0 μM) and a buffer solution containing increasing concentrations of HOCl (0–200 μM). The time course of the absorbance change was fitted to a single-exponential ($y=1-e^{-kt}$) or a double-exponential ($y=Ae^{-k_1t}+Be^{-k_2t}$) function as indicated. Signal-to-noise ratios for all kinetic analyses were improved by averaging at least six to eight individual traces. In some experiments, the stopped-flow instrument was attached to a rapid-scanning diode array device (Hi-Tech) designed to collect multiple numbers of complete spectra (200–800 nm) at specific time ranges. The detector was automatically calibrated relative to a holmium oxide filter, as it has spectral peaks at 360.8, 418.5, 446.0,