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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.

Keywords

Hypochlorous acid; Myeloperoxidase; Feedback inhibition; Inflammation; Free iron; Oxidative stress; Free radicals

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 ($\text{MPO-Fe(IV)=O}^{+\pi}$) [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 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 ($\cdot\text{OH}$) 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 (CH₃COONH₃), 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 Reinheitszahl value of >0.85 (A_{430}/A_{280}), SDS–PAGE analysis with Coomassie blue staining, and gel tetra-methylbenzidine 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].

H₂O₂-selective electrode measurements

Hydrogen peroxide consumption was measured using an H₂O₂-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. H₂O₂ (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 H₂O₂ 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 H₂O₂ (0–200 μM), in the absence and presence of sodium thiocyanate (NaSCN), sodium nitrite (NaNO₂), 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, 453.4, 460.4, 536.4, and 637.5 nm, which were used by the software to correctly align pixel positions with wavelength.

pH measurements

The effect of pH on HOCl-mediated MPO heme destruction was carried out using 50 mM sodium acetate buffer (pH 5) and 50 mM phosphate buffer (pH 6 to 9).

Free iron analysis

Free iron release was measured colorimetrically using ferrozine, with some modifications [35]. To 100 μ l of the sample (MPO–HOCl reaction mixture) 100 μ l of ascorbic acid (100 mM) was added. After 5 min of incubation at room temperature, 50 μ l of ammonium acetate (16%) and the same volume of ferrozine (16 mM) were added to the mixture and mixed well. Subsequently, the reaction mixture was incubated for 5 min at room temperature and the absorbance was measured at 562 nm. A standard curve prepared using ammonium Fe(III) sulfate was used for the calculation of free iron concentration. Final concentrations of the additives were as follows: ascorbic acid, 33.33 μ M; ammonium acetate, 5.3%; and ferrozine, 5.3 μ M.

HOCl preparation

Hypochlorous acid was prepared as previously described, with some modifications [36]. Briefly, a stock solution of HOCl was prepared by adding 1 ml of NaOCl solution to 40 ml of 154 mM NaCl and the pH was adjusted to around 3 by adding HCl. The concentration of active total chlorine species in solution, expressed as $[\text{HOCl}]_T$ (where $[\text{HOCl}]_T = [\text{HOCl}] + [\text{Cl}_2] + [\text{Cl}_3^-] + [\text{OCl}^-]$) in 154 mM NaCl, was determined by converting all the active chlorine species to OCl^- by adding a bolus of 40 μ l of 5 M NaOH and measuring the concentration of OCl^- . The concentration of OCl^- was determined spectrophotometrically at 292 nm ($\epsilon = 362 \text{ M}^{-1} \text{ cm}^{-1}$). As HOCl is unstable, the stock solution was freshly prepared on a daily basis, stored on ice, and used within 1 h of preparation. For further experimentation, dilutions were made from the stock solution using 200 mM phosphate buffer, pH 7, to give working solutions of lower HOCl concentrations.

Results

MPO self-inactivates by HOCl generated during steady-state catalysis

The aim of these experiments was to understand how self-generated HOCl regulates MPO catalytic activity. Our initial experiment utilized H_2O_2 -selective electrodes to determine whether the accumulation of HOCl in the reaction mixture caused MPO feed-back inhibition. After addition of an aliquot of H_2O_2 to the continuously stirred reaction mixture (100 mM Cl^- final), the H_2O_2 signal rose rapidly, achieved a maximum after ~ 30 s, and decreased gradually as H_2O_2 was depleted by autoreduction ($2 \text{H}_2\text{O}_2 + \text{O}_2 \rightarrow 2 \text{H}_2\text{O}$) (Fig. 2, inset, dashed line). Subsequent addition of MPO (40 nM) to the reaction mixture caused a rapid decay in the level of H_2O_2 (Fig. 2, inset, solid line), indicating that MPO is catalytically active, similar to prior reports [37,38]. We next investigated how multiple rounds of HOCl production would affect MPO catalytic activity and H_2O_2 consumption, by reversing the order of H_2O_2 and MPO addition to the reaction mixture. H_2O_2 -selective electrode measurements revealed that addition of H_2O_2 (10 μ M, final) to a continuously

stirred buffer containing MPO (40 nM)/Cl⁻ (100 mM) solution led to an instant disappearance in the H₂O₂ signal due to its consumption as a substrate by the enzyme during steady-state catalysis (Fig. 2A; first trace). Incremental additions of the same amount of H₂O₂ to the reaction mixture inhibited MPO activity, as judged by the accumulation of H₂O₂ (indicated by the amplitude of the signal) and the decreased rate of H₂O₂ consumption (indicated by the duration of the signal) (Fig. 2A). This reduction in MPO activity required the buildup of sufficient amounts of HOCl that was generated by multiple turn-over of H₂O₂ consumption.

Next, we examined whether HOCl, once generated, first diffuses out from the active site and equilibrates in the solution as a whole, or if the process of inhibition occurs internally without HOCl equilibrating in the solution. Similar experiments were repeated in the presence of a saturated amount of HOCl scavenger (methionine). The rates of MPO-mediated H₂O₂ consumption in the presence of saturated amounts of methionine were remarkably fast, suggesting that HOCl equilibrates with the solution first before inhibiting MPO (Fig. 2B). Alternatively, methionine may destabilize the MPO–Compound I–Cl complex that could be formed during catalysis generating MPO–Fe(III) and chlorinated methionine [39]. Thus, eliminating HOCl from the MPO milieu by scavenging HOCl and/or inhibiting MPO catalytic activity partially or completely prevent MPO from HOCl insults.

To quantitate the extent to which the enzyme was inhibited by self-generated HOCl during steady-state catalysis, we derived estimated standards for H₂O₂ consumption as a function of decreasing amounts of MPO (10.0–1.25 nM). As depicted in Fig. 2C, addition of 10 μM H₂O₂ to a continuously stirred buffer solution supplemented with 100 mM Cl⁻ caused a rapid buildup in H₂O₂ signal and achieved a maximum after ~30 s. Addition of 10 nM MPO to the buffer solution supplemented with 100 mM Cl⁻, resulting in the production of HOCl, caused a rapid consumption of H₂O₂ as judged by the disappearance of H₂O₂ signal. Repeating the same experiment by adding decreased amounts of MPO caused a decrease in the H₂O₂ consumption rate. By comparing the slope in Fig. 2A with the estimated standards, the degree of MPO inhibition (after five rounds of H₂O₂ additions) was remarkably high and calculated to be ~10–20% of the total enzyme activity.

HOCl inhibits MPO through a mechanism that involves MPO heme destruction

We next utilized UV–Vis spectrophotometry to determine whether the catalytic inhibition of MPO was due to HOCl-mediated MPO heme destruction. Myeloperoxidase as isolated displays a Soret absorbance peak centered at 430 nm, indicative of the heme content of the enzyme. The effect of incremental additions of H₂O₂ (20 μM) in the presence of Cl⁻ (100 mM) on the MPO Soret peak was monitored spectrophotometrically (Fig. 3A). With each subsequent addition of H₂O₂, there was a proportional decrease in the MPO Soret peak, indicating that HOCl-mediated MPO feedback inhibition is associated with MPO heme destruction.

To confirm that the MPO heme destruction is due to the buildup of HOCl concentration in the MPO solution mixture, we investigated the effects of exogenous HOCl on MPO heme destruction. Exposure of a fixed amount of MPO (2 μM) to increasing concentrations of HOCl caused MPO heme destruction, as judged by the loss and flattening of the Soret peak region (Fig. 3B). Incubation of MPO with 50 μM HOCl caused ~50% decrease in the Soret band, whereas the addition of 200 μM HOCl led to a complete flattening of the Soret absorbance peak, indicating total heme destruction.

As MPO heme iron is also known to exist in the ferrous dioxy form (e.g., MPO–Fe(II)–O₂; Compound III), we extended these studies to MPO Compound III. As shown in Fig. 3C, addition of molar excess of H₂O₂ (140 μM) to MPO–Fe(III) in the absence of cosubstrate

(Cl⁻) caused immediate MPO Compound III formation, as judged by a shift in the Soret absorption peak from 430 to 450 nm and the appearance of additional absorbance peaks in the visible range at 628 nm, as previously reported [8]. MPO Compound III is relatively stable and required 15 min to decay to the ferric form at 25 °C. Addition of 140 μM HOCl to the MPO solution mixture led to an immediate decrease in the Soret absorbance region, confirming that MPO heme destruction is mediated by HOCl, but not by H₂O₂.

To confirm that methionine prevents HOCl-mediated MPO heme destruction, a fixed amount of MPO was preincubated with 400 μM methionine followed by subsequent addition of 20 μM increments of H₂O₂ to the reaction mixture (Fig. 3D). Under these circumstances, addition of H₂O₂ (up to 200 μM in increments of 20 μM) in the presence of Cl⁻ (100 mM) had no significant effect on the MPO absorbance spectra (Fig. 3D), suggesting that methionine prevented HOCl-mediated MPO heme destruction. To test the ability of methionine in preventing HOCl-mediated MPO heme degradation, 1.5–2 μM MPO was preincubated with increasing concentrations of methionine (0–100 μM) in 1 ml (final volume) phosphate buffer solution, pH 7.4, supplemented with 100 mM Cl⁻, and then the reaction mixtures received successive aliquots of H₂O₂ (20 μM increments). After each addition, the full absorbance spectrum (from 300 to 700 nm) of the reaction mixture was collected, and the percentage recovery in the MPO Soret absorbance peak was plotted as a function of H₂O₂ concentration. Fig. 3D, inset, shows the plot of percentage destruction (as judged by the loss in absorbance at 430 nm) as a function of H₂O₂ for various methionine concentrations. As shown in the inset, at all concentrations tested methionine offered some protection early on, but as HOCl started to accumulate (at higher concentrations of H₂O₂), it overcame the protective effect of methionine, and heme destruction was observed. Thus, it can be concluded that methionine prevented self-generated HOCl-mediated MPO inactivation by preventing MPO heme destruction.

The effect of 1e⁻ substrate on HOCl-mediated MPO heme destruction

We next determined if the presence of a 1e⁻ substrate (e.g., SCN⁻ and NO₂⁻) can compete with HOCl and prevent HOCl-mediated MPO damage. To address this question, we performed experiments utilizing UV–visible spectroscopy to determine whether the MPO/H₂O₂ system can use SCN⁻ as a 1e⁻ substrate and prevent MPO heme destruction mediated by self-generated HOCl during catalysis. The experiment was carried out by incremental additions of H₂O₂ (10 μM) to a MPO–Fe(III) (1.5 μM) solution supplemented with 100 mM Cl⁻ and 100 μM SCN⁻. Remarkably, subsequent additions of 10 μM H₂O₂ to the enzyme solution mixture caused an immediate decrease in the Soret absorbance region at 430 nm, with an appearance of a new Soret absorbance peak at 450 nm, indicating that SCN⁻ is utilized as a 1e⁻ substrate by MPO during catalysis. The generated Compound II was unstable and gradually returned to MPO–Fe(III) after all H₂O₂ was consumed by the enzyme. Similar results were obtained when SCN⁻ was replaced with NO₂⁻ (100 μM), whereas replacement with Br⁻ (100 μM) did not show any sign of protection against HOCl destruction. These results indicate that SCN⁻/NO₂⁻, but not Br⁻, inhibits HOCl production.

The effect of Cl⁻ concentration on HOCl⁻ mediated MPO heme destruction

Change in Cl⁻ concentration within these ranges (80–500 mM) has little or no effect on MPO feedback inhibition. Higher concentration of Cl⁻ serves as a ligand for MPO–Fe(III) leading to enzyme inhibition through the formation of a low-spin six-coordination MPO–Fe(III)–Cl complex. Higher levels of Cl⁻ (1–5 M) caused a shift to the right in the curve of the percentage of MPO heme destruction versus H₂O₂ concentration, indicating that Cl⁻ is bound to the MPO heme iron moiety, and less HOCl is generated in the enzyme milieu.

The interaction of HOCl with MPO using rapid kinetic measurements

We next utilized diode array stopped-flow spectrophotometry to continuously monitor and identify species that were formed upon mixing the MPO solution with phosphate buffer supplemented with increasing concentrations of exogenous HOCl. All HOCl concentrations employed were in larger molar excess to MPO to ensure pseudo-first-order conditions. Fig. 4 shows spectra collected over time for the reaction of MPO with HOCl (12.5–200 μM , final). As shown in Fig. 4, the starting spectrum demonstrated a characteristic Soret peak at 430 nm with absorbance shoulders at 500 and 631 nm, indicative of ferric heme. Mixing the enzyme solution with 12.5, 50, 100, or 200 μM HOCl caused an immediate decrease in the Soret absorbance region, which was attributed to the formation of MPO–Fe(III)–OCl intermediate. The MPO–Fe(III)–OCl complex displayed a distinct Soret absorbance peak at 434 nm, which could be easily differentiated from the Soret absorbance peaks of MPO–Fe(III) (430 nm) and MPO Compounds I (428 nm) and II (450 nm). The stability and accumulation of this complex depends mainly on the concentration of HOCl. At low HOCl concentrations (12.5–50 μM) MPO–Fe(III)–OCl is converted to MPO Compound II via the formation of Compound I, by an intramolecular electron rearrangement as reported earlier [20]. Compound I/II is unstable and the majority of the complex that was formed slowly decays back to MPO–Fe(III), over a period of ~ 5 s after initiating the reaction. At higher HOCl concentrations (>50 μM), however, the spectral transformations were distinctly different. Diode array analysis revealed the accumulation of MPO–Fe(III)–OCl complex, its conversion to Compound II, and the subsequent heme destruction. Collectively our results showed that the pivotal first step in the reaction between MPO–Fe(III) and HOCl is the formation of a MPO–Fe(III)–OCl complex, which is then converted to Compound II. Compound II can subsequently decay either to MPO–Fe(III) (at low HOCl concentration) or to the irreversible heme degradation pathway (at higher HOCl concentrations).

Single-wavelength stopped-flow measurements were performed to determine the critical concentration of HOCl beyond which HOCl switches its role from mediating destabilization of Compound I/II to MPO heme destruction. The increase or decrease in absorbance followed at 430 and 450 nm was fitted to a one-exponential function, and the observed rate constant of Compound I/II decay to either MPO–Fe(III) or heme destruction was plotted as a function of HOCl concentration. As shown in Fig. 5, the rate constant of Compound II decay decreased with increasing HOCl concentration, with an inflection point at ~ 68 μM HOCl to a slower rate. The inflection point shows the ability of MPO to tolerate HOCl accumulation up to a certain level after which MPO heme destruction occurs.

The effects of pH on HOCl-mediated MPO heme destruction

Changes in the pH (from 4.0 to 9.0) did not affect the HOCl feedback mechanism, as judged by UV–visible and H_2O_2 -electrode measurements (data not shown). This observation may indicate that the amount of HOCl generated by the remaining active enzyme in this pH range is sufficient to compete with and destroy the MPO heme prosthetic group through a feedback mechanism.

Feedback inhibition of MPO by self-generated HOCl is associated with free iron release

To test whether HOCl-mediated MPO feedback inhibition, through heme destruction, is associated with free iron release, MPO (1.2 μM) was preincubated with 100 mM Cl^- , followed by the addition of aliquots of H_2O_2 (in increments of 20 μM) to the reaction mixture. The free iron released was measured using ferrozine as detailed under Materials and methods, before and after the addition of 200 μM H_2O_2 . As shown in Fig. 6 no free iron was detected before the addition of H_2O_2 , whereas after the addition of H_2O_2 , in the presence of Cl^- , ~ 0.36 μM free iron was detected.

Discussion

Our results suggest a complex and interdependent relationship between levels of self-generated HOCl and MPO catalytic activity during steady-state catalysis. Self-generated HOCl regulates MPO catalytic activity by heme degradation and subsequent free iron release, a process that is attenuated by HOCl scavengers. Given the toxicity of free iron, this observation has a wide application in biological systems in which MPO is expressed.

Incremental addition of H₂O₂ to a catalytic amount of MPO resulted in a remarkably high degree of MPO inhibition (~80 to 90%), allowing the enzyme to function at only a fraction of its maximum activity. This decrease in MPO activity was shown to correlate well with the loss and flattening of the MPO Soret peak, as well as the buildup of free iron, suggesting heme destruction as the mechanism of MPO inactivation. In contrast this process did not occur under conditions where MPO began reducing H₂O₂ without generating HOCl (e.g., in the absence of Cl⁻, in the presence of a 1e⁻ substrate that destabilizes compound II, or in the presence of melatonin, a potent inhibitor of MPO [6,40–42]), indicating that self-generated HOCl, and not H₂O₂, is the cause of MPO inactivation. The fact that HOCl scavengers prevent this process indicates that HOCl first equilibrates in the solution after generation, before heme destruction. Our rapid kinetic measurements clearly showed that Compound II, the long-lived intermediate that is formed through the reaction of HOCl and MPO–Fe(III), is the prime target for HOCl-mediated heme destruction and subsequent free iron release. These findings display an important application in biological systems because enhancement in MPO expression accompanied by increased free iron levels is a characteristic feature of many inflammatory and cardiovascular diseases, as well as various forms of cancers [11,12].

In general, the balance between the presence of a 1e⁻ substrate (e.g., SCN⁻, NO₂⁻, NO, and melatonin) versus a 2e⁻ substrate (e.g., H₂O₂) in the MPO milieu is very important for MPO action and prevention of the damage caused by HOCl. These results are consistent with work by Tabboub et al. [32], and Burner et al. [40] showed that SCN⁻ and NO₂⁻ can serve not only as ligands of MPO–Fe(III), but also as 1e⁻ substrates of MPO Compounds I and II [32,40]. The preincubation of SCN⁻ with MPO affects the enzyme catalytic site, alters heme iron reactivity, and decreases its affinity toward H₂O₂. Rapid kinetic measurements indicated that upon mixing MPO preincubated with SCN⁻, Compound II is the predominant species formed, allowing the enzyme to operate at only a fraction of its maximum activity [32]. Nitrite is an excellent substrate for MPO Compound I but reacts slowly with Compound II. It is not that “bad” a substrate for MPO Compound II at low pH [40]. Nitrite oxidation occurs during the peroxidatic cycle of MPO in a two-1e⁻ oxidation and yields nitrogen dioxide. Nitrite not only acts as electron donor for Compounds I and II, but also forms a low-spin complex with the ferric enzyme [40]. Collectively, these findings suggest that SCN⁻/NO₂⁻, but not Br⁻, inhibited HOCl production by competing with Cl⁻ and switching the MPO catalytic activity from a 2e⁻ oxidation to a 1e⁻ oxidation pathway.

The pH profile of MPO activity shows a bell-shaped pattern, with optimum pH ranging from 5.5 to 7.5, and drops sharply up to ~30% at pH 4.0 and to ~10% at pH 9.0 [43]. This pH range (4.0–9.0) has little or no effect on the HOCl feedback mechanism, as judged by UV–visible and H₂O₂-electrode measurements. This observation may indicate that the amount of HOCl generated by the remaining active enzyme in this pH range is sufficient to compete with and destroy the MPO heme prosthetic group through a feedback mechanism. Therefore, we predict the same to hold true under physiological and pathological conditions (pH 5–8) where HOCl scavengers are lacking.

A kinetic model of how HOCl generated by MPO modulates the classical peroxidase cycle is shown in Fig. 1. In general, the rapid reaction between MPO and H₂O₂ forming

Compound I is considered the first step in the catalytic cycle. Compound I forms HOCl through a one-step two-electron oxidation of Cl^- and is reduced back to the ferric state. When HOCl is generated, it first diffuses out of the active site and equilibrates with the solution as a whole, and may influence the steady-state catalysis by two distinct mechanisms: (1) by converting both MPO–Fe(III) and Compound I to Compound II and (2) by acting as a heme ligand to form the inactive MPO–Fe(III)–OCl complex that can only rejoin the productive cycle through the dissociation of OCl^- from the complex. Therefore, HOCl may act as a negative feedback modulator of MPO. Although previous studies have focused on the ability of HOCl to interact with MPO–Fe(III), Compound I, and Compound III to form Compound II, neither the role of HOCl as a ligand for MPO nor its role as a potential mediator of MPO heme destruction has been explored. The direct reaction between HOCl versus MPO–Fe(III) or Compound I is fast and occurs with second-order rate constants ranging from 2×10^8 [44] to $2\text{--}5 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ [45]. The present studies clearly showed that self-generated HOCl may serve as a ligand for MPO, leading to catalytic inhibition and formation of a MPO–Fe(III)–OCl complex, which sets the stage for MPO heme destruction and free iron release.

At HOCl concentrations less than the dissociation constant for OCl^- ($<75 \mu\text{M}$), accumulation of MPO–Fe(III)–OCl was not observed; instead the accumulation of Compounds I/II and their decay to MPO–Fe(III) was seen. In contrast, at higher HOCl concentrations, a significant increase in the stability and rate of MPO–Fe(III)–OCl formation was observed, which correlated with the proportional increase in the duration of the reaction as determined by the time elapsed during steady-state catalysis. Under these circumstances, OCl^- predominantly serves as a ligand for MPO–Fe(III), generating its inactive MPO–Fe(III)–OCl complex (Fig. 1), whereas the rate of MPO–Fe(III)–OCl complex formation significantly exceeds its rate of conversion to Compound I. As the dissociation rate constant of MPO–Fe(III)–OCl is slower than the formation of Compound I, accumulation of Compound I was not seen; instead accumulation of Compound II was observed. When HOCl concentrations were increased, the transition of the pathway of Compound II exhaustion was reflected by the inflection in the decay rate, with a HOCl critical concentration of $68 \mu\text{M}$ (Fig. 5). Below this concentration, HOCl was capable of destabilizing Compound I/II, but does not lead to heme destruction. Thus, under these circumstances, MPO tolerates HOCl accumulation by acting as a catalytic sink for HOCl. Alternatively, when HOCl accumulates in the reaction milieu, the rate-limiting step becomes the dissociation of OCl^- from the MPO–Fe(III)–OCl complex (Fig. 1). Collectively, this indicates that the loss of MPO catalytic activity is due to the buildup of inactive intermediates, Compound II, and MPO–Fe(III)–OCl, and/or MPO heme destruction.

Loss of MPO catalytic activity was correlated with MPO heme destruction and was also associated with the buildup of free iron. Experiments that utilized methionine to scavenge HOCl showed that the magnitude of heme destruction directly depends on the HOCl concentration achieved in the reaction mixture. Thus, the presence of HOCl scavengers at sites of infection may play an essential role in maintaining the catalytic activity of MPO and limiting free iron accumulation that is attributed to HOCl-mediated feedback heme destruction. The bioavailability of HOCl and its ability to compete with H_2O_2 on the active site of MPO–Fe(III) are key features that drive the enzyme to alter its function from peroxidation to a source of free iron.

A theoretical detailed chemical mechanism describing the involvement of HOCl in tetrapyrrole macrocyclic compound destruction (e.g., corrin ring, heme, and porphyrin) has been recently shown [18,20,25] and could also apply to MPO heme destruction and subsequent free iron release. In these models, we proposed that, after the formation of Compound II, HOCl can oxidatively cleave the heme ring, leading to the generation of

pyrrole derivatives of different chain lengths. Upon cessation of HOCl synthesis due to heme destruction, H₂O₂ buildup and slow autoreduction was observed. Moreover we have shown how hemoproteins such as hemoglobin and lactoperoxidase, in addition to heme degradation, undergo protein aggregation when they react with HOCl [19,20]. Extensive studies by Chapman et al. [46] showed that HOCl can cause aggregation in apo-hemoglobin by the formation of noncovalent interactions caused by the generation of protein carbonyls. Additionally, prevention of protein carbonyl formation prevented HOCl-induced protein aggregation [47]. However, the role of self-generated HOCl in MPO protein aggregation is still under investigation. Therefore, events that are associated with elevated levels of the MPO–HOCl system and/or decreased bioavailability of HOCl scavengers such as taurine, methionine, glutathione, and lycopene may highlight a new pathway for the generation of free iron in diverse inflammatory diseases [6,11,12,37,48]. For example, MPO and free iron were not only elevated in ovarian cancer, but they also correlated with the stages of ovarian cancer [12]. Therefore, the authors suggested that the combination of these biomarkers can be used to provide an accurate method for early detection and prognosis of the disease. Previous studies have shown catalytically active MPO and iron to be present in human atherosclerotic lesions [49–52]. Our current and recently published results provide a potential link between enhanced MPO activity and free iron accumulation [18–20]. Thus, inhibiting MPO and/or eliminating its final products may play a beneficial role by reducing free iron release in a wide variety of inflammatory conditions. MPO can be inhibited by multiple pathways, including destabilization of MPO Compound II (e.g., melatonin and NO) [6,7]; heme reduction that causes collapse or narrowing of heme pocket geometry, preventing the access of the substrate to the catalytic site of the enzyme (e.g., ascorbate) [53]; switching the MPO catalytic cycle from peroxidation to catalase-like activity (e.g., melatonin, tryptophan, tryptophan analogs) [6,37,54]; or direct scavenging of HOCl (e.g., lycopene) [48].

MPO plays an essential role in bacterial killing by generating lethal amounts of HOCl within the neutrophil phagosome that forms under pathophysiological conditions [55]. Although the total amount of MPO in unstimulated neutrophils is shown to be $3.3 \pm 0.3 \mu\text{g}$ ($44 \pm 4 \text{ pmol}$)/ 10^6 cells, the amount of MPO generated from phagosomes is mainly dependent on the number of granules and has been reported to reach a concentration of 1–2 mM [55]. Therefore, at the site of infection, generation of large amounts of cytotoxic HOCl is expected. High concentrations of HOCl could affect bacterial growth by an iron sequestration mechanism through its ability to mediate hemoprotein heme destruction and subsequent iron release that leads the invaded host to withhold iron from bacteria. The generation of free iron under pathological conditions may protect the host from frequent bacterial infections by $\cdot\text{OH}$ generation through an H₂O₂-driven Fenton reaction [55–57]. The effects of iron release may be much more important inside the phagolysosome than outside it, where the iron-binding capacity is much higher (because of the large pool) and the levels of both MPO and H₂O₂ are lower. Extracellular ferric iron is insoluble under aerobic conditions and neutral pH and hence cannot be accessed by invading pathogens, greatly decreasing the virulence of some pathogens [55].

In addition, a substantial proportion of neutrophil MPO (12 pmol with 210 min of stimulation) is released during neutrophil extracellular trap (NET) formation, which corresponds to 30% of the total cellular content, with a further 5% free in the medium. Parker and Winterbourn [58] have measured the amount of MPO retained by the stimulated neutrophils and found that it accounted for most of the difference between total and released MPO, implying that little of the MPO was inactivated during NET release. Several other groups have attempted to estimate the amount of MPO present on extracellular bacteria. Britigan et al. [59] have shown that incubation of MPO with certain strains of bacteria (e.g., *Escherichia coli* and *Pseudomonas aeruginosa*) resulted in stable association of MPO with

the bacteria, which enhanced their susceptibility to killing by H_2O_2 . MPO activity ranged from 60 to 204 *o*-dianisidine U/ 10^{10} organisms (median=100 U/ 10^{10} organisms, $n=7$) for the *E. coli* and 6.7 to 53 *o*-dianisidine U/ 10^{10} organisms for the *P. aeruginosa* strains (median=13.2, $n=7$). It is also important to remember that released heme would be free for a very short time, as hemopexin (a plasma protein) rapidly binds heme and delivers it to cells via a hemopexin receptor [59].

Neutrophil cytoplasm contains around 80 mM Cl^- , with a variety of Cl^- channels that provide access to the phagosome [60]. Soluble agonists, as well as particle ingestion, can cause rapid Cl^- efflux into the surroundings through specific channels [55,60]. The outward pumping of cytoplasmic Cl^- by stimulated neutrophils may be essential for preserving enough phagosomal Cl^- levels for HOCl production [55,61]. Chloride is also necessary for azurophil degranulation, and this may be a means of limiting MPO release when Cl^- is exhausted [55,62]. The Cl^- concentration used in our study was chosen to equal that of the plasma levels (100 mM). Change in Cl^- concentrations within these ranges (80–500 mM) has no effect on MPO feedback inhibition. High concentration of Cl^- may also serve as a ligand for MPO–Fe(III) leading to the enzyme inhibition through the formation of a low-spin six-coordination MPO–Fe(III)–Cl complex. Thus, the rate-limiting step in MPO catalysis becomes the relatively slow dissociation of Cl^- from the complex [54].

Examination of the H_2O_2 concentration dependence for MPO heme destruction mediated by self-generated HOCl revealed that the curve shifted to the right. A shift to the right indicates that Cl^- is bound to the MPO heme iron moiety, and less HOCl is generated in the enzyme milieu.

In summary, MPO binding of self-generated HOCl partially destroys its catalytic site, which in this case is the heme moiety. This alone may be important in biologic settings in which MPO is expressed. The fact that HOCl-mediated heme destruction is largely dependent on the H_2O_2 in solution adds another level of complexity because the cellular environment can control the extent of HOCl regulation of MPO. Thus, any changes that alter cellular HOCl scavengers or exposure to HOCl scavengers such as melatonin, taurine, glutathione, or methionine will potentially affect HOCl levels and, in turn, alter HOCl-mediated MPO heme destruction and subsequent free iron release. Hypochlorous acid-mediated changes in MPO function are irreversible and dynamic and occur in concert with changes in environmental H_2O_2 and HOCl concentrations.

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

Fig. 2.

MPO inactivation by self-generated HOCl and its prevention by methionine. (A) A typical recording by an H_2O_2 -selective electrode demonstrating the dramatic MPO feedback inhibition mediated by self-generated HOCl after addition of equal amounts of H_2O_2 (10 μM , 1–2 μl in 3 ml reaction mixture) five consecutive times (denoted by the arrows) to a continuously stirred phosphate buffer (200 mM, pH 7.4) containing 40 nM MPO and 100 mM Cl^- , at 25°C. The inset shows a typical recording by the H_2O_2 -selective electrode. The dashed line represents the auto-reduction of 10 μM H_2O_2 after addition to a stirred phosphate buffer, containing 100 mM Cl^- . Where indicated by the arrow, addition of a catalytic amount of MPO (40 nM) caused immediate consumption of H_2O_2 , indicating that the enzyme was catalytically active. (B) An identical experiment performed in the presence of HOCl scavenger (200 μM methionine). (C) The effect of MPO dilution on H_2O_2 consumption. H_2O_2 -selective electrode recording for the reactions of H_2O_2 consumption when various amounts of MPO (10, 5, 2.5, and 1.25 nM) were added at the time indicated by the arrows to a continuously stirred buffer solution containing identical amounts of H_2O_2 (10 μM) and 100 mM Cl^- . The data shown are representative of three independent experiments.

Fig. 3.

MPO heme destruction by self-generated HOCl and its prevention by methionine. MPO (2 μM) was first incubated with Cl^- (100 mM) and then up to 200 μM H_2O_2 was added in increments of 20 μM (2 μl) to the reaction mixture. After each H_2O_2 addition, the reaction mixture was left 5 min for reaction completion and absorbance spectra were recorded from 300 to 700 nm. (B) MPO (2 μM) was incubated with various HOCl concentrations (0–200 μM), for 1 h (for reaction completion), and excess HOCl was scavenged with methionine. Absorbance spectra were recorded from 300 to 700 nm. (C) Absorbance spectra of MPO (2 μM) before and after the addition of 140 μM H_2O_2 , to form MPO Compound III, and after bolus addition of HOCl (140 μM) to the solution mixture. (D) MPO (1.5–2.0 μM) was preincubated with Cl^- (100 mM) and methionine (400 μM), followed by sequential addition H_2O_2 (200 μM , in 20 μM increments) to the reaction mixture. Five minutes (for reaction completion) after each addition of H_2O_2 , absorbance spectra were collected from 300 to 700 nm. The spectrum labeled “Meth+ H_2O_2 ” indicates the spectrum collected after the last addition of H_2O_2 . The insets show the percentage of MPO heme destruction under the conditions where a fixed amount of MPO (1.0 μM) was first incubated with increasing concentrations of methionine (0, 25, 50, 70, and 100 μM) and the reaction mixture was then given increasing concentrations of H_2O_2 . The data are representative of three experiments.

Fig. 4.

Formation, duration, and decay of the transient's intermediates that formed through the reaction between MPO and HOCl. The diode array stopped-flow spectra for the MPO intermediates that are formed and their degradation by reacting MPO-Fe(III) (1.7 μM final; characterized by a Soret absorption peak centered at 430 nm) with various concentrations of HOCl (12.5, 50, 100, 200 μM final) at 10 $^{\circ}\text{C}$ are shown. The time of collected spectral traces after initiation of the reaction is indicated in seconds. Arrows indicate the direction of spectral change over time as each intermediate advanced to the next. The experiments shown are representative of three experiments.

Fig. 5.

Plot of the observed rate of Compound II exhaustion as a function of HOCl concentration. A buffer solution supplemented with 1.2 μM MPO was rapidly mixed with an equal volume of phosphate buffer (200 mM, pH 7.4) supplemented with varying concentrations of HOCl, at 10 °C. The formation of Compound II and its destruction were monitored at 430 and 450 nm. The data are the averages of at least six independent experiments. The standard error for each individual rate constant was estimated to be less than 5%.

Fig. 6.

H₂O₂/Cl⁻-mediated MPO inactivation is correlated with free iron release. The release of free iron in the MPO/H₂O₂/Cl⁻ reaction mixture as measured by the Ferrozine assay is shown. The data are the averages of three independent experiments with the error bars representing the standard error of measurement.