



# Catalase prevents myeloperoxidase self-destruction in response to oxidative stress

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## ABSTRACT

Catalase (CAT) and myeloperoxidase (MPO) are heme-containing enzymes that have attracted attention for their role in the etiology of numerous respiratory disorders such as cystic fibrosis, bronchial asthma, and acute hypoxemic respiratory failure. However, information regarding the interrelationship and competition between the two enzymes, free iron accumulation, and decreased levels of non-enzymatic antioxidants at sites of inflammation is still lacking. Myeloperoxidase catalyzes the generation of hypochlorous acid (HOCl) from the reaction of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and chloride (Cl<sup>-</sup>). Self-generated HOCl has recently been proposed to auto-inhibit MPO through a mechanism that involves MPO heme destruction. Here, we investigate the interplay of MPO, HOCl, and CAT during catalysis, and explore the crucial role of MPO inhibitors and HOCl scavengers in protecting the catalytic site from protein modification of both enzymes against oxidative damage mediated by HOCl. We showed that CAT not only competes with MPO for H<sub>2</sub>O<sub>2</sub> but also scavenges HOCl. The protective role provided by CAT versus the damaging effect provided by HOCl depends in part on the ratio between MPO/CAT and the affinity of the enzymes towards H<sub>2</sub>O<sub>2</sub> versus HOCl. The severity of such damaging effects mainly depends on the ratio of HOCl to enzyme heme content. In addition to its effect in mediating protein modification and aggregation, HOCl oxidatively destroys the catalytic sites of the enzymes, which contain porphyrin rings and iron. Thus, modulation of MPO/CAT activities may be a fundamental feature of catalysis, and functions to down-regulate HOCl synthesis and prevent hemoprotein heme destruction and/or protein modification.

## 1. Introduction

Catalase (CAT) and myeloperoxidase (MPO) are hemoproteins that have recently gained increasing attention for their potential role in the etiology of respiratory conditions related to oxidative stress [1–3]. Interestingly, many inflammatory disorders such as cystic fibrosis (CF), bronchial asthma, and acute hypoxemic respiratory failure, in which MPO/CAT are known to be elevated, and are also associated with significant free iron accumulation [4–9]. It has been postulated that variations in the levels or function of these two enzymes could modulate respiratory illness risk, especially under conditions of oxidative stress [1,2]. However, a detailed understanding of the interrelationship and competition between the two enzymes at sites of inflammation is still lacking. Catalase is an anti-inflammatory enzyme that predominantly competes with MPO by converting hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) to H<sub>2</sub>O

and oxygen [10]. Myeloperoxidase, the most abundant protein in neutrophils and monocytes, catalyzes the reaction between chloride (Cl<sup>-</sup>) and H<sub>2</sub>O<sub>2</sub> to form hypochlorous acid (HOCl) [11–13]. Hypochlorous acid is a powerful oxidant, and is extremely reactive with the heme moiety of MPO and other hemoprotein model compounds causing heme damage and free iron release [14–17]. Hypochlorous acid when released from the MPO cavity, first diffuses in the solution as a whole, binds to MPO heme iron forming MPO-Fe(III)-OCl complex, and then mediates MPO heme destruction, precluding the enzyme from functioning at the maximum activity (80–90% inhibition) [14].

Accordingly, inhibition of MPO-chlorinating activity and/or factors that influence rates of HOCl removal following its synthesis by MPO are of significant interest. In the biological system, a major pathway for HOCl removal is through its interactions with antioxidant compounds such as thiols (glutathione (GSH), taurine, and methionine), carotenoid

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(lycopene), derivatives of tetrapyrrole macrocyclic rings (heme and vitamin B<sub>12</sub>), and indole compounds (melatonin and tryptophan) [18–24]. These molecules compete with Cl<sup>-</sup>, the natural substrate of MPO, by serving as a one electron substrate of MPO Compounds I (ferryl porphyrin  $\pi$  cation radical, Fe(IV) = O(+ $\pi^*$ )) and Compound II (ferryl complex; Fe(IV) = O), and/or directly scavenging HOCl [18–24].

Several respiratory diseases with underlying inflammation have been linked to abnormal MPO to CAT ratios, enhanced iron levels, and decreased levels of antioxidants [1,2,9,25–27]. Increased MPO activity combined with decreased CAT activity creates an environment of increased reactive oxygen species (ROS) that are implicated in various cancers and in chronic respiratory inflammation [28]. Synthetic compounds with superoxide dismutase (SOD) and CAT activities have shown promising results in animal models against a variety of oxidant exposures including cigarette smoke in the lung [27].

In this work, we utilized H<sub>2</sub>O<sub>2</sub>-selective electrode analysis and direct spectroscopic measurements to determine the interdependence between CAT and MPO, and the role of HOCl in down-regulating the catalytic activity of both enzymes. Our recently published [14,16,17] and the current work highlight the link between free iron accumulation, decreased levels of antioxidant, and the catalytic activity of both enzymes.

## 2. Materials and methods

### 2.1. Materials

All the materials used were of the highest-grade purity and used without further purification. Catalase powder from bovine liver, sodium hypochlorite (NaOCl), melatonin, L-methionine, and taurine were obtained from Sigma–Aldrich (St. Louis, MO, USA).

### 2.2. MPO purification

Myeloperoxidase was initially purified from detergent extracts of human leukocytes by sequential lectin affinity and gel-filtration chromatography, as previously reported [29–31]. Trace levels of contaminating eosinophil peroxidase were then removed by passage over a sulfopropyl Sephadex column [30]. Purity of isolated MPO was established by demonstrating a Reinheitszahl (RZ) value of > 0.87 ( $A_{430}/A_{280}$ ), 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<sup>-1</sup> cm<sup>-1</sup>/heme of MPO at 430 nm [32]. Catalase, from the manufactures, was used without further purification. Catalase concentration was determined spectrophotometrically using extinction coefficients of 120,000 M<sup>-1</sup> cm<sup>-1</sup>/heme at 406 nm [33].

### 2.3. H<sub>2</sub>O<sub>2</sub>-selective electrode measurements

Hydrogen peroxide consumption was measured using an H<sub>2</sub>O<sub>2</sub>-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. Hydrogen peroxide (10  $\mu$ M) was added to a continuously stirred buffer solution containing fixed amount of MPO (40 nM, final) and various concentrations of CAT (40–160 nM), or fixed amount of CAT (40 nM final) and various concentrations of MPO (40–160 nM, final), and/or Cl<sup>-</sup> (100 mM) during which the change of H<sub>2</sub>O<sub>2</sub> concentration was continuously monitored.

### 2.4. Absorbance measurements

The absorbance spectra were recorded using a Cary 100 Bio

UV–visible photometer, at 25 °C, pH 7.4. Experiments were performed in 1 mL phosphate buffer solution supplemented with fixed amounts of MPO (~0.6  $\mu$ M) and Cl<sup>-</sup> (100 mM) and increasing concentrations of H<sub>2</sub>O<sub>2</sub> (0–200  $\mu$ M), in the absence and presence of different ratios of CAT relative to MPO and/or various antioxidants (100  $\mu$ M methionine, taurine, or melatonin).

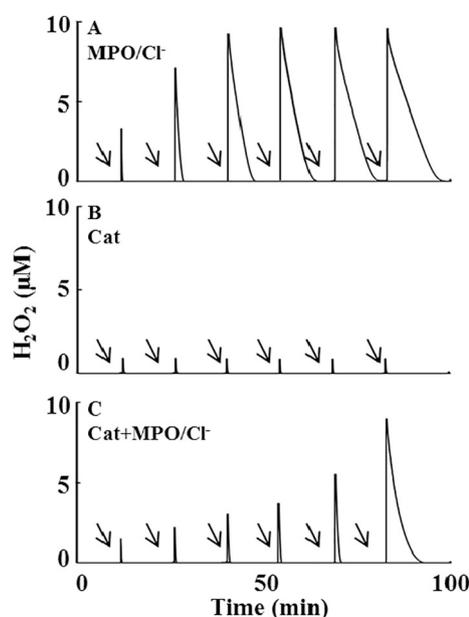
### 2.5. HOCl preparation

Hypochlorous acid was prepared as previously described, with some modifications [34]. 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 hydrochloric acid. The concentration of active total chlorine species in solution, expressed as [HOCl]<sub>T</sub> (where [HOCl]<sub>T</sub> = [HOCl] + [Cl<sub>2</sub>] + [Cl<sub>3</sub><sup>-</sup>] + [OCl<sup>-</sup>]) in 154 mM NaCl, was determined by converting all the active chlorine species to OCl<sup>-</sup> by adding a bolus of 40  $\mu$ L of 5 M NaOH and measuring the concentration of OCl<sup>-</sup>. The concentration of OCl<sup>-</sup> was determined spectrophotometrically at 292 nm ( $\epsilon = 362 \text{ M}^{-1} \text{ cm}^{-1}$ ) [35]. As HOCl is unstable, the stock solution was freshly prepared on a daily basis, stored on ice, and used within 1 h of preparation.

## 3. Results

### 3.1. Influence of HOCl generation on MPO/CAT catalytic functions

We first investigated if multiple rounds of H<sub>2</sub>O<sub>2</sub> addition to the MPO/Cl<sup>-</sup> system could affect MPO performance and if the generated HOCl would affect the MPO peroxidase activity. Fig. 1, depicts an experiment in which six cycles of MPO activity were promoted by repeatedly adding a limiting amount of H<sub>2</sub>O<sub>2</sub> (10  $\mu$ M) to trigger transient HOCl synthesis in a single sample of MPO. The amount of H<sub>2</sub>O<sub>2</sub> accumulated in the solution mixture (amplitude H<sub>2</sub>O<sub>2</sub> signal) and the time of its consumption (duration of the signal) were continually measured



**Fig. 1.** MPO inactivation by self-generated HOCl and its prevention by CAT. (A) A typical recording by an H<sub>2</sub>O<sub>2</sub>-selective electrode demonstrating the dramatic MPO feedback inhibition mediated by self-generated HOCl after incremental consecutive addition of H<sub>2</sub>O<sub>2</sub> (10  $\mu$ M, 3  $\mu$ L in 3 mL reaction mixture) (denoted by the arrows) to a continuously stirred phosphate buffer (200 mM, pH 7.4) containing 40 nM MPO and 100 mM Cl<sup>-</sup>, at 25 °C. Identical experiment performed (B) By replacing MPO/Cl<sup>-</sup> with 40 nM CAT. (C) In the presence of combination of 40 nM MPO, 40 nM CAT and 100 mM Cl<sup>-</sup>. The data shown are representative of three independent experiments.

after the addition of the  $\text{H}_2\text{O}_2$  solution to a continuously stirred buffer containing MPO (40 nM)/ $\text{Cl}^-$  (100 mM). The first  $\text{H}_2\text{O}_2$  addition to the enzyme solution led to an instant disappearance in the  $\text{H}_2\text{O}_2$  signal due to its consumption as a substrate during catalysis, indicating that MPO is catalytically active, similar to prior reports [14]. Sequential additions of the same amount of  $\text{H}_2\text{O}_2$  to the reaction mixture inhibited MPO activity, as judged by the accumulation of  $\text{H}_2\text{O}_2$  (indicated by the amplitude of the signal) from the decreased rate of  $\text{H}_2\text{O}_2$  consumption (indicated by the duration of the signal) (Fig. 1A). This reduction in MPO activity required the buildup of sufficient amounts of HOCl generated by multiple rounds of  $\text{H}_2\text{O}_2$  consumption. We also utilized the  $\text{H}_2\text{O}_2$ -selective electrode to show that CAT alone can rapidly consume  $\text{H}_2\text{O}_2$ . As shown in Fig. 1B, the first  $\text{H}_2\text{O}_2$  (10  $\mu\text{M}$ ) addition to the enzyme solution led to an instant disappearance of  $\text{H}_2\text{O}_2$  signal due to its consumption as a substrate by the enzyme during steady-state catalysis, indicating that CAT is catalytically active. However, sequential addition of the same amount of  $\text{H}_2\text{O}_2$  to the reaction mixture demonstrated a similar instant consumption of  $\text{H}_2\text{O}_2$  (Fig. 1B).

To determine whether CAT possesses the ability to compete with MPO on  $\text{H}_2\text{O}_2$  and prevent HOCl-mediated heme destruction of both enzymes, identical experiments were repeated in the presence of both MPO and CAT. Experiments were carried out by either employing a fixed amount of MPO (40 nM) and varying concentrations of CAT (40, 80, 120, 160 nM) (Fig. 2A) or a fixed amount of CAT (40 nM) and varying concentrations of MPO (40, 80, 120, 160 nM) (Fig. 2B). In each MPO/CAT cycle,  $\text{H}_2\text{O}_2$ , the common substrate of both enzymes, was the limiting factor and would be reduced completely after each addition to the solution mixture by both enzymes. Remarkably, addition of  $\text{H}_2\text{O}_2$  to a continuously stirred reaction mixture supplemented with 40 nM MPO, 100 mM  $\text{Cl}^-$ , and 40 nM CAT, demonstrated a modest brief increase in  $\text{H}_2\text{O}_2$  signal, indicating rapid  $\text{H}_2\text{O}_2$  disappearance (Fig. 1C). However, after several additions of  $\text{H}_2\text{O}_2$  to the solution mixture the  $\text{H}_2\text{O}_2$  signal rose rapidly, achieved its maximum level after  $\sim 30$  s, and then gradually returned to baseline when all the  $\text{H}_2\text{O}_2$  was consumed (Fig. 1C).

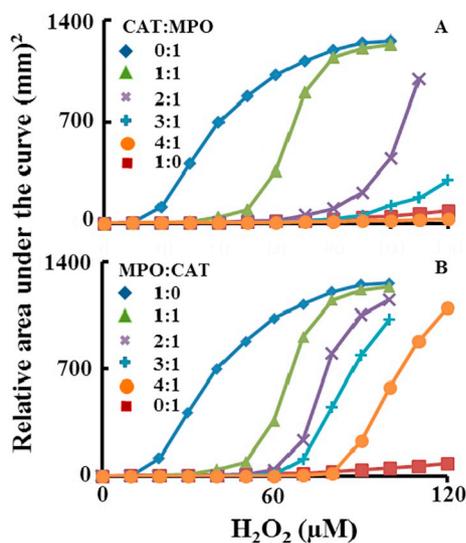


Fig. 2. Area under the curves of the  $\text{H}_2\text{O}_2$  selective-electrode traces reflecting the maximum signal intensity to time of  $\text{H}_2\text{O}_2$  consumption by both MPO and CAT, were determined and plotted as a function of  $\text{H}_2\text{O}_2$  added to the same solution mixture. A) When an incremental  $\text{H}_2\text{O}_2$  solution (10  $\mu\text{M}$ , 3  $\mu\text{L}$ ) was added to a continuously stirred phosphate buffer (200 mM, pH 7.4) supplemented with fixed amount of MPO (40 nM) and increasing concentration of CAT (0, 40, 50, 120, and 160 nM) in the presence of fixed amount of  $\text{Cl}^-$  (100 mM), at 25  $^\circ\text{C}$ . B) When an incremental  $\text{H}_2\text{O}_2$  solution (10  $\mu\text{M}$ , 3  $\mu\text{L}$ ) was added to a continuously stirred phosphate buffer (200 mM, pH 7.4) supplemented with fixed amount of CAT (40 nM) and increasing concentration of MPO (0, 40, 50, 120, and 160 nM) in the presence of fixed amount of  $\text{Cl}^-$  (100 mM), at 25  $^\circ\text{C}$ . The data shown are representative of three independent experiments.

Thus, the accumulation of free  $\text{H}_2\text{O}_2$  in MPO/CAT milieu requires more addition of  $\text{H}_2\text{O}_2$  compared to the MPO alone (Fig. 1B). Area-under-the-curves (AUC), which reflect the maximum signal intensity and the duration of  $\text{H}_2\text{O}_2$  consumption by both enzymes, were determined and plotted as a function of  $\text{H}_2\text{O}_2$  concentration. In both cases, no significant increase in the AUC was observed when incremental addition of  $\text{H}_2\text{O}_2$  was added to CAT alone (Fig. 2 A&B). MPO was not immediately inhibited by self-generated HOCl and remained active until a sufficient amount of HOCl was built-up in the solution mixture (Fig. 2A &B). When a 1:1 ratio of MPO:CAT was used, this condition caused a right shift in the curve compared to MPO alone. Increasing CAT concentrations caused a further shift in the curves to the right (Fig. 2A). A similar trend was also observed by increasing the MPO to CAT ratio, but to a lesser degree (Fig. 2B). Another factor that caused the MPO inactivation curve to shift to the right in addition to MPO:CAT ratio, is the presence of an HOCl scavenger.

### 3.2. Effect of MPO inhibitor and/or HOCl scavengers

Melatonin, methionine, and taurine react with HOCl at rates from  $3.4 \times 10^7$  to about  $10^4 \text{ M}^{-1} \text{ s}^{-1}$  and have been used to scavenge HOCl or inhibit MPO chlorinating activity and protect MPO from inhibition and destruction by HOCl [36–38]. We therefore examined if these would protect MPO/CAT from HOCl in our system. Fig. 3B shows the effect of melatonin (100  $\mu\text{M}$ ) on MPO and CAT activities during HOCl synthesis. In this experiment, 10  $\mu\text{M}$  (3  $\mu\text{L}$ ) of  $\text{H}_2\text{O}_2$  was added six consecutive times (at the arrow indicators in Fig. 3) to initiate six rounds of MPO/CAT catalysis at 25  $^\circ\text{C}$ . Hydrogen peroxide accumulation and its decay were monitored as an increase and decrease in  $\text{H}_2\text{O}_2$  signal over time. The amount of  $\text{H}_2\text{O}_2$  accumulated in the solution mixture (amplitude of the  $\text{H}_2\text{O}_2$  signals) and the time of its exhaustion reaction (duration of  $\text{H}_2\text{O}_2$  signal) were nearly identical within experimental error for all six trials. The data showed that consumption of  $\text{H}_2\text{O}_2$  can occur at multiple times without slowing the rate of  $\text{H}_2\text{O}_2$  consumption by MPO and CAT. Panel 3A is the control reaction, in which both MPO/ $\text{Cl}^-$  (40 nM/100 mM) and CAT (40 nM) were added to the reaction at time zero to initiate concurrent  $\text{H}_2\text{O}_2$  consumption as  $\text{H}_2\text{O}_2$  is added to the reaction (at the arrow indicators in Fig. 3) and subsequent HOCl synthesis. In this experiment (Fig. 3B), melatonin is a  $1e^-$  substrate of MPO that competes with  $\text{Cl}^-$ , and this shifts the MPO

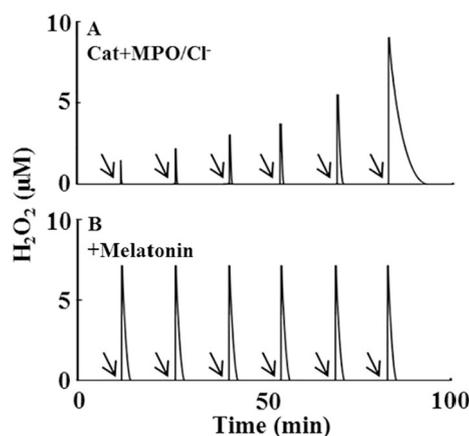


Fig. 3. Melatonin inhibits the MPO chlorinating activity and prevents MPO/CAT inactivation mediated by HOCl. (A) A typical recording by an  $\text{H}_2\text{O}_2$ -selective electrode demonstrating the dramatic effect of CAT in protecting MPO from HOCl insults after incremental consecutive addition of equal amounts of  $\text{H}_2\text{O}_2$  (10  $\mu\text{M}$ , 3  $\mu\text{L}$  in 3 mL reaction mixture) (denoted by the arrows) to a continuously stirred phosphate buffer (200 mM, pH 7.4) containing 40 nM MPO, 40 nM CAT, and 100 mM  $\text{Cl}^-$ , at 25  $^\circ\text{C}$ . (B) An identical experiment performed in the presence of 100  $\mu\text{M}$  melatonin. The data shown are representative of three independent experiments.

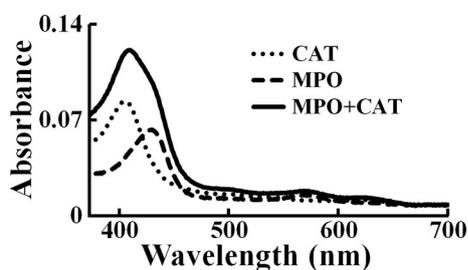


Fig. 4. Absorbance spectra of MPO (0.6  $\mu\text{M}$ ) (dashed line), CAT (0.6  $\mu\text{M}$ ) (dotted line), and combination of both (solid line) recorded in 200 mM phosphate buffer, (pH 7.4) from 350 to 700 nm, at 25  $^{\circ}\text{C}$ . The data shown are representative of three independent experiments.

catalysis paradigm from a  $2e^{-}$  to a  $1e^{-}$  oxidation pathway [21]. As such, MPO consumed  $\text{H}_2\text{O}_2$  more slowly without producing HOCl, and the  $\text{H}_2\text{O}_2$  signal in the presence of melatonin correspondingly had much higher amplitude and lower decay rate relative to the control. Similar results have been observed for methionine and taurine (data not shown). Thus, these compounds, such as melatonin, methionine, and taurine, could also prevent HOCl-mediated heme destruction of both enzymes.

### 3.3. Effect of HOCl generation on MPO and CAT heme during steady-state catalysis

Our next experiments utilized light absorbance spectroscopy to examine if a significant amount of the MPO and CAT heme contents would be destroyed during catalysis of HOCl synthesis from MPO- $\text{Cl}^{-}$ - $\text{H}_2\text{O}_2$  system. Fig. 4 contains UV-Visible spectra of MPO alone ( $\sim 0.6 \mu\text{M}$ ) (dashed line), CAT alone ( $\sim 0.6 \mu\text{M}$ ) (dotted line), and MPO and CAT in combination (solid line). The starting MPO spectrum demonstrated a characteristic Soret peak at 430 nm with absorbance shoulders at 500 and 631 nm, indicative of ferric heme, whereas the absorption spectrum of the purified CAT exhibited a Soret peak at 406 nm and an additional minor peak at 610 nm. We next utilized the absorbance spectra to monitor MPO/CAT heme destruction during HOCl synthesis to see how HOCl levels correlate with MPO/CAT heme destruction. Enzyme reactions reported here were run at 25  $^{\circ}\text{C}$  in 1 mL cuvettes, under conditions in which  $\text{H}_2\text{O}_2$  was the limiting substrate and would be consumed completely by MPO/CAT after several minutes of reaction. For clarity, a cuvette containing phosphate buffer supplemented with CAT ( $\sim 0.6 \mu\text{M}$ ) and 100 mM  $\text{Cl}^{-}$  was mixed well and used to make the baseline of the absorbance spectra, from 300 to 500 nm, zero (Fig. 5 dotted line). The dashed line is the prominent Soret absorbance centered at 430 nm after addition of MPO ( $\sim 0.6 \mu\text{M}$ ) to the solution mixture (Fig. 5). Sequential incremental addition of 20  $\mu\text{M}$   $\text{H}_2\text{O}_2$  (3  $\mu\text{L}$ ) to the solution mixture caused MPO and CAT heme destruction in a dose dependent manner, as judged by the loss of visible absorbance at 430 nm (MPO-Soret peak) and 406 nm (CAT-Soret peak) (Fig. 5A, B). Spectra were recorded immediately after the addition of  $\text{H}_2\text{O}_2$  (Fig. 5A) as well as 10 min. After the addition of  $\text{H}_2\text{O}_2$  (Fig. 5B) to demonstrate the slight accumulation of MPO Compound II as the stable form of MPO. To examine the potential physiological significance of these observations, we also determined the effect of HOCl scavengers on HOCl-mediated heme destruction utilizing similar methods. Fig. 5C shows the same experiment in the presence of methionine (100  $\mu\text{M}$ ), an MPO inhibitor and HOCl scavenger, which showed no alteration of MPO or CAT spectra, indicating that methionine completely prevented MPO/CAT heme destruction and/or modification. When similar experiments were performed with a MPO:CAT ratio of 5:1, less  $\text{H}_2\text{O}_2$  was needed to achieve an increased degree of heme alteration, and at the conclusion of the experiment the MPO heme was completely destroyed, as indicated by flattening of the MPO Soret absorption peak at 430 nm (data not shown). Additionally, the buildup of the absorbance shoulder

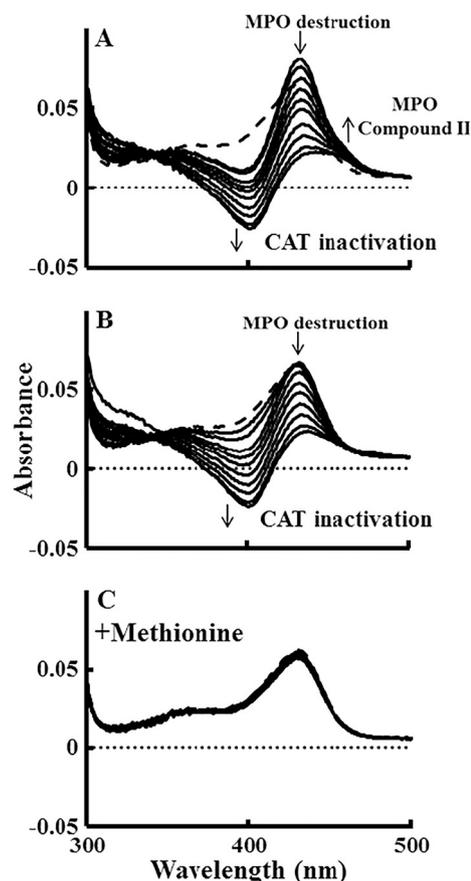
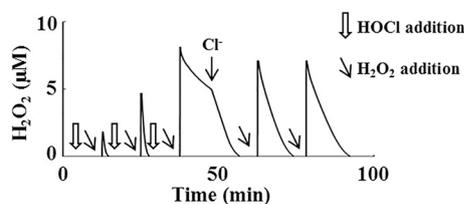


Fig. 5. Effects of methionine in inhibiting MPO/CAT heme destruction mediated by MPO-generated HOCl. A cuvette containing phosphate buffer supplemented with CAT ( $\sim 0.6 \mu\text{M}$ ) and 100 mM  $\text{Cl}^{-}$  was mixed well and used to zero the absorbance reading from 300 to 500 nm (dotted line), at 25  $^{\circ}\text{C}$ . The dashed line is the prominent Soret absorbance centered at 430 nm after adding of MPO ( $\sim 0.6 \mu\text{M}$ ) to the solution mixture, followed by sequential incremental addition of  $\text{H}_2\text{O}_2$  (20  $\mu\text{M}$ , 3  $\mu\text{L}$ ) to the solution mixture. The spectra were recorded immediately after each  $\text{H}_2\text{O}_2$  addition (A). Spectra collected 10 min. after each  $\text{H}_2\text{O}_2$  addition (B). The same experiments in the presence of methionine (100  $\mu\text{M}$ ) (C). The data shown are representative of three independent experiments.

at 450 nm is identical to that partial formation of authentic MPO Compound II, a long lived intermediate, where its accumulation is the first step in HOCl-mediated heme destruction in hemoprotein [14,16,17]. Furthermore, when MPO:CAT ratio was 5:1, HOCl-mediated heme destruction was, to a large extent, prevented in the presence of excess amounts of methionine (100  $\mu\text{M}$ ) with slight formation of MPO compound II. Methionine, like taurine and melatonin, is an important scavenger for HOCl that eliminates the impact of it on both CAT and MPO [36–38]. Similar experiments were also done using exogenously added HOCl to the enzyme solution mixtures; they showed the same effect, confirming that the alteration in the spectra of Fig. 5 comes from HOCl. Together, these results suggest that a significant amount of MPO and CAT were destroyed during aerobic HOCl synthesis under these conditions, and increasing the ratio between MPO and CAT increased the degree of heme destruction and/or modification, a process that was substantially inhibited by the presence of HOCl scavengers.

Finally, we utilized  $\text{H}_2\text{O}_2$ -selective electrodes to determine whether HOCl mediated MPO heme destruction occurs first and CAT destruction/modification second or vice versa. During continuous monitoring of  $\text{H}_2\text{O}_2$  levels with an  $\text{H}_2\text{O}_2$ -selective electrode, rates of  $\text{H}_2\text{O}_2$  consumption from the reaction were dramatically attenuated by addition of HOCl (10  $\mu\text{M}$ ) to MPO/CAT mixture with prior  $\text{H}_2\text{O}_2$  addition to the



**Fig. 6.** The susceptibility of MPO versus CAT heme destruction by HOCl. A typical recording by an  $\text{H}_2\text{O}_2$ -selective electrode when three consecutive addition of exogenous HOCl ( $10\ \mu\text{M}$ )/ $\text{H}_2\text{O}_2$  ( $10\ \mu\text{M}$ ) to a continuously stirred phosphate buffer (200 mM, pH 7.4) supplemented with 40 nM MPO and 40 nM CAT at  $25\ ^\circ\text{C}$  to insure MPO/CAT inactivation. The reaction mixture then received 100 mM  $\text{Cl}^-$  followed by two consecutive  $\text{H}_2\text{O}_2$  additions (denoted by the arrows). The data shown are representative of three independent experiments.

solution mixture. Under these experimental conditions, the  $\text{H}_2\text{O}_2$  signal rose rapidly, achieved a maximum after  $\sim 30$  s, and fell gradually to the base line as  $\text{H}_2\text{O}_2$  was depleted. To ensure MPO/CAT inactivation, an additional consecutive two HOCl aliquots ( $10\ \mu\text{M}$ )/ $\text{H}_2\text{O}_2$  ( $10\ \mu\text{M}$ ) were added to a continuously stirred phosphate buffer (200 mM, pH 7.4) supplemented with 40 nM MPO and 40 nM CAT at  $25\ ^\circ\text{C}$ . Here, the  $\text{H}_2\text{O}_2$  signal rose rapidly, achieved a maximum after  $\sim 30$  s, and fell very slowly not reaching the base line as  $\text{H}_2\text{O}_2$  was depleted slowly. The reaction mixture then received 100 mM  $\text{Cl}^-$  followed by two consecutive  $\text{H}_2\text{O}_2$  additions (denoted by the arrows). The fact that the addition of  $\text{Cl}^-$  led to acceleration of  $\text{H}_2\text{O}_2$  consumption (Fig. 6) indicates that MPO is still active and that the slow reaction could be due to the formation of Compound II, the stable form of MPO, partial MPO heme destruction, or a combination of both. Therefore, our experiment shows that CAT is inactivated prior to MPO in the presence of HOCl. Collectively, these results may suggest that CAT is more susceptible to HOCl-mediated destruction than MPO under these experimental conditions.

#### 4. Discussion

Our central finding is that CAT competes with MPO for the substrate  $\text{H}_2\text{O}_2$  and thus prevents HOCl-mediated heme degradation and/or protein modification. The protective role provided by CAT versus the damaging effect of HOCl depends on both the MPO/CAT ratio and the enzymes' affinity towards  $\text{H}_2\text{O}_2$  versus HOCl. In addition to mediating

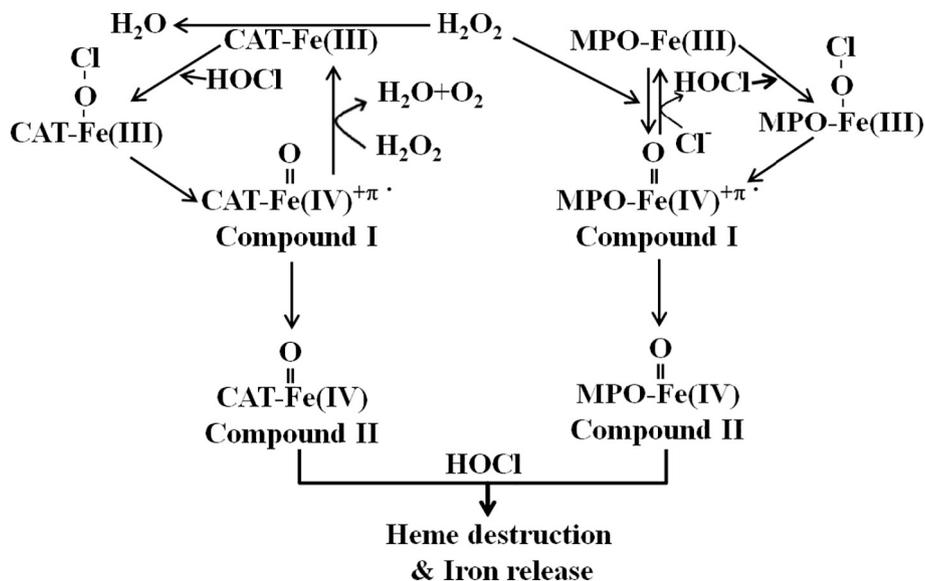
protein modification and protein aggregation [39–41], HOCl's damaging effect is also due to its ability to oxidatively destroy the catalytic sites, in this case the heme group, of the enzymes to different degrees. The severity of this process depends mainly on the HOCl level in the solution milieu. Collectively, increased MPO/CAT ratio may lead to accumulation of free iron, in addition to modifications of the structure and function of proteins that are biologically relevant to the initiation and maintenance of inflammation. On the other hand, increasing CAT:MPO ratio, scavenging HOCl/ $\text{H}_2\text{O}_2$ , and/or inhibiting MPO minimize instant HOCl-mediated damage, thereby keeping both enzymes catalytically active. Understanding the mechanism of the mutual influences of individual enzyme activities should be useful for elucidating disease mechanisms and developing clinically useful therapy.

##### 4.1. Catalase modulates the toxicity of the MPO- $\text{H}_2\text{O}_2$ system

The toxicity of the MPO/HOCl system could be controlled by multiple factors including CAT and other related enzymes that catalyze the conversion of  $\text{H}_2\text{O}_2$  to  $\text{H}_2\text{O}$ , HOCl scavengers, and MPO inhibitors (e.g. taurine, methionine, and melatonin). Disturbance of this balance may play a crucial role in catalytic function of both MPO and CAT. HOCl may influence MPO/CAT steady-state catalysis by acting either as a heme ligand [14], or as a mediator of heme destruction [14,15], protein modification [40,41], and protein aggregation [39]. The interaction of  $\text{OCl}^-$  with both MPO and CAT includes its ability to form the Fe(III)-OCl complex and subsequently generate compounds I and II [14,39,42]. Moreover, the ability of both MPO-Fe(III)-OCl and CAT-Fe(III)-OCl complexes to decay to higher oxidative states, namely Compounds I and II, was also illustrated by diode array stopped-flow measurements [14,42–44].

##### 4.2. A model showing competition between MPO and CAT for $\text{H}_2\text{O}_2$ and resulting HOCl-mediated heme destruction and/or modification

HOCl is a potent oxidant that under normal physiological conditions operates as an antimicrobial agent [14,39,43,44]. However, under pathological conditions, the rate of HOCl production becomes excessive, and the compound may mediate protein modification and/or hemo-protein heme destruction leading to the generation of free iron [14,39,43,44]. A kinetic model that describes the interaction of HOCl with MPO/CAT intermediates is shown in Fig. 7. Under normal conditions, CAT catalyzes the conversion of  $\text{H}_2\text{O}_2$  to  $\text{H}_2\text{O}$ , whereas MPO



**Fig. 7.** Working kinetic model for the  $\text{H}_2\text{O}_2$  and HOCl interaction with both MPO and CAT.

employs  $\text{Cl}^-$  in the presence of  $\text{H}_2\text{O}_2$  to generate HOCl through the formation of MPO Compound I (ferryl porphyrin  $\pi$  cation radical,  $\text{Fe(IV)} = \text{O}(\pi^+)$ ). The partitioning of  $\text{H}_2\text{O}_2$  consumption between the two pathways depends mainly on two enzymes' concentration and their relative affinity to  $\text{H}_2\text{O}_2$ . Rapid kinetic measurements have shown that the reaction between CAT and  $\text{H}_2\text{O}_2$  followed a second order reaction with a rate constant of  $5.0 \times 10^6 \text{ s}^{-1} \text{ M}^{-1}$  [45]. In parallel, the second order rate constant for the reaction of MPO –  $\text{Fe(III)}$  with  $\text{H}_2\text{O}_2$  to form MPO Compound I was found to be  $1.8 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$  at 25 °C, whereas the second order rate constant of Compound I with  $\text{Cl}^-$  to form HOCl was found to be  $4.7 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$  [46,47]. HOCl, when accumulated in the MPO/CAT solution mixture, competes with  $\text{H}_2\text{O}_2$  for the catalytic sites of both enzymes and binds to the heme iron at a near diffusion rate forming the corresponding  $\text{Fe(III)-OCl}$  complexes.  $\text{Fe(III)-OCl}$  complexes then decay to Compound II through the formation of Compound I. Compound II is formed through the reaction of HOCl with CAT- $\text{Fe(III)}$ /MPO- $\text{Fe(III)}$  and is the prime target of HOCl-mediated heme destruction and subsequent free iron release [14–17]. Alternatively, CAT Compound I reacts with  $\text{H}_2\text{O}_2$  to complete its natural catalytic cycle that could partially protect against HOCl-mediated heme destruction but not against protein aggregation [48]. Consistent with this notion, Bonini et al. have demonstrated that the reaction of CAT with HOCl leads to protein oxidation both in vitro and in cell cultures, which is a primary event leading to CAT aggregation and consequent loss of function [39]. Taking into consideration that heme groups in the catalase molecule are deeply buried [49], Krych-Madej and Gebicka have shown that the sulfur-containing amino acid residues on the protein surface are the main targets for HOCl modification [42]. Irreversible CAT heme damage was detected by the authors when the enzyme was treated with higher concentrations of HOCl [42]. Regardless of enzymatic inactivation through either heme destruction, protein aggregation, or a combination of both, HOCl mediates the inhibition of both enzymes [39,42,50]. The degree of CAT/MPO inactivation is significantly higher at lower CAT: MPO ratios because only a small portion of total enzymes is estimated to remain active after sequential addition of  $\text{H}_2\text{O}_2$ .

With  $\text{H}_2\text{O}_2$  serving as a limiting substrate of HOCl synthesis under such circumstances, modulation in the ratio between both enzymes provides significant protection against HOCl insults. This protection is due to the increase in  $\text{H}_2\text{O}_2$  consumption by CAT and CAT compound I, increase in the heme content provided by MPO and CAT, or a combination of both. This process is reflected by the shift to the right in the MPO inactivation curve (Fig. 2A). In marked contrast, increasing the MPO/CAT ratio could lead to more HOCl production, but the MPO inactivation curve still shifts to the right (Fig. 2B), indicating that the HOCl/heme content ratio plays a crucial role in hemoprotein inhibition, with  $\text{H}_2\text{O}_2$  serving to limit HOCl synthesis under such circumstances. MPO contains two heme prosthetic groups while CAT is a tetramer with four heme groups, which allows the CAT heme groups to be more susceptible to HOCl attacks [51,52]. However, the ability of CAT Compound I to utilize  $\text{H}_2\text{O}_2$  as a substrate could favor protein modification and enzyme inactivation rather than CAT heme destruction, especially at low HOCl concentrations, consistent with other reports [39,42]. The loss of catalytic activity of both enzymes by HOCl mediated heme destruction and/or protein modification is also supported by evaluating different MPO inactivation curves that display the same heme content (e.g., 2:1 CAT:MPO (Fig. 2A) and 3:1 MPO:CAT (Fig. 2B) but display different degrees of protection. Our UV-visible studies showed that higher  $\text{H}_2\text{O}_2$  concentration was required to inactivate a correspondingly higher concentration of MPO and CAT (Fig. 3A), a process that could be prevented in the presence of MPO inhibitors and/or HOCl scavengers. Taken together, the ability of HOCl to mediate MPO/CAT inhibition through multiple pathways may provide new insight into biological roles, particularly in organs that experience exposure to wide variations of MPO/CAT levels. The prevention of free iron generation by catalase suppresses reactions between metals and

highly reactive free radicals such as the hydroxyl radical ( $\cdot\text{OH}$ ) produced through the Fenton reaction, which are extremely damaging to tissues and further perpetuate the inflammatory environment [53,54].

#### 4.3. The crosstalk between MPO and CAT

CAT is an enzymatic antioxidant, which under normal conditions, serves a signaling function and plays an important role in protecting the cell from oxidative damage mediated by reactive oxygen species (ROS) [55,56].  $\text{H}_2\text{O}_2$  is freely diffusible through membranes, and under pathological conditions, such as during atherosclerosis, asthma, and other inflammatory processes, rates of  $\text{H}_2\text{O}_2$  production become excessive. CAT deficiency or its inactivation by HOCl plays an important role in up-regulating the catalytic activity of MPO that occurs at sites of inflammation. CAT and MPO crosstalk with each other through the diffusion of  $\text{H}_2\text{O}_2$  and HOCl, whether the enzymes are in different locations or are co-localized in the same cell [57]. In our current work, we have shown that HOCl, the final product of MPO, promotes CAT inactivation and MPO heme destruction resulting in the release of oxidants. Taken together, our findings support a biologically relevant mechanism, which delineates a novel role for MPO in the pathogenesis of inflammatory diseases.

#### 4.4. Alterations of MPO/CAT ratio in respiratory and cardiovascular pathologies

Specific interrelationships between variants of CAT and MPO and their role in the etiology of respiratory conditions related to oxidative stress have been infrequently explored. Significantly increased concentrations of plasma MPO, nitric oxide metabolites, and iron were detected in asthmatic individuals [58]. It is believed that the loss of SOD and CAT activities is related to oxidative modifications, while other antioxidant gene polymorphisms are linked to the susceptibility to develop asthma. In support of this, CAT activity was lower in the red blood cells of asthmatic children and in bronchoalveolar lavage specimens of asthmatic lungs, as compared to healthy individuals [59]. In addition to asthma, the crosstalk between CAT and MPO also plays an important role in cystic fibrosis (CF). Although there are no significant differences in the concentrations or activities of  $\text{H}_2\text{O}_2$  in breath condensate of CF patients compared to healthy individuals, sputum specimens from CF patients were found to contain large amounts of active MPO ( $5.93 \pm 4.8 \mu\text{M}$ ) and CAT ( $0.31 \pm 0.18 \mu\text{M}$ ) [1]. Epithelial lining fluid and plasma in CF patients were characterized by a marked deficiency of non-enzymatic antioxidants such as GSH, taurine, and methionine compared to controls [1,60]. An abnormal accumulation of total iron, and iron-regulatory cytokines such as interleukin-1 beta and tumor necrosis factor alpha were found in the sputum of the patients with chronic bronchitis and, to a greater extent, in patients with CF [61,62]. The involvement of HOCl in mediating the generation of free iron through heme destruction in disease pathology might highlight its involvement in the production of  $\cdot\text{OH}$  in the presence of  $\text{H}_2\text{O}_2$ . Accumulation of free iron may promote oxidative injury and enhance bacterial growth [63]. Additionally, overload of free iron is also associated with many inflammatory disorders such as arthritis, diabetes, psychiatric illness, and liver disease, and is also a risk factor for cancer and heart diseases [4,5,7,64,65].

Furthermore, MPO and its oxidant products, which predict increased cardiovascular risk, have been implicated in the pathogenesis of atherosclerosis [66]. MPO mediates lipid peroxidation and conversion of LDL to an atherogenic form [66]. Recently, it has been shown that MPO-derived oxidants can also modify HDL, causing an impairment of cholesterol efflux [66]. Thus, MPO has been considered a useful biomarker for cardiovascular disease [67]. CAT, among other antioxidant enzymes, has been shown to protect the endothelium of human aorta against apoptosis associated with oxidized forms of LDL [68]. Such results highlighted the key role of  $\text{H}_2\text{O}_2$  in atherogenesis as well as CAT

and other related antioxidant enzymes' role in insulating against oxidative stress of the cardiovascular system by impacting H<sub>2</sub>O<sub>2</sub>-mediated signaling and HOCl-production [69,70]. Therefore, blocking the MPO/HOCl chlorination machinery might be a useful therapeutic approach in vascular diseases and other inflammatory conditions, where iron overload is a significant etiopathological factor.

## 5. Conclusion

In conclusion, it is conjectured that CAT and MPO crosstalk with each other, independent of their localization, through the diffusion of the substrate (H<sub>2</sub>O<sub>2</sub>) and product (HOCl) in a common pro-oxidant mechanism. Therefore, HOCl overproduction, which occurs in many inflammatory diseases, could lead to the imbalance of MPO and CAT through protein modification and/or heme destruction. Inactivation of both enzymes leads to H<sub>2</sub>O<sub>2</sub> accumulation, which, when combined with free iron, causes further oxidative damage to the host by generation of •OH. Thus, we describe a HOCl-based regulatory mechanism that enables hemoprotein instant release of free iron and protein modification that can be attenuated by CAT. This may provide new insights into biological roles for MPO and CAT, particularly in organs that experience elevated MPO/HOCl levels. The crosstalk between MPO and CAT is remarkable and suggests that there are biological roles for their interaction which remain to be considered. These are under current investigation in our laboratories.

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