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Mechanism of hypochlorous acid mediated heme destruction and free iron release

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Abstract

Here, we show that hypochlorous acid (HOCl), a potent neutrophil generated oxidant, can mediate destruction of free heme (Ht) and the heme precursor, protoporphyrin IX (PPIX). Ht display a broad Soret peak centered at 365 and 394 nm, indicative of the presence of monomer and μ -oxo-dimer. Oxidation of Ht by HOCl was accompanied by a marked decrease in the Soret absorption peak and release of free iron. Kinetic measurements showed that the Ht-HOCl reaction was triphasic. The first two phases were HOCl concentration dependent and attributable to HOCl binding to the monomeric and dimeric forms. The third phase was HOCl concentration independent, and attributed to Ht destruction with the release of free iron. HPLC and LC-ESIMS analyses of the Ht-HOCl reaction revealed the formation of a number of degradation products, resulting from the cleavage or modification of one or more carbon-methene bridges of the porphyrin ring. Similar studies with PPIX showed that HOCl also mediated tetra-pyrrole ring destruction. Collectively, this work demonstrates the ability of HOCl to modulate destruction of heme, through a process that occurred independent of the iron molecule that resides in the porphyrin center. This phenomenon may play a role in HOCl-mediated oxidative injury in pathological conditions.

Keywords

Free iron; heme deficiency; heme degradation; mammalian peroxidases; mass spectrometry; oxidative stress; porphyrin; stopped-flow

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INTRODUCTION

Heme is a planar metalloporphyrin compound with an iron (Fe) atom chelated at the center of the porphyrin macrocycle (Fig. 1A) [1]. The Fe atom plays a pivotal role in the functioning of the heme, such as molecular recognition and chemical selectivity [1]. The important role of free heme is its ability to serve as a signaling molecule and a regulator of growth and differentiation of hematopoietic and non-hematopoietic cells [2]. It also serves as a prosthetic group in several hemoproteins [2]. The heme moiety of hemoproteins forms the catalytic site where the stepwise oxidation of substrate takes place [3, 4]. During enzymatic catalysis, the heme prosthetic group plays a critical role in supporting one or more of following functions: substrate binding and storage (hemoglobin), electron transfer (mitochondrial electron transport system), bond formation and breaking (catalase and peroxidases), product release (nitric oxide synthases), and protein conformational change (hemoglobin) [2]. The reduced form of hemoprotein reacts with molecular oxygen and generates superoxide anion ($O_2^{\bullet-}$) [5, 6]. Due to the absence of steric hindrance effects from the neighboring protein, these redox reactions are faster with free heme than with hemoprotein model compounds and, therefore, free heme mediates the generation of more toxic components than hemoprotein model compounds.

In the biological system, heme degradation occurs in two different pathways: the enzymatic pathway that requires the presence of heme oxygenase system; and non-enzymatic pathway mediated through reactive oxygen species (ROS) such as $O_2^{\bullet-}$ and hydrogen peroxide (H_2O_2) [7]. Heme deficiency mediated by the acceleration in heme destruction and consequent dysregulation of the cellular heme homeostasis may lead to pathological conditions such as Alzheimer's disease and aging [8]. Heme destruction is extremely toxic to different organs and cells leading to serious pathological consequences [2]. The toxicity of heme destruction is mainly due to the free iron release, which can generate ROS and mediate cellular mitochondrial dysfunctional lipid peroxidation, and uncoupling of oxidative phosphorylation [9-12]. Free Fe damages blood vessels and causes vasodilation with increased vascular permeability, leading to hypotension and metabolic acidosis [12, 13].

Under many pathological conditions such as atherosclerosis, endometriosis, and cancer, where myeloperoxidase (MPO) has been known to play a role, there have been reports of significant free Fe accumulation [12-16]. The source of this Fe is remains unclear, but it is thought to be hemoglobin [13]. Myeloperoxidase uses H_2O_2 to catalyze the two-electron oxidation of chloride (Cl^-) to generate hypochlorous acid (HOCl) [17]. Hypochlorous acid and its conjugate base (OCl^-) are potent oxidants that function as powerful antimicrobial agents [18]. However, under a number of pathological conditions such as inflammatory diseases, atherosclerosis, pulmonary fibrosis, acute vasculitis, rheumatoid arthritis, glomerulonephritis, and cancer, HOCl is implicated in damaging the host tissue by the same mechanism used to destroy invading pathogens [19-22]. We believe that there is a mechanistic link between high HOCl and higher free Fe levels. In the current work, we studied the reaction between purified bovine hematin (Ht-Fe(III)) and protoporphyrin IX (PPIX) (Fig. 1B) with HOCl utilizing a variety of spectroscopic and analytical techniques. Our rapid kinetic measurements demonstrate that HOCl can mediate free Fe release through a mechanism that involves heme destruction ultimately resulting in oxidative cleavage of heme moiety generating fluorescent and non-fluorescent porphyrin derivatives. This mechanism may contribute to vascular endothelial dysfunction that is induced by oxidative stress in various inflammatory diseases.

Materials and methods

Materials

All the materials used were of highest purity grade and used without further purification. Sodium hypochlorite (NaOCl), ammonium acetate (CH₃COONH₃), ferrozine, L-methionine, ascorbic acid, hematin (Ht), protoporphyrin IX (PPIX), dimethylformamide, methanol and trifluoroacetic acid (TFA) - HPLC grade, were obtained from Sigma Aldrich (St. Louis, MO, USA). HPLC grade acetonitrile (CH₃CN) was obtained from EMD Chemicals Inc. (Gibbstown, NJ, USA).

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 a 1-mL phosphate buffer solution supplemented with fixed amount of Ht (2.5 μM) and increasing concentration of HOCl (0–100 μM). After 10 min incubation for reaction completion, methionine (5-fold of 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 Ht destruction were performed using a dual syringe stopped-flow instrument obtained from Hi-Tech, Ltd. (Model SF-61). Measurements were carried out under an aerobic atmosphere at 10 °C following rapid mixing of equal volumes of a buffer solution containing a fixed amount of Ht (2.5 μM) and a buffer solution containing increasing concentration of HOCl. 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.

High performance liquid chromatography (HPLC) analysis

HPLC analyses was carried out using a Shimadzu HPLC system equipped with a SCL-10A system controller, with a binary pump solvent delivery (LC-10 AD) module and a SIL-10AD auto-injector connected to a SPD-M10A diode array detector (DAD) and a RF-10A XL fluorescence detector. An Alltech 5 μm particle size, 4.6 × 150 mm reverse-phase octadecylsilica (C18) HPLC column was used. The column was kept at 27 °C. The photodiode array detector was set at 400 nm and the fluorescent detector was set at excitation 321 nm and emission 465 nm to monitor the chromatogram. The column was eluted at a flow rate of 1.0 mL/min with linear gradients of Buffers A and B (A, 0.1% TFA in water; B, 0.1% TFA in 80% acetonitrile). The solvent gradient was as follows: 0 to 10 min, 55–65%B; 10 to 14 min, 65–90% B; then the buffer B composition dropped down to 55% within 14 to 24 min. After treatment of Ht with HOCl, 500 μL of the reaction mixture was diluted with 500 μL of injection solvent (55% B and 45% A) and 50 μL were injected. At the end of the run, the system was equilibrated with 45% solvent A. Under these conditions, Ht eluted at around 19 min and was identified from the characteristic spectral signal from the Diode Array Detector. For fluorescence detection, the excitation and emission wavelength were set at 321 nm and 465 nm, respectively. Each sample was analyzed in triplicate.

Mass spectrometric analysis of heme degradation products

Mass spectrometry (MS) experiments were performed using an Agilent 6410 Triple Quadrupole mass spectrometer coupled with an Agilent 1200 HPLC system (Agilent Technologies, New Castle, DE), equipped with an electrospray source. A Waters symmetry C18 column (particle size 3.5 μm ; 2.1 $\times$ 100 mm) (Milford, MA) was used to separate reaction products. Solvent A was H_2O with 0.1% formic acid and Solvent B was acetonitrile with 0.1% formic acid. The column was equilibrated with 80% solvent A and 20% solvent B. The gradient was: 20-95% solvent B over 10 min; 95% solvent B for 10 min; 95-20% solvent B for 1 min; and 80% solvent A for 14 min. Five μL of the sample was injected at a flow rate of 250 $\mu\text{L}/\text{min}$.

Liquid chromatography electrospray ionization (LC/ESI) MS in the positive mode was performed using the following parameters: spray voltage 4000 V, drying gas flow 10 L/min, drying gas temperature 325 $^{\circ}\text{C}$, and nebulizer pressure 40 psi. Fragmentor voltage was optimized using Flow Injection Analysis with Ht. Optimal fragmentor voltage was 300 V in MS2 scan mode. Mass range between m/z 200 and m/z 900 was scanned to obtain full scan mass spectra.

Free iron analysis

Free iron release was measured colorimetrically using ferrozine, following a slight modification of a published method [23]. To 100 μL of the sample (Ht-HOCl reaction mixture) 100 μL of ascorbic acid (100 mM) was added. After 5 min of incubation at room temperature, 50 μL 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 by using ammonium Fe(III) sulfate was used for the calculation of free iron concentration. Final concentrations of the additives are as follows: ascorbic acid - 33.33 μM , ammonium acetate - 5.3%, and ferrozine - 5.3 μM .

Solution Preparation

HOCl preparation—HOCl was prepared following a slight modification of a published method [24]. Briefly, a stock solution of HOCl was prepared by adding 1 mL NaOCl solution to 40 mL of 154 mM 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 μL 5 M NaOH and measuring the concentration of OCl^- . The concentration of OCl^- was determined spectrophotometrically at 292 nm ($= 362 \text{ M}^{-1} \text{ cm}^{-1}$). As HOCl is unstable, the stock solution was freshly prepared on a daily basis, stored on ice, and used within one hour of preparation. For further experimentations, dilutions were made from the stock solution using 200 mM phosphate buffer pH 7, to give working solutions of lower HOCl concentration.

Hematin solution—Hematin solution was prepared by dissolving 11.3 mg of porcine Ht in 100 mL of 100 mM NaOH [25]. The solution was stored in a dark bottle at 10 $^{\circ}\text{C}$ and was stable for a month. For experiments, the stock solution was diluted in 200 mM phosphate buffer pH 7 to get working solutions of lower concentration.

Protoporphyrin IX solution—Approximately 1 mg of solid protoporphyrin IX was dissolved in methanol:dimethylformamide (1:1). Since protoporphyrin IX is light sensitive, care was taken not to expose the stock solution to light and was used within 1 h of preparation. For experiments, the stock solution was diluted in 200 mM phosphate buffer pH 7 to get working solutions of lower concentration.

Results

HOCl mediated heme destruction is a triphasic process

To examine the kinetics of interaction between HOCl and Ht, we utilized diode array stopped flow spectrophotometry. Rapid kinetic studies were initially performed under pseudo first order condition where the HOCl concentrations employed were in large molar excess of Ht. The visible spectrum of Ht displayed a broad Soret absorbance peak with two shoulders centered on 360 and 394 nm indicative of a mixture of two forms, Ht monomer and Ht μ -oxo dimer, respectively, as previously reported [26]. The influence of HOCl on the kinetics of Ht-OCl complex formation and subsequent heme destruction were examined following rapid mixing of a buffer solution supplemented with 2.5 μ M (final) Ht with equal volume of a buffer solution supplemented with increasing concentration of HOCl (0- 400 μ M, final). Fig. 2 shows the time course for the formation of Ht-Fe(III)-OCl and heme destruction by monitoring the absorbance changes from 300-700 nm. The Figure shows spectral traces collected at 3, 7, 19, 51, 121, 237 and 595 seconds after mixing. The decrease in absorbance that takes place at either 360 or 394 nm indicated that the reaction is triphasic in nature (Fig. 3). The change in absorbance that takes place in the first 1 s of the reaction is shown in Fig. 3 inset and is attributed to the binding of the HOCl to the heme iron in the monomeric form of Ht, leading to the formation of Ht-Fe(III)-OCl complex. The build-up of Ht-OCl complex was best fitted to a single exponential function, giving an apparent second order combination rate constant (k_{on}) of 0.095 μ M⁻¹s⁻¹ and dissociation rate constant (k_{off}) of 3.9 s⁻¹ calculated from the slope and y-intercept, respectively. The second phase of the reaction that takes place in the next 30 s was attributed to the formation of Ht(III)-OCl complex through the binding of HOCl to the heme iron of the dimeric form of Ht. Plotting the pseudo first order rates for this phase as a function of HOCl concentration revealed that this phase was also reversible in nature but with a comparatively slower rate than the first phase, k_{on} = 0.0037 μ M⁻¹s⁻¹ and k_{off} = 0.3 s⁻¹. The subsequent decrease in absorbance observed was also fit to a single exponential function and found to be HOCl independent with a rate constant of 0.005 s⁻¹. This decrease in absorbance was attributed to the heme destruction/cleavage of the tetrapyrrole ring (Fig. 4). Together, these results indicate that the build-up of Ht-Fe(III)-OCl complex is relatively fast, biphasic, and occur with a much faster than the heme destruction. The change in absorbance at 394 nm shows change differed in the amplitude of the three phases but otherwise it follows the same kinetics.

HOCl treatment causes release of free iron from hematin

Ht (25 μ M) was incubated with different molar ratios of HOCl (1:1, 2:1, 4:1, 8:1, 16:1, 32:1 and 40:1; Ht:HOCl) for 10 min, and free iron released was measured using ferrozine assay as detailed in *Materials and methods* section. The free iron increased in a linear manner and showed signs of saturating only when the ration of HOCl:Ht of 16:1. The inflection point in the two straight lines was at 11:1 indicating eleven molecules of HOCl are required to completely destroy one molecule of Ht and release the iron (Fig. 5).

HPLC analysis of heme degradation products obtained after HOCl treatment

Ht does not have an intrinsic fluorescence, but previous reports have shown that different porphyrin degradation products generated due to cleavage of the tetrapyrrole moiety have an intrinsic fluorescence. We decided to exploit this property to study and characterize the degradation products of the Ht-HOCl reaction. Ht (25 μ M) was treated with increasing molar ratios of HOCl (1:1, 1:2, 1:6) and HPLC separation coupled with fluorescence detection (excitation 321 nm and emission 465 nm) was employed to monitor the formation of the fluorescent heme degradation products (Fig. 6). From the HPLC chromatograms we can see that reaction of Ht with HOCl lead to the progressive accumulation of at least five major compounds eluting at earlier time. The retention times of the five different fluorescent

degradation products were 1.9, 2.9, 3.3, 5.2 and 8.5 min, respectively. The appearance of newer earlier eluting peaks in the chromatograms could be due to the formation of degradation products with lesser hydrophobicity generated by fragmentation of the tetrapyrrole ring of the heme.

Role of iron in HOCl mediated cleavage of the tetrapyrrole-ring

To understand the role of the metal center (iron in our case) in HOCl mediated cleavage of the tetrapyrrole ring, we analyzed the reaction between PPIX with HOCl by HPLC. PPIX (25 μ M) was reacted with different molar ratios of HOCl (1:16 and 1:60) and HPLC was performed in similar manner (Fig. 7). Comparison of the retention times of the fluorescent products observed from PPIX with those obtained when Ht was treated with HOCl revealed that they are very similar. Thus, it can be concluded that the presence or absence of the metal center does not play a role in the pattern of the cleavage of the porphyrin ring.

LC-ESI-MS of the heme degradation obtained from Ht and PPIX after HOCl treatment

The majority of the heme degradation products were tentatively identified by detecting the corresponding molecular weight and comparing the proposed structures with the previously identified products and/or owing to the chemical reactivity of HOCl with carbon-carbon double bonds [18,28,29]. Mass spectrometric analysis revealed that HOCl can randomly cleave the tetrapyrrole ring at any C=C double bond including the carbon-methyne bridge, the terminal carbon in the side chain and even in the pyrrole ring itself. Oxidative modification of the C=C resulted in the formation of epoxide, carboxylic acid, chlorination, hydroxylation and methyl ester formation. We have been able to tentatively identify 6 fragments from Ht and 10 fragments from PPIX. m/z = 659, 449, 437, 425, 421, 409 were identified from both Ht and PPIX. Whereas, m/z = 615, 581, 577, 563 were identified only from PPIX. Table 1 shows the structures for all the different degradation products that we have been able to identify. Figure 8 shows the Extracted Ion Chromatogram (EIC) and MS spectra for m/z 421. This product was observed from both Ht and PPIX and is an example where HOCl treatment induced formation of methyl ester. We propose two alternative structures that match this molecular weight. Figure 9 shows the chlorinated oxidation fragment that was obtained from PPIX. Figure 9A shows the EIC for m/z 615 with two different products eluting around 11 min and 12 min. The MS spectrum for both the peaks (Fig. 9B and 9C) revealed the presence of one chlorine atom as the ion intensity of the $[M + H + 2]^+$ ion is approximately 40% of $[M + H]^+$, indicating a chlorine isotope pattern. One chlorinated product matching this molecular weight was identified. m/z 581 is an example of hydroxylated fragment generated from HOCl treatment of PPIX (Fig. 10). The EIC for this product is shown in Fig. 10A, which shows three isomers of these products. Figure 10B, 10C and 10D shows the MS spectra for the three individual peaks. We have been able to assign structures two of the three isoforms for this molecular weight.

Discussion

Although increased levels of HOCl are typically observed in the plasma and tissues of individuals with various inflammatory diseases [19-21], the link between elevated levels of HOCl and heme destruction has yet to be fully elucidated. Using a variety of spectrophotometric and mass spectrometric techniques, we have shown that HOCl not only binds to the monomeric and dimeric forms of Ht to generate a penta-coordinate high-spin complex Ht-Fe(III)-OCl, but also mediates Ht destruction and subsequent iron release. HPLC and LC-MS analysis showed that exposure of Ht to increasing concentrations of HOCl gave a range of metabolites resulting from oxidative cleavage of one or more C=C (Table 1). HOCl may directly mediate the destruction of Ht by nonselective cleavage at any double bond position in nonenzymatic manner. HOCl-mediated Ht destruction occurred

independent of Ht iron as similar products are observed for PPIX. A total of 16 cleavage products have been identified based on their mass signals through the treatment of Ht/PPIX solutions with a range of HOCl concentrations. The degree of oxidation, the amount of Ht and PPIX degradation mainly depend on the HOCl concentration, suggesting that multiple molecules of HOCl are required to destroy one molecule of Ht or PPIX.

Using rapid kinetic measurements, OCl^- was shown to bind to both monomeric and dimeric forms of Ht, consistent with the formation of the corresponding high-spin penta-coordinate Fe(III)-OCl complex. Stopped-flow analyses demonstrated that the binding of OCl^- to both forms followed a simple reversible single-step mechanism with a remarkable decreased rate of OCl^- binding to monomeric *versus* dimeric form. A simple explanation that could account for this result would be that the dimeric form dissociates and releases oxygen that allows accessibility of OCl^- to the heme iron. Exposure of Ht to saturating amounts of HOCl caused a decrease and flattening of the Soret absorbance peak region, suggesting Ht destruction. Since the formation of the Ht-Fe(III)-OCl occur faster than heme destruction, the porphyrin destruction could occur in two distinct pathways.

First, through the subsequent effect of OCl^- binding to the iron center, in this case through the formation of ferryl complex followed by the binding of OCl^- to the Ht iron center [27] (Scheme 1). In this case, the heterolytic cleavage of the O-Cl bond in an Ht-Fe-OCl intermediate preferentially occur, at neutral conditions to degrade HOCl and form a ferryl porphyrin radical cation $\text{Ht-Fe(IV)=O}^\bullet$ intermediate. This intermediate is highly unstable and decayed to form Ht-Fe(IV)=O complex. Ht-Fe(IV)=O complex is also unstable in the presence of HOCl which could be destroyed through the formation of $\text{Ht-Fe(III)-OO}^\bullet$ radical (Scheme 1). Under these circumstances, the formation rate of the ferryl intermediates are comparable or slower than the decay, therefore, the buildup of these intermediates can not been seen, and the conversion of Ht-Fe(IV) to $\text{Ht-Fe(III)-OO}^\bullet$ is the rate limiting step and occurs independent of the HOCl concentration. This pathway could be excluded since the heme destruction take place independent of the presence of Fe. Indeed, our results showed that the treatment of PPIX with increasing concentration of HOCl generates a number of PPIX degradation products. This pathway could apply for a number of hemoprotein model compounds, where the heme serves as the catalytic sites of the proteins [28-30]. The Ht destruction process is relatively slow, irreversible in nature, and occurred independent of HOCl concentration.

Second, Ht destruction can occur through the direct attack of HOCl to the tetrapyrrole ring. Scheme 2 shows a possible chemical mechanism to through which HOCl can randomly attack any of the four carbon methyne bridges between the adjacent pyrrole ring and forms the chlorinated adducts, which by releasing of chloride, forms an epoxide or amination. The epoxide gives rise to a hydroxylated compound with the hydroxyl group being attached to the carbon-methyne bridge of the tetrapyrrole moiety, where the initial attack by HOCl occurred. Attack by another hydroxyl group to this carbon leads to the formation of vicinyl diol (two hydroxyl groups attached to two adjacent carbon atoms). Cleavage of the vicinyl diol leads to the formation of two carbonyl compounds. The cleavage of the vicinyl diol can either occur through a hemolytic cleavage forming two carbonyl compounds, or in presence of iron, through the formation of dioxetane intermediate by a heterolytic two electron process (shown in red, Scheme 2A). The aldehydes that are generated may be further oxidized by HOCl to form carboxylic acid, through a mechanism as shown previously [17]. Cleavage of the C=C bond is not only limited to the carbon-methyne bridge, but it can also occur at the terminal C=C bond, leading to the formation of formaldehyde. This single carbon aldehyde can be oxidized to formic acid by the electrophilic addition of HOCl. The formaldehyde thus generated is used for the formation of methyl esters as seen in some of the structures (Scheme 2B).

Our current results clearly show that HOCl can non-enzymatically mediated heme destruction and subsequent liberation of free iron. Indeed, our HPLC and mass spectrometric results show a direct link between Ht oxidation and the formation of fluorescent and non-fluorescent degradation products as well as free iron accumulation. Ferrozine assays showed that 11 molecules of HOCl required for the destruction of one molecule of Ht. Based on the pattern of cleavage, we are able to categorize the compounds we have been able to identify tentatively by LC-MS to four major groups. Group 1 consists of compounds which have the intact tetrapyrrole ring with modification (methyl ester formation) only in the terminal C=C bond, e.g. m/z 577. Group 2 consist of compounds resulting from subsequent oxidative modification of the Group 1 compounds, and they include compounds with modification (epoxide, hydroxylation, chlorohydrin formation) in one or more carbon methene bridge and a terminal C=C bond. Examples of compounds in this group are m/z 659, 615 and 581. The compound with m/z 581, has three isomers with different retention times, we have been able to assign structures to two of them (Table 1). Group 3 consists of dipyrrolic compounds that are formed by the oxidative cleavage of the tetrapyrrole moiety at two carbon methyne bridges, e.g. m/z 437, 425, 421, 409, 405. From our LC-MS studies it can be said that there are two isomers for m/z 425, and we have been able to propose three different possible structures, which are shown in Table 1. Group 4 consists of compounds resulting from extensive oxidative modifications of Group 3 compounds, resulting in opening of the tetrapyrrole ring, e.g. m/z 449, 409.

H₂O₂, like HOCl, can mediate heme destruction unselectively at any of the four-meso carbon bridges, but to lesser degree [31, 32]. Several fluorescent and non-fluorescent heme degradation products have been identified using NMR and/or mass spectrometric techniques upon the treatment of hematin with H₂O₂ solution [31, 32]. Previous studies by Schaefer *et. al.* and He *et. al.* have shown that several oxidants such as cumene hydroperoxide, NADPH and O₂ (in presence of NADPH-P450 reductase) and H₂O₂ can mediate destruction of free heme as well as heme from hemoprotein [31, 32]. These oxidants have been shown to cleave the tetrapyrrole ring in the carbon-methyne bridges forming the corresponding mono, dipyrrole derivatives. Our LCMS studies also support these observations. Our results show that in addition to the cleavage occurring at the carbon methyne bridges, HOCl can also cleave the C=C in the pyrrole ring itself, causing ring opening. Examples of such extensive modifications are observed for m/z 449 and m/z 425. One possible structure proposed for m/z 425 is a di-pyrrole derivative where one pyrrole ring has been cleaved open, but the other one is intact (Table 1), whereas m/z 449 is a dipyrrole derivative where both the pyrrole rings have been cleaved opened include m/z 449 (Table 1). Additionally, HOCl mediated oxidative modifications can also occur at the terminal C=C of the leading to the release of a single carbon, formaldehyde molecule, which is used to form the methyl esters as observed in some of the structures (e.g. m/z 659, 437, 421 and 405). These distinctive features (cleavage of the pyrrole ring and oxidative modification of the terminal C=C) points to the fact that HOCl is a more potent oxidant, causing extensive destruction of the heme tetrapyrrole moiety. In the enzymatic pathway, heme oxygenase system catalyzes heme cleavage and subsequently releases the heme iron in the ferrous form, and in a specific manner it eliminates the -methene bridge carbon of heme as carbon monoxide (CO) to form either biliverdin, or if the heme is still attached to a globin, verdohemoglobin [7, 33]. In sum HOCl is a more potent oxidant than H₂O₂ and heme oxygenase in mediating heme destruction and extensive modification of the heme degradation products.

Heme deficiency mediated by the enhancement in heme destruction or altered cellular heme homeostasis may lead to pathological conditions such as Alzheimer's disease and aging [8, 34, 35]. Heme destruction is extremely toxic to various organs and cells leading to serious pathological consequences [36, 37]. Heme insufficiency promotes collapse of mitochondrial membrane potential, oxidative stress, disruption of Ca⁺⁺ homeostasis, and release of

cytochrome C from mitochondria, events that induce cell aging and programmed cell death (apoptosis) [38]. Heme deficiency can also affect the function of translational initiation factor 2 kinase (heme-regulated eIF2a kinase; HRI), a functional hemoprotein that severely modifies the phenotype of both erythropoietic porphyria and beta-thalassemia [39]. Our results show that heme deficiency mediated through the HOCl heme destruction pathway is associated with accumulation of free iron. The toxicity of cellular free iron is due to its capacity to participate in the further generation of ROS, such as the $O_2^{\bullet-}$, H_2O_2 , and the hydroxyl radical ($\bullet OH$) [9-11]. Free iron can also lead to an increase in bacterial infection. Molecular iron is an essential micronutrient for bacteria, and it is used for several vital life processes such as DNA replication and aerobic respiration [40, 41]. To acquire iron from the environment, bacteria secrete siderophores, which bind free iron and are then captured by the bacterial cell surface receptors that transport the siderophore bound iron across the cell membrane [42]. Thus, the deficiency of several host proteins such as lactoferrin that sequester iron [43-45] and reduce its concentration and/or the decrease in the bioavailability of HOCl scavenger within tissues can increase bacterial growth.

In this respect, inhibiting MPO and/or eliminating its final products may play a beneficiary role in biological systems in reducing the free iron release mediated by HOCl. Recently, we have shown that melatonin, tryptophan, and tryptophan analogs display the potential capacity in inhibiting MPO, the major source of HOCl [46-49]. Mechanistic studies indicate that melatonin and other indole compounds inhibit MPO activity by switching the catalytic pathway from peroxidase to catalase-like activity by acting as $1e^-$ substrates for MPO Compounds I and II [46-49]. We have also shown that lycopene can function as a potent scavenger of HOCl at a wide range of concentrations that span various physiological and supplemental ranges [17]. HPLC and LC-MS analysis showed that the exposure of lycopene to increasing concentrations of HOCl gave a range of metabolites resulting from oxidative cleavage of one or more $C = C$. The degree of degradation of lycopene depends mainly on the ratio of HOCl to lycopene, suggesting that multiple molecules of HOCl are consumed per molecule of lycopene [17]. In related studies, we have also shown that peroxynitrite in combination of H_2O_2 in a low chloride concentration environment inhibit MPO through a mechanism that involves heme destruction and iron release [50].

To summarize, the present studies demonstrate a heretofore unrecognized bidirectional relationship Ht/PPIX and HOCl. Using a combination of biochemical and kinetic approaches, we show that Ht may serve as a catalytic sink for HOCl, limiting its bioavailability and function. HOCl not only served as a ligand for heme iron, but also mediated Ht destruction by direct interaction with the tetra-pyrrole ring. The damage caused to heme by HOCl can be greatly amplified by the liberation of free redox-active iron. However, it should be noted that HOCl can also mediate destruction of the heme moiety of hemoproteins [28, 29]. Thus, HOCl generated from MPO may participate in similar reactions at sites of inflammation where leukocytes, peroxidases, and enhanced HOCl concentration are observed. Taken together, this work may elucidate in part the mechanism of heme deficiency and free iron accumulation under certain pathological conditions where MPO activity is elevated. It may also explain the increased bacterial infection at sites of inflammation through increasing the availability of free iron.

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List of Abbreviations

Fe	Iron
H₂O₂	Hydrogen Peroxide
HPLC	High Performance Liquid Chromatography
HOCl	Hypochlorous Acid
Ht	Hematin
LC-ESI-MS	Liquid-Chromatography-Electrospray Ionization-Mass Spectrometry
MPO	Myeloperoxidase
MS	Mass Spectrometry
O₂^{•-}	Superoxide
PPIX	Protoporphyrin IX
ROS	Reactive Oxygen Species

References

1. Han SW, Cho KJ, Ihm JS. Ab initio study on the molecular recognition by metalloporphyrins: CO interaction with iron porphyrin. *Physical Review E*. 1999; 59:2218–2221.
2. Tsiftoglou AS, Tsamadou AI, Papadopoulou LC. Heme as key regulator of major mammalian cellular functions: molecular, cellular, and pharmacological aspects. *Pharmacol Ther*. 2006; 111:327–345. [PubMed: 16513178]
3. Tahboub YR, Galijasevic S, Diamond MP, Abu-Soud HM. Thiocyanate modulates the catalytic activity of mammalian peroxidases. *J Biol Chem*. 2005; 280:26129–26136. [PubMed: 15894800]
4. Galijasevic S, Saed GM, Diamond MP, Abu-Soud HM. Myeloperoxidase up-regulates the catalytic activity of inducible nitric oxide synthase by preventing nitric oxide feedback inhibition. *Proc Natl Acad Sci U S A*. 2003; 100:14766–14771. [PubMed: 14657339]
5. Misra HP, Fridovich I. The generation of superoxide radical during the autoxidation of hemoglobin. *J Biol Chem*. 1972; 247:6960–6962. [PubMed: 4673289]
6. Wever R, Oudega B, Van Gelder BF. Generation of superoxide radicals during the autoxidation of mammalian oxyhemoglobin. *Biochimica et Biophysica Acta (BBA) - Enzymology*. 1973; 302:475–478.
7. Nagababu E, Rifkind JM. Heme degradation by reactive oxygen species. *Antioxid Redox Signal*. 2004; 6:967–978. [PubMed: 15548894]
8. Atamna H, Killilea DW, Killilea AN, Ames BN. Heme deficiency may be a factor in the mitochondrial and neuronal decay of aging. *Proceedings of the National Academy of Sciences of the United States of America*. 2002; 99:14807–14812. [PubMed: 12417755]
9. Clark RA. Oxidative stress and “senescent” fibroblasts in non-healing wounds as potential therapeutic targets. *J Invest Dermatol*. 2008; 128:2361–2364. [PubMed: 18787545]
10. Crichton RR, Wilmet S, Legssyer R, Ward RJ. Molecular and cellular mechanisms of iron homeostasis and toxicity in mammalian cells. *Journal of Inorganic Biochemistry*. 2002; 91:9–18. [PubMed: 12121757]
11. Kumar S, Bandyopadhyay U. Free heme toxicity and its detoxification systems in human. *Toxicology Letters*. 2005; 157:175–188. [PubMed: 15917143]
12. Ong W-Y, Halliwell B. Iron, Atherosclerosis, and Neurodegeneration: A Key Role for Cholesterol in Promoting Iron-Dependent Oxidative Damage? *Annals of the New York Academy of Sciences*. 2004; 1012:51–64. [PubMed: 15105255]
13. Trinder D, Fox C, Vautier G, Olynyk JK. Molecular pathogenesis of iron overload. *Gut*. 2002; 51:290–295. [PubMed: 12117898]

14. Defrère S, Lousse JC, González-Ramos R, Colette S, Donnez J, Van Langendonck A. Potential involvement of iron in the pathogenesis of peritoneal endometriosis. *Molecular Human Reproduction*. 2008; 14:377–385. [PubMed: 18508952]
15. Chau LY. Iron and atherosclerosis. *Proc Natl Sci Counc Repub China B*. 2000; 24:151–155. [PubMed: 11087066]
16. Yamaguchi K, Mandai M, Toyokuni S, Hamanishi J, Higuchi T, Takakura K, Fujii S. Contents of Endometriotic Cysts, Especially the High Concentration of Free Iron, Are a Possible Cause of Carcinogenesis in the Cysts through the Iron-Induced Persistent Oxidative Stress. *Clinical Cancer Research*. 2008; 14:32–40. [PubMed: 18172249]
17. Pennathur S, Maitra D, Byun J, Sliskovic I, Abdulhamid I, Saed GM, Diamond MP, Abu-Soud HM. Potent antioxidative activity of lycopene: A potential role in scavenging hypochlorous acid. *Free Radic Biol Med*. 49:205–213. [PubMed: 20388538]
18. Pullar JM, Vissers MCM, Winterbourn CC. Living with a killer: The effects of hypochlorous acid on mammalian cells. *IUBMB Life*. 2000; 50:259–266. [PubMed: 11327319]
19. Malech HL, Gallin JI. Current concepts: Immunology - Neutrophils in human diseases. *New England Journal of Medicine*. 1987; 317:687–694. [PubMed: 3041216]
20. Malle E, Buch T, Grone HJ. Myeloperoxidase in kidney disease. *Kidney Int*. 2003; 64:1956–1967. [PubMed: 14633118]
21. Ohshima H, Tatemichi M, Sawa T. Chemical basis of inflammation-induced carcinogenesis. *Archives of Biochemistry and Biophysics*. 2003; 417:3–11. [PubMed: 12921773]
22. Schiller J, Fuchs B, Arnhold J, Arnold K. Contribution of reactive oxygen species to cartilage degradation in rheumatic diseases: Molecular pathways, diagnosis and potential therapeutic strategies. *Current Medicinal Chemistry*. 2003; 10:2123–2145. [PubMed: 12871089]
23. Carter P. Spectrophotometric determination of serum iron at the submicrogram level with a new reagent (ferrozine). *Anal Biochem*. 1971; 40:450–458. [PubMed: 5551554]
24. Wang L, Bassiri M, Najafi R, Najafi K, Yang J, Khosrovi B, Hwang W, Barati E, Belisle B, Celeri C, Robson MC. Hypochlorous acid as a potential wound care agent: part I. Stabilized hypochlorous acid: a component of the inorganic armamentarium of innate immunity. *J Burns Wounds*. 2007; 6:e5. [PubMed: 17492050]
25. Zhang G, Dasgupta PK. Hematin as a peroxidase substitute in hydrogen peroxide determinations. *Analytical Chemistry*. 1992; 64:517–522. [PubMed: 1575321]
26. de Villiers KA, Kaschula CH, Egan TJ, Marques HM. Speciation and structure of ferriprotoporphyrin IX in aqueous solution: spectroscopic and diffusion measurements demonstrate dimerization, but not mu-oxo dimer formation. *J Biol Inorg Chem*. 2007; 12:101–117. [PubMed: 16972088]
27. Ozaki SI, Dairaku C, Kuradomi Y, Inoue J. The reaction of hemoproteins with hypochlorous acid. *Chemistry Letters*. 2008; 37:666–667.
28. Bonini MG, Siraki AG, Atanassov BS, Mason RP. Immunolocalization of hypochlorite-induced, catalase-bound free radical formation in mouse hepatocytes. *Free Radic Biol Med*. 2007; 42:530–540. [PubMed: 17275685]
29. Floris R, Wever R. Reaction of myeloperoxidase with its product HOCl. *Eur J Biochem*. 1992; 207:697–702. [PubMed: 1321719]
30. Iakutova E, Osipov AN, Kostenko OV, Arnkhol'd I, Arnol'd K, Vladimirov Iu A. Interaction of hypochlorite with oxyhemoglobin leads to liberation of iron in a catalytically active form]. *Biofizika*. 1992; 37:1021–1028. [PubMed: 1338491]
31. Schaefer WH, Harris TM, Guengerich FP. Characterization of the enzymatic and nonenzymatic peroxidative degradation of iron porphyrins and cytochrome P-450 heme. *Biochemistry*. 1985; 24:3254–3263. [PubMed: 3927975]
32. He K, Bornheim LM, Falick AM, Maltby D, Yin H, Correia MA. Identification of the heme-modified peptides from cumene hydroperoxide-inactivated cytochrome P450 3A4. *Biochemistry*. 1998; 37:17448–17457. [PubMed: 9860860]
33. Kikuchi G, Yoshida T, Noguchi M. Heme oxygenase and heme degradation. *Biochem Biophys Res Commun*. 2005; 338:558–567. [PubMed: 16115609]

34. Dwyer BE, Stone ML, Zhu X, Perry G, Smith MA. Heme deficiency in Alzheimer's disease: a possible connection to porphyria. *J Biomed Biotechnol.* 2006; 2006:24038. [PubMed: 17047301]
35. Chernova T, Nicotera P, Smith AG. Heme deficiency is associated with senescence and causes suppression of N-methyl-D-aspartate receptor subunits expression in primary cortical neurons. *Mol Pharmacol.* 2006; 69:697–705. [PubMed: 16306232]
36. Ferreira GC. Heme biosynthesis: biochemistry, molecular biology, and relationship to disease. *J Bioenerg Biomembr.* 1995; 27:147–150. [PubMed: 7592561]
37. Wijayanti N, Katz N, Immenschuh S. Biology of heme in health and disease. *Curr Med Chem.* 2004; 11:981–986. [PubMed: 15078160]
38. Ye W, Zhang L. Heme deficiency causes apoptosis but does not increase ROS generation in HeLa cells. *Biochem Biophys Res Commun.* 2004; 319:1065–1071. [PubMed: 15194476]
39. Han AP, Fleming MD, Chen JJ. Heme-regulated eIF2 α kinase modifies the phenotypic severity of murine models of erythropoietic protoporphyria and beta-thalassemia. *J Clin Invest.* 2005; 115:1562–1570. [PubMed: 15931390]
40. Braun V. Protein structure - Pumping iron through cell membranes. *Science.* 1998; 282:2202–2203. [PubMed: 9890828]
41. Weinberg ED. Microbial pathogens with impaired ability to acquire host iron. *Biometals.* 2000; 13:85–89. [PubMed: 10831229]
42. Hantke K. Iron and metal regulation in bacteria. *Current Opinion in Microbiology.* 2001; 4:172–177. [PubMed: 11282473]
43. Lizzi AR, Carnicelli V, Clarkson MM, Di Giulio A, Oratore A. Lactoferrin Derived Peptides: Mechanisms of Action and their Perspectives as Antimicrobial and Antitumoral Agents. *Mini-Reviews in Medicinal Chemistry.* 2009; 9:687–695. [PubMed: 19519494]
44. Orsi N. The antimicrobial activity of lactoferrin: Current status and perspectives. *Biometals.* 2004; 17:189–196. [PubMed: 15222464]
45. Valenti P, Berlutti F, Conte MP, Longhi C, Seganti L. Lactoferrin functions - Current status and perspectives. *Journal of Clinical Gastroenterology.* 2004; 38:S127–S129. [PubMed: 15220678]
46. Sliskovic I, Abdulhamid I, Sharma M, Abu-Soud HM. Analysis of the mechanism by which tryptophan analogs inhibit human myeloperoxidase. *Free Radic Biol Med.* 2009; 47:1005–1013. [PubMed: 19596067]
47. Galijasevic S, Abdulhamid I, Abu-Soud HM. Melatonin is a potent inhibitor for myeloperoxidase. *Biochemistry.* 2008; 47:2668–2677. [PubMed: 18237195]
48. Galijasevic S, Abdulhamid I, Abu-Soud HM. Potential role of tryptophan and chloride in the inhibition of human myeloperoxidase. *Free Radic Biol Med.* 2008; 44:1570–1577. [PubMed: 18279680]
49. Lu T, Galijasevic S, Abdulhamid I, Abu-Soud HM. Analysis of the mechanism by which melatonin inhibits human eosinophil peroxidase. *Br J Pharmacol.* 2008; 154:1308–1317. [PubMed: 18516076]
50. Galijasevic S, Maitra D, Lu T, Sliskovic I, Abdulhamid I, Abu-Soud HM. Myeloperoxidase interaction with peroxynitrite: chloride deficiency and heme depletion. *Free Radic Biol Med.* 2009; 47:431–439. [PubMed: 19464362]

B

Fig. 1.
Structures of Ht (A) and PPIX (B).

Fig. 2. HOCl causes heme degradation. Diode array stopped-flow experiment was carried out by rapid mixing of a buffer solution containing 5 μM Ht with an equal volume of buffer solution containing 140 μM HOCl, at 10 $^{\circ}\text{C}$, and successive full wavelength scans (from 300-700 nm). The arrows indicate the position of the Soret absorbance peaks of both the monomeric and dimeric forms of Ht (360 and 394 nm, respectively), as well as, the direction of the spectral changes. The time of each collected spectrum after initiation of the reaction is indicated in seconds. The data are representative of three independent experiments.

Fig. 3. Stopped-flow traces for the reaction of Ht with HOCl. The reaction was monitored by following the decrease in absorbance at 394 nm over time. Experiment was initiated by rapid mixing a buffer solution containing 5 μM Ht with an equal volume of buffer solution containing 160 μM HOCl, at 10 $^{\circ}\text{C}$. The inset depicts the absorbance change that occurred within the first 60 s of the reaction. The results shown are representative of three independent experiments.

Fig. 4. Rates of Ht-OCI complex formation from Ht monomer (A) and from Ht μ -oxo dimer (B) along with the rate of HOCl mediated heme degradation (C), as in Fig. 3, as a function of HOCl concentration. Experiments were performed in triplicate. The error bars represent the standard error of measurements.

Fig. 5.

HOCl-mediated free iron release in Ht. Ht (25 μM) was incubated with increasing molar ratios of HOCl for 10 minutes and the accumulation of free iron was measured by ferrozine method. The inflection point (which occurs at 11:1 HOCl:Ht ratio) is marked by the dotted line. The data are average of three independent experiments and the error bars represent the standard error of measurement.

Fig. 6. HOCl mediated Ht destruction and subsequent generation of fluorescent heme degradation products. Ht (25 μ M) was reacted with a range of molar ratios of HOCl and the reaction products were analyzed by HPLC with fluorescent detection (excitation 321 nm and emission 465 nm). The HOCl:Ht ratios are mentioned in each panel. The data shown are representative of three independent experiments.

Fig. 7.

Treatment of PPIX with HOCl led to the generation of fluorescent degradation products similar to those obtained from Ht. PPIX (25 μ M) was reacted with a range of molar ratios of HOCl and the reaction products were analyzed by HPLC with fluorescent detection (excitation 321 nm and emission 465 nm). The HOCl:PPIX ratios are mentioned in each panel. The data shown are representative of three independent experiments.

Fig. 8. EIC and MS spectrum of the product m/z 421 identified by LC-ESI-MS (positive mode) from HOCl treatment of Ht and PPIX. The reaction mixture was separated by reverse-phase HPLC and subjected to ESI/MS as described under Materials and Methods. The major reaction product produced an intense peak at 11 min. Examination of the MS spectrum revealed that the $[M + H]^+$ ion had a m/z 421. (A) The extracted ion chromatogram and (B) the MS spectrum of this peak are depicted.

Fig. 9.

EIC and MS spectrum of the product m/z 615 identified by LC-ESI-MS (positive mode) from HOCl treatment of PPIX. The reaction mixture was separated by reverse-phase HPLC and subjected to ESI/MS as described under Materials and Methods. The major reaction product produced an intense peak at 11 min (labeled as 1) and 12 min (labeled as 2). Examination of the MS spectrum revealed that the $[M + H]^+$ ion had a m/z 615. (A) The extracted ion chromatogram and (B) and (C) the MS spectrum of peak 1 and 2, respectively, are depicted.

Fig. 10.

EIC and MS spectrum of the product m/z 581 identified by LC-ESI-MS (positive mode) from HOCl treatment of PPIX. The reaction mixture was separated by reverse-phase HPLC and subjected to ESI/MS as described under Materials and Methods. The major reaction product produced three intense peak at 10.25, 11.25 and 13.5 min, respectively. Examination of the MS spectrum revealed that the $[M + H]^+$ ion had a m/z 581. (A) The extracted ion chromatogram and (B) the MS spectrum of this peak are depicted.

Scheme 1.

Kinetic model describing the interaction of HOCl with Ht that leads to heme destruction and subsequent free iron release.

A: Mechanism for tetra-pyrrole ring cleavage **B: Mechanism for methylation** **Scheme 2.**

Proposed chemical mechanism for the HOCl mediated cleavage and subsequent methylation of the heme degradation products.

Table 1

Structures of heme degradation products tentatively identified by LC-ESI-MS after HOCl treatment of Ht and PPIX.

m/z=659, C ₃₄ H ₃₄ N ₄ O ₁₀	m/z=615, C ₃₄ H ₃₅ ClN ₄ O ₅	m/z=581, C ₃₄ H ₃₆ N ₄ O ₄

m/z=577, C ₃₅ H ₃₆ N ₄ O ₄	m/z=563, C ₃₄ H ₃₄ N ₄ O ₄	m/z=449, C ₁₅ H ₁₆ N ₂ O ₁₄	m/z=437, C ₁₅ H ₁₆ N ₂ O ₁₄

m/z=405, C ₁₉ H ₂₀ N ₂ O ₈		m/z=425, C ₁₇ H ₁₆ N ₂ O ₁₁

m/z=421, C ₁₉ H ₂₀ N ₂ O ₄		m/z=409, C ₁₇ H ₁₆ N ₂ O ₁₀	