Potential and Mechanism of Peroxynitrite to Mediate Heme Degradation

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Abstract: The reaction of heme with peroxynitrite was studied. The results showed that peroxynitrite was able to degrade heme in solution with optimum pH of 7.4 and resulted in the release of iron from heme. Degradation depended on the peroxynitrite concentration with perferryl reactive species such as sodium sulfide and peroxidase substrate (phenol), demonstrated that perferryl reactive species formation was required for heme degradation. It was inhibited by superoxide dismutase, implicating the involvement of $O_2 \cdot^-$ in the process of heme degradation. [Life Science Journal. 2006;3(2):55-60] (ISSN: 1097-8135).

Keywords: heme degradation; peroxynitrite; reaction mechanism

Abbreviations: BSA: bovine serum albumin; DTPA: diethylenetriaminepentaacetic acid; Hb: hemoglobin; metHb: methemoglobin; oxyHb: oxyhemoglobin; HSA: human serum albumin

1 Introduction

The most sizable and concentrated store of heme in the body resides is erythrocytes Hb. As long as heme is bound tightly to its hydrophobic pocket in globin, it mediates its normal physiological role in oxygen transport. However, in some pathological conditions or under oxidative stress, heme may be released and exert various noxious actions[1]. The normal red cell is endowed with efficient mechanisms to remedy these physiological deviations, but variant erythrocytes such as sickle and thalassemic cells are unable some times to do so, either because their Hb has a higher tendency to auto-oxidization or is less stable. Unstable metHb readily forms hemichromes which have a tendency to bind to the cell membrane and sometimes may release their heme. Normal Hb were also shown to release their heme to cell membranes and to liposomes made of aminophospholipids, although at a reduced extent compared with metHb or hemichromes. Heme has been shown to rapidly destabilize the bilayer structure, thus increasing its permeability to ions and leading to hemolysis[2], and to induce the peroxidation of the membrane lipids[3]. Its binding to membrane proteins diminishes their reduced thiol content and leads to cross-linking. Both latter processes are apparently due to the chronic effect of increased membrane heme. Hence, efficient mechanisms must exist to prevent the build-up of heme in membranes. Since heme is able to translocate across membranes[4], it's fate and membrane concentration are determined by the presence of various ligands, such as serum's albumin and hemopexin[5]. Altogether, it is presumed that the concentration of free heme in the membrane is physiologically kept at low (micromolar) levels[6]. In conditions that favor higher membrane heme association, the concentrations of non-heme iron also increase in the membrane[7]. The origin of this iron is not well established, but it has been suggested that it could result from the destruction of heme by organic and lipid peroxides and by H_2O_2.

Recently, peroxynitrite chemistry is of considerable interest as a result of the increasing evidence for the role of peroxynitrite in the development of oxidative damage in various pathologies[8]. Peroxynitrite is formed *in vivo* from the diffusion-controlled reaction between the nitrogen monoxide and superoxide radicals[9]. Although these precursors are relatively unreactive, peroxynitrite is a powerful oxidizing and nitrating agent that can react with biological substances such as protein[10], nucleic acid[11], lipids[12] and carbonate[13] in cells and tissues. Peroxynitrite is relatively long-lived, which allows it to reach critical targets in the cell. Our

previous studies have shown peroxynitrite can promote the conversion of oxyHb to metHb and mediate damage to porphyrin ring of Hb active center, subsequent instability and heme loss from the protein[14]. But the studies on the direct reaction between peroxynitrite and free heme have not been reported yet.

In the present work, the interactions between free heme and peroxynitrite were investigated. The experimental results showed that co-incubation of these compounds led to the destruction of heme and the generation of oxidative radicals. These results indicated that peroxynitrite interacted with heme to produce an oxidative stress and increased the iron content.

2 Materials and Methods

2.1 Chemicals

Materials were obtained from the following sources: diethylenetriaminepentaacetic acid (DTPA), heme, catalase, superoxide dismutase, bovine serum albumin (BSA), human serum albumin (HSA), EDTA, Ferrozine (3-(2-pyridyl)-5, 6-bis(4-phenylsulfonic acid)-1, 2, 4-triazine) and neocuproine (2, 9-dimethyl-1, 10-phenanthroline) from Sigma. All other chemicals were of the best available grade and have been used without further purification, unless stated otherwise, and doubly distilled water was used throughout.

2.2 Preparation for peroxynitrite

Peroxynitrite were synthesized by reaction of 0.6 mol/L nitrite with 0.7 mol/L H_2O_2 at pH 13 and characterized according to the method reported previously[14]. Excess H_2O_2 was removed by passage through a column of manganese dioxide. The desired concentration of peroxynitrite was prepared daily by the dilution of the stock solution in aqueous 0.5−1.0% NaOH and this solution was kept in an ice bath (less than 10% of peroxynitrite had decomposed over 8 h). Peroxynitrite was stored at −20 ℃ and the concentration of peroxynitrite was determined spectrally in 0.1 mol/L NaOH at 302 nm ($\varepsilon_{302} = 1,670\ mol^{-1} \cdot L \cdot cm^{-1}$) immediately prior to each experiment[15]. After 3 − 5 days at room temperature the peroxynitrite had completely decomposed; this solution was called "decomposed peroxynitrite" and has been used in blank experiments.

2.3 Investigation of the destruction of the heme molecule by peroxynitrite

Heme was prepared fresh at the beginning of each experiment as a stock solution of 1 mmol/L in of 20 mmol/L. Fresh peroxynitrite solution was prepared as a stock of in 0.1 mol/L NaOH. First, DTPA was added (1 mmol/L final concentration) to the PBS buffer pre-warmed to 25°C. Then heme was added to the desired final concentration. Finally, different amount of peroxynitrite were added to give a serials of peroxynitrite concentration. The spectral changes between 300 nm and 800 nm were measured immediately after gentle mixing in a TU-1800pc UV-vis spectrophotometer (Beijing Purkinje General Instrument Co., Ltd, China) and thereafter at 100-s intervals using 1 cm light path quartz cuvette. All pH values were measured with a pH S-301 digital ion meter.

The absorbance at 390 nm was recorded after 30 min reaction. The pH dependence of heme degradation by peroxynitrite was measured as described above, in PBS adjusted to the desired pH. The pH was determined at the beginning and at the end of the reaction and was found to be constant. It was ascertained that heme did not precipitate out of solution during the measurement.

The effect of sodium sulfide, phenol, DTPA, EDTA, benzoic acid, BSA, HSA, superoxide dimutase and catalase on the heme degradation was also studied. These agents were added to heme 15min before initiating the reation with peroxynitrite.

2.4 Iron release from the heme molecule during the degradation reaction

A reaction mixture of the heme decomposing system was prepared in a final volume of 3 ml at 25 ℃. The heme concentration was 10 μmol/L, and different amounts of peroxynitrite were added. DTPA was not added to this reaction because it interfered with iron determination. Free iron was measured by the Ferrozine method[16]. Briefly, a 0.45 ml sample taken from the reaction mixture was mixed with 50 ml of 100% (*w*/*V*) trichloroacetic acid. Then, 0.5 ml of 0.02% ascorbic acid in 0.1 N HCl was added, the system was incubated for 5 min at room temperature, and 0.4 ml of ammonium acetate (10%) and 0.1 ml of Ferrozine solution (75 mg of Ferrozine and 75 mg of neocuproine in 25 ml water) were added. After an additional incubation for 5 min at room temperature, the color developed was measured at 562 nm.

3 Results

3.1 Degradation of heme by peroxynitrite

Mixing of heme with peroxynitrite results in a rapid decline in peak absorbance, indicating the

that degradation succeeds through various intermediate products. The final product of this reaction was obtained after substantially longer incubations (>2 h). The chemical nature of the end product (s) has not been investigated.

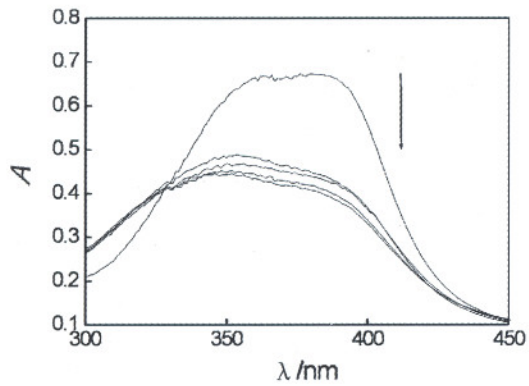

Figure 1. Heme degradation by peroxynitrite.

Heme (15 μmol/L) was mixed with 335 μmol/L peroxynitrite + 1 mmol/L DTPA in PBS, pH 7.4, and incubated at 25 ℃. Absorption spectra (300 − 450 nm) were taken at 120 sec intervals starting immediately after mixing.

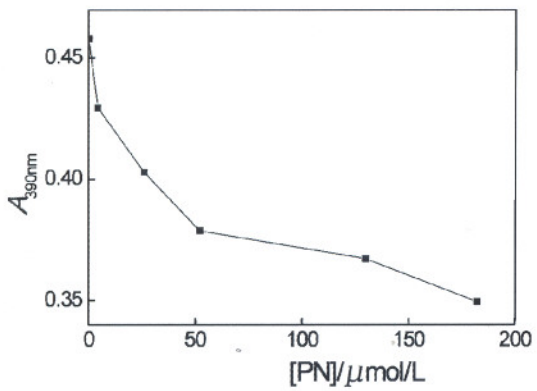

Figure 2. The dependence of heme degradation on peroxynitrite concentration.

Heme and peroxynitrite were mixed + 1 mmol/L DTPA at the desired concentrations in PBS, pH 7.4, at 25 °C, and heme degradation was monitored for 600 sec at 390 nm. [heme] = 10 μmol/L.

As shown in Figure 2, the rate of heme degradation was peroxynitrite-dependent, which are well consistent with those described for heme degradation by H_2O_2[17], but no further attempts were made to determine the precise molecular mechanism.

3.2 Effect of pH on peroxynitrite-mediated heme degradation

Heme degradation was pH-dependent in 0.1 mol/L PBS, peaking at pH 7.4 (Figure 3). The technique used for the assay of heme degradation precludes the possibility that the low rates observed at acid pH were due to precipitation of heme.

3.3 The liberation of iron from degraded heme

The decomposition of the heme molecule led to the liberation of the heme iron as determined by the Ferrozine method[16] (Figure 4). The release of heme iron was peroxynitrite-dependent.

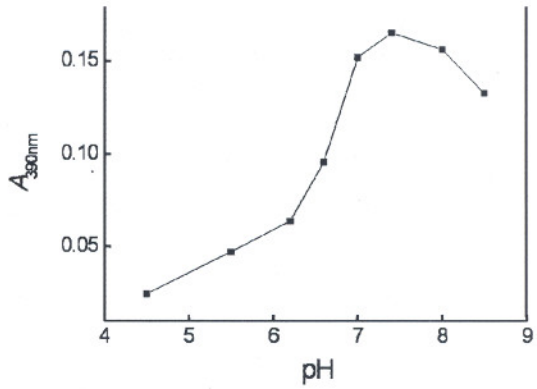

Figure 3. The dependence of heme degradation on pH.

Heme (10 μmol/L) was mixed with 250 μmol/L peroxynitrite and 1 mmol/L DTPA in PBS preset to the indicated pH. The heme degradation was determined by the decrease of the absorption at 390 nm.

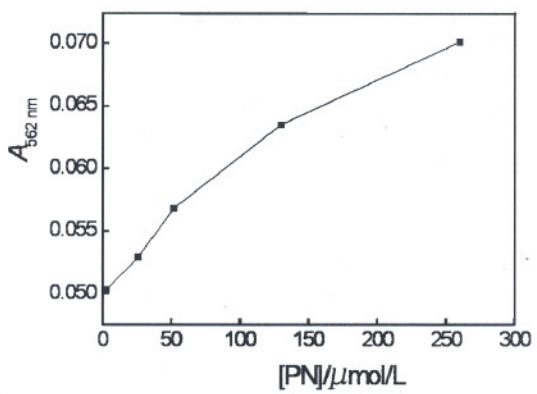

Figure 4. Iron releases from heme in the presence of peroxynitrite.

The reaction conditions were the same as described in Figure 2. The iron concentration was determined by the Ferrozine method.

3.4 Effect of various treatments on peroxynitrite-mediated heme degradation

The effect of prior addition of various reagents on the heme absorption was investigated. Reagents which reacted with ferryl reactive species were inhibited. Thus, sodium sulfide inhibited about 52% heme degradation. While phenol inhibited about 68% heme degradation. DMSO and benzonic acid, putative ·OH radical quencher, had no significant effect on the absorption. Two iron chelators, EDTA and DTPA, had no effect on the absorption.

This implies that ·OH radicals and free iron were not necessarily involved in the heme degradation. In spite of this fact, we included DTPA regularly in order to discard any nonspecific heavy metal-catalyzed peroxynitrite oxidation reactions.

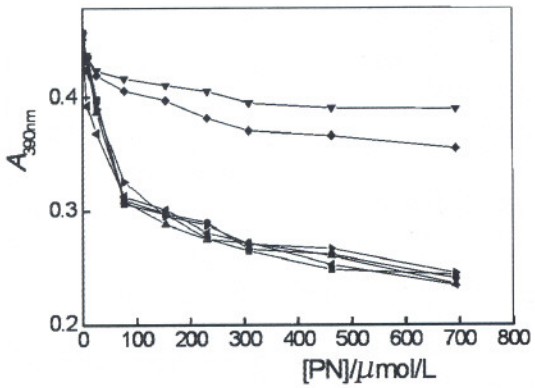

Figure 5. Effect of various treatments on the heme degradation.

The assay mixture contained 10 μmol/L and different amount of peroxynitrite in 0.1 mmol/L PBS (pH 7.4) in a total volume of 3 mL. The reaction was performed at pretreatment, (■) no treatment, (●) EDTA (0.1 mmol/L), (▲) DTPA (0.1 mmol/L), (▼) phenol (1 mmol/L), (◆)Na_2S (1 mmol/L), (▶) benzoic acid (1 mmol/L), (◀)DMSO (1 mmol/L). Absorption at 390 nm was recorded after reaction for 10 min.

Degradation also occurred when heme was bound nonspecifically to protein. Heme was complexed with defatted BSA and HSA in PBS by mixing the two compounds for 30 min at room temperature at a molar ratio of 70:1 (heme:albumin) and then subjected to the same spectrophotometric assay in the presence of peroxynitrite as done with free heme. The degradation of protein-bound heme was somewhat lower than that of free heme (Figure 6).

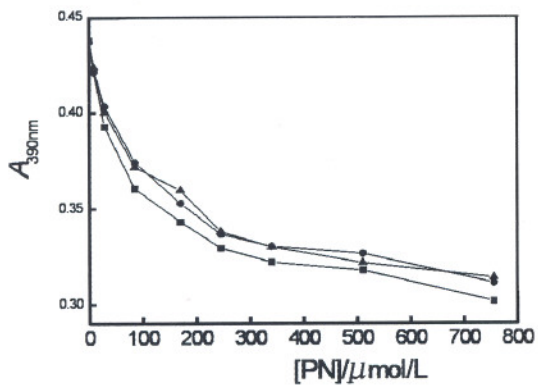

Figure 6. Effect of albumin on the heme degradation mediated by peroxynitrite.

The reaction conditions were the same as described in Figure 5. The reaction was performed at pretreatment, (■) no treatment, (●) BSA, (▲) HSA. Absorption at 390 nm as recorded after reaction 10 min.

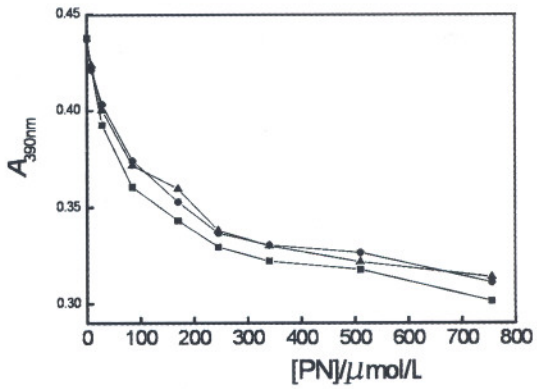

Figure 7. Effect of CAT and SOD on the heme degradation.

The reaction conditions were the same as described in Figure 5. The reaction was performed at pretreatment, (■) no treatment, (●) SOD (40mg/mL), (▲) CAT (128 U/mL). Absorption at 390 nm was recorded after reaction 10 min.

Superoxide dismutase (40 mg/mL) inhibited heme degradation by 46%. While catalase (128 U/mL) had no significant influence on the heme degradation (Figure 7).

4 Discussions

Structural changes in the globin molecule may lead to serious modifications of the hydrophobic heme binding pocket[18], ensuing in the loss of heme to other cell components, mainly to the membrane compartment of the red cell. High heme levels were detected in the membrane of abnormal red cell, such as b-thalassemic[19] and sickle cells[6,7]. In parallel, a significant phospholipid-bound fraction of non-heme iron was detected in the membranes of these cells[7,20]. Iron decompartmentalization was recently suggested to be an important feature of abnormal red cell and an important cause for cell lysis[21]. Knowledge about the mechanism which leads to the release of free iron detected in abnormal red cell is scarce. Iron can be released from hemoglobin or heme molecules by hydroperoxides[21]. The parallel increase of membrane heme and non-heme iron concentrations suggests that iron derives from heme[7], but the mechanism responsible for this phenomenon, or the elimination of excess intracellular heme altogether, remains an enigma.

Our previous studies have shown that heme moiety is partially degraded during the reaction of peroxynitrite with oxyHb. We have now established the reactions responsible for free heme degradation. The interaction between hemeproteins and peroxynitrite has been known for some time[22], but here we show directly, and for the first time,

that the interaction of free heme and peroxynitrite leads to a destruction of the tetrapyrrole ring of heme. This is evidenced by the unique absorbance of the final product of the reaction, the peroxynitrite dependence of this process (Figure 2), and the release of iron from the destroyed tetrapyrrole ring (Figure 4). We have observed that the degradation of heme is maximal at pH 7.4 and almost undetectable in PBS at pH 5 (Figure 3). The conclusion of these experiments is that peroxynitrite-dependent heme degradation occurs at physiological conditions, even when heme is bound nonspecifically to protein.

The decomposition of heme by peroxynitrite was not affected by traces of free iron which usually contaminate various chemicals, since no effect was seen in the presence of the iron chelators DTPA and EDTA, suggesting that iron released due to heme decomposition does not participate in the destruction of the tetrapyrrole ring.

At the present time, one can only speculate about the mechanism of peroxynitrite-dependent heme degradation. The oxidation of the various substrates by peroxynitrite ($OONO^-$/ONOOH) can take place via multiple pathways[15] (Scheme 1): (i) Peroxynitrite may directly oxidize the substrates. (ii) Peroxynitrite may decompose firstly into highly reactive species ($\cdot OH$, $\cdot NO_2$), which subsequently oxidizes the substrate or hydroxylates and nitrates aromatic compound. In the present study, the possible mechanism of the oxidation of heme by peroxynitrite was studied. Firstly, DMSO and benzoic acid (1 mmol/L), the specific scavenger for $\cdot OH$, was introduced to the reaction mixture before the addition of peroxynitrite, and the absorption of the system was almost unchanged compared with that in the absence of DMSO or benzoic acid, proving that $\cdot OH$ does not mediate the absorbance decrease of the system. Second, when SOD (40 mg/mL), a specific scavenger for $O_2^{\cdot -}$, was added to the reaction system, we found that the absorbance decrease of the reaction mixture was inhibited 46%, indicating that $O_2^{\cdot -}$, contribute to the absorbance increase of the system. As mentioned above, peroxynitrite predominantly reacted with hemoglobin in peroxynitrite anion form.

$$ONOO^- + H^+ \rightleftharpoons ONOOH \quad (1)$$

$$ONOO^- \rightleftharpoons \cdot NO + O_2^{\cdot -} \quad (2)$$

$$ONOOH \rightleftharpoons \cdot OH + \cdot NO_2^- \quad (3)$$

Scheme 1

Ferrylheme, is an intermediate of reaction. The requirement for ferrylheme is indicated by the dramatic inhibition of heme degradation when ferrylheme reacts with peroxidase substrates phenol or sodium sulfide. Thus, the peroxidase substrates, phenol, which reduce ferrylheme to metheme, completely inhibit the heme degradation, and sodium sulfide, which reacts with ferrylheme to produce sulfheme, results in an inhibition of heme degradation. These results indicated that heme degradation involved reactions of ferrylheme and was not the primary reaction of peroxynitrite with heme.

In conclusion, the present investigation indicates that heme is decomposed by peroxynitrite, either when heme is free or bound to proteins. During this process, heme is oxidized, oxidative radicals are produced, and iron is released. Further research is needed in order to verify the nature of the degradation products of the tetrapyrrole ring, and the types of the free radicals generated during peroxynitrite-dependent heme degradation. However, the elucidation of this mechanism and the identification of the reaction products are not pertinent to the present work which seeks to investigate the ability of peroxynitrite mediates heme degradation and effect of various pretreatments on heme degradation mediated by peroxynitrite.

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