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Tyrosine residues play an important role in heme detoxification by serum albumin

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ABSTRACT

Background: Serum albumin binds avidly to heme to form heme–serum albumin complex, also called methemalbumin, and this binding is thought to protect against the potentially toxic effects of heme. However, the mechanism of detoxification has not been fully elucidated.
Methods: SDS-PAGE and Western blot were used to determine the efficiency of methemalbumin on catalyzing protein carbonylation and nitration. HPLC was used to test the formation of heme to protein cross-linked methemalbumin.
Results: The peroxidase activity of heme increased upon human serum albumin (HSA) binding. Methemalbumin showed higher efficiency in catalyzing tyrosine oxidation than free heme in the presence of H_2O_2. Methemalbumin catalyzed self-nitration and significantly promoted the nitration of tyrosine in coexistent protein, but decreased the carbonylation of coexistent protein compared with heme. The heme to protein cross-linked form of methemalbumin suggested that HSA trapped the free radical accompanied by the formation of ferryl heme. When tyrosine residues in HSA were modified by iodination, HSA lost of protection effect on protein carbonylation. The low concentration of glutathione could effectively inhibit tyrosine nitration, but had no effect on protein carbonylation.
Conclusion: HSA protects against the toxic effect of heme by transferring the free radical to tyrosine residues in HSA, therefore protecting surrounding proteins from irreversible oxidation, rather than by direct inhibiting the peroxidase activity. The increased tyrosine radicals can be reduced by endogenic antioxidants such as GSH.
General significance: This investigation indicated the important role of tyrosine residues in heme detoxification by HSA and suggested a possible novel mechanism.

1. Introduction

In some pathological states, including intravascular or massive extravascular hemolysis, heme is released from hemoglobin and causes damaging effects. Free heme promotes lipid peroxidation, and has been suggested to contribute to protein and DNA damage [1]. Heme detoxification serves as a protective mechanism for cell survival in the redox environment produced by heme and is mediated by the heme oxygenase (HO) and extra-HO (albumin, hemopexin, etc.) systems. Hemopexin is the strongest heme-binding protein in plasma [2], but its concentration is low (10–20 μM). Thus, heme binding to serum albumin (SA) occurs frequently under conditions of excessive hemolysis when hemopexin is saturated.

Serum albumin is the most abundant protein in plasma and has antioxidant properties arising from its extraordinary ligand-binding capacity [3]. Ferric heme binds strongly ($K_d = 10^{-8}$ M; pH = 7 and 24 °C) to HSA at a specific binding site located within subdomain IB of the molecule [4,5]. Albumin conserves the porphyrinic iron and channels it to a specific catabolism site and is thought to detoxify free heme by forming a non-toxic heme-complex [1,6]. Grinberg et al. [6] proposed that albumin complexation with heme could prevent the toxic effects of extracellular heme by inhibiting its peroxidative activity. It was found that heme–albumin infusions were very safe and effective in replenishing deficient heme pools [7]. Kamal et al. [8] showed that the heme–HSA complex exhibited higher peroxidative activity compared to heme alone. The (pseudo-)enzymatic activity of methemalbumin has been wildly studied [9–13] and shown that methemalbumin exhibits weak catalase and peroxidase activities [9] as reported for sperm whale myoglobin (Mb) and human hemoglobin (Hb) [14]. However, the relevance of the peroxidase activity of methemalbumin to the preventive role of HSA on the toxic effect of heme has not been fully elucidated. This work was to explore how the tight binding of HSA prevents heme toxicity.

Abbreviations: 3-NT, 3-Nitrotyrosine; AAP, 4-Aminoantipyrine; DNP, Dinitrophenol; DNPH, 2,4-Dinitrophenylhydrazine; DTNB, 5,5′-Dithiobis-(2-nitrobenzoic acid); Hb, Hemoglobin; HRP, Horseradish peroxidase; HSA, Human serum albumin; HSA-T, HSA treated with iodine to modify tyrosine residues; Mb, Myoglobin; PBS, Phosphate buffered saline; SA, Serum albumin; SDS-PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis; TMB, 3,3′,5,5′-Tetramethylbenzidine

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The reaction of ferric heme with H_2O_2 generated higher oxidation ferryl and, presumably, a cation radical on the porphyrin ring. In myoglobin (Mb), catalase and horseradish peroxidase (HRP), transient formation of an oxidized porphyrin cation radical has been detected [15]. However, the free radical can migrate within the protein, forming globin free radicals detectable on tyrosine residues [16]. Uncontrolled free radical chain reactions can result in oxidative modification to heme protein itself or nearby biomolecules.

In the present work, to investigate the mechanism by which HSA detoxifies free heme, enolase was used as a coexistent model protein to determine the efficiencies of heme and methemalbumin in catalyzing protein carbonylation and nitration reactions in the presence of H_2O_2 and nitrite. The heme to protein cross-linked forms of methemalbumin was identified by HPLC. To explore the role of tyrosine residues in heme detoxification by SA, the tyrosine residues of HSA were modified by iodine to block the generation of tyrosyl radicals. From these studies, a novel mechanism was proposed for heme detoxification by HSA.

2. Materials and methods

2.1. Materials

Hemin (Ferriprotoporphyrin IX chloride), human serum albumin (fatty acid and globulin free, Sigma A3782), 3,3′,5,5′-tetramethylbenzidine (TMB), enolase from Baker's yeast, 2,4-dinitrophenylhydrazine (DNPH), glutathione (GSH), rabbit polyclonal antibody against 3-nitrotyrosine (3-NT) and dinitrophenol (DNP) were purchased from Sigma. Horseradish peroxidase-conjugated ImmunoPure goat anti-rabbit IgG was purchased from Pierce. HPLC grade acetonitrile and trifluoroacetic acid were purchased from Fisher Scientific. All solvents and other reagents were the highest purity commercially available.

2.2. UV–visible absorption spectroscopy

Heme stock solutions were prepared in dimethyl sulfoxide and were further diluted in 100 mM phosphate buffered saline (PBS, pH 7.4) immediately before use. The precise concentration of heme in PBS was determined using an extinction coefficient of $5.84 \times 10^4\ cm^{-1}\ M^{-1}$ at 410 nm [17]. Methemalbumin was prepared by incubating 10 μM heme and HSA in a 1:1 molar ratio in 100 mM PBS (pH 7.4) at 37 °C for 15 min. The scanning spectra of heme in the absence or presence of HSA were recorded between 300 and 700 nm on a UV2550 spectrophotometer at 25 °C using a quartz cell with 1 cm path length. The spectral changes were monitored to confirm the formation of the complex.

2.3. Determination of peroxidative activities

TMB, a classical substrate for peroxidases, was employed for peroxidative activity assays. Activity was measured by monitoring the increase in TMB oxidation product absorbance at 652 nm ($\varepsilon_{625} = 3.9 \times 10^4\ M^{-1}\ cm^{-1}$) [18]. Reactions were initiated by the addition of H_2O_2. The reaction mixtures contained the following reagents in 50 mM citric acid buffer (pH 5.0): 400 nM heme, 1 mM TMB and 20–500 mM H_2O_2 for assays with heme alone or 400 nM heme–HSA, 0.1 mM TMB and 1–10 mM H_2O_2 for assays with heme–HSA.

2.4. Fluorescence measurements

Dityrosine fluorescence was determined by excitation at 325 nm and monitored from 350–500 nm with 5 nm gap widths. The fluorescence intensity was measured at 414 nm every 5 s. Heme-induced dityrosine formation was monitored upon the addition of 200 μM H_2O_2 to a solution of 1 μM heme or heme–HSA and 200 μM tyrosine in 100 mM PBS, pH 7.4, at room temperature.

2.5. Detection of protein nitration and carbonylation by Western blot

A heme (or heme–HSA)–H_2O_2–NO_2^- system was employed to study the modification of enolase, an enzyme that is the target of protein nitration in many diseases [19,20]. Samples were prepared by incubating 1 μM heme or heme–HSA with 500 μM NO_2^-, 500 μM H_2O_2 and 0.1 μg/μl enolase in 100 mM PBS (pH 7.4) for 60 min at 37 °C. To test the antioxidant activity, enolase was pre-incubated (5 min at 37 °C) with different concentrations of GSH.

To detect protein carbonylation, samples were denatured and derivatized with 10 mM DNPH in the presence of 3% SDS. After incubation for 30 min at room temperature with occasional stirring, an equal volume of neutralization solution (2 M Tris, 30% glycerol) was added to stop the reaction. Then, samples were mixed with loading buffer and subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). For detection of protein tyrosine nitration, samples (2.8 μg of enolase) were directly mixed with loading buffer and subjected to 12% SDS-PAGE. After electrophoresis, proteins were transferred to a nitrocellulose membrane using a semi-dry transfer apparatus. Proteins were immunoblotted first with a rabbit polyclonal antibody against 3-NT (1:1000) or DNP (1:3000) and then with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5000). Finally, chemiluminescence was measured using the ECL system (Pierce) to identify specific proteins.

2.6. Measurement of heme to protein cross-linking

The formation of covalent heme: protein cross-links was determined by reversed phase HPLC as described previously [21]. Methemalbumin (100 μM) was reacted with H_2O_2 (100 μM) at 37 °C pH 5 (25mM sodium acetate, containing 100 μM DTPA) for 30 min. Samples were analyzed on an Agilent HP1100 HPLC fitted with a diode array spectrophotometer. A Zorbax StableBond 300 C3 reverse phase column was used (150 × 4.6 mm, 5 μm pore size). Solvents used were: A, 0.1% trifluoroacetic acid (TFA); B, Acetonitrile containing 0.1% TFA. Elution profile was 0–10 min; 35% B, 10–15 min; 35% B increasing to 37% B, 15–16 min; 37% B increasing to 40% B, 16–20; min 40% B increasing to 43% B, steady for 5 min then increasing to 95% B 25–30 min. Column temperature was 25 °C and pump flow rate was 1 ml min^{-1}. Injections of sample were 25 μl.

2.7. Modification of the tyrosine residues by iodine

Tyrosine residues in HSA were iodinated with iodine according to the protocol outlined in reference [22]. HSA (250 mg) was dissolved in 25 ml of 0.1 M borate/0.01 M carbonate buffer (pH 9.5). The protein solution was cooled down in an ice bath, and 2.98 ml of ice-cold iodine solution (0.05 M I_2 in 0.24 M KI) was added. This concentration of iodine was selected on the basis of the amino acid composition of HSA (18 mol tyrosine per mol protein) to ensure that all tyrosine residues were modified. The reaction was allowed to proceed on ice for 30 min, after which excess iodine was removed by centrifugation (4000 *g*, 15 min) through Centricon filters (30,000 MW cut off, Millipore). The protein samples were subsequently washed twice with 10 ml of buffer and centrifuged in an identical fashion. The concentrated protein solution was eluted with PBS buffer on an AKTA purifier (GE Healthcare). The concentrations of protein samples were determined using the BCA Protein Assay (Pierce, BCA Protein Assay Reagent).

2.8. Statistical analysis

Experimental values are means ± SD of the number of experiments indicated in the legends. Significance was assessed by using the Student's *t*-test ($P < 0.05$ as significant).

3. Results

3.1. Peroxidase activity

To further study the mechanism by which HSA modulates the peroxidase activity of heme, we carried out kinetic studies on the oxidation of TMB by H_2O_2 under various conditions. The apparent K_M and k_{cat} values extrapolated from a Lineweaver–Burk plot obtained over a limited range of H_2O_2 concentrations are given in Table 1. The K_M for H_2O_2 binding to methemalbumin was much lower than for binding to free heme, indicating that H_2O_2 has more affinity for the former species. Accordingly, methemalbumin has a higher molar catalytic activity than free heme, although it is very small relative to that of HRP [23]. These kinetic data suggest that HSA enhances, rather than inhibits, the peroxidase activity of heme.

3.2. Oxidation/nitration of tyrosine induced by heme/heme–HSA complex

The enzymatic oxidation of tyrosine by peroxidases in the presence of hydrogen peroxide produces tyrosyl radicals, which react to yield dityrosine. By monitoring the formation of dityrosine, it was shown that the rate of heme-induced oxidation of tyrosine by H_2O_2 increased in the presence of HSA (shown in Fig. 1). This result also indicated that the intrinsic pro-oxidative activity of heme was enhanced by its interaction with HSA. Given that the dimerization of HSA tyrosine residues was not detected in the absence of free tyrosine (data not shown), the contribution of HSA to increases in fluorescence intensity can be ignored.

Catalysis by heme-containing proteins in the presence of H_2O_2 and nitrite is considered to be the primary mechanism of tyrosine nitration in vivo [24,25]. Therefore, we measured the catalytic efficiencies of heme and the heme–HSA complex for protein nitration in the presence of H_2O_2 and nitrite. As shown in Fig. 2A, after incubation of heme–HSA with H_2O_2 and nitrite, pronounced protein bands characteristic of HSA (~65 kDa) and enolase (~47 kDa) were detected in the immunoblot, and the band density of HSA was significantly greater than that of enolase (lane 5). This observation suggests that methemalbumin can catalyze tyrosine nitration both on itself and on other proteins in solution. Based on its total number of tyrosine residues, enolase was used at a final concentration of 0.1 mg/ml to ensure that the total tyrosine content of enolase equaled to that of HSA in the reaction. Thus, the higher degree of nitration in HSA than in enolase indicates that tyrosine residues of HSA are the principal targets of oxidation.

3.3. Carbonylation of protein induced by heme or heme–HSA complex

Protein carbonylation, the most common irreversible reaction of protein oxidation, occurs inevitably in the oxidative damage in the heme–H_2O_2–NO_2^- system and is induced by the oxidation of amino acid side chains or by oxidative cleavage of proteins. Protein carbonylation was detected using an anti-DNP antibody to test for carbonyl/DNPH adducts. As shown in Fig. 2A, incubation with H_2O_2 and nitrite induced the carbonylation of enolase (lane 2). The enolase carbonylation may be associated with Fenton chemistry due to the presence of traces amount of transition metals (although we used deionized water from Milli Q system in all experiments, traces of transition metal may be inevitable) (Fig. 2A, lane 2). The addition of heme significantly increased the enolase carbonylation (Fig. 2A, lane 4). It seems that the overall carbonylation (total in lane 5 > total in lane 3, Fig. 2C) increased with elevated peroxidase activity. The presence of HSA both decreased the carbonylation of enolase induced by heme or only by H_2O_2 and nitrite (band of 47 kDa in lane 5 < lane 4 and that of lane 3 < lane 2, Fig. 2C). As a major antioxidant HSA has free radical-trapping properties. Therefore the decrease in carbonylation on enolase probably appears due to two reasons: (1) distribution of radicals formed in Fenton chemistry towards HSA; and (2) heme detoxification played by HSA. This result indicated that the elevated peroxidase activity specifically increased the tyrosine nitration of other proteins in solution but that oxidative damage on other amino acid (i.e., protein carbonylation) was reduced in the presence of HSA.

Table 1
Steady-state kinetic data with H_2O_2 at pH 5.0 and at 25 °C.

	Heme	Heme–HSA	HRP [23]
K_m (mM)	218.3	10.8	3.7
k_{cat} (s^{-1})	0.356	0.522	3.48×10^3

3.4. Dose effects of $NaNO_2$ and H_2O_2 on protein carbonylation and nitration induced by the heme–HSA complex

The carbonylation and nitration of enolase and HSA were detected with different concentrations of $NaNO_2$ and H_2O_2. As shown in Fig. 3, increasing the concentration of $NaNO_2$ increased the formation of 3-NT in a dose-dependent manner. In agreement with our previous study [26], carbonylation was increased at the relatively low concentrations of $NaNO_2$ but was reduced at high $NaNO_2$ concentrations (1 mM). Upon increasing the concentration of H_2O_2 from 0.2 mM to 1 mM, protein carbonylation increased while tyrosine nitration decreased, both in dose-dependent manners. This phenomenon may be due to the increasing of heme decomposition caused by increased H_2O_2, leading to more free iron ion released from heme, causing Fenton chemistry, which is closely related to protein carbonylation. The heme decomposition may also account for the decrease in 3-NT formation with increasing H_2O_2 concentrations. This result suggests that tyrosine nitration showed a stronger dependence on heme than did carbonylation.

3.5. Formation of heme to protein cross-linked heme–HSA complex

Hydrogen peroxide reacts with heme to form (initially) ferryl heme and a free radical. Under acidic conditions the protein radical can react with the protonated form of the oxo-ferryl heme (Fe^{4+}-OH^{-1}), resulting in the formation of a covalent bond between the heme porphyrin ring and the protein [27]. Fig. 4 shows the reverse phase HPLC chromatograms of heme–HSA complex measured optically at 280 nm and 400 nm. Heme and HSA separated on the column, giving two distinct elution bands. The unmodified heme component eluted at 9.9 min and could be detected at both 280 nm and 400 nm (Fig. 4A). The protein eluted at 18 min with a peak at 280 nm and no absorbance in the visible region of the spectrum. Following addition of H_2O_2 to

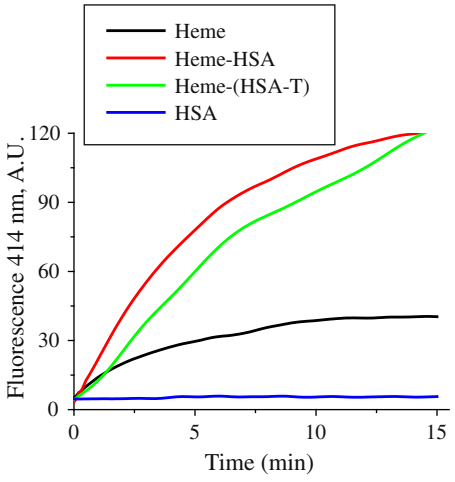

Fig. 1. Kinetics of dityrosine formation by heme or heme–HSA in the presence of Tyr and H_2O_2. The fluorescence response was monitored at 414 nm (excitation at 322 nm) upon addition of 200 μM H_2O_2 to a solution of 1 μM heme or heme–HSA and 200 μM tyrosine in 100 mM phosphate buffer, pH 7.4 at room temperature.

heme–HSA complex (1:1 ratio), the unmodified heme content decreased due to oxidation of heme. The difference was that a peak appeared at 400 nm during the protein elution time. This compound was the heme to protein cross-linked forms of heme–HSA complex. This result indicated that HSA trapped the free radical accompanied by the formation of ferryl heme. The free radical may migrate within the protein, forming tyrosyl radicals as the above detection.

3.6. Effect of HSA tyrosine modification on protein carbonylation and nitration

To study the role of tyrosine residues, the tyrosine residues in HSA were modified with iodine (abbreviate "modified HSA" to HSA-T). Iodine has the potential to react with all tyrosine residues in a protein and shows the highest affinity for those located in a hydrophobic environment [28]. Tyrosine iodination affected neither the interaction between HSA and heme (as deduced from the identical spectra before and after treatment, data not shown) nor the peroxidative activity (shown in Fig. 1). As shown in Fig. 5, modification the tyrosine residues of HSA remarkably reduced the band density of 3-NT in HSA (lane 2), indicating that HSA tyrosine residues were successfully modified by iodination. However, nitration was increased in the co-incubated enolase.

Carbonylation was also detected in the same protein samples. In the absence of heme, protein carbonylation levels in HSA-T and HSA were similar (lanes 4 and 5; lanes 6 and 7 in Fig. 6, respectively). After incubation with heme, the intensity of the band at ~65 kDa was much greater in the treated sample (HSA-T) than in the untreated sample (lane 2 in Fig. 6). This result indicated that tyrosine residue modification redirected the elevated peroxidase activity towards the protein carbonylation. The SDS-PAGE gels shown in Figs. 5 and 6 reveal that incubation with heme–H_2O_2–NO_2^- system resulted in significant degradation of HSA-T. In addition, the band intensity of enolase was greater in the reaction with heme-(HSA-T) (lane 2) than that with heme (lane 1) or methemalbumin (lane 3). Tyrosine residue modification in HSA resulted in increased carbonylation in both HSA and enolase in the heme–H_2O_2–$NaNO_2$ system. To prevent interference by sulfhydryl groups, DTNB was used to block the sulfhydryl side chains of cysteine in both HSA and HSA-T. An identical result was obtained when sulfhydryls were blocked (data not shown).

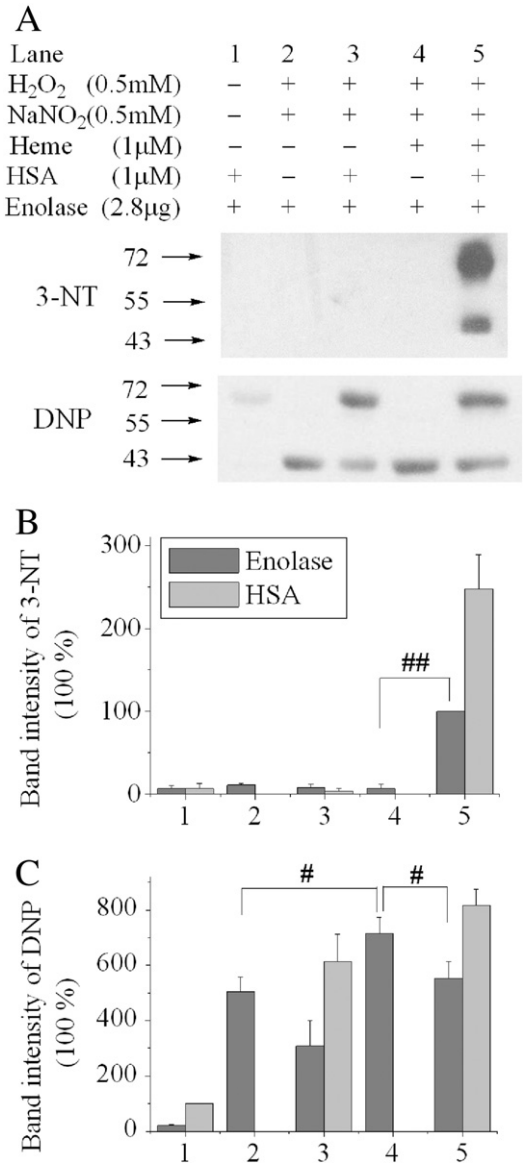

Fig. 2. Protein carbonylation and nitration induced by the heme–H_2O_2–NO_2^- or (heme–HSA)–H_2O_2–NO_2^- systems. (A) A representative immunoblot showing 3-NT formation and carbonylation. The band intensities of nitration (B) and DNP (C) are compared to control. Statistics were obtained from multiple experiments (means ± SD; n = 3; $^{\#}p < 0.05$; $^{\#\#}p < 0.01$).

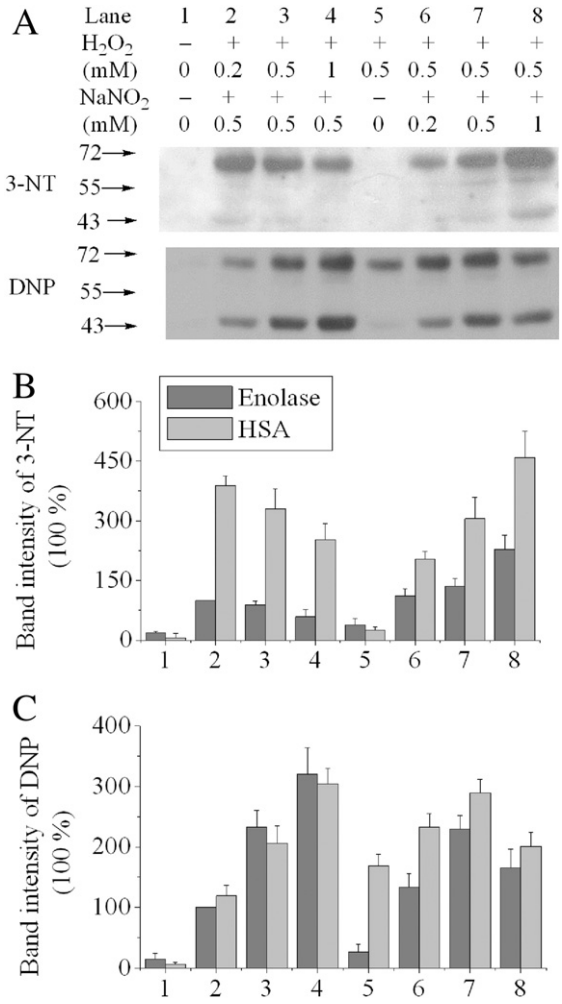

Fig. 3. Dose effects of $NaNO_2$ and H_2O_2 on protein carbonylation and nitration induced by the heme–HSA complex (A) A representative immunoblot showing 3-NT formation and carbonylation. The band intensities of nitration (B) and DNP (C) are compared to the control. Statistics were obtained from multiple experiments (means ± SD; n = 3).

3.7. Inhibition of protein nitration by GSH

The elevated tyrosine nitration induced by methemalbumin also has deleterious effect. Then we test the inhibition of protein nitration and carbonylation by glutathione, a major intracellular antioxidant. As shown in Fig. 7, protein nitration was inhibited by GSH significantly in a dose-dependence manner. However, the significantly protective effect on protein carbonylation was observed only when the concentration of GSH was up to 1 mM. This result suggested that the production of protein nitration could be efficiently inhibited by GSH at low concentration, while the high concentration of GSH was needed for the protection from protein carbonylation.

4. Discussion

The present study shows that the mechanism of HSA-mediated heme detoxification is not mediated by peroxidase inhibition. The apparent K_M and k_{cat} values for H_2O_2 indicated that heme binding to HSA increased the affinity of H_2O_2 for the iron center of heme. It is noted that heme alone has poor solubility in aqueous solutions at physiological pH, tends to form dimers or higher-order aggregates at concentrations as low as 1 μM, and rapidly undergoes oxidative degradation in the absence of albumin [29]. Heme may have two distinct fates after dissociation from Hb: insertion into hydrophobic sites such as phospholipid bilayers and hydrophobic pockets of proteins or, alternatively, heme molecules can get out of the solution by the formation of heme aggregates [30–32]. It is possible that the activity of heme in vivo could be influenced by surrounding environment. However, the toxic damage induced by heme may appear when heme inserts into the phospholipid bilayers or hydrophobic pockets of proteins. It has been demonstrated that in aqueous solutions heme frequently aggregates in the membrane and promotes oxidation [1,33]. Therefore, binding to HSA increases the solubility of heme in PBS, and, the accessibility of H_2O_2 to the iron center is possibly increased as compared with heme alone. Nevertheless, the peroxidase activity of methemalbumin is very small relative to that of HRP [23]. As the main factor limiting peroxidase activity of methemalbumin is the binding circumstance provided by HSA [9]. This result suggests, contrary to the earlier suggestion [6], that the binding of heme to HSA enhances its peroxidase activity. The measurement made in the previous study [6] was determined by monitoring the H_2O_2-induced co-oxidation of 4-aminoantipyrine (AAP) and phenol. The color products were generated by coupling AAP and phenol. However, Teng et al. [34] found that there was an interaction between AAP and SA, and we suppose that this interaction may lead to the opposite conclusion in peroxidase activity study.

The enhanced peroxidase activity is expected to generate more free radical in the presence of H_2O_2. Monzani et al. [9] showed that methemalbumin exhibits peroxidase activity in the oxidation of phenolic compounds related to Tyr (i.e., p-cresol, 3-(p-hydroxyphenyl) propionic acid, tyramine, and tyrosine). Our results with tyrosine oxidation and nitration confirm this finding. As heme is tightly bound to HSA in the solution, tyrosine residues within HSA are the first sites of oxidation, resulting in a higher degree of nitration in HSA than in enolase.

It should be noted that in heme–H_2O_2–NO_2^- system, the formation of carbonyl is inevitable [26]. Contrary to our expectations, although the peroxidase activity of heme increased significantly upon binding to HSA, protein carbonylation degree of enolase was decreased instead of enhanced. This result means that upon binding to heme, HSA can protect enolase from heme catalyzed oxidation. The explanation for this result could be the distribution of radicals towards HSA under oxidative stress. However, as the peroxidase activity was largely increased, the

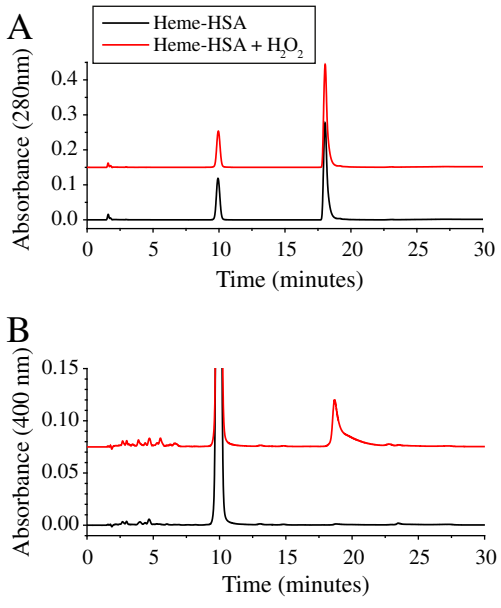

Fig. 4. Reverse phase HPLC chromatograms of methemalbumin (black line) and the one after treatment with H_2O_2 (red line). Methemalbumin (100 μM) was injected onto the HPLC. Chromatograms were measured at 280 nm (A) and 400 nm (B). Heme and HSA were separated on the column, giving two distinct elution bands (at 9.9 min and 18 min). After treatment with H_2O_2, heme to protein cross-linked methemalbumin elutes at 18 min with a peak observed at 400 nm.

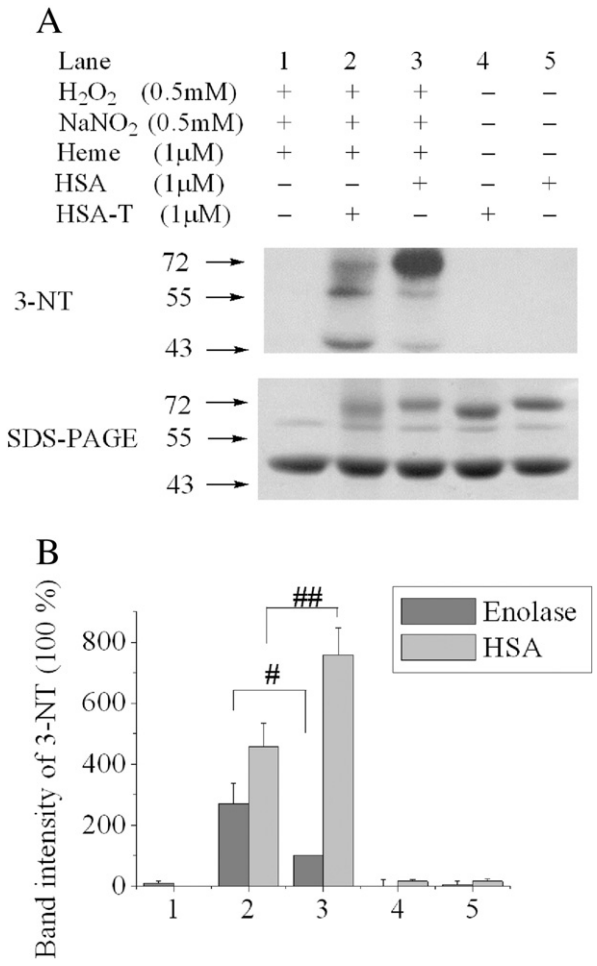

Fig. 5. Effect of HSA tyrosine modification on protein nitration. (A) An SDS-PAGE gel and a representative immunoblot of 3-NT. (B) The band intensities of nitration are compared to the control. Statistics were obtained from multiple experiments (means ± SD; n = 3; $^{\#}p < 0.05$; $^{\#\#}p < 0.01$).

level of overall carbonylation in the reaction system was not increased enough as expected. There must be another protective mechanism in the heme detoxification by HSA.

Recently study showed that haptoglobin protects against Hb-induced damage, not by preventing the primary reactivity of heme oxidants, but by stabilizing the free radical on βTyr145 [16]. The key features of heme detoxification by HSA seem to be similar to that of detoxification by haptoglobin, indicating an underlying mechanism, with tyrosine residues playing an important role. Then, to demonstrate this hypothesis, the tyrosine residues in HSA were modified with iodine. Iodination has been shown previously to result in the loss of EPR signals from tyrosine-derived radicals [35,36]. The commonly accepted mechanism for peroxidase-catalyzed tyrosine nitration involving the formation of tyrosyl radicals was consistent with the observation that tyrosine nitration in HSA was inhibited by iodination (shown in Fig. 5). When the tyrosine residues of HSA were iodinated, the effects of heme-catalyzed oxidation were redirected towards other amino acids and proteins in solution, leading to the increase of carbonylation and degradation of HSA, as well as the nitration and carbonylation of enolase (as shown in Figs. 5 and 6). It means that the detoxification role of HSA may fade out when all HSA tyrosine residues have been modified. The commonly accepted oxidation-sensitive amino acids in albumin are cysteine and methionine residues. However, there are only one cysteine and six methionine residues in HSA, the amounts of which are much less than the amount of tyrosine residues (18 tyrosine residues in HSA) [3]. Thus, tyrosine residues may be a previously unrecognized contributor to the antioxidant property of HSA. Our results suggest that the presence of the cysteine thiol in HSA has a minimal effect on the carbonylation of HSA or enolase in the presence of excess H_2O_2, perhaps because the thiol reacts with only a single molar equivalent of H_2O_2 [37,38].

The ferric (Fe^{3+}) form of the heme group reacts with the H_2O_2 to form the higher oxidation ferryl form and an associated free radical. The free radical can migrate within the heme protein or form an uncontrolled free radical chain reactions resulting oxidative modification to heme protein itself or nearby biomolecules. Peroxide-induced oxidative modifications of heme proteins can lead to the formation of a covalent bond between the heme and globin, which required both a protein-based radical and the ferryl form of the heme iron [27]. Therefore, the detectable heme to protein cross-links indicated the formation of the ferryl heme and a nearby protein radical in HSA. It has been proposed that, in the case of myoglobin, there is equilibrium between the ferryl Mb/tyrosine state and the ferric Mb/tyrosyl radical state [39]. It suggested that tyrosine functions as a redox-active center in electron transfer to ferryl heme in globin proteins. Although we cannot detect the location of protein-based radical, we postulate that the free radical may migrate within HSA and form tyrosyl radicals, thereby forbidding undesirable free radical chain reaction.

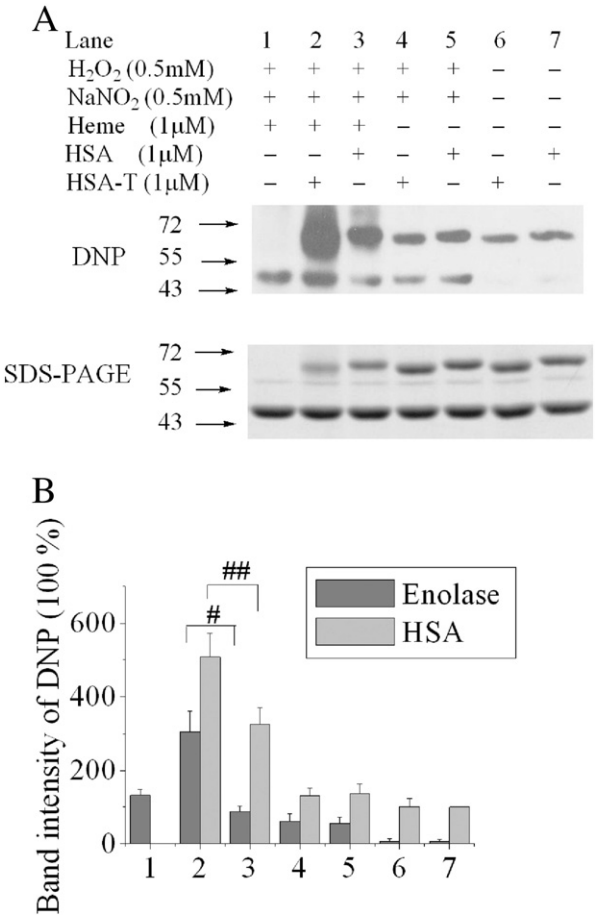

Fig. 6. Effect of HSA tyrosine modification on protein carbonylation. (A) An SDS-PAGE gel and a representative immunoblot of DNP. (B) The band intensities of DNP are compared to the control. Statistics were obtained from multiple experiments (means ± SD; n = 3; $^{\#}p < 0.05$; $^{\#\#}p < 0.01$).

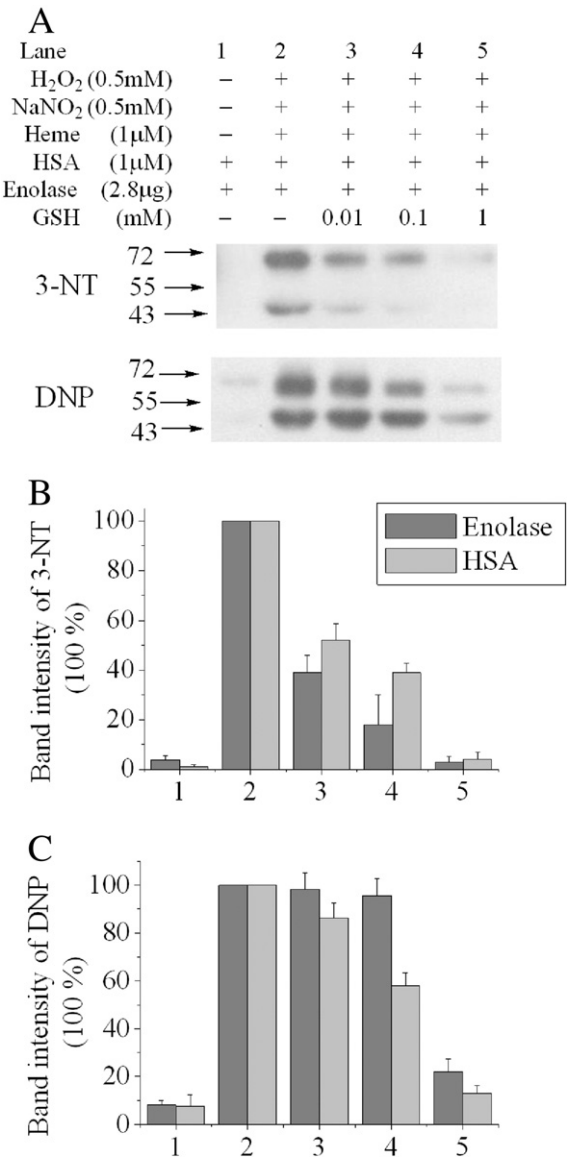

Fig. 7. Inhibition of protein nitration and carbonylation by GSH. (A) A representative immunoblot showing 3-NT formation and carbonylation. The band intensities of nitration (B) and DNP (C) are compared to the control. Statistics were obtained from multiple experiments (means ± SD; n = 3).

Since methemalbumin enhances the tyrosine nitration (probably also tyrosine oxidation) level of coexistent proteins and may damage their structure or function, the theory of heme detoxification by HSA seems incomplete. However, endogenic antioxidants, such as glutathione, are effective at quenching free tyrosyl radicals [40] and protein-tyrosyl radicals [41]. We found that tyrosine nitration can be efficiently inhibited by GSH, at concentration of as low as 10 μM (Fig. 7). Since GSH concentration is millimolar in cells and micromolar in plasma [42,43], extracellular or intracellular protein nitration can be inhibited efficiently.

In summary, although heme binding with HSA increases the reactivity with peroxide, the toxicity of elevated peroxidase activity can be ameliorated by HSA. Here we propose a novel mechanism for heme detoxification by HSA, that is, HSA inhibits destructive protein oxidation by using its tyrosine residues to scavenge the free radicals generated during heme-catalyzed peroxidation, rather than by inhibiting the peroxidative activity of heme. Meanwhile, the forming tyrosine radicals can be recovered to tyrosine by the endogenic antioxidant, such as GSH. Without tyrosine residues, the formation of protein carbonylation induced by heme is irreversible and become a permanent damage. Thus, HSA renders the resultant protein products impermanent and prevents surrounding protein from irreversible carbonylation.

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