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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.

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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 5.84×10^4 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).

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