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Peroxynitrite induced structural changes result in the generation of neo-epitopes on human serum albumin

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ABSTRACT

Human serum albumin (HSA), the most abundant plasma protein, is quite vulnerable to oxidizing and nitrating agents. In this study, peroxynitrite induced nitration and oxidation of HSA was assessed by various physicochemical techniques. Cross-linking of HSA was evident on polyacrylamide gel electrophoresis. The carbonyl content was markedly elevated in peroxynitrite-modified HSA as compared to the native protein. Dityrosine and 3-nitrotyrosine were present only in peroxynitrite-modified HSA. The peroxynitrite-modified HSA induced high titre antibodies in experimental animals showing high specificity towards the immunogen. Spectroscopic studies showed structural alterations in the HSA molecule upon peroxynitrite treatment which result in the generation of neo-epitopes and enhanced immunogenicity. The possible role of damaged HSA in various diseases has been suggested.

1. Introduction

A major and aggressive reaction of nitric oxide ($NO^{\bullet}$) is with superoxide anion ($O_2^{\bullet -}$) to form peroxynitrite ($ONOO^-$) which has a physiological half-life of approximately 1 s, as it decomposes spontaneously to give nitrate [1].

$$O_2^{\bullet -} + NO^{\bullet} \rightarrow ONOO^-$$

The possible biological significance of this reaction was first realized by Beckman and co-workers who pointed out that peroxynitrite may be formed under pathophysiological conditions, where both $NO^{\bullet}$ and $O_2^{\bullet -}$ are produced at high rates by phagocytic cells such as macrophages, and that $ONOO^-$ is a potent oxidant with the potential to destroy critical cellular components [2]. Peroxynitrite can alter a variety of biomolecules but possesses high affinity for tyrosine residues in proteins, and 3-nitrotyrosine is a relatively specific marker of peroxynitrite-mediated damage to proteins [3]. Reaction of peroxynitrite with tyrosine residues is a covalent modification that results in attachment of a nitro ($-NO_2$) group on the aromatic ring of tyrosine residues [4]. Nitrotyrosine formation is considered as a marker of nitrosative stress. More than 60 human disorders are now known to be associated with protein nitration. Indeed, the formation of protein 3-nitrotyrosine *in vivo* has been shown in a number of inflammatory conditions in human and experimental animals [5]. Peroxynitrite also causes oxidation of sulfhydryls [6]. Being an oxidizing agent, peroxynitrite also causes protein oxidation; the most prominent marker of protein oxidation is increase in the carbonyl content of proteins which is the outcome of oxidative modifications of the side chains of lysine, proline, arginine and threonine [7]. Increase in protein carbonyls has been observed in many diseases like diabetes, Alzheimer's, systemic lupus erythematosus, rheumatoid arthritis, sepsis, chronic renal failure, cancer, *etc.* [8]. Local pH and microenvironment affect the reactions of peroxynitrite, with hydrophobic membrane compartments favouring nitration and aqueous environments favouring oxidation.

Human serum albumin (HSA) is the most abundant protein found in human plasma. It is a heart-shaped single polypeptide of 66 kDa. It has 585 amino acid residues with eighteen tyrosines, six methionines, one tryptophan, seventeen disulfide bridges and only one free cysteine (Cys^{34}). HSA carries out several clearly defined physiological functions like maintenance of colloidal osmotic pressure, free radical scavenging, binding and transport of important solutes, *etc.* [9]. Several studies have shown that HSA is quite vulnerable to modification by reactive oxygen species and an elevated level of oxidized albumin is found in various diseases [10]. It has been shown that peroxynitrite preferentially reacts with the cysteine residue of HSA and causes thiol oxidation [11]. HSA is continuously exposed to oxidative stress conditions due to its abundance in the plasma, leading to the conformational and functional alterations of the protein molecule. The altered HSA may contribute to the progression of many diseases. In the present study,

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we have investigated the quantitative and qualitative changes in the structure of HSA upon reaction with peroxynitrite causing the generation of neo-epitopes in protein which in turn induce the production of highly specific antibodies in experimental animals.

2. Materials and methods

2.1. Chemicals

Human serum albumin, 3-nitrotyrosine, sodium nitrite, 1-anilinonaphthalene-8-sulphonic (ANS) acid, tyrosine, tryptophan, phenylalanine, protein A-agarose affinity column, p-nitrophenyl phosphate, Tween-20, Freund's complete and incomplete adjuvants, and 2,4-dinitrophenyl hydrazine (DNPH) were obtained from Sigma–Aldrich (St. Louis, MO). Hydrogen peroxide, sodium hydroxide, silver nitrate and guanidine hydrochloride were from Qualigens (Mumbai, India). Flat bottom polysorp ELISA modules were purchased from NUNC, Denmark. All other reagents were of the highest analytical grade available.

2.2. Peroxynitrite-modification of HSA

Peroxynitrite was synthesized by rapid quenched flow process using sodium nitrite and acidified hydrogen peroxide [12] and stored in 1.2 M NaOH at −20 °C. Before each use, concentration of stored peroxynitrite was determined from absorbance at 302 nm using molar extinction coefficient of 1670 M^{-1} cm^{-1}. HSA, at a concentration of 5 μM in phosphate buffered saline (PBS) (10 mM sodium phosphate buffer, 150 mM NaCl, pH 7.4), was incubated with 60, 125, 250, 500 and 750 μM peroxynitrite at 37 °C for 3 h. The reaction volume was made up to 3 ml with PBS. After incubation, the solutions were extensively dialyzed against PBS to remove excess peroxynitrite. To maintain the same condition for the control, unmodified HSA was also dialyzed in the same manner. The HSA concentration in both the cases was determined spectrophotometrically using a molar extinction coefficient of 35,219 M^{-1} cm^{-1} at 280 nm [13].

2.3. Absorbance spectroscopy

The absorption profiles of native and peroxynitrite-modified samples were recorded on Shimadzu UV-1700 spectrophotometer in the 200–400 nm wavelength range.

2.4. Electrophoresis

Native and peroxynitrite-modified HSA were analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) under non-reducing conditions on a 10% polyacrylamide gel, as described previously [14]. Electrophoresis was performed at 80 V for 4 h at room temperature and the protein bands in the gel were visualized by silver staining.

2.5. Fluorescence studies

Fluorescence spectra were recorded on Hitachi F-200 spectrofluorimeter at 25 ± 0.1 °C. Fluorescence of tyrosine residues in native and peroxynitrite-modified HSA was monitored after excitation at 275 nm and recording the emission in the 300–400 nm range. Loss in fluorescence intensity (FI) was calculated from the following equation:

$$\%\ \text{loss of}\ FI = \left(\frac{FI_{\text{native sample}} - FI_{\text{modified sample}}}{FI_{\text{native sample}}}\right) \times 100$$

2.6. Effective protein hydrophobicity

Binding of ANS to native and peroxynitrite-modified HSA was evaluated in terms of fluorescence. The ANS fluorescence is affected by exposure or masking of hydrophobic patches in proteins. A fresh stock solution of ANS was prepared in distilled water and its concentration was determined spectrophotometrically using a molar extinction coefficient of 5000 M^{-1} cm^{-1} at 350 nm [15]. The molar ratio of protein and ANS was 1:10 and emission spectra were recorded in the range of 400–600 nm using an excitation wavelength of 380 nm. Decrease in fluorescence intensity (FI) was calculated as follows:

$$\%\ \text{loss of}\ FI = \left(\frac{FI_{\text{native sample}} - FI_{\text{modified sample}}}{FI_{\text{native sample}}}\right) \times 100$$

2.7. Estimation of dityrosine

The dityrosine content of peroxynitrite-modified HSA was determined spectrophotometrically using a molar extinction coefficient of 4000 M^{-1} cm^{-1} at 330 nm [16]. Dityrosine formation was also confirmed by fluorescence spectra after excitation of native and peroxynitrite-modified HSA at 330 nm and measuring emission in the 350–450 nm range.

2.8. Nitrotyrosine determination

The concentration of nitrotyrosine in peroxynitrite-modified HSA was determined by measuring the absorbance at 420 nm using a molar extinction coefficient of 4300 M^{-1} cm^{-1} [17].

2.9. Determination of protein-bound carbonyl groups

Carbonyl content of native and peroxynitrite-modified HSA was determined after reaction with DNPH [18]. The final absorbance was read at 360 nm against appropriate blank. The carbonyl content was determined using a molar extinction coefficient of 22,000 M^{-1} cm^{-1} and expressed as nmol/mg protein.

2.10. Circular dichroism

CD measurements were carried out on a Jasco spectropolarimeter (J-815) equipped with a Jasco Peltier-type temperature controller (PTC-424S/15). The instrument was calibrated with D-10-camphorsulphonic acid. Spectra were taken in a cell of 1 and 10 mm path length and protein concentrations used were 4.5 and 12 μM for far and near-UV CD, respectively. The results were expressed as mean residue ellipticity (MRE) in deg cm^2 $dmol^{-1}$ which is defined as:

$$MRE = \frac{\theta_{obs}(m\,deg)}{10 \times n \times C \times l}$$

where θ_{obs} is the CD in milli-degree, n is the number of amino acid residues (585 − 1 = 584), l is the path length of the cell, and C is the concentration of protein in moles/litre. Helical content was calculated from the MRE values at 222 nm using the following equation [19]:

$$\%\ \alpha\text{-helix} = \left(\frac{MRE_{222\ \text{nm}} - 2340}{30,300}\right) \times 100$$

2.11. FTIR spectroscopy

Native and peroxynitrite-modified HSA samples were first lyophilized and prepared as KBr pellets and then subjected to spectral recording on a Tensor 37 FTIR spectrometer (Bruker).