

Regular Article

The Structural and Pharmacokinetic Properties of Oxidized Human Serum Albumin, Advanced Oxidation Protein Products (AOPP)

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Summary: To determine the pharmacokinetic properties of advanced oxidation protein products (AOPP), we prepared oxidized human serum albumin (oxi-HSA) using chloramine-T (a hypochlorite analogue) *in vitro*. The AOPP and dityrosine content of oxi-HSA (AOPP content, $244.3 \pm 12.3 \mu\text{M}$; dityrosine content, $0.7 \pm 0.11 \text{ nmol of dityrosine/mg protein}$) were similar to those of uremic patients. In structural analysis, the increases in AOPP and dityrosine content of HSA induced slight decreases in its α -helical content. In pharmacokinetic analysis, oxi-HSA left the circulation rapidly, and organ distribution of oxi-HSA 30 min after intravenous injection was 51% for the liver, 23% for the spleen, and 9% for the kidney, suggesting that the liver and spleen were the main routes of plasma clearance of oxi-HSA. The liver and spleen uptake clearance of oxi-HSA were significantly greater than those of normal HSA (CL_{liver}, 5058 ± 341.6 vs $24 \pm 4.2 \mu\text{L/hr}$ [$p < 0.01$]; CL_{spleen}, 2118 ± 322.1 vs $32 \pm 2.7 \mu\text{L/hr}$ [$p < 0.01$]). However, uptake by other organs was not significantly affected by oxidation. These results suggest that the liver and spleen play important roles in elimination of AOPP.

Key words: human serum albumin; structural change; advanced oxidation protein products; pharmacokinetics

Introduction

Recent evidence indicates that oxidative stress plays an important role in the pathogenesis of chronic renal failure (CRF).¹⁾ Oxidative stress is defined as increased production of reactive oxygen species (ROS) due to an imbalance of oxidant/antioxidant systems. Activated phagocytes are a major source of ROS, and play a fundamental role in host defense.²⁾ Neutrophils contain the heme enzyme myeloperoxidase (MPO), which catalyzes the reaction of chloride ion with hydrogen peroxide (H_2O_2), to generate the large amounts of hypochlorous acid (HOCl) produced by neutrophils.³⁾ HOCl-modified proteins have been detected in atherosclerotic lesions⁴⁾ and plasma from glomerular nephritis patients.⁵⁾ To estimate the degree of oxidant-mediated protein damage in plasma of CRF patients, researchers have assayed levels of advanced oxidation protein

products (AOPP) in CRF plasma. Witko-Sarsat *et al.* showed that *in vivo* levels of AOPP strongly correlate with creatinine clearance, indicating that AOPP are excellent markers of progression of CRF.⁶⁾ HOCl-treated HSA and *in vivo*-generated AOPP can trigger oxidative bursts in neutrophils and monocytes *in vitro*, indicating that they both can act as true inflammatory mediators.⁷⁾ Results of *in vitro* studies of mechanisms of AOPP production indicate that HOCl-treated HSA can trigger an oxidative burst.⁷⁾ However, the mechanisms by which AOPP are degraded and eliminated from circulating blood remain unclear.

Recently, there have been reports of several types of receptors that bind to modified albumin, including SR-A (human scavenger receptor class A, which binds LDL)^{8,9)} and CD36 (scavenger receptor class B family).^{10,11)} Despite such important findings, it remains unclear whether physiologically oxidized HSA (oxi-

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HSA) containing AOPP behaves in the same way as other modified albumins.

In the present study, using normal HSA and chloramine-T-treated HSA (oxi-HSA), we examined the mechanisms by which AOPP are degraded and eliminated from circulating blood.

Material and Methods

Materials and animals: Chloramine-T (CT) was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). The fluorescence probe 1, 1-bis-4-anilino-naphthalene-5,5-sulfonic acid (bis-ANS) was purchased from Sigma (St Louis, MO, USA). $^{111}\text{InCl}_3$ (74 MBq/mL in 0.02 N HCl) was donated by Nihon Medi-Physics (Takarazuka, Japan). All chemicals used were of the highest grade commercially available, and all solutions were prepared using deionized, distilled water. Male ddY mice (24–26 g) were purchased from the Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka, Japan). Animals were maintained under conventional housing conditions. All animal experiments were conducted in accordance with the principles and procedures outlined in the National Institute of Health Guide for the Care and Use of Laboratory Animals.

Preparation of oxi-HSA: HSA (300 μM) was incubated for 1 h at 37°C in an oxygen-saturated solution containing 100 mM CT in phosphate buffer (pH 8.0). After incubation, the oxidation reaction was stopped by extensive dialysis of the solution against water. As a negative control, albumin was incubated in buffer alone. The oxi-HSA and negative control HSA were stored at –20°C until used.

Western blot analysis: Oxi-HSA was detected by performing Western blot analysis. The antibodies used were an anti-HSA primary antibody raised in a rabbit and an anti-rabbit secondary antibody conjugated to horseradish peroxidase.

Determination of AOPP and dityrosine content: AOPP content of oxi-HSA was determined using a previously reported semi-automated method.⁶ Dityrosine content was assayed by measuring fluorescence after the sample was diluted to 2 μM . Fluorescence emission spectra of dityrosine were recorded at 410 nm after excitation at 325 nm, using a spectrofluorometer (FP-6200 Jasco) as described previously.¹²

Amino acid analysis: The amino acid composition of oxi-HSA was quantified by performing amino acid analysis after acid hydrolysis with 6 M HCl for 24 h at 110°C, using an amino acid analyzer (L-8500A, Hitachi, Tokyo, Japan) as described previously.¹³

Structural properties of oxi-HSA: CD spectra were obtained using a JASCO J-720 spectropolarimeter (JASCO, Tokyo, Japan) at 25°C. The effective hydrophobicity of oxi-HSA was estimated using the

fluorescent characteristics of bis-ANS (10 μM) at 25°C. Each compound was excited at 394 nm, and fluorescence spectra were recorded on a Jasco FP-770 fluorescence spectrometer (Tokyo, Japan).

In Vivo Experiments: All proteins were radiolabeled with ^{111}In using the bifunctional chelating reagent DTPA anhydride, according to the method of Hnatowich *et al.*^{14,15} Mice received tail vein injections of ^{111}In -labeled proteins in saline, at a dose of 1 mg/kg, and were housed in metabolic cages to allow the collection of urine samples. At appropriate intervals after the injection, blood was collected from the vena cava under ether anesthesia, and plasma was obtained by centrifugation. The liver, kidney, spleen, lung, heart and muscle were excised, rinsed with saline and weighed. The radioactivity of each sample was measured using a well-type NaI scintillation counter (ARC-500, Aloka, Tokyo). Pharmacokinetic analyses were performed as follows. The plasma ^{111}In radioactivity concentrations were normalized with respect to the percentage of the dose per mL, and were analyzed using the nonlinear least-square program MULTI.¹⁶ Tissue distribution was evaluated using the organs uptake clearance method (CLorgans), as described previously.¹⁷

Cellular assays: Endocytic uptake was determined as described previously.^{18,27} RAW 264.7 cells were seeded in each well of a 24-well culture plate in 1.0 mL RPMI 1640 medium, containing 10% FCS, 100 U/mL penicillin, and 100 $\mu\text{g}/\text{mL}$ streptomycin, and cultured for 12 hr to subconfluence. The cells were washed with 1.0 mL PBS and replaced with Dulbecco's modified Eagle's medium containing 3% BSA, 100 U/mL penicillin, and 100 $\mu\text{g}/\text{mL}$ streptomycin (medium A). The cells in each well were incubated at 37°C for 6 hr in 0.5 mL medium A with various concentrations of ^{125}I -control- or oxi-HSA in the presence (non-specific) or absence (total) of 50-fold unlabeled each ligands. At the indicated time, 0.375 mL of the culture medium was taken from each well and mixed with 0.15 mL 40% trichloroacetic acid (TCA) in a vortex mixer. We added 0.1 mL of 0.7 mol/L AgNO_3 to this solution, which was followed by centrifugation. The resulting supernatant (0.25 mL) was used to determine TCA-soluble radioactivity, which was taken as an index of cellular degradation, since proteins are endocytosed by the cells and delivered to lysosomes where they are degraded and excreted into the culture medium in a TCA-soluble form. Then, each well was washed three times with 1.0 mL ice-cold PBS. The cells were lysed with 1.0 mL of 0.1 N NaOH for 1 hr at 37°C to determine the cell-associated radioactivity. Specific cell-association or degradation were determined by subtracting non-specific from total.

Statistics: Statistical analyses were performed using

Student's t-test.

Results

Western blot analysis of oxi-HSA: After exposure to CT, protein aggregation or cross-linking was clearly indicated by the presence of a smear in the high molecular weight range. The high-molecular-weight fragments were recognized by the anti-HSA antibody (**Fig. 1**). These results suggest that treatment of HSA with CT causes aggregation and cross-linking due to oxidation (oxi-HSA).

The characteristics of oxi-HSA: The AOPP content of oxi-HSA was significantly greater than that of control-HSA (244.3 ± 12.3 vs 10.3 ± 6.3 μM ; $p < 0.01$; **Table 1**). Dityrosine content of oxi-HSA was also significantly greater than that of control-HSA (0.7 ± 0.11 vs 0.32 ± 0.13 nmol dityrosine per mg protein; $p < 0.01$). These characteristics of oxi-HSA are similar to those of proteins in uremic plasma (**Table 1**). The results of amino acid analysis indicate that oxidation of HSA induced significant changes in its content of certain amino acid residues including tyrosine and basic

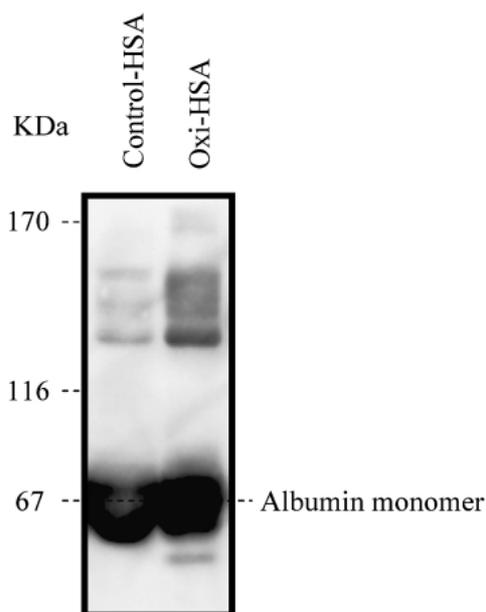


Fig. 1. Western blot analysis of control-HSA and oxi-HSA.

amino acids (**Table 2**).

Structural properties of oxi-HSA: To obtain information about protein structure, CD measurements were performed in the far-UV regions (**Fig. 2A**). The molar ellipticity of oxi-HSA was significantly less than that of control-HSA in the far-UV CD spectrum. The effects of oxidation on the structural properties were examined using the fluorescence probe bis-ANS. The spectra indicate that oxidation of HSA causes a decrease in its accessible hydrophobic areas (**Fig. 2B**). These results suggest that the structure of HSA is significantly changed by oxidation.

Pharmacokinetic properties of oxi-HSA: **Figure 3A** shows the time courses for radioactivity of ^{111}In -labeled preparations of oxi-HSA. Oxi-HSA had a shorter half-life than control-HSA. To determine the reasons for the decreased plasma half-life of oxi-HSA, we examined organ uptake. Liver, spleen and kidney uptake of oxi-HSA increased with time, and were all much greater than those of control-HSA (**Fig. 3B-D**, **Table 3**). Uptake by other organs was not significantly affected by oxidation (data not shown).

Discussion

Although *in vivo* studies indicate that the constant plasma half-life of radioisotope-labeled native albumin is 2 to 2.5 days in rats,¹⁹⁾ modified albumin has been

Table 2. Changes in amino acids composition of oxi-HSA.

Amino acids	Literature value	Control-HSA (n=3)	Oxi-HSA (n=3)
Thr	28	22.5 ± 0.3	22.4 ± 0.4
Ser	24	24.5 ± 0.2	24.6 ± 0.3
Gly	12	12.0 ± 0.3	12.8 ± 0.3
Ala	62	62.0 ± 0.4	62.0 ± 0.1
Val	41	38.6 ± 0.2	39.4 ± 0.4
Met	6	3.2 ± 0.1	2.14 ± 0.2
Ile	8	7.7 ± 0.5	7.8 ± 0.1
Leu	61	61.3 ± 0.4	61.8 ± 0.1
Tyr	18	16.0 ± 0.3	$6.7 \pm 0.4^*$
Lys	59	53.0 ± 0.1	$26.9 \pm 0.4^*$
His	16	15.1 ± 0.4	14.5 ± 0.4
Arg	24	23.9 ± 0.4	$18.6 \pm 0.3^*$

*Significantly different ($P < 0.01$) from control-HSA. Data are mean \pm SD.

Table 1. AOPP and dityrosine content of HSA treated with chloramine T.

	Control-HSA	Oxi-HSA	Normal subjects	HD patients
AOPP (μM)	10.3 ± 6.3	$244.3 \pm 12.3^*$	$33.9 \pm 3.4^{\text{a)}$	$267.5 \pm 16.5^{\text{a)}$
Dityrosine (nmol/mg protein)	0.32 ± 0.13	$0.7 \pm 0.11^*$	$0.36 \pm 0.05^{\text{a)}$	$1.03 \pm 0.12^{\text{a)}$

*Significantly different ($P < 0.01$) from control-HSA. Data are mean \pm SD.

^{a)}Ref. 6.

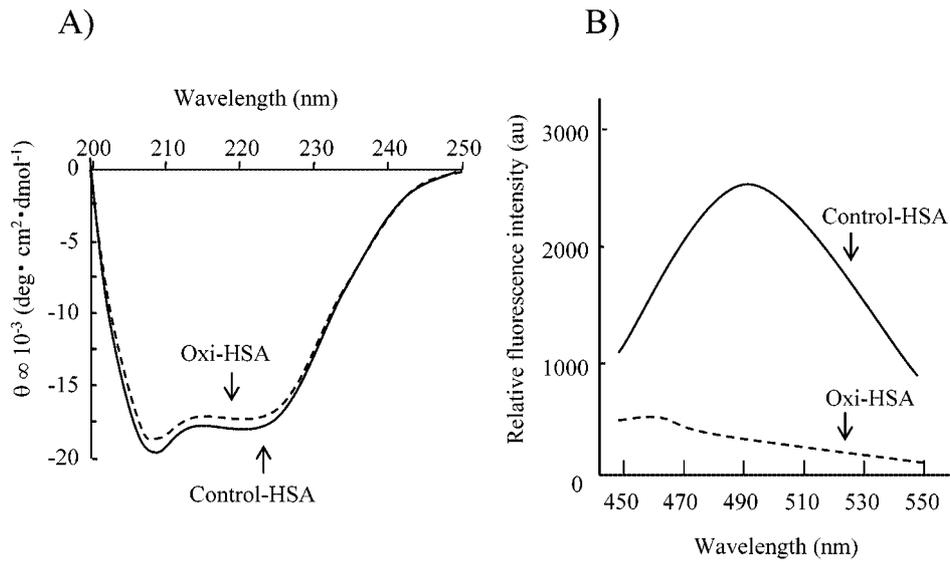


Fig. 2. Far-UV CD spectra (A) and relative fluorescence intensity of hydrophobicity using bis-ANS (B) of control-HSA (—) and oxi-HSA (---). Data are mean \pm SD.

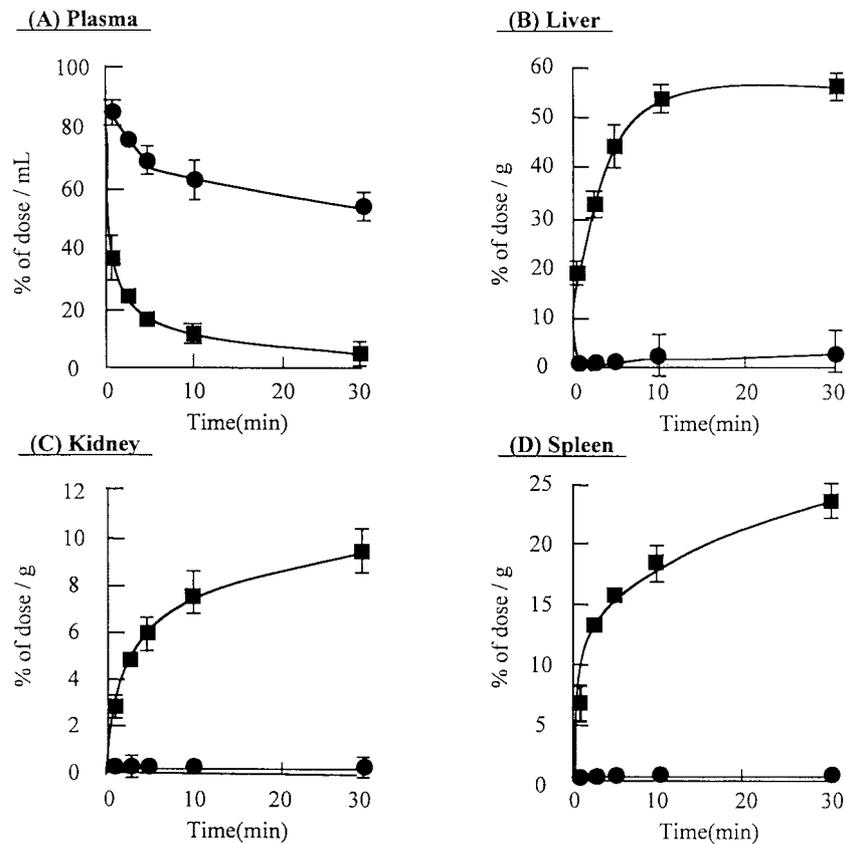


Fig. 3. Plasma and organ levels of ^{111}In -labeled control-HSA (●) and oxi-HSA (■) after i.v. administration to mice. Data are mean \pm SD.

shown to have an extremely short half-life in circulating blood.²⁰ As one of reasons for the formation of modified albumin *in vivo*, free radical species have been found to induce conformational changes, degradation

and aggregation of proteins in several diseases.²¹ In uremic patients, AOPP are produced by HOCl-modified albumin cross-linking *in vivo*. Further, AOPP can act as mediators of oxidative stress, and they have

been implicated in the immune dysregulation associated with chronic uremia.^{6,7} However, the mechanisms by which AOPP is degraded or eliminated from circulating blood remain unclear. Therefore, in the present study, we used CT-treated HSA (oxi-HSA) to examine the mechanisms involved in elimination of AOPP.

The AOPP and dityrosine content of oxi-HSA were similar to those of proteins from uremic plasma (Table 1). In the Western blot analysis, oxi-HSA was mostly present in the form of multimolecular aggregates, probably resulting from dityrosine cross-linking and/or disulfide bridges (Fig. 1). Further, the amino acid analysis indicates that oxidation of HSA induced significant modification of tyrosine and basic amino acid residues such as lysine and arginine (Table 2). These results suggest that the modifications of those amino acid residues are responsible for the conformational changes observed in HSA (Fig. 2).

The pharmacokinetic analysis consistently showed that oxi-HSA left the circulation rapidly. The organ distribution 30 min after the intravenous injection was 51% for the liver, 23% for the spleen, and 9% for the kidney, suggesting that the liver was the main route for plasma clearance (Fig. 3). Since many of the scavenger receptors are expressed in sinusoidal endothelial cells and Kupffer cells in the liver,^{22,23} plural scavenger receptors may be responsible for the hepatic uptake of oxi-HSA. In general, most scavenger receptors can bind a variety of polyanionic ligands, including negatively charged albumins.^{24,25} In some diseases including diabetes, AGE-albumins have well known as major

negatively charged proteins in plasma. AGE-albumins are nonenzymatically glycosylated proteins that accumulate in vascular tissue with aging and accumulate at accelerated rates in diabetic patients. Schmidt *et al.*²² reported that AGE-albumins plasma clearance in mice was rapid, with 70% of the injected dose removed from the circulation within 5 min. In their study, liver was also the main route for plasma clearance of AGE-albumin. Their data are very similar to the present findings for oxi-HSA. Interestingly, oxi-HSA has a negatively charged molecule due to the modifications of basic amino acid residues in structural analysis (Table 2). Further, oxi-HSA has known to contain a structural motif of AGE-proteins as a result of oxidation.^{6,7} Therefore, oxi-HSA are possible to be distributed for liver nonparenchymal cells (endothelial and Kupffer cells) as same as those of AGE-albumins. However, the evidence for scavenger receptor involved in the elimination of modified albumin, including AGE-albumin and oxi-HSA, on liver nonparenchymal cells has been rarely found.^{26,27} Unfortunately, we were also unable to clarify the receptor responsible for hepatic uptake in this study, but we should examine the elimination mechanism of oxi-HSA by using cell line highly expressed scavenger receptors in the future.

On the other hands, scavenger receptors are expressed not only on liver nonparenchymal cells but also various macrophages. Given the fact that the organ distribution of oxi-HSA was 23% for the spleen, oxi-HSA may be also taken up by macrophage of spleen via scavenger receptor-mediated endocytosis. Macrophage has known to express highly SR-A, one of the scavenger receptor.⁸ To examine the relationship between oxi-HSA and SR-A in macrophage, we used the macrophage-derived cell line RAW 264.7. Significant amounts of ¹²⁵I-oxi-HSA were associated with these cells and underwent endocytic degradation within the cells, whereas no such findings were observed for control-HSA (Fig. 4). Thus, SR-A may be involved in the spleen clearance of oxi-HSA.

Table 3. Uptake clearance of control- and oxi-HSA labeled with ¹¹¹In after i.v. administration to mice.

($\mu\text{L/hr}$)	Liver	Kidney	Spleen
Control-HSA	24 \pm 4.2	48 \pm 5.3	32 \pm 2.7
Oxi-HSA	5058 \pm 341.6*	1188 \pm 208.2*	2118 \pm 322.1*

*Significantly different ($P < 0.01$) from control-HSA. Data are mean \pm SD.

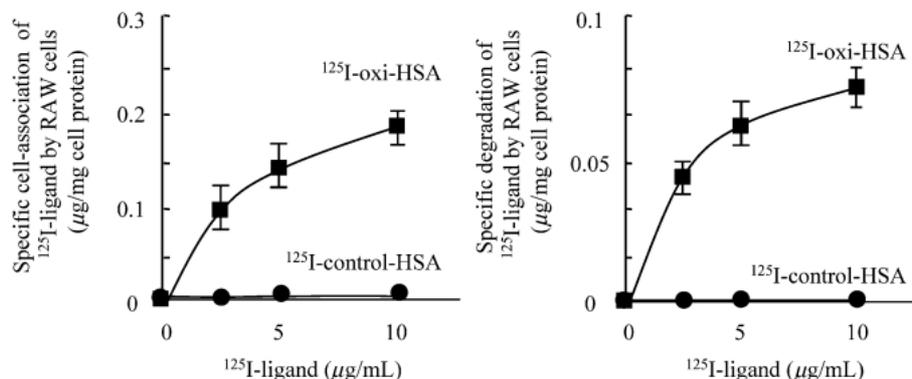


Fig. 4. Endocytic uptake of ¹²⁵I-labeled control-HSA (●) or oxi-HSA (■) by RAW 264.7 cells. Data are mean \pm SD.

In summary, the kinetics of chloramine-T-treated HSA (oxi-HSA) are similar to those of AOPP in uremic patients. Oxi-HSA left the circulation rapidly and accumulated in the liver, spleen and kidney. The liver and spleen appear to play particularly important roles in elimination of AOPP.

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