

## Albumin and Hydroxyethyl Starch Modulate Oxidative Inflammatory Injury to Vascular Endothelium

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**Background:** Human serum albumin is used clinically to maintain colloid osmotic pressure and is viewed to serve an antioxidant role in the vascular compartment *via* binding of redox-active metal complexes, transport of nitric oxide, and the oxidant-scavenging reactions of the single thiol of human serum albumin, cys34. Because of these potentially desirable adjunctive actions, we evaluated the purity and thiol redox state and compared the relative effects of clinically available 25% human serum albumin preparations with a starch-derived colloid, 6% hydroxyethyl starch, in *in vitro* models of inflammatory vascular injury.

**Methods:** Bovine aortic endothelial cell responses to chemical, enzymatic, and cell-derived reactive inflammatory mediators in the presence of human serum albumin or hydroxyethyl starch were assessed.

**Results:** The cys34 thiol of fresh human serum albumin preparations was 70–85% oxidized and contained a population of human serum albumin (approximately 25% of total) having the cys34 resistant to reduction by 2-mercaptoethanol and NaBH<sub>4</sub>. Treatment of bovine aortic endothelial cells with human serum albumin dose-dependently protected from HOCl-mediated <sup>14</sup>C-adenine release, with this protective effect of human serum albumin not dependent on protein thiol status. Addition of human serum albumin to cell media provided no protection from the cytotoxic actions of peroxyxynitrite and xanthine oxidase-derived reactive species. Binding of activated polymorphonuclear leukocytes to bovine aortic endothelial cells was significantly amplified by hydroxyethyl starch and inhibited by human serum albumin administration. The binding of neutrophil-derived myeloperoxidase to bovine aortic endothelial cells, a mediator of multiple oxidative and nitric oxide-consuming reactions, was also inhibited by human serum albumin and enhanced by hydroxyethyl starch.

**Conclusions:** Clinical human serum albumin preparations show modest intrinsic non-thiol-dependent antiinflammatory properties *in vitro*, a phenomenon that was not observed with hydroxyethyl starch.

HUMAN serum albumin (HSA), the most abundant protein in plasma, has numerous functions, including maintenance of plasma volume, transport of endogenous lipophilic molecules, drug binding, and the scavenging of reactive inflammatory mediators.<sup>1</sup> Albumin is a 66-kDa protein containing 585 amino acids and accounting for approximately 50% of the total protein in the plasma of healthy people. It is typically present in approximately 0.6 mM or 45 mg/ml concentrations. Essential to the structure, function, and potential redox activity of HSA are 18 tyrosines, 6 methionines, 1 tryptophan, 59 lysines, 17 disulfide bridges, and 1 free cysteine.<sup>2</sup> This latter amino acid, cys34, accounts for the greater-than 75% contribution of HSA to the free thiol content of plasma. Because cysteine is biochemically notable as a facile scavenger of reactive species, it has been frequently assumed that the plasma HSA pool and component thiol substituents serve as a key element of intravascular antioxidant defenses.<sup>3-6</sup>

Clinically, colloids are used for volume replacement when attempting to maintain or improve tissue perfusion in patients experiencing sepsis, trauma, shock, or surgical stress.<sup>7,8</sup> Although maintenance of intravascular volume for these clinical problems is of paramount importance, the relative immunomodulatory effects of clinical colloid preparations, specifically HSA and its non-protein analog, hydroxyethyl starch (HES), is a topic of speculation and active investigation. HSA and HES have been shown to enhance and mitigate the up-regulation of inflammatory processes, with several comparisons showing divergent actions.<sup>9-12</sup> For example, cultured vascular endothelial cell exposure to HES, but not HSA, showed dose-dependent inhibition of stimulated von Willebrand factor release.<sup>11</sup> Random batches of HSA have also been shown to directly stimulate vascular cell adhesion molecule expression.<sup>12</sup> In contrast is the finding of significantly increased polymorphonuclear neutrophil (PMN) activation in human blood treated with HES.<sup>10</sup>

A significant aspect of the putative inflammatory-modulating properties of HSA is its action as an expendable antioxidant in the intravascular compartment. This behavior is expressed in scavenging the diverse free radical and oxidizing species generated by basal aerobic metabolic processes and produced at increased rates by activated vascular and reticuloendothelial cells during tissue inflammatory responses. Three principal elements lend antioxidant character to HSA: the nitric oxide-reactive

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and oxidant-reactive thiol of cys34, the ability to bind or otherwise catalytically sequester redox-active transition metals (e.g., iron and copper), and the capacity of HSA to bind amphipathic species (e.g., fatty acids, heme, and bilirubin), which themselves may catalyze injurious redox reactions.<sup>1,2,13</sup> Detailed kinetic analyses support the precept that HSA, in particular the thiol in cys34, react with inflammatory oxidants.<sup>4</sup> Also, the hydrophobic core of HSA can mediate micellar catalysis of nitric oxide oxidation, yielding secondary adducts (e.g., S-nitrosothiols) that may also have antiinflammatory activity.<sup>14</sup>

Hydroxyethyl starch is a colloidal, synthetically modified polymer of amylopectin. It is pharmacologically classified as a plasma expander and is intended to support oncotic pressure and provide electrolytes.<sup>15</sup> Its physical and chemical characteristics are defined by the degree of hydroxylation, which is the major determinant of circulating half-life, and by its molecular weight, which determines osmolarity of the solution and thus the colloidal activity of HES. The metabolism of HES remains a topic of investigation, with molecular weight being a factor in relative extents of clearance by renal excretion and hydrolysis by amylase.<sup>16</sup> Because of its uptake by reticuloendothelial cells, HES may also influence inflammatory responses.<sup>17,18</sup> For example, blood from healthy volunteers diluted with 6% HES showed an increased PMN respiratory burst, as compared with 5% and 25% HSA solutions.<sup>10</sup> Expression of PMN CD18 receptors were also significantly increased in HES-treated blood preparations in this study, compared with treatment with 25% HSA.

Herein, we evaluated the ability of HSA to modulate target cell injury by reactive oxygen-derived and nitric oxide-derived inflammatory oxidants that are recognized to show unique and overlapping target molecule selectivities. We also determined whether the thiol of cys34, seen to be predominantly oxidized in clinical preparations of HSA, was essential for mitigation of oxidant-induced endothelial injury. Finally, the relative influences of HSA and HES on activated PMN and myeloperoxidase (MPO) adhesion and injury to vascular endothelium was evaluated.

## Materials and Methods

### Chemicals

Human serum albumin (25% HSA; molecular weight, 66 kDa) was purchased from Baxter Healthcare Corporation (Deerfield, IL), the American Red Cross (Washington, DC), and Alpine Biologics (Orangeburg, NY). Hetastarch (6% HES; average molecular weight, 670 kDa) was purchased from Abbott Laboratories (North Chicago, IL). Xanthine oxidase came from Calbiochem (San Diego, CA). HOCl was from Aldrich Laboratories (Toronto, Canada). Spermine NONOate was from Alexis Laboratories (San Diego, CA). Medium 199, Hanks bal-

anced salt solution, and antibiotic-antimycotic were from Gibco Laboratories (Carlsbad, CA). Fetal calf serum and iron-supplemented calf serum were from Hyclone (Logan, UT). 5,5'-Dithio-bis-(2-nitrobenzoic acid), NaBH<sub>4</sub>, thymidine, lumazine, dextran, and histopaque were from Sigma (St. Louis, MO), and 2-mercaptoethanol was obtained from Fisher Laboratories (St. Louis, MO). Peroxynitrite was synthesized as previously described.<sup>3</sup>

### Albumin Preparation

Defatting of HSA to exclude actions of fatty acids was performed before electrophoretic analysis and was achieved by treatment with charcoal in acidic solution.<sup>19</sup> To reduce sulfhydryls oxidized during purification and storage, HSA was treated with 10 mM 2-mercaptoethanol for 16 h and subsequently with 10 mM NaBH<sub>4</sub> for 1 h, both at 4°C. Excess reductant was removed by gel exclusion filtration on Sephadex G-25 using 0.01 M potassium phosphate and 0.1 mM diethylenetriaminepentaacetate to bind metals, at pH 7.4. Reduction of HSA thiols was performed immediately before experiments to minimize reoxidation in air-equilibrated buffers. In some cases, HSA cys34 thiols were blocked by treatment with a 10-fold molar excess of N-ethylmaleimide, followed by desalting with Sephadex G-25 gel filtration.

### Electrophoresis

Assorted HSA lots obtained from commercial vendors were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 10% gels. Equal amounts of HSA were loaded in each lane. The gel was then stained with Coomassie Blue for protein detection.

### Biochemical Analysis

Albumin concentrations were measured by absorbance at 279 nm ( $0.531 \text{ [g/l]}^{-1} \cdot \text{cm}^{-1}$ ) and by Coomassie Blue dye binding. For polyacrylamide gel electrophoretic analyses, 1  $\mu\text{g}$  albumin was loaded into each lane of the gel. The molecular weight used for HSA was 66,486.<sup>20</sup> Sulfhydryls were quantified using 5,5'-dithio-bis-(2-nitrobenzoic acid) at a wavelength of 412 nm ( $E = 13.6 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ ).<sup>21</sup> Extents of thiol reduction in HSA preparations were expressed as a molar ratio (sulfhydryl/albumin).

### Endothelial Cell Culture

Bovine aortic endothelial cells were isolated as previously described.<sup>22</sup> Cells were propagated by subculturing at a 1:4 ratio in Medium 199, 5% fetal calf serum, 5% iron-supplemented calf serum, 1% antibiotic-antimycotic, and 10  $\mu\text{M}$  thymidine. Total volumes in cell wells were maintained to be constant, and approximately  $2 \times 10^5$  cells per well were obtained.

### Exposure of Bovine Aortic Endothelial Cells to Oxidants

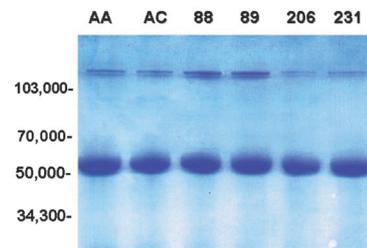
In all cytotoxicity assessments, bovine aortic endothelial cells were preincubated for 16 h with  $^{14}\text{C}$ -adenine in serum-containing medium. HSA (0.03–2.0 mg/ml) was always present before the initiation of oxidant stress. Unincorporated  $^{14}\text{C}$ -adenine was removed by washing with Hanks balanced salt solution immediately before bolus addition of inflammatory oxidants, such as 150  $\mu\text{M}$  HOCl. All cell exposures to oxidants were performed in Hanks balanced salt solution, supplemented in some cases with HSA or HES. For exposure to superoxide ( $\text{O}_2^-$ ), hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), and hydroxyl radical ( $\cdot\text{OH}$ )/perferryl ( $\text{Fe}_{\text{IV}} = \text{O}$ ), cell monolayers were exposed to 10 mU/ml xanthine oxidase, with repeated additions of 100  $\mu\text{M}$  xanthine. Cell exposure to peroxynitrite ( $\text{ONOO}^-$ ) was accomplished by addition of 10 mU/ml xanthine oxidase, 100  $\mu\text{M}$  lumazine, and 100  $\mu\text{M}$  spermine NONOate. Rates of xanthine oxidase-derived  $\text{O}_2^-$  and spermine NONOate-derived  $\cdot\text{NO}$  generation were measured daily, by cytochrome *c* reduction and electrochemical detection, respectively, permitting calibration of the concentrations of spermine NONOate required for giving relative rates of  $\text{O}_2^-$  and  $\cdot\text{NO}$  production of approximately 1:1, with  $\text{O}_2^-$  and  $\cdot\text{NO}$  generation maintained at approximately 2  $\mu\text{M}/\text{min}$  for up to 6 h by repeated addition of spermine NONOate and xanthine. All experiments were repeated at least three times.

### Determination of Cell Injury

The extent of cell lysis induced by oxidants was measured by the release of  $^{14}\text{C}$ -adenine from bovine aortic endothelial cells.<sup>23</sup> Aliquots of the medium were removed at 6 h, to allow for immediate necrotic cell lysis and early apoptosis to occur, and released radioactivity, consisting of typically 80% acid-soluble  $^{14}\text{C}$ -adenine derivatives being released. The percentage of cell lysis was calculated as a percentage of total lysis of cell monolayers induced by  $^{14}\text{C}$ -adenine release from 10% CHAPS (3-[(3-Cholamidopropyl)dimethylammonio]-1-propane-sulfonate)-treated cell monolayers.

### Polymorphonuclear Neutrophil Isolation

Isolation of human PMNs was performed as previously described.<sup>24</sup> In brief, blood was drawn from healthy volunteer donors after informed consent was obtained. The plasma was removed, and the cellular fraction was placed onto an equal volume of lymphocyte separation medium. After centrifugation and discarding lymphocyte-containing layers, dextran solution (molecular weight, 485,000) was added to promote aggregation and sedimentation of erythrocytes. The PMN-enriched supernatant was collected, and remaining erythrocytes were separated by hypotonic lysis. The PMN fraction was suspended in Hanks balanced salt solution containing 1 mg/ml glucose, at pH 7.4, and activated with N-formyl-methionyl-leucyl-phenylalanine (f-MLP, 1  $\mu\text{M}$ ).



**Fig. 1.** Sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis of 25% human serum albumin preparations. Gels were run under reducing conditions on a 10% gel. Lots AA and AC were purchased from the American Red Cross, lots 88 and 89 from Alpine Biologics, and lots 206 and 231 were purchased from Baxter Healthcare.

### Polymorphonuclear Neutrophil Binding

Bovine aortic endothelial cells were washed, and Hanks balanced salt solution,  $\text{NaHCO}_3$  (0.35 g/l), and glucose (2 mg/ml) were added to each well. HSA or HES plus 0.9% NaCl was added to bring the total volume per well to 1 ml. Activated (f-MLP, 1  $\mu\text{M}$ ) or resting (nonactivated) PMNs ( $6 \times 10^5$ ) were added into the wells with bovine aortic endothelial cells for a total of 3 h. The wells were then washed twice with Hanks balanced salt solution. PMNs adherent to the bovine aortic endothelial cells were counted using microscopy at  $\times 10$  and at five different fields.

### Myeloperoxidase Activity Assay

Myeloperoxidase activity in cell lysates was determined by adding aliquots of cell lysate to 43 mM  $\text{NaH}_2\text{PO}_4$  (pH 5.4), 1.2 mM tetramethylbenzidine, and 100  $\mu\text{M}$   $\text{H}_2\text{O}_2$ .<sup>24</sup> Absorbance kinetics were assessed spectrophotometrically at 655 nm.

### Statistical Analysis

Data were subjected to one-way ANOVA and multiple comparison analysis between groups performed by Dunnett *post hoc* test. Data are reported as mean  $\pm$  SEM measurement, with  $P < 0.05$  considered significant.

## Results

### Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis Analysis of Commercially Available Human Serum Albumin Preparations

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed under reducing conditions to determine the purity of HSA in commercially available 25% HSA (fig. 1). All six preparations contained the stated concentrations of HSA (data not shown) and were observed to predominantly include a species of approximately 66.5 kDa, confirmed as HSA by matrix-assisted laser desorption/ionization time-of-flight mass spectroscopic analyses. Additional high-molecular-weight (approximately 125 kDa) nondisulfide, covalently cross-

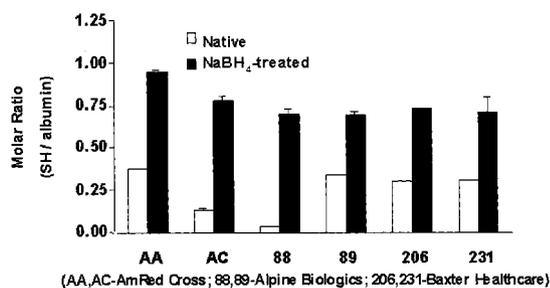


Fig. 2. The oxidation status of freshly opened and 2-mercaptoethanol/NaBH<sub>4</sub>-reduced 25% human serum albumin preparations. Lots AA and AC were purchased from the American Red Cross, lots 88 and 89 from Alpine Biologics, and lots 206 and 231 were purchased from Baxter Healthcare. Data are expressed as mol sulfhydryl (SH) groups/mol.

linked, presumed multimers of HSA were also evident at the top of lanes run for all HSA lots.

#### Oxidation Status of Commercially Available Human Serum Albumin Preparations

The oxidation state of the cys34 thiol was determined for randomly selected lots of commercial 25% HSA preparations available for clinical use. All HSA lots, analyzed immediately after opening, were significantly thiol-oxidized, with some intermanufacturer and intramanufacturer variability evident (fig. 2). Cys34 reduction with 2-mercaptoethanol and NaBH<sub>4</sub> increased HSA thiol content to maximal sulfhydryl/albumin molar ratios ranging from 0.71 to 0.95 ( $P < 0.05$ ).

#### Influence of Native and Reduced Human Serum Albumin on HOCl-induced Endothelial Injury

Cultured bovine aortic endothelial cells exposed to HOCl manifested significant cell lysis, with native (approximately 0.25 sulfhydryl/albumin) and reduced (approximately 0.75 sulfhydryl/albumin) HSA significantly limiting the extent of <sup>14</sup>C-adenine release in a dose-dependent fashion (fig. 3). There were no significant differences in HOCl cytotoxicity for cells treated with various concentrations of native versus reduced HSA. Treatment of cells with only 0.25 mg/ml HSA resulted in

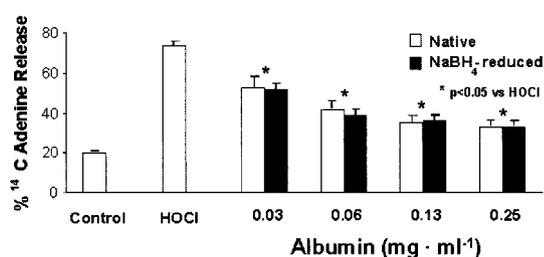


Fig. 3. The influence of HOCl and human serum albumin (HAS) thiol oxidation state on endothelial <sup>14</sup>C-adenine release. Increasing concentrations of HSA significantly reduced <sup>14</sup>C-adenine release when compared with HOCl-treated cells. (\*  $P < 0.05$ ). There were no significant differences between native HSA and reduced HSA. Administration of 0.25 mg/ml HSA induced the greatest extent of reduction in <sup>14</sup>C-adenine release.

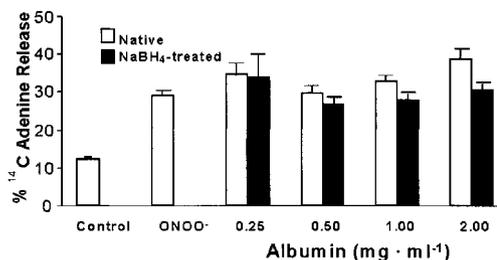


Fig. 4. The influence of ONOO<sup>-</sup> and human serum albumin (HSA) thiol oxidation state on endothelial <sup>14</sup>C-adenine release. Exposure of bovine aortic endothelial cells to ONOO<sup>-</sup> (xanthine oxidase-derived O<sub>2</sub><sup>-</sup> and spermine NONOate-derived NO) increased <sup>14</sup>C-adenine release. Increasing concentrations of native and reduced HSA showed no protective effect. Similar cell responses (e.g., no protective effect of HSA) were seen for bovine aortic endothelial cells exposed to only xanthine plus xanthine oxidase-derived species.

the greatest extent of reduction in <sup>14</sup>C-adenine release, with no further protection evident with greater concentrations of HSA.

#### Influence of Native and Reduced Human Serum Albumin on ONOO<sup>-</sup>-induced Endothelial Injury

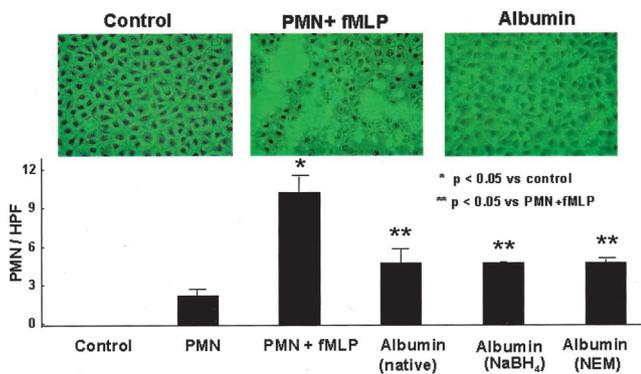
Cultured bovine aortic endothelial cells exposed to enzymatically generated ONOO<sup>-</sup> showed significant increases in cell lysis (fig. 4). Cells, though injured, were not protected by up to 30 mg/ml concentrations of native and reduced HSA (data not shown).

#### Human Serum Albumin Inhibition of Activated Polymorphonuclear Neutrophil-Endothelial Cell Binding

The activation of human-derived PMNs (fMLP-treated) resulted in a significant increase in endothelial cell binding compared with control or nonactivated PMNs (activated, 10 per high-power field vs. 3 per high-power field vs. control, 0 per high-power field) (fig. 5). The coadministration of 30 mg/ml HSA resulted in a significant decrease in endothelial cell binding. This observation was consistent when treated with native, re-reduced, and blocked HSA solutions.

#### Hydroxyethyl Starch Amplification of Activated Polymorphonuclear Neutrophil-Endothelial Cell Binding

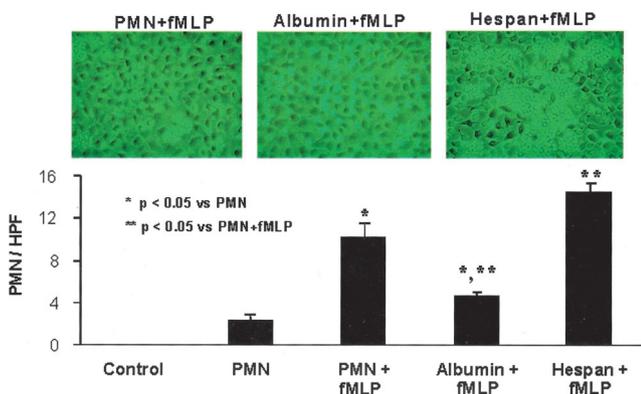
The activation of human PMNs by fMLP resulted in a significant increase in endothelial cell-PMN binding compared with nonactivated PMNs (fig. 5). Administration of 30 mg/ml HSA resulted in a significant reduction in endothelial cell-PMN binding that occurred to a similar extent with native, reduced, and alkylated (e.g., thiol-blocked) HSA solutions (fig. 5). Administration of 30 mg/ml HES to endothelial cell-PMN cocultures induced intense PMN aggregation and significantly enhanced endothelial binding of fMLP-activated PMNs, compared with native HSA-treated conditions (fig. 6).



**Fig. 5.** The influence of human serum albumin (HSA) (native, thiol-reduced, and N-ethylmaleimide [NEM]-alkylated) on endothelial binding of activated polymorphonuclear leukocytes (PMNs). Human PMNs were activated with N-formyl-methionyl-leucylphenyl-alanine (fMLP) and added to bovine aortic endothelial cells in a 10:1 effector-to-target cell ratio. This resulted in a significant increase in PMN binding to bovine aortic endothelial cells, compared with control, nonactivated PMNs (\* *P* < 0.05). Administration of 30 mg/ml HSA significantly reduced PMN-endothelial cell binding, compared with the activated human-derived PMN group (\*\* *P* < 0.05). There was no significant impact of thiol oxidation state on PMN-endothelial cell interactions. The microscopic images represent the typical monolayer appearance of control, fMLP-treated, and native HSA-treated PMN-endothelial cell cocultures.

*Enhancement of Polymorphonuclear Neutrophil-derived Myeloperoxidase Binding to Endothelial Cells by Hydroxyethyl Starch*

Myeloperoxidase from degranulation of activated PMNs avidly binds to and is transcytosed by the endothelium *in vitro* and *in vivo*. In the presence of HES, bovine aortic endothelial cells exposed to fMLP-activated



**Fig. 6.** The influence of native human serum albumin and hydroxyethyl starch (HES) on endothelial binding of activated polymorphonuclear leukocytes (PMNs). Human PMNs were activated with N-formyl-methionyl-leucylphenyl-alanine (fMLP) and added to bovine aortic endothelial cells in a 10:1 effector-to-target cell ratio. This resulted in a significant increase in PMN binding to bovine aortic endothelial cells, compared with control, nonactivated PMNs (\* *P* < 0.05). Administration of 30 mg/ml human serum albumin significantly reduced PMN-endothelial cell binding, compared with the activated derived PMN and HES-treated PMN-endothelial cell cocultures (\*\* *P* < 0.05). The microscopic images represent the typical monolayer appearance of control, fMLP-treated, and HES-treated PMN-endothelial cell cocultures.

**Table 1.** Influence of Native HSA and HES on Endothelial Binding of Myeloperoxidase Released by Activated PMNs

Condition	MPO Activity (nmol · min <sup>-1</sup> · well <sup>-1</sup> )
Nonactivated PMNs	14.6 ± 3.2
Activated PMNs	20.9 ± 4.7
HSA	19.3 ± 3.3
HES	34.6 ± 4.5†

\* *P* < 0.05 vs. untreated controls. † *P* < 0.05 vs. N-formyl-methionylleucylphenyl-alanine-activated PMNs.

HES = hydroxyethyl starch; HSA = human serum albumin; MPO = myeloperoxidase; PMNs = polymorphonuclear leukocytes.

PMNs showed significantly greater bound PMN and MPO activity (table 1). In contrast, bovine aortic endothelial cell binding of MPO was not affected by HSA, regardless of the cys34 thiol redox state.

**Discussion**

The key findings of the current study are as follows: Native, commercially available clinical preparations of HSA were predominantly oxidized at the sole cys34 thiol, with a significant fraction of this cys34 thiol oxidation product resistant to re-reduction. Chemical re-reduction of the cys34 of HSA did not influence extents of oxidative and PMN-induced cell injury. HSA attenuated HOCl-induced cultured vascular cell injury, but did not alter that induced by O<sub>2</sub><sup>-</sup>, H<sub>2</sub>O<sub>2</sub>, and ONOO<sup>-</sup> generating systems. The PMN regulatory actions of HSA and HES were different, with HES enhancing and HSA limiting endothelial binding by activated PMNs and with HSA reducing and HES enhancing vascular cell binding of PMN-derived MPO.

*Vascular Cell Function and Inflammatory Modulatory Effects of Human Serum Albumin*

The cys34 of HSA, as opposed to lipids and other plasma biomolecules, is a key intravascular scavenger of the traditional family reactive inflammatory species, including O<sub>2</sub><sup>-</sup>, H<sub>2</sub>O<sub>2</sub>, ·OH, and its oxidative equivalent Fe<sub>IV</sub>-O, ·NO, ONOO<sup>-</sup>, PMN-derived oxidants, such as HOCl and ·NO<sub>2</sub>, and lipid peroxy radicals (LOO·).<sup>2-5</sup> Recently, it has also been shown that HSA can enhance lung cell glutathione content and, through this action, may protect lung cells against H<sub>2</sub>O<sub>2</sub>-mediated injury, activation of nuclear factor-κB and expression of tumor necrosis factor α.<sup>25</sup>

Support that HSA may manifest clinically beneficial oxidant-scavenging properties comes from biochemical test systems showing that ONOO<sup>-</sup>, the ·NO- and O<sub>2</sub>-derived oxidant, is highly thiol-reactive and preferentially reacts with the cys34 of HSA, as opposed to other amino acids.<sup>3,4</sup> Also, addition of physiologic concentrations of HSA (4%) to activated PMNs resulted in up to 80% diminution of luminol-dependent chemilumines-

cence, a general indicator of oxidative and electron transfer reactions.<sup>26,27</sup> Unfortunately, this finding is difficult to interpret from an oxidative chemistry perspective, because it did not include a control analysis of the expected quenching actions of HSA toward luminol-dependent light emission. Several crucial issues pertain to virtually all *in vitro* studies of HSA and HES modulation of indices of vascular cell function and inflammatory injury. First, isolation of inflammatory cells and their density gradient centrifugation in Ficoll and balanced salt solution-based buffers can result in partial activation. Thus, the activation state of PMNs *in vitro* and their response to secondary stimuli can differ from that of resting cells *in vivo*. Also, culture of vascular cells on plastic surfaces in the presence of HSA-free and serum-free conditions, before the addition of HES as a test substance, creates metabolic stress and is a potentially nonphysiologic reflection of cell status in the vascular compartment. Thus, cell responses to the restoration of HSA, rather than a manifestation of clinically relevant antiinflammatory actions of HSA, may simply represent a return of cells to the more favorable ionic and metabolic conditions facilitated by protein addition. This precept may apply to observations of HSA-mediated inhibition of PMN respiratory burst,<sup>9,10,28,29</sup> adhesion of PMNs and MPO to vascular cells (figs. 5 and 6), cytokine<sup>29</sup> and integrin expression,<sup>10</sup> the attenuation of transendothelial macromolecule flux,<sup>30</sup> and restoration of intracellular low-molecular-weight thiol content that is observed *in vitro*.<sup>25</sup>

Our analyses confirmed a high level of protein purity in the 25% HSA solutions typically supplied by hospital pharmacies for patient use (fig. 1). They also showed a high degree of basal thiol oxidation (> 65% oxidized) in the principal oxidant-scavenging amino acid of HSA, cys34. Moreover, approximately 25% of the oxidized thiols in a solution of HSA, all attributable to cys34, were not re-reducible by 2-mercaptoethanol and the more potent reductant, NaBH<sub>4</sub> (fig. 2). This suggests an oxidation of the cys34 to a higher and irreversible oxidation state, such as sulfonic acid (RSO<sub>3</sub>H) and shows that reduction of this population of HSA thiols would not occur biologically. This significant basal level of HSA oxidation also implies that much of the putative antioxidant quality of intravenously administered HSA is already spent, unless some HSA cys34 reduction by plasma reductants occurs after HSA infusion. This issue has been addressed in an inconclusive study of human plasma thiol content before and after administration of HSA to critically ill patients and volunteers.<sup>6</sup> In this study, 200 ml 20% HSA (noted to be 40% reduced) was given to patients, with healthy individuals typically having approximately 280 g endogenous HSA. A calculated net increase of 12% plasma HSA and 5% plasma thiol content matched well with the measured 12% and 11% increases for healthy volunteers. Unexpectedly, when HSA was

given to patients with sepsis syndrome, the plasma HSA and thiol content increases after HSA infusion were 80% and 38%, respectively, much greater than expected. There were also unexpected discrepancies in net plasma thiol/HSA ratios in patients with sepsis, in whom the plasma thiol/HSA ratio decreased, rather than remaining the same or increasing after HSA administration (from 0.76 to 0.57  $\mu\text{M}$  thiol/ $\mu\text{M}$  HSA). This raises concern that lysed erythrocytes, damaged vascular endothelium, or interfering chromophoric substances were affecting measured HSA and thiol concentrations after HSA infusion in patients with sepsis. Importantly, from the observation that administration of 200 ml 20% HSA (40 g) increases the endogenous and relatively low plasma thiol content by only 5%, a key question arises: Is a minimal increase in a modestly oxidant-reactive plasma thiol of biochemical and clinical significance, with respect to redox chemistry and inflammatory cell function? We believe this is probably not the case, unless nonspecific effects of osmolality or plasma protein content are mitigating the influence of HSA on more subtle inflammatory cell signaling processes, rather than thiol redox chemistry.

In this study, the ability of HSA to protect endothelial cells from <sup>14</sup>C-adenine release induced by PMN-derived or exogenously administered inflammatory oxidants depended on the nature of the reactive inflammatory mediator. Also, the conceptual disadvantage lent by low extents of thiol reduction of clinical HSA preparations did not translate into an impairment of the relative extents of native *versus* reduced HSA to limit endothelial cell injury in our model system. For example, endothelial injury induced by a key PMN-derived oxidant, HOCl, was blunted by low concentrations of HSA (fig. 3). It was viewed that this potent scavenging of HOCl by HSA was a result of not only thiol-dependent HOCl scavenging but also the avid reactivity of HOCl with amines; there are 59 lysines in HSA. Although HSA inhibited necrotic and apoptotic endothelial injury induced by HOCl administration, up to 30 mg/ml HSA did not affect oxidative cell injury induced by reactive species derived from activated PMNs (fig. 4), xanthine oxidase plus purine administration (data not shown), and ONOO<sup>-</sup> generated by fluxes of  $\cdot\text{NO}$  and O<sub>2</sub><sup>-</sup> (data not shown). The reasons for this differential effect of HSA are speculative, but it may be the result of the greater relative reactivity of HOCl for the thiols, amines, and other oxidant-reactive amino acids of HSA, as compared with the net HSA reactivity of other reactive species. Thus, it appears that various reactive mediators of oxidative tissue injury, although all appreciated to be chemically scavenged to a greater or lesser extent by thiols, are not equally or readily scavenged by HSA to an extent that target cell necrosis and apoptosis is attenuated. In the case of HOCl-induced endothelial injury, it is notable that the

thiol redox state was not a factor in HSA-induced cytoprotection.

As a counterpoint to the observation that HSA lends minimal protection to endothelial cells exposed to PMNs and reactive species *in vitro*, HSA significantly inhibited and HES amplified activated PMN and MPO binding to endothelial cells (fig. 6). Again, the thiol redox state of HSA was not a factor in this attenuation of PMN-cell binding (fig. 5). Moreover, PMN-derived MPO binding to endothelium was similarly enhanced by HES, but not affected by HSA (table 1). This antiinflammatory property of HSA may have clinically meaningful manifestations not observable with *in vitro* test systems, such as an impairment of vascular relaxation and barrier function,<sup>31</sup> and deserves further investigation in animal models of inflammatory vascular injury in which the influence of endogenous ionic and plasma protein levels can be better studied.

From a clinical perspective, HSA seems to be worth considering as a resuscitation fluid because of an ability to at least transiently restore physiologic oncotic pressure, participate in solute transport, provide plasma pH buffering and anticoagulant activity, and, finally, scavenge inflammatory-derived reactive species and bind prooxidant transition metals and porphyrin derivatives.<sup>32,33</sup> However, in pathologic situations such as sepsis, high concentrations of HSA may accumulate in the interstitium and lead to the formation of interstitial edema, thus stealing from the intravascular space. Although previous reports have emphasized the central roles of the cys34 of HSA in antioxidant and antiinflammatory actions,<sup>4,6,34</sup> we did not observe this to be a critical factor in inflammatory and vascular cell model systems designed to evaluate the protective actions of various synthetic and biologic oxidant scavengers toward oxidative inflammatory mediators.

#### *Vascular Cell Inflammatory Influences of Hydroxyethyl Starch*

Hydroxyethyl starches have shown contrasting effects on markers of inflammation, whereas we observed HES to have no effect or an apparent proinflammatory action (fig. 6, table 1). When administered during resuscitation of patients who have experienced trauma, 10% HES significantly reduced the expression of plasma endothelial leukocyte adhesion molecules and intercellular adhesion molecules, compared with patients administered 20% HSA and pentoxifylline.<sup>34</sup> Other apparent antiinflammatory manifestations of HES include reduced PMN chemotaxis, decreased microvascular permeability, attenuation of the PMN respiratory burst, and a reduction in myocardial infarct size.<sup>35-37</sup> Again, characteristics of *in vitro* model systems may influence basal cell conditions and the impact of HES. Clinically, however, improvements in cardiopulmonary and oxygen delivery parameters occurred when HES was used as the exclu-

sive resuscitation fluid in patients experiencing septic shock and trauma.<sup>38</sup> Nonetheless, in a model of hemorrhagic shock, resuscitation with HES increased leukocyte adhesion to the hepatic sinusoidal wall and resulted in microcirculatory luminal narrowing.<sup>39</sup> Inflammatory vascular injury has been shown to include an important role for PMN MPO, which directly mediates biomolecule oxidation and catalytically consumes  $\cdot\text{NO}$ , thus impairing the vascular relaxation, eicosanoid-regulatory, cell adhesion, and antiinflammatory actions of this reactive signaling species.<sup>31,40</sup> Other than the current *in vitro* study, the influence of HSA *versus* HES administration on vascular PMN adhesion and vessel wall association of MPO has never been appraised and would be a fruitful area for further investigation.

#### *Study Limitations*

The use of systems lacking pulsatile flow and an intact circulatory system cannot take into account mechanical stress and, thus, stimulation of the vascular endothelium. Vascular endothelial stimulation by shear-dependent mechanisms leads to up-regulated cellular and humoral responses, such as integrin expression, resulting in an influence on PMN-endothelial cell interactions, including neutrophil binding properties. Therefore, extents of <sup>14</sup>C-adenine release, PMN-endothelial cell binding, and PMN-derived MPO binding to the vascular endothelium might have differed in extent, with no likely trend differences. Inclusion of additional fluids (*e.g.*, lactated Ringer's solution) would have allowed for added discrimination of solute effects on inflammation.

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