Abstract—Advanced oxidation protein products (AOPPs) are carried by oxidized plasma proteins, especially albumin and accumulate in subjects with renal disease and coronary artery disease. AOPPs represent an excellent novel marker of oxidative stress and their roles in the development of cardiovascular disease might be of great importance. Here, we show that in vitro–generated AOPP-albumin binds with high affinity to the high-density lipoprotein (HDL) receptor scavenger receptor class B type I (SR-BI). Already an equimolar concentration of AOPP-albumin to HDL blocked HDL association to SR-BI and effectively inhibited SR-BI–mediated cholesterol ester (CE) uptake. Interestingly, albumin extensively modified by advanced glycation end products (AGE-albumin), which is an established SR-BI ligand known to accumulate in renal disease, only weakly interfered with HDL binding to SR-BI. Furthermore, AOPP-albumin administration increased the plasma half-life of [3H]CE-HDL in control mice 1.6-fold (P=0.01) and 8-fold (P=0.0003) in mice infected with adenoviral vectors encoding human SR-BI. Moreover, albumin isolated from hemodialysis patients, but not albumin isolated from healthy controls, markedly inhibited SR-BI–mediated HDL-CE transfer in vitro dependent on the AOPP content of albumin. These results indicate that AOPP-albumin effectively blocks SR-BI in vitro and in vivo. Thus, depressed plasma clearance of HDL-cholesterol may contribute to the abnormal composition of HDL and the high cardiovascular risk observed in patients with chronic renal failure. (Circ Res. 2009;104:750-757.)

Key Words: AOPP ■ hemodialysis ■ myeloperoxidase ■ oxidative stress ■ HDL
Materials and Methods

**Materials**

NaOCl, organic solvents, potassium bromide, potassium iodide, chloramine-T, and fatty acid–free human serum albumin (HSA) were obtained from Sigma, isoflavone was from Pharmacia & Upjohn SA (Gyancourt, France). OxyBlot oxidized protein detection kit was from Millipore Corp (Molsheim, France). Ham’s-F12K medium was from Gibco (Life Technologies, Vienna, Austria). DMEM and FCS were obtained from Boehringer Ingelheim Bioproducts (Mannheim, Germany). HiTrap Blue HP, 1-mL columns were from (GE Healthcare, Vienna, Austria). Radiochemicals were purchased from PerkinElmer Life Sciences. All other reagents were obtained from Sigma (Vienna, Austria).

**Methods**

**HDL and LDL Preparation**

HDL (density range, 1.125 to 1.21 g/mL) and LDL (density, 1.063 g/mL) were prepared by discontinuous density ultracentrifugation of plasma obtained from HD patients or normalolipidemic blood donors.8

**Albumin Preparation**

Albumin from HD patients (HD-albumin) and controls was separated from other plasma proteins by affinity chromatography using HiTrap Blue HP, 1 mL columns (GE Healthcare) according to the instructions of the manufacturer.

**AOPP Assay**

The AOPP assay included a sample preparation procedure to precipitate lipoproteins in the plasma to avoid assay interference (mostly by triglycerides, which are usually markedly elevated in chronic kidney failure) as recently described.16 MgCl₂ (5 μL of a 2 mol/L stock solution) and phosphotungstate (20 μL of a 4% stock solution in 0.19 mol/L NaOH) were mixed with 200 μL of EDTA plasma, centrifuged at 1000 g for 20 minutes, on which the supernatant was carefully removed. AOPP were immediately measured in the supernatant at 340 nm under acidic conditions and expressed as chloramine-T equivalents.1

**Modification of Albumin and LDL**

AOPP-albumin was prepared in the absence of free amino acids/carbohydrates/lipids to exclude formation of advanced glycation end products (AGE)-like structures as described.17,18 Briefly, 10 mg of fatty acid–free HSA per millilitre of PBS was incubated with HOCl solution at 4°C for up to 1 hour at pH 7.4. The modified HSA preparations were passed over a PD10 column to remove unreacted HOCl and used immediately for experiments.

AGE-albumin was prepared as described previously.19 Briefly, 0.5 g of fatty acid–free HSA was dissolved with 3.0 g of d-glucose in 10 mL of 0.5 mol/L sodium phosphate buffer (pH 7.4) containing 0.05% NaN₃. Each solution was deoxygenated with nitrogen, sterilitized by ultrafiltration, and incubated for 90 days at 37°C in the dark. The samples were then passed over a PD 10 column and used for experiments.

Copper-induced oxidation of LDL was performed as described.9

**Amino Acid Analysis**

Aliquots of native, AOPP, and AGE-modified albumin (500 μg of protein) were lyophilized in 5-mL ampoules and purged with nitrogen before hydrolysis in constant boiling 6 N HCl (24 hour, 120°C). Amino acid analysis was performed on a Biotronics analyzer as described.18

**Labeling Procedures**

HDL and albumin labeling with ¹²⁵I-Na iodination was performed as described using N-bromosuccinimide as the coupling agent. HDL was labeled with [cholesteryl-1,2,6,7-³H]palmitate by CE transfer protein-catalyzed transfer from donor liposomes as described.8

**Cell Culture**

Chinese hamster ovary (CHO) cells: LdIA cells (LDL receptor-deficient CHO cell line) and stable transfectants expressing murine SR-BI (LdIA(SR-BI)) were cultured and maintained as described.8 Both cell lines were kindly provided by Dr Monty Krieger (Massachusetts Institute of Technology, Boston).

**SDS-PAGE and Western Blotting**

SDS-PAGE and subsequent Western blotting experiments of plasma and albumin preparations were performed with 3.75% to 20% polyacrylamide gradient gels as described.19

**Detection of Carbonylated Proteins**

Carboxylylated albumin was detected using the chemical and immunological reagents of the OxyBlot oxidized protein detection kit according to the instructions of the manufacturer.

**Cell Association Studies**

LdIA(SR-BI) and control LdIA7 cells were incubated for 2 hours at 37°C with ¹²⁵I-labeled albumin/HDL (0 to 800 μg/mL) in DMEM. Specific cell association of ¹²⁵I-labeled proteins to SR-BI was determined by subtracting association to control LdIA7 cells from LdIA(SR-BI) cells as described.8

**Recombinant Adenovirus Preparation**

Generation of adenoviral vectors encoding hSR-BI (Ad/hSR-BI) or lacZ cDNA (Ad/lacZ) have been described previously.20

**¹²⁵I-AOPP-Albumin and ³H-CE-HDL Turnover**

**In Vivo**

Three-month-old male BALB/c mice were anesthetized with isoflurane and infected via the tail vein with 2×10⁹ virus particles of Ad/hSR-BI and Ad/lacZ.

**¹²⁵I-AOPP-Albumin Turnover**

Four days after infection, 5 mice of Ad/hSR-BI and Ad/lacZ mice were injected via tail vein with 100 μg of ¹²⁵I-AOPP-albumin (1×10⁵ cpm) or control ¹²⁵I-albumin in 100 μL of PBS. Blood samples were drawn from anesthetized mice starting at 30 seconds up to 60 minutes by retroorbital puncture.

**³H-CE-HDL Turnover**

Four days after infection, 5 mice of Ad/hSR-BI and Ad/lacZ mice were injected via tail vein with a mixture comprising 50 μg of [³H]CE-HDL (5×10⁵ cpm) and either native albumin or AOPP-albumin (5 mg) in 100 μL of PBS. Blood samples were drawn from anesthetized mice at 30 seconds up to 60 minutes by retroorbital puncture. Plasma samples were analyzed by liquid scintillation counting.

Liver samples were harvested from mice killed by an overdose of isoflurane to measure uptake of ¹²⁵I-albumin preparations or ³H-CE-HDL and 50 μg liver homogenates were analyzed by Western blotting to determine SR-BI expression. All animal experiments were approved by the Austrian Ministry of Education, Science and Culture according to the Regulations for Animal Experimentation.
AOPP-albumin and AGE-albumin, an amino acid analysis was performed. It is evident that lysine, tyrosine, histidine, arginine, and cysteine were oxidized by HOCl dose-dependently up to 20% to 70% at the highest HOCl:albumin molar ratio, whereas 40% to 70% of lysine, arginine, and histidine residues were modified in AGE-albumin (Table 1).

### Cell Association Studies

To obtain specific binding data of AOPP-albumin preparations to SR-BI, SR-BI–overexpressing CHO cells (LdlA[SR-BI]) and control LdlA7 cells that show only minimal SR-BI expression (see insert, Figure 2A) were incubated with increasing concentrations of in vitro–modified $^{125}$I-labeled AOPP-albumin and native albumin. As shown in Figure 2A, SR-BI specifically mediated binding of $^{125}$I-AOPP-albumin, and binding curves exhibited a dose-dependent saturation pattern (Figure 2A). Nonlinear regression analysis revealed a marked increase in binding affinity ($K_d$ values are listed in Table 2) for AOPP-albumin related to the increased AOPP content of albumin (Table 2). Next, we investigated whether AOPP-albumin interferes with HDL association to SR-BI and subsequently inhibits HDL-CE delivery. For this purpose, we performed competition experiments with a constant amount of $^{125}$I-HDL or $^3$H-CE–labeled HDL (10 μg/mL) in the presence of increasing concentrations of native and modified albumin. As shown in Figure 2B, AOPP-albumin effectively displaced $^{125}$I-HDL from LdlA(SR-BI) cells and attenuated SR-BI–mediated CE transfer (Figure 2C), whereas native albumin showed no effect. Thus, AOPP-albumin is a potent SR-BI antagonist. In contrast, the known SR-BI ligand AGE-albumin$^{21}$ showed only weak inhibitory activity on SR-BI–mediated CE transfer (Figure 2C) compared to AOPP-albumin. The calculated $IC_{50}$ values are listed in Table 2. Of note, AOPP-albumin more effectively blocked SR-BI–mediated HDL-CE-transfer than $^{125}$I-HDL binding. This is in line with the observation that iodination of HDL increases the affinity of HDL to SR-BI (G. Marsche, unpublished data, 2008).

### 125I-AOPP-Albumin and 3H-CE-HDL Clearances In Vivo

Prompted by the high affinity binding of AOPP-albumin to SR-BI, we analyzed the fate of $^{125}$I-AOPP-albumin injected into mice. To test whether SR-BI is directly involved in AOPP-albumin clearance, we induced SR-BI expression by infecting mice with Ad/hSR-BI virus. Control mice were infected with Ad/lacZ. SR-BI expression in livers of Ad/
hSR-BI and Ad/LacZ mice is shown in the inset of Figure 3A. hSR-BI expression in livers decreased plasma total cholesterol levels from 52 ± 8 (Ad/LacZ) to 15 ± 2.5 mg/dL. Plasma decay curves analyzed during a 1-hour period after injection (Figure 3A) showed that in control mice the calculated plasma half-life of 125I-AOPP-albumin is decreased (40.8 ± 2.2 versus 123.3 ± 1.7 minutes, P < 0.0001) compared with 125I-albumin. Importantly, half-life of 125I-AOPP-albumin was further decreased in Ad/hSR-BI–treated mice (23.5 ± 1.9 versus 40.8 ± 2.2 minutes; P = 0.0043). In contrast, hSR-BI expression did not exert any effect on 125I-albumin clearance. The decreased half-life of AOPP-albumin was reflected in its increased uptake by the liver as shown in Figure 3B.

To assess whether AOPP-albumin interferes with SR-BI mediated clearance of plasma HDL-CE in vivo, we examined the plasma clearance of [3H]-CE-HDL coinjected with either native albumin or AOPP-albumin in Ad/hSR-BI and Ad/LacZ-infected mice. Plasma decay curves analyzed during a 1-hour period post injection showed, as expected, an hSR-BI–mediated decrease in the plasma half-life of [3H]-CE-HDL coinjected with the native albumin, when compared with control Ad/lacZ mice (3.2 ± 0.3 versus 48.0 ± 2.3 minutes, P < 0.0001) (Figure 3C). Most importantly, AOPP-albumin administration increased the plasma half-life of [3H]-CE-HDL in control mice 1.6-fold (78.24 ± 6.2 versus 48.0 ± 2.3 minutes, P = 0.01) and 8-fold (25.3 ± 1.9 versus 3.2 ± 0.3 minutes, P < 0.0001) (Figure 3C).

Table 2. Binding Properties of Modified Albumin Preparations to SR-BI

<table>
<thead>
<tr>
<th>Preparation</th>
<th>AOPP (µmol/L)</th>
<th>Kd (µg/mL)</th>
<th>Bmax (ng/mg cell protein)</th>
<th>IC50 (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alb</td>
<td>1.1 ± 0.2</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>HDCl-Alb (25:1)</td>
<td>3.9 ± 0.4</td>
<td>99.3 ± 14</td>
<td>181.6 ± 14.6</td>
<td>33.8 ± 2.9</td>
</tr>
<tr>
<td>HOCl-Alb (50:1)</td>
<td>12.9 ± 0.8</td>
<td>45.3 ± 2.4</td>
<td>210.0 ± 4.7</td>
<td>8.5 ± 2.6</td>
</tr>
<tr>
<td>HOCl-Alb (100:1)</td>
<td>33.2 ± 5.2</td>
<td>10.5 ± 1.9</td>
<td>183.3 ± 9.1</td>
<td>4.1 ± 1.3</td>
</tr>
<tr>
<td>AGE-Alb</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>143.4 ± 4.1</td>
</tr>
</tbody>
</table>

AOPP values for albumin preparations (1 mg/mL) are expressed as chloramine-T equivalents. Calculated Kd and Bmax values for binding of 125I-labelled albumin preparations to SR-BI and IC50 values for inhibition of [3H]-CE-HDL (10 µg/mL) binding to SR-BI by albumin (Alb), AOPP-albumin (at indicated oxidant: protein molar ratio), and AGE-albumin are given. Calculations were performed by nonlinear regression analysis (GraphPad Prism). ND indicates not determined.
mediated HDL-CE clearance, we isolated albumin from HD patients with high plasma AOPP values and healthy age matched control subjects. The plasma content of AOPPs found in delipidated uremic plasma was significantly higher compared to controls (168.3±7.3 versus 41.0±1.5 μmol/L, mean±SEM, P<0.0001), respectively. To examine whether HD-albumin is able to interfere with SR-BI mediated CE uptake, LdlA(SR-BI) and control LdlA7 cells were incubated with 3H-CE–labeled HDL in the presence of albumin isolated from HD patients and controls, respectively. The calculated molar excess of albumin over HDL in plasma is 50 to 125 fold; therefore, we used a 70-fold molar excess of HD-albumin (1 mg/mL) over 3H-CE-HDL (25 μg/mL). As shown in Figure 4A,
albumin isolated from HD patients contained a high carbonyl content (insert Figure 4A) and profoundly decreased [3H]-CE-HDL association up to 50%. In contrast, albumin isolated from controls did not alter [3H]-CE-HDL association. The inhibitory activity of uremic albumin was dose dependent and also operative at higher, receptor saturating concentrations of both [3H]-CE-HDL (125 μg/mL) and HD-albumin (5 mg/mL) (data not shown). Next, we examined the relationship between the AOPP content of HD-albumin and its SR-BI inhibitory activity. For that purpose, albumin was isolated from HD patients with low and high plasma AOPP content. The AOPP content of 1 mg/mL isolated HD-albumin ranged from 2 to 10 μmol/L and was similar to the AOPP content of in vitro–generated AOPP-albumin (Table 2). As shown in Figure 4B, the SR-BI inhibitory activity of HD-albumin measured as the efficacy to attenuate [3H]-CE-HDL association with LdlA7(SR-BI) cells correlated highly significantly with the AOPP content of HD-albumin (r = 0.91, P < 0.001). Increased oxidative stress in HD patients might also lead to formation of oxidized HDL, which binds with increased affinity to SR-BI. Therefore, we tested whether HD-albumin is able to block SR-BI association of HDL isolated from HD patients (HD-HDL). Indeed, HD-albumin (1 mg/mL; AOPP content, 7.6 μmol/L) displaced HD-HDL isolated from two patients (35% to 45%) to approximately the same extent as found for HDL isolated from controls (40%) (Figure 4C). This finding clearly indicates that the affinity of HD-HDL to SR-BI is similar to that of control HDL.

### Binding Properties of HD-Albumin and AOPP-Albumin to SR-BI

To determine the binding properties of HD-albumin to SR-BI, binding studies with LdlA(SR-BI) and control LdlA7 cells were performed (Figure 5A). Binding capacity of [125I]-HD-albumin to LdlA(SR-BI) cells was higher compared to LdlA7 cells, indicating that SR-BI mediates binding of HD-albumin. Nonlinear regression analysis revealed a Kd value of 336 ± 48 μg/mL and a Bmax value of 324 ± 35 ng/mg cell protein. To further demonstrate that HD-albumin and in vitro–generated AOPP-albumin bind to the same binding site(s) of SR-BI, competition experiments with known SR-BI ligands were performed. For that purpose, LdlA(SR-BI) and LdlA7 cells were incubated with 20 μg/mL [125I]-HDL isolated from 1 control and 2 HD patients in the presence of 200 μg/mL competitors. As shown in Figure 5B, HOCI-albumin, HDL, and oxidized LDL effectively displaced [125I]-HD-albumin, as well as [125I]-AOPP-albumin in the presence of 200 μg/mL competitors. As shown in Figure 5B, HOCI-albumin, HDL, and oxidized LDL effectively displaced [125I]-HD-albumin, as well as [125I]-AOPP-albumin. Importantly, AGE-albumin did not affect [125I]-AOPP-albumin binding and only weakly interfered with [125I]-HD-albumin binding, indicating that HD-albumin and in vitro–generated AOPP-albumin, but not AGE-albumin, share the same binding site on SR-BI. Only minimal competition was observed in LdlA7 cells.

### Discussion

In the present study, we demonstrate that albumin isolated from nondiabetic HD patients effectively blocks SR-BI-mediated HDL-CE transfer. We provide evidence that the inhibitory activity of albumin from uremic patients is
Our results are in line with a recent study showing that AGE-albumin had no effect on HDL binding to SR-BI,21 indicating that the binding domain(s) of AGE-albumin and HDL are not identical. Furthermore, it has been recently shown that the affinity of AGE-proteins to SR-BI strictly depends on the extent of modification by AGEs. Only highly modified AGE-albumin is significantly recognized by SR-BI, whereas mildly, more physiological modified AGE-albumin shows no ligand activity.22

The inhibitory activity of in vitro–generated AOPP-albumin is stronger compared to albumin isolated from HD patients, despite similar AOPP content of albumin. Our binding data show that in vitro–generated AOPP-albumin with an AOPP content of 3.9 μmol/L binds with higher affinity (Kd value of ≈100 μg/mL) (Table 2) to SR-BI compared to isolated HD-albumin (AOPP content of 7.2 μmol/L; Kd value of ≈340 μg/mL) (Figure 5A). The reason for this difference is probably because ≈50% of the AOPP generation in HD patients results from MPO-independent modifications.7 Therefore, it is likely that non MPO-derived modifications, like the AGE-product pentosidine,1 with weak or no SR-BI inhibitory activity, may contribute to the AOPP content of HD-albumin. However, the observed inhibitory activity of HD-albumin is of clear physiological relevance. The calculated molar excess of albumin over HDL in plasma is ≈50- to 125-fold. Therefore, our data indicate that a physiological molar excess of HD-albumin over HDL (≈70 fold) may block up to 50% of HDL-CE delivery to SR-BI.

Our findings do not clarify the molecular mechanism responsible for AOPP-albumin binding to SR-BI. All of the identified modified proteins recognized by the multiligand receptor SR-BI hold in common a negative charge, suggesting that SR-BI recognizes the negative charge of proteins. In line with this finding, modification of albumin-lysine residues by the MPO product HOCl (leading to a decrease of positive charge) markedly affects albumin interaction with SR-BI (Tables 1 and 2). This observation is also supported by the finding that HOCl-modification of HDL and LDL also increases the affinity to SR-BI up to 10-fold.8,9 Thus, it can be speculated that under proinflammatory conditions, AOPP-albumin and oxidized lipoproteins may markedly impair SR-BI–mediated reverse cholesterol transfer.

It has been recently shown that a decreased clearance of HDL, as shown in SR-BI–deficient mice, renders HDL dysfunctional and proinflammatory.23 Another study showed that the HDL inflammatory index correlates with poor outcome in HD patients.24 This is of particular interest because besides transferring excess cholesterol to the liver, HDL has antioxidant, antiinflammatory, vasodilating, and antithrombotic properties.25 Moreover, hepatic expression of SR-BI has been shown to be a positive regulator of cholesterol efflux from macrophages26; hence, blockade of SR-BI by AOPP-albumin might directly contribute to foam cell formation in the arterial wall.

The risk for cardiovascular disease in a 30-year-old end-stage renal disease patient is similar to the calculated risk of a 70- to 80-year-old subject from the nonrenal population.27,28 Most importantly, AOPPs are risk factors for cardiovascular events in nondiabetic patients with renal disease.29 Chronic renal failure in humans results in profound lipid disorders, which arise largely from dysregulation of HDL and triglyceride-rich lipoprotein metabolism.13,15 Accordingly, an AOPP–albumin–induced decrease in SR-BI–mediated HDL-CE clearance, as observed in the present study, could promote the shift/redistribution of CE from HDL to the proatherogenic apoB containing lipoprotein pool.

Besides acting as a high-affinity ligand for SR-BI, as observed in the present study, AOPP-albumin is also recognized by CD3640 and the receptor for advanced glycation end products (RAGE).18,31,32 Furthermore, AOPP-albumin stimulates the oxidative burst and the synthesis of proinflammatory
cytokines in neutrophils and monocytes, indicating that oxidized albumin may be a potent inflammatory mediator in vivo. Together these observed effects of in vitro–generated AOPP-albumin were not mediated by AGE-like structures, because the content of AGEs does not increase when albumin is modified by HOCl. In summary, we provide strong in vivo and in vitro evidence that AOPPs, which arise from the reaction between chlorinated oxidants and plasma proteins, are proinflammatory mediators that directly impair HDL metabolism and might therefore be potential key players in the development of cardiovascular disease.

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**Disclosures**
None.

**References**
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