CD36 is one of important receptors promoting renal tubular injury by advanced oxidation protein products

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PROTEINURIA IS A PROMINENT feature of many renal diseases. It is a consequence of glomerular capillary wall breakdown, causing abnormal transglomerular passage of plasma protein. Excessive plasma proteins can gain access to the proximal tubular cells (PTC), causing tubulointerstitial inflammation, tubular atrophy, and tubulointerstitial fibrosis (2, 41). The most prevalent protein in the urine of nephritic patients is human serum albumin (HSA). Several authors have postulated that excessive filtration of HSA into proximal tubules may have a detrimental effect on tubulointerstitial function (18, 44). In in vitro experiments, albumin stimulates various intracellular signaling pathways in PTC and induces them to produce various chemokines, such as that caused by HSA. Recently, Li et al. (26) showed that treatment with AOPPs-HSA enhanced AOPPs levels in HK-2 cells, whereas anti-CD36 antibody neutralizes the upregulation of TGF-B1. These results suggest that AOPPs-HSA may cause renal tubular injury via the CD36 pathway.

HSA is the most abundant plasma protein and serves as a carrier of endogenous and exogenous compounds (39). In addition, HSA is quite vulnerable to reactive oxygen species (ROS) (9). In chronic kidney disease (CKD), oxidative stress is increased because there is an imbalance between excessive generation of oxidant compounds and insufficient antioxidant defense mechanisms. This results in the generation of large amounts of ROS, such as O2, H2O2, and HOCl, by activation of neutrophils. In particular, HOCl, which is a powerful oxidizing agent, reacts with a wide variety of biological molecules, such as DNA, amino acids, peptides, and proteins (1, 40, 47). Recently, Witko-Sarsat et al. (48) reported the presence of elevated levels of advanced oxidation protein products (AOPPs) in the plasma of uremia, and Capeillere-Blandin et al. (5) identified albumin as the main AOPPs in plasma. Furthermore, plasma concentration of AOPPs was closely correlated with the levels of dityrosine, a hallmark of oxidized protein, and pentosidine, a marker of protein glycation closely related to oxidative stress (19). Thus AOPPs might be formed during oxidative stress by the reaction of albumin with chlorinated oxidants and considered novel markers of oxidant-mediated protein damage (48).

A more recent finding is that AOPPs are highly correlated with carotid intima media thickness (13) and may even be related to atherosclerotic cardiovascular events (10). Chronic administration of AOPPs accelerated atherosclerosis in a hyperlipidemic rabbit model (29). A clinical study revealed a close relationship between levels of AOPPs and serum markers of monocyte activation (49). These data suggest that these oxidized proteins contribute to the inflammatory processes associated with several diseases. On the basis of this information, we hypothesized that a large amount of plasma AOPPs may access PTC across the glomerulus and cause PTC injury, such as that caused by HSA. Recently, Li et al. (26) showed that treatment with AOPPs-HSA enhanced AOPPs levels in plasma and renal tissue and upregulated expression of monocyte chemoattractant protein-1 and transforming growth factor (TGF)-B1 in the renal cortex, demonstrating that chronic accumulation of AOPPs promotes renal fibrosis. However, the underlying mechanisms of induced progressive renal damage by AOPPs at the cellular level remain largely unresolved.

In the present study, chloramine-T (HOCl analog) was used to prepare AOPPs-HSA. Chloramines are primarily produced by HOCl reactions (43). Because chloramines retain the oxidizing equivalents of HOCl and are longer lived than HOCl...
chloramines are one of the important oxidants involved in the progression of CKD. Therefore, we examined the mechanisms of AOPPs-HSA and the detrimental effects induced by AOPPs-HSA in HK-2 cells (in vitro model as PTC).

MATERIALS AND METHODS

Chemicals. HSA was donated by the Chemo-Sera-Therapeutic Research Institute (Kumamoto, Japan). BSA (fraction V) was purchased from Wako Pure Chemical Industries (Osaka, Japan). Chloramine T and protease inhibitor cocktail were purchased from Nacalai Tesque (Kyoto, Japan). Keratinocyte serum-free medium (K-SFM) and epidermal growth factor were purchased from Gibco Life Technologies. Bovine pituitary extract was purchased from Kurabo. DMEM was obtained from Sigma (St. Louis, MO). Penicillin G (1,650 IU/mg), streptomycin sulfate (750 IU/mg), G418, and Ham’s F-12 medium were purchased from Life Technologies, and Na125I (3.7 GBq/ml in NaOH) was purchased from Amersham Pharmacia Biotech (Little Chalfont, Buckinghamshire, UK). Goat anti-CD36 polyclonal antibody (L-17), goat anti-actin polyclonal antibody (I-19), and horseradish peroxidase (HRP)-rabbit anti-goat IgG (H + L) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-human CD36 monoclonal antibody (FA6-152) was purchased from Immunotech. Humanized mouse anti-human lectin-like oxidized low-density lipoprotein (LDL) receptor (LOX-1) monoclonal antibody (JTX92) was donated by Dr. T. Sawamura (National Cardiovascular Center Research Institute, Osaka, Japan). All reagents used were of the highest grade available from commercial sources.

AOPPs-HSA preparation and determination. Fraction V HSA (96% pure) was defatted using the charcoal procedure described by Chen (6), deionized, freeze dried, and then stored at −20°C until used. AOPPs-HSA was prepared in vitro as described previously (22). Briefly, HSA (300 μM) was incubated for 1 h in phosphate buffer (pH 8.0) at 37°C in an oxygen-saturated solution containing 100 mM chloramine-T, an HOCl analog. After incubation, the oxidation reactions were stopped by extensive dialysis of solutions against water. The control involved incubating HSA dissolved in buffer alone, and in all cases the proteins were stored at −20°C until use. The endotoxin levels in HSA and AOPPs-HSA were measured with the Limulus Amoebocyte lysate test (the endospecy test) and were found to be 0.072 ng/ml protein in HSA and 0.095 ng/ml protein in AOPPs-HSA. Since Valencia et al. (45) demonstrated that low levels of endotoxin (below 0.2 ng/ml protein) did not show any effects on the induction of cellular responses, we used those AOPPs-HSA preparations in the present study. The content of AOPPs, which was thought as a useful oxidative stress marker, was determined as described previously (49). Briefly, 200 μl of samples were placed in a 96-well microtiter plate (Becton Dickinson Labware, Lincoln Park, NJ) and mixed with 20 μl of acetic acid. In standard wells, 10 μl of 1.16 mol/l potassium iodide was added to 200 μl of chloramine-T solution, followed by 20 μl of acetic acid. The absorbance of the reaction mixture at 340 nm was read immediately in a microplate reader. The contents of AOPPs in our AOPPs-HSA and HSA samples (1.34 mg/ml solutions) were 244.3 ± 12.3 μM and 10.3 ± 6.3 μM, respectively. Since we used 0.0025–5 mg/ml of AOPPs-HSA or HSA throughout the present study, the ranges of the AOPPs content in our AOPPs-HSA and HSA were 0.46–911.5 μM and 0.019–38.5 μM, respectively. Dityrosine content, which is also a useful oxidative stress marker, of AOPPs-HSA was also significantly greater than that of HSA (0.7 ± 0.11 vs. 0.32 ± 0.13 nmol dityrosine per miligram of protein; P < 0.01). The contents of AOPPs and dityrosine in the plasma of patients undergoing hemodialysis are reported as 267.5 ± 16.5 μM and 1.03 ± 0.12 nmol dityrosine per miligram of protein, respectively (48), thus indicating that the degree of modification of our AOPPs-HSA preparation is comparatively similar to that of AOPPs and dityrosine levels in the plasma of patients undergoing hemodialysis. To determine whether the AOPPs-HSA contained advanced glycation end products (AGE), we measured the content of Ncarboxymethyl)lysine (CML), pyrrole, pentosidine, and imidazolone in both AOPPs-HSA and unmodified HSA by ELISA, as described elsewhere (32). Briefly, each well of a 96-well microtiter plate was coated with 100 μl of the sample to be tested in 50 mM sodium carbonate buffer (pH 9.6). Each well was then blocked with 0.5% gelatin and washed three times with PBS containing 0.05% Tween 20 (washing buffer). The wells were incubated for 1 h with each monoclonal antibody against these AGE structures dissolved in washing buffer. The wells were then washed with washing buffer three times, incubated with an HRP-conjugated antiserum IgG antibody, and finally incubated with 1,2-phenylenediamine dihydrochloride. The reaction was terminated by the addition of 0.1 ml of 1.0 M sulfuric acid, and the absorbance at 492 nm was read on a micro-ELISA plate reader. As a result, there were no significant differences in the content of AGE structures between AOPPs-HSA and unmodified HSA. In addition, agarose gel electrophoresis was performed to examine whether there was unmodified HSA in AOPPs-HSA using the Universal Gel/8 electrophoresis kit (Ciba-Corning, Tokyo, Japan), followed by staining with Coomassie brilliant blue (33). As a result, the electrophoretic mobility of the AOPPs-HSA toward the anode was higher than that of unmodified HSA, and there was no unmodified HSA in the AOPPs-HSA (data not shown).

Cell culture of HK-2. The immortalized human PTC line HK-2 (American Type Culture Center, Manassas, VA) was cultured at 37°C in 5% CO2 in K-SFM, supplemented with 5 ng/ml human recombinant epidermal growth factor and 0.05 μg/ml bovine pituitary extract. HK-2 cells were seeded to tissue culture flasks (Greiner Bio-One) and further grown to confluence until cellular assay.

Cell culture and isolation of a transfected cell line (CD36-CHO cells and mock-CHO cells). The cDNA of human CD36 was amplified from a human placenta cDNA library by polymerase chain reaction using the primers as described previously (37). The amplified human CD36 cDNA was transfected into Chinese hamster ovary (CHO)-K1 cells by the electroporation method. CD36-CHO cells were selected and maintained as described previously (37).

RNA interference of megalin to HK-2 cells. Human megalin siRNA consisted of three target-specific 20- to 25-nt siRNAs, and the siRNA negative control that contained a scrambled sequence was obtained from Santa Cruz Biotechnology. HK-2 cells, 1 × 105, in log phase were plated into six-well tissue culture plates with normal growth medium 24 h before transfection or when the cells reached 60–80% confluence. For each transfection well, the siRNA-transfection reagent complexes were overlaid onto the washed cells. Cells were incubated in normal cell culture conditions for 7 h, and then fresh normal growth medium was added to each well to maximize cell growth and prevent potential cytotoxicity. After 24 h of transfection, assays for target gene expression by Western blot were performed using goat anti-human megalin antibody (C19, Santa Cruz Biotechnology).

Cellular assays. Cellular endocytosis experiments were performed at 37°C in a humidified atmosphere of 5% CO2 in air. HK-2 cells were cultured in supplemented K-SFM. Cells (1 × 105) were seeded in a 24-well plate and cultured for 1 day in 1.0 ml of this medium, which was then replaced by un-supplemented K-SFM (medium A). After culture for 1 h (cells cultured to 90–100% confluence), each well received 0.5 ml of medium A containing various concentrations of...
125I-HSA or 125I-AOPPs-HSA in either the presence or the absence of 50-fold excess amounts of the unlabeled ligands. For inhibition experiments, the incubation medium also included anti-human CD36 monoclonal antibody (FA6-152, 80 μg/ml) or control IgG (anti-human IgG monoclonal antibody, 80 μg/ml). CD36-CHO cells and mock-CHO cells were maintained with Ham’s F-12 medium (Gibco) supplemented with 10 μg/ml of blasticidin S (Funakoshi, Tokyo, Japan) and 10% fetal calf serum (medium B) in humidified air with 5% CO₂. Cells (1 × 10⁶) were seeded in a 24-well plate and cultured for 1 day in 1.0 ml of medium B, which was then replaced by DMEM containing 3% BSA (medium C). After being cultured for 1 h, each well received 0.5 ml of medium C containing various concentrations of 125I-AOPPs-HSA in either the presence or the absence of 50-fold excess amounts of the unlabeled ligand to be tested. After incubation for the indicated times, an aliquot (0.375 ml) of the culture medium was mixed with 0.15 ml of 40% trichloroacetic acid (TCA). To this solution was added 0.1 ml of 0.7 M AgNO₃, followed by centrifugation at 1,500 g for 10 min. 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. Each well was then 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 h at 37°C to determine the cell-associated radioactivity. Specific cell association or degradation was determined by subtracting the nonspecific values from the totals.

**Immunoblotting technique.** HK-2 cells were solubilized with 1% Triton X-100/PBS buffer containing 50× protease inhibitor cocktail. These samples (10 μg) were run on 10% SDS-polyacrylamide gels, followed by electrophoretic transfer to nitrocellulose membrane. The membranes were exposed to a goat anti-CD36 polyclonal antibody (L-17, Santa Cruz Biotechnology), mouse anti-scavenger receptors class A-I/II (SR-A) monoclonal antibody (SRA-E5), humanized anti-human LOX-1 antibody (JTX92), rabbit anti-scavenger receptor class B type 1 (SR-B1) polyclonal antibody (Novus Biologicals), rabbit anti-human receptor for advanced glycation end products (RAGE) polyclonal antibody (5505), or rabbit anti-human galectin-3 polyclonal antibody and visualized by HRP-conjugated anti-goat, -mouse, -human, or -rabbit IgG antibody using the ECL Western blotting detection reagent (Amersham Biosciences). This membrane was reblotted with goat anti-actin polyclonal antibody (I-19, Santa Cruz Biotechnology) as an internal normal. The molecular sizes of CD36,

**Fig. 1. Dose-dependent endocytic association (A and B) and degradation (C and D) by HK-2 cells of 125I-human serum albumin (HSA) (A and C) and 125I-advanced oxidation protein products (AOPPs)-HSA (B and D).** HK-2 cells were incubated at 37°C for 24 h with the indicated concentrations of 125I-HSA and 125I-AOPPs-HSA in the presence (nonspecific: □) or absence (total: ○) of 30-fold excess amounts of the unlabeled ligands. The specific cell association or degradation (●) was plotted after subtracting the nonspecific values from the total values. Data represent the means ± SD (n = 6). *P < 0.05, **P < 0.01 vs. each specific cell association or degradation of HSA.
SR-A, LOX-1, SR-B1, RAGE, and galectin-3 detected by this immuno blot were 88, 50, 43, 82, 55, and 31 kDa, respectively.

Assessment of intracellular ROS. Intracellular ROS generation was assessed in HK-2 cells by means of an oxidant-sensitive dye, CM-H$_2$DCFDA. Suspensions of the cells (1 × 10$^6$ cells) were incubated with 10 μM CM-H$_2$DCFDA for 15 min at 37°C in the medium. After centrifugation and being washed to remove the unincorporated probe, cells were treated with several concentrations of normal-HSA or AOPPs-HSA medium for 30 min at 37°C and placed on ice. Accumulation of dichlorofluorescein (DCF) in HK-2 cells was measured with a flow cytometer (FACSCalibur; Becton Dickinson Biosciences) by monitoring the fluorescence at 526 nm. Intracellular ROS formation was expressed as a ratio of the mean fluorescence intensity of control cells incubated in ligand-free medium.

Measurement of active TGF-β1 proteins. Cells were plated at 1 × 10$^6$ cells/well in 12-well plates. The next day, cells were rested in serum-free assay medium for 24 h (cells cultured to 90–100% confluence) and then treated with or without normal-HSA or AOPPs-HSA for 24 h. TGF-β released into media was measured with an enzyme immunoassay system (Promega, Madison, WI) according to

![Graph A](image1.png)

**A**: effects of HSA on the endocytic association of 125I-AOPPs-HSA with HK-2 cells and of megalin siRNA transfection on the expression of megalin transcript and on the endocytic association of 125I-AOPPs-HSA with HK-2 cells.  

![Graph B](image2.png)

**B**: immunoblot analyses of megalin in HK-2 cells transfected with 80 pmol megalin siRNA was performed using goat anti-human megalin antibody (C19). The graphic representation of an immunoblot analysis demonstrated the effect of megalin siRNA on their protein expression in HK-2 cells. PSL, photo-stimulated luminescence.  

![Graph C](image3.png)

**C**: cells transfected with 80 pmol megalin siRNA were incubated at 37°C for 24 h with 0.5 ml of K-SFM containing 2.5 μg/ml of 125I-AOPPs-HSA, followed by the determination of any endocytic association. Data represent the means ± SD (n = 4).
the manufacturer’s instructions. The inhibitory effect of TGF-β1 secretion in HK-2 cells was induced by treatment with anti-human CD36 monoclonal antibody (FA6-152, 80 μg/ml), N-acetylcysteine (NAC, 20 mM), diphenyleneiodonium (DPI, 10 μM), or control IgG (anti-human IgG monoclonal antibody, 80 μg/ml).

Statistics. Statistical analyses were performed using the Student’s t-test. A probability value of P < 0.05 was considered to indicate statistical significance.

RESULTS

Interaction of AOPPs-HSA with HK-2 cells. To examine whether AOPPs-HSA interacts with HK-2 cells, endocytosis experiments were conducted using 125I-labeled HSA or AOPPs-HSA in HK-2 cells. Upon incubation at 37°C, cell association of 125I-AOPPs-HSA with HK-2 cells significantly increased in a dose-dependent manner, by more than 125I-HSA (Fig. 1, A and B). In addition, endocytic degradation of 125I-AOPPs-HSA by HK-2 cells occurred at a higher level than HSA (Fig. 1, C and D). These results suggest that AOPPs-HSA undergoes endocytosis at a higher level than does HSA in HK-2 cells. Furthermore, we performed the ligand-binding study of AOPPs-HSA in HK-2 cells. The total binding of 125I-AOPPs-HSA to these cells at 4°C was inhibited by >60% by the presence of an excess amount of unlabeled AOPPs-HSA. The specific binding, which was obtained by subtracting nonspecific binding from the total binding, showed a saturation curve for which Scatchard analysis revealed a binding site with an apparent Kd of 1.93 μg/ml and a maximal binding of 111.2 ng/mg of cell protein (data not shown). These results indicated that HK-2 cells possess a high-affinity binding site for AOPPs-HSA. The recognition site for AOPPs-HSA in HK-2 cells was also examined. The cell association of 125I-AOPPs-HSA with HK-2 cells was effectively replaced by a 50-fold excess of unlabeled AOPPs-HSA (>80%, P < 0.01 vs. control), whereas a 50-fold excess of unlabeled HSA had only a slight effect (<5%, not significant vs. control) (Fig. 2A). The endocytic degradation of 125I-AOPPs-HSA was also inhibited almost completely by the presence of a 50-fold excess of unlabeled AOPPs-HSA (>80%, P < 0.01), whereas a 50-fold excess of unlabeled HSA had only a slight effect (<10%) (data not shown). These results indicated that AOPPs-HSA was endocytosed differently from HSA. Although it has been suggested that the receptors involved in the uptake of HSA are mainly the megalin/cubilin receptor complex (3, 8), those are unlikely to contribute to the endocytosis of AOPPs-HSA because megalin is negatively charged, thus showing a preferential affinity for positively charged proteins (31), whereas AOPPs-HSA shows a negative charge. To further elucidate the role of megalin/cubilin complex in the endocytosis of AOPPs-HSA in HK-2 cells, we evaluated the effect of silencing the megalin gene on the endocytosis of AOPPs-HSA. As a result, although the
transfection of megalin siRNA (80 pmol) resulted in more than 99% suppression of megalin protein expression in HK-2 cells, the association of 125I-AOPPs-HSA did not decrease in these cells (Fig. 2, B and C). In addition, the endocytic degradation of 125I-AOPPs-HSA was not also affected by megalin siRNA transfection (data not shown). Li et al. (27) demonstrated that transfection by megalin siRNA (50 pmol) resulted in up to 90% suppression of not only megalin but also cubilin protein and mRNA expression in HK-2 cells, suggesting that cubilin protein and mRNA expression are most probably knocked down in cells transfected with megalin siRNA. Therefore, these data demonstrated the evidence that the megalin-cubulin complex is not associated with the endocytosis of AOPPs-HSA in HK-2 cells.

CD36 was involved in the endocytosis of AOPPs-HSA in HK-2 cells. Recently, there have been reports of several types of receptors that bind to modified albumin (20). However, it is not yet clear which scavenger receptor is involved in the endocytosis of AOPPs-HSA by HK-2 cells. The expression of SR-A, scavenger receptors class B (CD36), LOX-1, SR-B1, RAGE, and galectin-3 was examined using a Western blotting analysis in HK-2 cells. As shown in Fig. 3, CD36, SR-B1, and galectin-3 were expressed in HK-2 cells, whereas SR-A, LOX-1, and RAGE were poorly expressed in HK cells. Specifically, CD36 is one of important receptors that is involved in the endocytosis of oxidized protein in adipocytes (25), thus suggesting that CD36 may be involved in the uptake of AOPPs-HSA in HK-2 cells. To elucidate this association, the cellular endocytosis of 125I-AOPPs-HSA was also examined using CD36-CHO cells (overexpressed CD36 in CHO cells) and mock-CHO cells (in which only the vector was transfected to CHO cells) (Fig. 4). The specific cell association and degradation of 125I-AOPPs-HSA with CD36-CHO cells exhibited a dose-dependent effect (Fig. 4, A and C). However, the specific cell association and degradation of 125I-AOPPs-HSA were not observed in mock-CHO cells (Fig. 4, B and D). These results demonstrated that AOPPs-HSA served as a ligand for CD36.

Fig. 4. Dose-dependent endocytic association (A and B) and degradation (C and D) of 125I-AOPPs-HSA by CD36-CHO cells (A and C) or mock-CHO cells (B and D). Cells were incubated at 37°C for 5 h in 0.5 ml DMEM containing 3% BSA with concentrations of 125I-AOPPs-HSA in the presence (nonspecific: ○) or absence (total: □) of 50-fold excess amounts of unlabeled ligands. The specific cell association and degradation (●) were plotted after subtracting the nonspecific values from the total values. Data represent the means ± SD (n = 3). *P < 0.01 vs. each specific cell association or degradation of AOPPs-HSA in mock-CHO cells.
To examine the involvement of CD36 in endocytosis of AOPPs-HSA in HK-2 cells, neutralizing anti-CD36 monoclonal antibody (FA6-152) was used for the endocytic association and degradation of $^{125}$I-AOPPs-HSA in HK-2 cells (Fig. 5). Nonspecific association, which was measured with a 50-fold excess of unlabeled AOPPs-HSA (Fig. 2), was subtracted from each experiment. As shown in Fig. 5, the receptor-mediated specific cell association and degradation of $^{125}$I-AOPPs-HSA in HK-2 cells was significantly inhibited by addition of anti-CD36 antibody (inhibitory effect of association: 35%, $P < 0.05$; inhibitory effect of degradation: 45%, $P < 0.01$), whereas nonimmune IgG and excess unlabeled HSA had no effect on this process (<5%, considered not significant vs. control). These results indicate that some endocytic pathways of AOPPs-HSA in HK-2 cells are mediated by CD36.

**AOPPs-HSA enhances the expression of CD36 in HK-2 cells.** It has been shown that oxidized LDL (ox-LDL) and AGE proteins, such as CD36 ligands, induce CD36 protein synthesis in macrophages (17, 23). Therefore, the effects of AOPPs-HSA on CD36 protein expression in HK-2 cells were examined. Exposure of cells to 100 $\mu$g/ml AOPPs-HSA at 37°C for 24 h, but not exposure to HSA, significantly increased levels of CD36 protein expression in cell lysates (Fig. 6). This result suggested that proteinuria, including a large amount of AOPPs-HSA, causes a series of adverse effects in PTC, resulting from upregulation of CD36 expression and increased uptake of AOPPs-HSA.

**AOPPs-HSA increased the generation of ROS in HK-2 cells.** To assess whether AOPPs-HSA could also induce oxidative stress like HSA, ROS in HK-2 cells was measured by the CM-H$_2$DCFDA method and fluorescence-activated cell-sorter analysis. After addition of HSA or AOPPs-HSA (1.0, 2.5, and 5.0 mg/ml) to HK-2 cells, ROS was generated in a dose-dependent manner by AOPPs-HSA. To a lesser extent, HSA also generated ROS in HK-2 cells (Fig. 7). To examine the mechanism of ROS generation by AOPPs-HSA, the authors examined the effects of the following: NAC (20 mM), a ROS scavenger; DPI (10 $\mu$M), an inhibitor of membrane NADPH oxidase; and staurosporine (100 nM), a PKC inhibitor for ROS generation. As shown in Fig. 7, all three inhibitors were able to block ROS generation induced by AOPPs-HSA, suggesting that AOPPs-HSA produced intercellular ROS by the activation of PKC and membrane NADPH oxidase.
AOPPs-HSA activated secretion of TGF-β1 by the CD36 pathway in HK-2 cells. To confirm whether AOPPs-HSA actually causes injury of HK-2 cells via the CD36 pathway, the secretion of TGF-β1 proteins, which causes fibrosis on PTC, was examined using an enzyme immunoassay. After the incubation of HK-2 cells with 100 μg/ml of HSA or AOPPs-HSA for 24 h, TGF-β1 proteins in the medium were measured. Higher levels of TGF-β1 proteins were secreted in the medium by cells exposed to AOPPs-HSA. To a lesser extent, HSA also produced TGF-β1 (Fig. 8). The involvement of CD36 in the secretion of TGF-β1 by AOPPs-HSA was also examined, using an anti-CD36 monoclonal antibody (FA6-152). As expected, FA6-152 significantly inhibited secretion of TGF-β1 induced by AOPPs-HSA in HK-2 cells. In addition, we examined whether NAC and DPI could suppress the secretion of TGF-β1 induced by AOPPs-HSA. These inhibitors significantly inhibited the secretion of TGF-β1 induced by AOPPs-HSA, thus suggesting that ROS generated by AOPPs-HSA act as signaling molecules in HK-2 cells and lead to the activation of TGF-β1.

DISCUSSION

CKD is a worldwide health problem, and the number of patients with CKD is increasing rapidly (7). Progressive renal disease is caused by the development of glomerulosclerosis and interstitial fibrosis. Therefore, it is important to explore the factor(s) that promote the process to develop new strategies for suppressing CKD. Recent studies have found that plasma concentration of AOPPs significantly increased with the progression of renal dysfunction in patients (48) and that AOPPs may contribute to the progression of CKD (26). However, the mechanisms by which AOPPs accelerate renal fibrosis remain poorly understood. Therefore, we examined the mechanisms of AOPPs and the detrimental effects induced by AOPPs in HK-2 cells. Taking this approach, the novel findings of the present study were as follows. 1) AOPPs-HSA undergoes endocytic uptake and subsequent lysosomal degradation by PTC and HK-2 cells. 2) These processes were effectively inhibited by anti-CD36 antibody. 3) AOPPs-HSA upregulated the generation of intracellular ROS via a mechanism that involves PKC and membrane NADPH oxidase signaling pathways, and the secretion of TGF-β1 in HK-2 cells. 4) AOPPs-HSA-induced secretion of TGF-β1 is mediated by CD36. Thus this study provides new evidence for a detailed mechanism by which AOPPs cause PTC injury via CD36.

CD36 is an 88-kDa transmembrane glycoprotein of the class B scavenger receptor family, and is involved in multiple biological processes (15). It is broadly expressed by renal tubular cells, platelets, monocytes, adipocytes, endothelial cells, erythroblasts, epithelial cells, and several tumor cell lines (15, 50). In addition, CD36 interacts with multiple extracellular ligands, including thrombospondin-1, long-chain free fatty acids, modified (oxidized) ox-LDL, collagens I and IV, and AGE (15). Previous reports showed that the expression of CD36 is upregulated by its own ligand. For instance, Iwashima et al. (23) showed that AGE induced the expression of CD36 in human monocyte-derived macrophages and in THP-1 cells (23). Susztak et al. (42) reported that CD36 expression was induced by d-glucose in PTC. In this study, AOPPs-HSA, a ligand for CD36, significantly induced protein expression of CD36 in a dose-dependent manner. Interestingly, HSA (100 μg/ml), not a ligand for CD36, slightly increased the expression of CD36 in HK-2 cells in this study. Recently, Yang et al. showed that high concentrations of normal albumin (1.0 and 10 mg/ml) induced the expression of CD36 in LLC-PK1 cells and enhanced the secretion of bioactive TGF-β1 and fibronectin with the upregulation of CD36 (50). Thus this report speculates that high concentrations of HSA (>1.0 mg/ml) may increase the expression of CD36 in HK-2 cells.

Fig. 7. Assessment of reactive oxygen species (ROS) by fluorescence-activated cell sorter analysis. After CM-H2DCFDA incubation, HK-2 cells were treated with HSA or AOPPs-HSA for 15 min. Accumulation of dichlorofluorescein (DCF) was measured with a flow cytometer by monitoring fluorescence at 526 nm. Intracellular ROS formation is expressed as a ratio of the mean fluorescence intensity (MFI) of control cells incubated in an albumin-free medium. Results are the means ± SD (n = 5). N-acetylcysteine (NAC) = 20 mM; diphenyleneiodonium (DPI) = 10 μM; staurosporine = 100 nM. *P < 0.05, **P < 0.01 vs. control cells. #P < 0.01 vs. cells treated with 5 mg/ml HSA.

Fig. 8. Effect of anti-CD36 antibody (FA6-152) or ROS inhibitors on the secretion of TGF-β1. Cells were treated with HSA, AOPPs-HSA, or AOPPs-HSA in the presence of anti-CD36 antibody (FA6-152), NAC, or DPI at 37°C for 24 h. Results are the means ± SD (n = 3). *P < 0.01 vs. control cells.
findings suggested the possibility that CD36 may be an important receptor in proximal tubular injury and that AOPPs and normal albumin may be deleterious mediators in this disease.

Recent evidence has clearly shown rapid and significant increases in intracellular ROS after growth factor and cytokine stimulation. These types of ROS appear to be essential for a host of downstream signaling events, including cell proliferation, apoptosis, and fibrosis, and thus contribute to the development of disease (16). Nakajima et al. (34) reported that albumin upregulated ROS production in PTC and that this was mediated by NADPH oxidase and PKC, indicating that albumin has the ability to increase the generation of ROS in PTC. In the present study, we used 1–5 mg/ml of HSA or AOPPs-HSA to investigate ROS production. The content of AOPPs in the plasma of patients undergoing hemodialysis is reported to be 267.5 μM (48), and the AOPPs content in 5 mg/ml AOPPs-HSA used in the present study is 911.5 μM, thus indicating that we used a 3.4-fold higher concentration of AOPPs protein than the pathophysiologically relevant concentration. We performed these experiments with short-term incubation in such a comparatively high concentration of AOPPs-HSA because AOPPs is continuously generated in vivo. The results of the present study clearly showed that AOPPs-HSA also upregulated increases in intracellular ROS in a dose-dependent manner, and, in particular, 5 mg/ml AOPPs-HSA induced increases in intracellular ROS about 1.8 times higher than did the control. In contrast, 5 mg/ml HSA slightly elevated intracellular ROS, about 1.3 times higher than did the control. A previous report showed that 30 mg/ml HSA significantly induced intracellular ROS about 1.8 times higher than did the control (34). These results demonstrated that AOPPs-HSA potentially activated the generation of ROS as well as HSA. Interestingly, the increases in ROS induced by AOPPs-HSA were significantly decreased by inhibitors of NADPH oxidase and PKC. It is highly possible that interaction of CD36 and AOPPs-HSA results in increased production of intracellular ROS that may take place via a mechanism that involves NADPH oxidase and PKC.

Since anti-CD36 antibody only partially suppresses the uptake of AOPPs-HSA by HK-2 cells, other receptors in PTC may also be involved in the uptake of AOPPs-HSA. In this study, SR-B1 and galectin-3 were also expressed in HK-2 cells (Fig. 3), thus suggesting that those are likely to be involved in the endocytosis of AOPPs-HSA in HK-2 cells. Recently, Marsche et al. (30) reported that CHO cells overexpressing class B scavenger receptors such as CD36 and SR-B1 recognize HOCl-modified LDL. SR-B1 also has a multiligand specificity for various forms of native and modified (lipo)proteins (24), and the protein expression of SR-B1 has been observed in PTC (51). Therefore, SR-B1 may also be involved in the uptake of AOPPs-HSA in the PTC. In addition, Nishiyama et al. (36) reported that galectin-3, a multifunctional β-galactoside-binding lectin, is expressed by epithelial cells of the kidney and participates in development, oncogenesis, cell-to-cell attachment, and inflammation, suggesting that galectin-3 may also play an important role in acute tubular injury. Furthermore, galectin-3 was identified as a component of the AGE receptor complex (28). These observations suggested that SR-B1 and galectin-3 might also be involved in PTC injury. Although further studies using CHO cells overexpressing SR-B1 and galectin-3 and those neutralizing antibodies would be required to determine whether SR-B1 and galectin-3 are also involved in the proximal tubular injury by AOPPs, there are no reports using the optimal neutralizing antibodies against SR-B1 and galectin-3, thus suggesting that we have to conduct another approach such as the silencing strategies for SR-B1 and galectin-3. However, there is only commercial reagent for SR-B1 knockdown, not for galectin-3. Therefore, for the time being, we performed the transfection of SR-B1 siRNA to HK-2 cells. As a result, although transfection by SR-B1 siRNA (80 pmol) resulted in more than 60% suppression of SR-B1 protein expression in HK-2 cells, the endocytic association and degradation of 125I-AOPPs-HSA did not decrease in these cells (data not shown). This result directly demonstrated that SR-B1 is not involved in the endocytosis of AOPPs-HSA in HK-2 cells. Further studies using siRNA knockdown against galectin-3 in HK-2 cells would therefore shed some light on the mechanisms of renal tubular injury as well as the extent of the contribution of each factor identified in this study.

In conclusion, a new functional role of CD36 was identified as an essential mediator of tubulointerstitial diseases induced by AOPPs in PTC. Furthermore, because AOPPs-HSA by itself stimulates CD36 expression in human PTC, it is speculated that uptake of AOPPs-HSA by PTC would be increased, causing further progression of renal disease. Therefore, CD36 appears to be closely associated with renal tubular dysfunction. Modulating the expression of CD36 or blocking this receptor may be a good approach in the treatment of renal tubular fibrosis.

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