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The receptor of advanced glycation end products plays a central role in advanced oxidation protein products-induced podocyte apoptosis

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The accumulation of plasma advanced oxidation protein products (AOPPs) is prevalent in chronic kidney disease. We previously showed that accumulation of AOPPs resulted in podocyte apoptosis and their deletion by a cascade of signaling events coupled with intracellular oxidative stress. The transmembrane receptor that specifically transmits the AOPPs' signals to elicit cellular activity, however, remains unknown. Using co-immunoprecipitation and immunofluorescence, we found that AOPPs colocalized and interacted with the receptor of advanced glycation end products (RAGE) on podocytes. Blocking RAGE by anti-RAGE immunoglobulin G or its silencing by siRNA significantly protected podocytes from AOPPs-induced apoptosis both *in vitro* and *in vivo* and ameliorated albuminuria in AOPPs-challenged mice. AOPPs-induced activation of nicotinamide adenine dinucleotide phosphate oxidase and the excessive generation of intracellular superoxide were largely inhibited by anti-RAGE immunoglobulin G or RAGE siRNA. Moreover, blockade of RAGE decreased the activation of the p53/Bax/caspase-dependent proapoptotic pathway induced by AOPPs. Thus, AOPPs interact with RAGE to induce podocyte apoptosis and this, in part, may contribute to the progression of chronic kidney disease.

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Chronic kidney disease (CKD) is a worldwide health problem, and the population of CKD patients is increasing rapidly.[1] The pathogenesis of CKD is characterized by widespread glomerulosclerosis and interstitial fibrosis. There is a growing body of literatures showing that podocyte loss is related to the increased proteinuria and contributes to the renal progression in both diabetic and nondiabetic CKD.[2–8] Therefore, exploring factors implicated in the pathogenesis of podocyte injury is of great importance to the development of new strategies for the treatment of CKD.

AOPPs are a family of oxidized, dityrosine-containing protein products generated during excessive production of oxidants and often carried by albumin *in vivo*.[9,10] Accumulation of plasma AOPPs was first found in patients undergoing dialysis[9] and subsequently demonstrated in patients with diabetes[11,12] and metabolic syndrome.[13] Clinical studies have shown that AOPPs level is a strong predictor for the prognosis of immunoglobulin-A nephropathy.[14] In experimental models, chronic accumulation of plasma AOPPs is associated with aggravated glomerulosclerosis in remnant kidney and diabetic nephropathy.[15,16] These data indicate that AOPPs may not merely be a marker for oxidative stress, they are actually a new class of renal pathogenic mediators as well.[9,14]

Most recently, we demonstrated that AOPPs induce podocyte apoptosis and deletion via the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-dependent, Rodox-sensitive signaling, for the first time, establishing a causative link between oxidative stress and podocyte loss. However, the transmembrane receptor that specifically transmits the signal of AOPPs to elicit cellular activity has not yet been addressed. Previous studies have shown that various scavenger receptors (SRs) such as RAGE (receptor of advanced glycation end products), SR class A, and CD36 are expressed on the surface of podocytes.[17–19] Marsche *et al.*[20,21] and our group have shown that class B type I receptor (SR-BI) and RAGE mediate AOPP-induced inflammatory response in endothelial cells.[20–22] Thus, the present

study was aimed to characterize the receptor responsible for AOPPs-induced podocytes apoptosis.

RESULTS

RAGE mediated AOPPs-induced apoptosis in cultured podocytes

To clarify which receptor on podocyte is responsible for AOPPs-induced podocyte apoptosis, we preincubated podocytes with various concentrations of anti-RAGE, anti-CD36, anti-SR class A, anti-LOX1, anti-AGE-R3, anti-SR-BI or anti-TLR4 antibody before AOPPs exposure. As shown in Figure 1a and b, Supplementary Figures S1 and S2 online, only anti-RAGE significantly blocked AOPPs-induced podocyte apoptosis. Preincubation with anti-RAGE antibody significantly abrogated the apoptotic effect of AOPPs in a dose-dependent manner with 70% reduction at 20 μg/ml concentration, whereas 20 μg/ml of nonimmune immunoglobulin G (IgG) had no detectable effect.

To further confirm the role of RAGE on AOPPs-induced podocyte apoptosis, we next knocked down endogenous RAGE expression using a RAGE-specific small interference RNA (siRNA). As shown in Figure 1c, RAGE siRNA reduced the RAGE protein expression by 60–70%. We then transfected RAGE siRNA or scramble siRNA into podocytes before AOPPs treatment. Flow cytometric analysis showed that RAGE-specific siRNA transfection resulted in a significant decrease in AOPPs-induced apoptosis as compared with scramble siRNA transfection (Figure 1d and Supplementary Figure S3 online). Similar result was obtained using terminal deoxyuridine nick-end labeling staining (TUNEL; (Figure 1e)).

To test whether AOPPs interact with RAGE on podocyte, we performed co-immunoprecipitation assay. As shown in Figure 1f, AOPPs were detected by anti-AOPPs antibody in the anti-RAGE immunoprecipitate in AOPPs-treated cells but not in control cells. The colocalization of AOPPs and RAGE on podocyte was further confirmed by co-immunofluorescence staining in AOPPs-treated cells (Figure 1g).

RAGE mediated AOPPs-induced intracellular superoxide generation *in vitro*

Our previous study has demonstrated that NAD(P)H oxidase-dependent O_2^- production is the key step of AOPPs-induced podocyte apoptosis. We thus examined whether this key step is mediated by RAGE. As shown in Figure 2a, AOPPs-induced O_2^- production could be blocked by both anti-RAGE antibody and RAGE siRNA transfection.

Similarly, AOPPs-induced activation of NAD(P)H oxidase, such as $p47^{phox}$ phosphorylation, binding of $p47^{phox}$ with $p22^{phox}$, and the expression of NADPH subunits $p47^{phox}$ and $gp91^{phox}$, could be inhibited by blocking RAGE using either anti-RAGE IgG or RAGE siRNA transfection (Figures 2b and c).

We previously demonstrated that AOPPs-induced NADPH oxidase-dependent O_2^- production could be blocked by total protein kinase C (PKC) inhibitor,[23] suggesting that PKC activation is the upstream event of NADPH oxidase activation. However, which isoform of PKC is responsible for this effect remains unclear. Therefore, we tested the effect of AOPPs on the phosphorylation of the main subunits of PKC such as PKCα, PKCβ1, PKCβ2, and PKCζ. As shown in Figure 3a and b, AOPPs exposure significantly increased the phosphorylation of PKCα ($P<0.01$), but had no effect on the phosphorylation of PKCβ1, PKCβ2, and PKCζ.

To test whether the activation of PKCα is mediated by RAGE, we pretreated podocytes with anti-RAGE or RAGE siRNA transfection before AOPPs exposure. As shown in Figure 3c and d, blocking RAGE dramatically attenuated AOPPs-induced PKCα phosphorylation, indicating that the effect of AOPPs on PKCα activation is mediated by RAGE.

RAGE mediated AOPPs-induced activation of p53/Bax/caspase-3 pathway *in vitro*

We previously demonstrated that AOPPS-induced podocyte apoptosis is associated with increased activity of the p53/Bax/caspase-3 pathway. To examine whether AOPPS-induced activation of p53/Bax/caspase-3 pathway was RAGE dependent, we preincubated podocytes with anti-RAGE antibody or nonimmune IgG before AOPPs exposure. As shown in Figure 4a, AOPPs-enhanced expression of p53 and Bax proteins was dramatically inhibited by anti-RAGE antibody. Similar result was obtained when podocytes were transfected with specific RAGE siRNA (Figure 4b).

Similarly, AOPPs challenge resulted in increased cleaved caspase-3 followed by decrease in procaspase-3. The levels of intact 113-kDa poly (adenosine diphosphate-ribose) polymerase (PARP), a substrate of activated caspases, were decreased after AOPPs treatment, coincident with the appearance of 85-kDa PARP cleavage products (Figure 4c and d). AOPPs-enhanced expression of cleaved caspase-3 and PARP (85 kDa) could be blocked by anti-RAGE antibody or RAGE siRNA (Figure 4c and d), suggesting that AOPPs-induced activation of proapoptotic pathway is mainly mediated by RAGE.

AOPPs enhanced RAGE expression on podocyte *in vitro* and *in vivo*

Previous studies have shown that RAGE expression is linked to the situations in which its ligands are abundant.[24,25] We therefore examined the effect of AOPPs on RAGE expression in podoctyes. Podocytes were treated with indicated concentrations of AOPPs for 12 h. As shown in Figure 5a–d, AOPPs challenge significantly augmented RAGE expression in cultured podocytes at both mRNA and protein levels in a dose- and time-dependent manner.

To determine whether AOPPs could induce RAGE expression *in vivo*, normal C57BL/6 mice were administrated with intravenous injection of AOPPs modified mouse serum albumin (AOPPs-MSA) for 5 weeks. We first examined the effect of AOPPs on RAGE expression by western blot. As shown in Figure 5e, RAGE expression was significantly increased in AOPPs-challenged mice compared with control mice. We next examined the localization of RAGE using immunohistochemistry staining. Co-staining of RAGE with antibody against podocyte marker Wilms's tumor-1 indicated

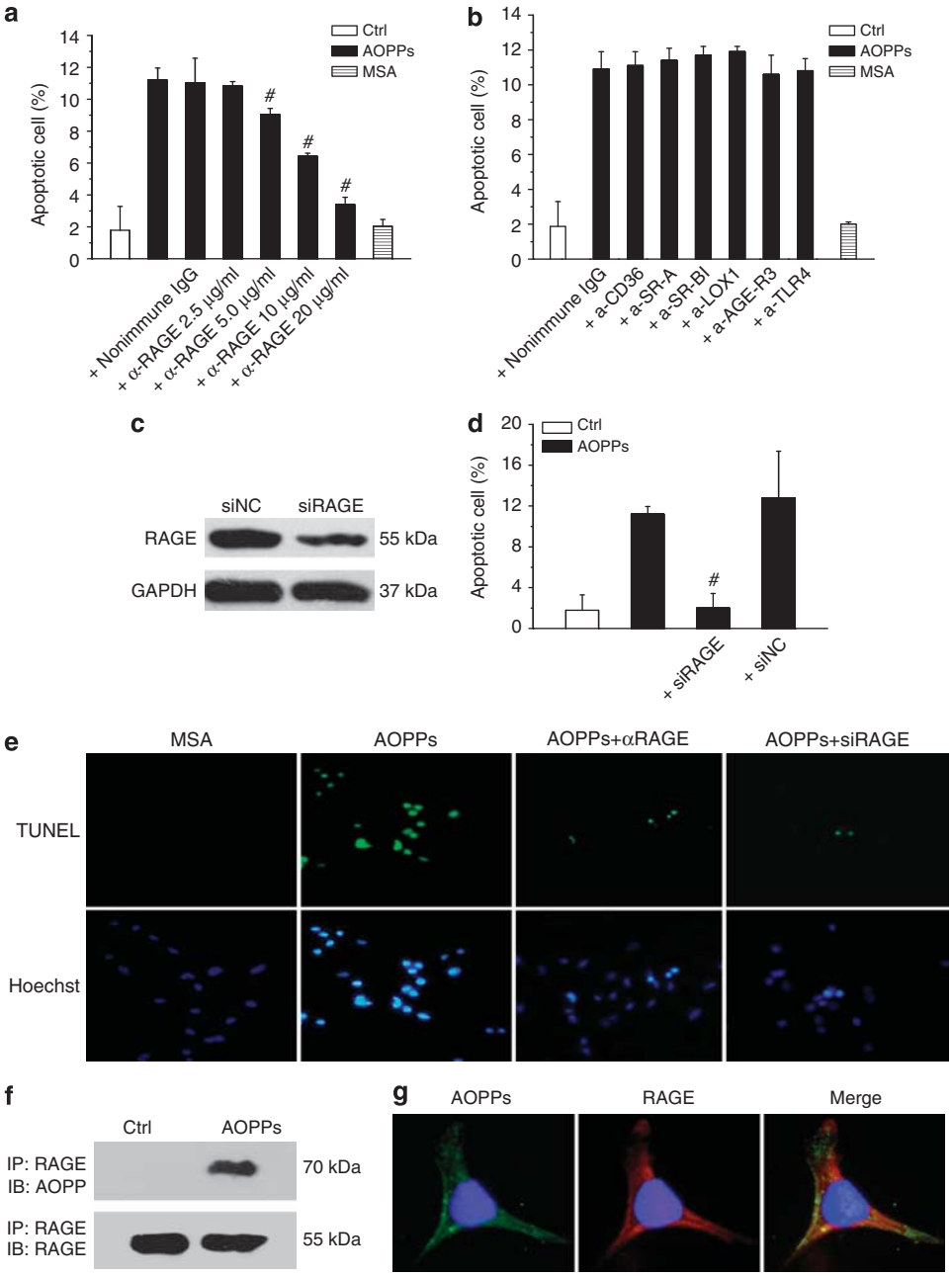

Figure 1 | AOPPs-induced podocyte apoptosis is RAGE-dependent. Mouse podocytes were treated with indicated amount of RAGE neutralizing antibody (**a**) or indicated neutralizing antibody (**b**) followed by AOPPs treatment for 24 h. Cells treated with medium alone (control, Ctrl) or unmodified mouse serum albumin (MSA) were served as controls. Podocytes apoptosis was analyzed by flow cytometry. (**c**) Podocytes were transfected with RAGE siRNA or scramble siRNA. Western blot was performed to analyze the expression of endogenous RAGE in podocytes. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to verify equivalent loading. (**d**) Podocytes were transfected with either RAGE siRNA or scramble siRNA. Cells were harvested and analyzed for apoptosis by flow cytometry 24 h after transfection. The percentage of apoptotic cells was identified with Annexin V^+PI^-. Flow cytometry data are expressed as mean ± s.d. of three independent experiments. Analysis of variance, $P<0.001$, $^{\#}P<0.05$ vs. nonimmune IgG-treated cells or scramble siRNA-transfected cells. (**e**) Podocytes were pretreated with RAGE neutralizing antibody or transfected with RAGE siRNA before AOPPs treatment. The apoptosis of podocytes were analyzed by TUNEL assay. Representative photographs showing double immunofluorescence labeling of Hoechst (blue) and TUNEL (green) in podocytes. (**f**) Co-immunoprecipitation of AOPPs and RAGE in cultured podocytes. Podocytes were treated with AOPPs for 30 min, the binding of AOPPs to RAGE was analyzed by immunoprecipitation using anti-RAGE antibody and followed by immunoblotting using anti-AOPPs antibody. (**g**) Colocalization of AOPPs and RAGE in cultured podocyte cells. Podocytes were treated with AOPPs for 30 min, and then the cells were co-stained with antibodies against AOPPs (green) and RAGE (red). Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI; blue). Images were taken by confocal microscopy. Original magnification × 400. AOPPs, advanced oxidation protein products; IB, immunoblot; Ig, immunoglobulin; IP, immunoprecipitation; RAGE, receptor of advanced glycation end products; siNC, scramble siRNA; siRNA, small interference RNA; TUNEL, terminal deoxyuridine nick-end labeling staining.

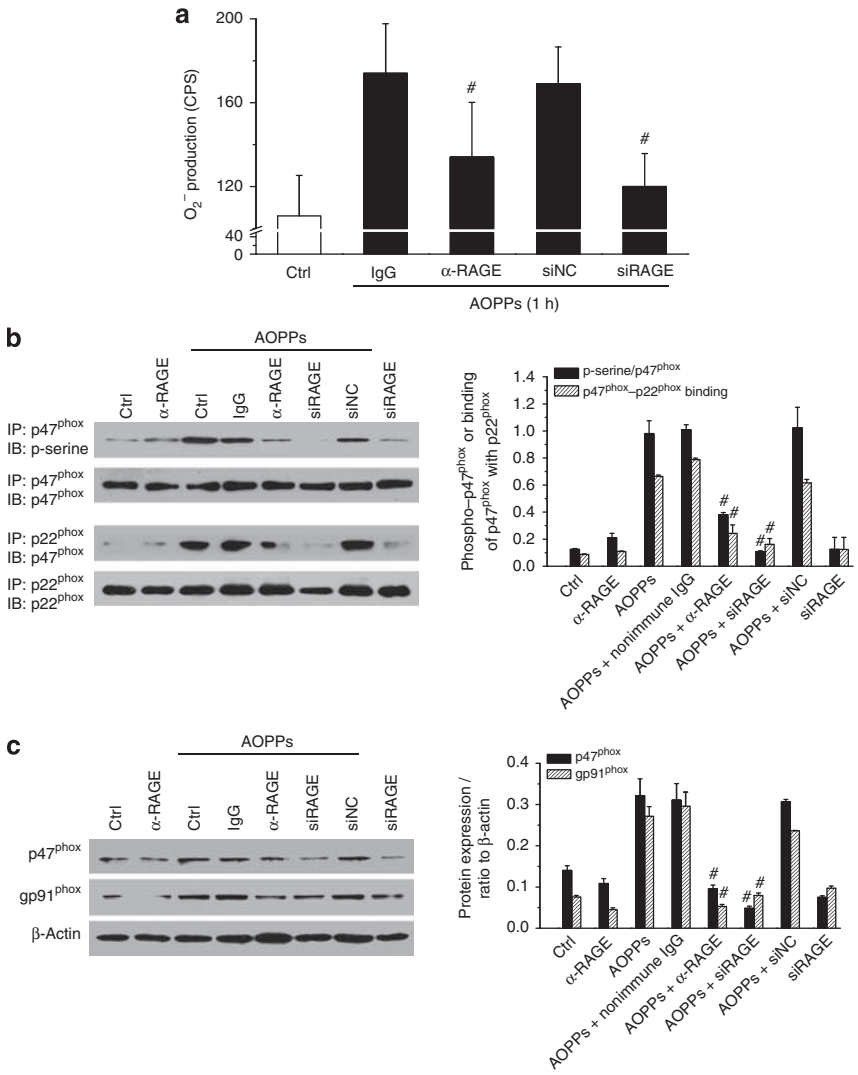

Figure 2 | AOPPs-induced nicotinamide adenine dinucleotide phosphate oxidase activation and O_2^- generation is RAGE-dependent. (**a**) AOPPs-augmented O_2^- production was inhibited by RAGE neutralizing antibody or RAGE siRNA. O_2^- production in podocyte homogenates was determined using the lucigenin chemiluminescence method. (**b**) Podocytes were preincubated with anti-RAGE antibody or transfected with RAGE siRNA followed by incubation with 200 μg/ml of AOPPs-MSA for 30 min. Phosphorylation of $p47^{phox}$ was assayed by immunoprecipitation using anti-$p47^{phox}$ antibody followed by immunoblotting with antibody against phosphoserine. The interaction of $p47^{phox}$ with $p22^{phox}$ was determined by immunoprecipitation using anti-$p22^{phox}$ antibody followed by immunoblotting with anti-$p47^{phox}$ antibody. (**c**) Podocytes were preincubated with anti-RAGE antibody or transfected with RAGE siRNA followed by incubation with 200 μg/ml of AOPPs-MSA for 12 h and then the cell lysates were harvested. The protein level of $p47^{phox}$ and $gp91^{phox}$ was analyzed by western blot. Data are expressed as mean ± s.d. of three independent experiments. Analysis of variance, $P<0.001$, $^{\#}P<0.05$ vs. IgG-treated or scramble siRNA-transfected cells. AOPPs, advanced oxidation protein products; Ctrl, control; IB, immunoblot; Ig, immunoglobulin; IP, immunoprecipitation; RAGE, receptor of advanced glycation end products; siNC, scramble siRNA; siRNA, small interference RNA.

that increased RAGE expression was mainly localized on podocytes in AOPPs-challenged mice (Figure 5f).

Blocking RAGE attenuated AOPPs-induced podocyte apoptosis and albuminuria *in vivo*

To determine the role of RAGE in AOPPs-induced podocyte apoptosis *in vivo*, normal C57BL/6 mice were administrated with intraperitoneal injection of anti-RAGE IgG at 5 mg/kg body weight or vehicle together with intravenous injection of AOPPs-MSA for 5 weeks. We first examined podocyte apoptosis using double-immunofluorescence labeling of Wilms's tumor-1 and TUNEL assay. AOPPs treatment resulted in a significant increase of podocyte apoptosis. Anti-RAGE treatment dramatically decreased the number of TUNEL-positive podocytes compared with vehicle control mice (Figure 6a and b). Likewise, AOPP-induced increase in urinary albumin excretion was significantly attenuated by RAGE blockade (Figure 6c).

Blocking RAGE inhibited AOPPs-induced apoptotic signaling *in vivo*

Consistent with *in vitro* data, AOPPs-induced O_2^- production in renal cortex homogenates was blocked by anti-RAGE IgG (Figure 7a). RAGE blockade inhibited AOPPs-induced

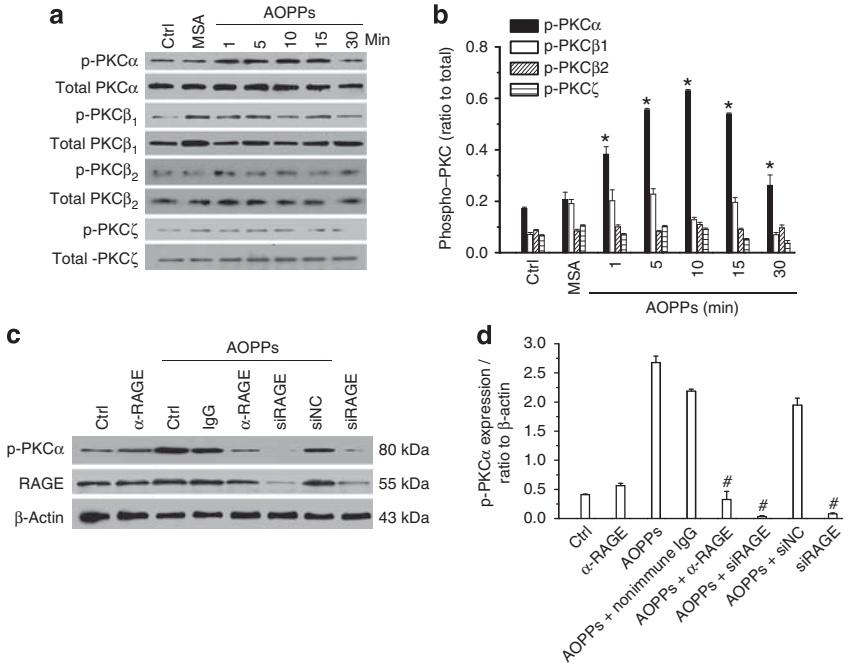

Figure 3 | AOPPs-induced PKCα activation is RAGE-dependent. (**a**) AOPPs activated PKCα in cultured podocytes. Podocytes were treated with 200 μg/ml of AOPPs for indicated time period. The expression level of phosphorylated and total PKCα, PKCβ1, PKCβ2, and PKCζ were detected by western blot. (**b**) Graphic representation of the ratio of phosphorylated PKCα, $PKC\beta_1$, $PKC\beta_2$, and PKCζ to total PKC. Data are expressed as mean ± s.d. of three independent experiments. Analysis of variance, $P<0.001$, $^{*}P<0.05$ vs. MSA-treated cells. (**c**) AOPPs-induced PKCα phosphorylation was blocked by pretreatment with RAGE neutralizing antibody or RAGE siRNA transfection. (**d**) Graphic representation of the ratio of phosphorylated PKCα to β-actin. Data are expressed as mean ± s.d. of three independent experiments. Analysis of variance, $P<0.001$, $^{\#}P<0.05$ vs. nonimmune IgG-treated cells or scramble siRNA-transfected cells. AOPPs, advanced oxidation protein products; Ctrl, control; Ig, immunoglobulin; MSA, mouse serum albumin; PKC, protein kinase C; RAGE, receptor of advanced glycation end products; siNC, scramble siRNA; siRNA, small interference RNA.

overexpression of $p47^{phox}$ and $gp91^{phox}$ in renal cortex (Figure 7b). Similarly, AOPPs-induced activation of p53/Bax-cleaved caspase-3 pathway was significantly inhibited by anti-RAGE (Figure 7c). Taken together, these data demonstrated that AOPPs-induced proapoptotic signaling in podocyte was mediated by RAGE *in vivo*.

DISCUSSION

We previously demonstrated that AOPPs accumulation resulted in podocyte apoptosis and depletion, establishing a causative link between oxidative stress and podocyte injury.[23,26] However, the transmembrane receptor that specifically transmits the signal of AOPPs remains unknown. In this study, we provided *in vitro* and *in vivo* evidence that RAGE plays a central role in AOPPs-induced podocyte apoptosis. Blocking RAGE inhibited AOPPs-induced activation of intracellular proapoptotic pathway and protected podocytes from apoptosis. To the best of our knowledge, this is the first study demonstrating that AOPPs trigger podocytes apoptosis through interaction with RAGE.

Our previous study demonstrated that accumulation of AOPPs induce podocytes apoptosis via a cascade of signaling events coupled with intracellular oxidative stress. AOPPs activate NADPH oxidase through a PKC-dependent pathway, thereby leading to an excessive generation of intracellular superoxide, which in turn promotes the classical p53/Bax/caspase-dependent apoptosis pathway.[23] One fundamental issue to be addressed is exactly how AOPPs transmit their signals across the plasma membrane to elicit cellular activities. One can assume that this probably requires a transmembrane receptor for AOPPs. In this study, we provided several lines of evidence demonstrating that AOPPs-induced podocyte apoptosis is mainly mediated by RAGE. First, AOPPs interacted with RAGE and colocalized with RAGE in AOPPs-treated podocyte, suggesting that AOPPs might be ligands of RAGE. Second, blocking RAGE or knocking down the expression of RAGE significantly protected podocyte from AOPPs-induced apoptosis both *in vitro* and *in vivo*. Third, activation of apoptotic signaling triggered by AOPPs was largely inhibited by RAGE blockade. These observations support the hypothesis that activation of RAGE is a common pathway that transduces signals from the diverse biochemical and molecular species, leading to propagation of inflammation and cellular perturbation.[22,27] The bioactive structure of AOPPs, which interact with RAGE, has not been defined yet and is in active study.

RAGE is a member of the IG superfamily and a multiligand signal–transduction receptor. The advanced glycation end products (AGEs), the first identified ligand of RAGE, have been implicated not only in diabetes but also in

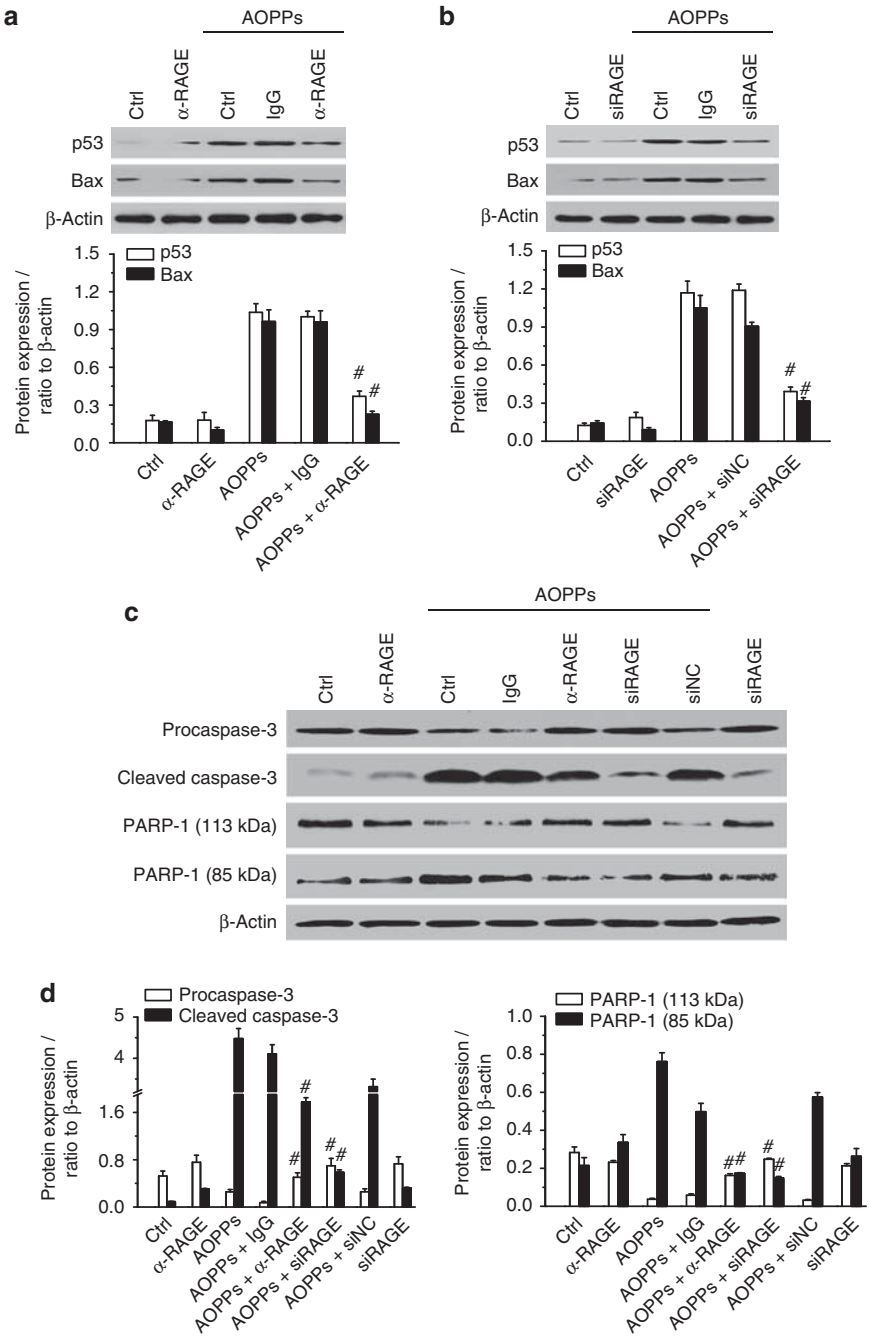

Figure 4 | The expression of apoptotic proteins induced by AOPPs was RAGE-dependent. Podocytes were pretreated with RAGE neutralizing antibody or transfected with RAGE siRNA before 200 μg/ml of AOPPs stimulation for 24 h. The protein level of p53, Bax (**a**, **b**), PARP (113 and 85 kDa), procaspase-3, and cleaved caspase-3 (**c**, **d**) was determined by western blot. Data are expressed as mean ± s.d. of three independent experiments. Analysis of variance, $P < 0.001$, $^{\#}P < 0.05$ vs. scramble siRNA-transfected cells. AOPPs, advanced oxidation protein products; Ctrl, control; Ig, immunoglobulin; PARP, poly (adenosine diphosphate-ribose) polymerase; RAGE, receptor of advanced glycation end products; siNC, scramble siRNA; siRNA, small interference RNA.

such settings as inflammation, hypoxia, and ischemia/reperfusion injury.[28,29] In addition to AGEs, RAGE interacts with at least four other classes of ligands: the S100/calgranulins, high-mobility group box-1, amyloid-β peptide and other amyloid species, and Mac-1.[30] These classes of ligands are linked to diabetes, inflammation, chronic neurodegenerative disorders, tumors, and amyloidoses.[30,31] Highly intriguing is the observation that podocyte is the principal RAGE-expressing cell in the glomerulus, with expression also noted in the endothelium.[17,32] In this study, we found that interaction of AOPPs with RAGE triggered podocyte apoptosis. This is consistent with Marsche *et al.*[20,21]

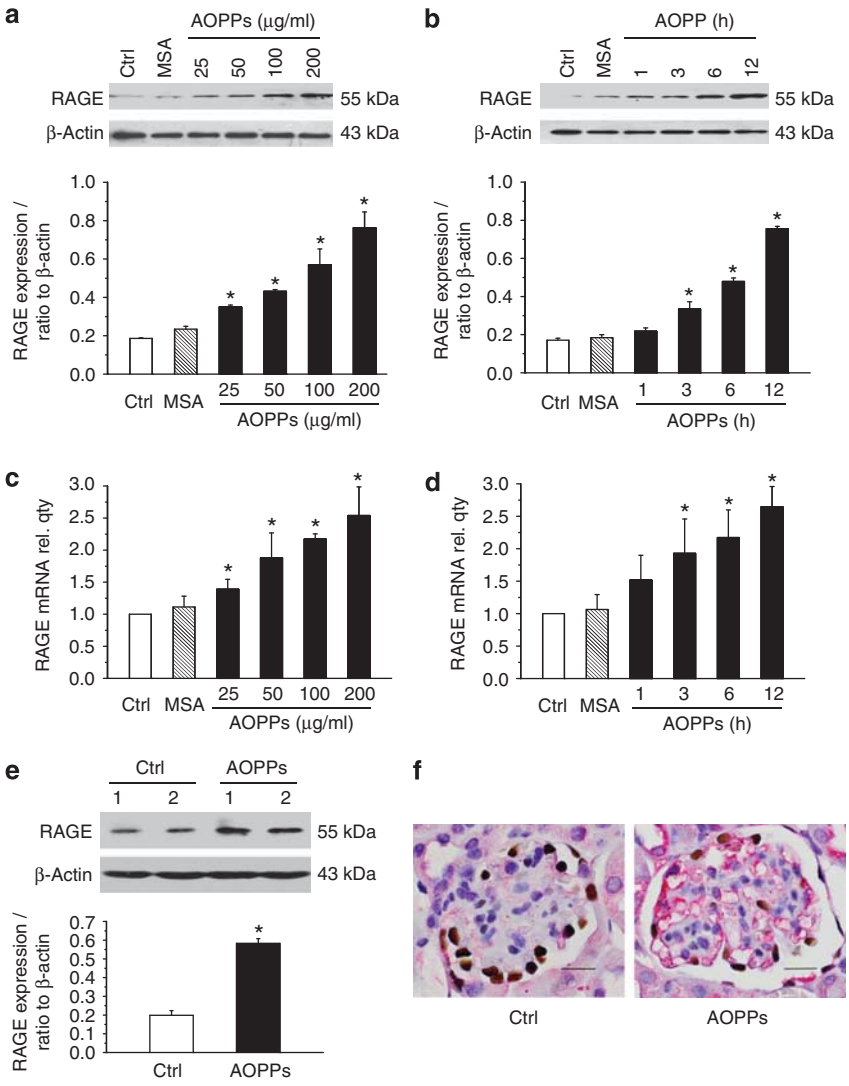

Figure 5 | AOPPs enhanced RAGE expression in podocytes both *in vitro* and *in vivo*. AOPPs induced RAGE expression in a time- and dose-dependent manner. Mouse podocytes were treated with indicated amount of AOPPs for 12 h (**a**, **c**), or 200 μg/ml of AOPPs for indicated time period (**b**, **d**). The protein level of RAGE was analyzed by western blot (**a**, **b**). The mRNA level of RAGE was analyzed by real-time PCR (**c**, **d**). Data are expressed as mean ± s.d. of three independent experiments. Analysis of variance, $P<0.001$, $^{*}P<0.05$ vs. MSA-treated cells. (**e**) Western blot of RAGE expression in the kidney of control and AOPPs-treated mice. Data presented are means ($n=6$ in each group) ± s.d. Analysis of variance, $P<0.001$, $^{*}P<0.05$ vs. control mice. Ctrl, mice treated with phosphate-buffered saline; AOPPs, mice treated with AOPPs. (**f**) Immunohistochemistry staining analysis of RAGE localization in AOPPs-challenged mice. Renal cortex sections of AOPPs-treated mice were paraffin-fixed and then co-stained with antibodies against Wilms's tumor-1 (brown) and RAGE (red). AOPPs, advanced oxidation protein products; Ctrl, control; MSA, mouse serum albumin; RAGE, receptor of advanced glycation end products.

and our previous finding that AOPPs induce inflammation in endothelial cells through interacting with RAGE.[20–22] Supporting our results, AGEs, via a RAGE-dependent signaling in podocytes, lead to generation of monocyte chemoattractant peptide-1, production of transforming growth factor-β, and other matrix-modifying species.[33,34] Pharmacological antagonism of RAGE or its genetic deletion imparts marked protection from podocyte effacement, albuminuria, and glomerular sclerosis in diseases models.[34,35] These data provide evidence linking RAGE to the pathogenesis of renal diseases, particularly the diseases related to podocyte injury. Although one might posit that interaction of AOPPs with RAGE in podocyte is expected based on observation concerning interaction of RAGE with other oxidized proteins, this is the first study examining the interaction of AOPPs with RAGE on kidney cells. AOPPs exert their pathobiological effect via different receptors on renal cells. Previous studies showed that AOPPs are capable of using CD36, a class B transmembrane multiligand SR, for transmitting their signals in renal tubular epithelial cells[36] AOPPs can use RAGE and SR-BI for their signaling in endothelial cells.[20–22] It is noteworthy that both RAGE and SR-BI are expressed on endothelial cells, but the expression level of SR-BI on podocytes is very low (data not shown).

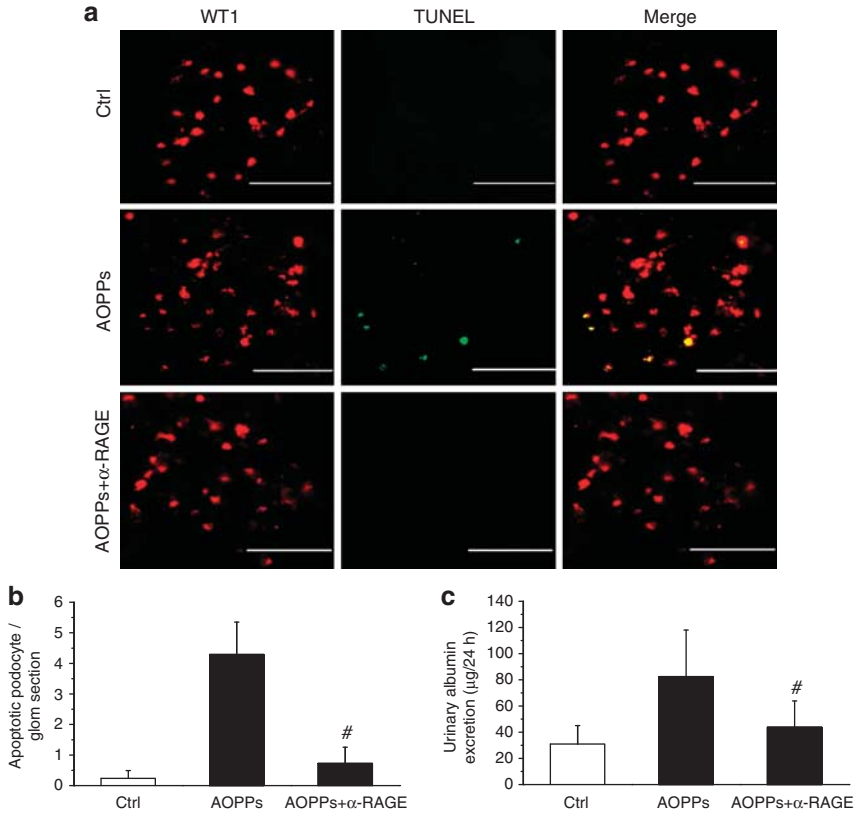

Figure 6 | RAGE neutralizing antibody attenuated podocyte apoptosis and albuminuria in AOPPs-challenged mice. (**a**) Representative image of Wilms's tumor-1 (WT-1) and TUNEL staining in the glomeruli. Frozen kidney tissue sections were stained with WT-1 (red) and TUNEL (green). Images (bar = 50 μm) were taken by confocal microscopy. (**b**) Bar graphs show the mean number of WT-1 and TUNEL-positive podocytes per glomerular (glom) cross-section. (**c**) The urine albuminuria in 24 h was collected and analyzed using mouse Albumin ELISA quantitation kit. Data are expressed as mean ± s.d. of six animals per group. Analysis of variance, $P < 0.001$, $^{\#}P < 0.05$ vs. AOPPs. Ctrl, mice treated with phosphate-buffered saline; AOPPs, mice treated with AOPPs; AOPPs + α-RAGE, mice treated with AOPPs together with RAGE neutralizing antibody. AOPPs, advanced oxidation protein products; Ctrl, control; RAGE, receptor of advanced glycation end products; TUNEL, terminal deoxyuridine nick-end labeling staining.

Furthermore, anti-SR-BI antibody was not able to block AOPPs-induced podocytes apoptosis, suggesting that the receptors mediating AOPPs' effect could be cell-type specific. Therefore, it is important to determine in each situation the impact of AOPPs on the affected cells, as well as the ability to bind to cellular surface.

Although present at low levels in homeostasis, RAGE expression is increased during disease.[28,29,37] RAGE expression is upregulated in situations in which its ligands are abundant.[24,25] Immune/inflammatory disorder is another situation in which RAGE expression is enhanced.[38,39] This study demonstrated that AOPPs accumulation upregulated expression of RAGE *in vivo* and *in vitro*. AOPPs accumulation in plasma and in renal tissue has been found in diabetic and non-diabetic CKDs.[9,11–17] Meanwhile, the upregulation of RAGE has been demonstrated in diabetic kidney and lupus nephropathy, particularly in podocytes.[17] Therefore, the increased activity of AOPPs–RAGE axis may promote renal inflammation and sclerosis and thus accelerate the progression of renal diseases. Stopping the cycle of AOPPs–RAGE-dependent stress in the glomerulus can hold significant promise in treating renal disease of varied etiologies.

MATERIALS AND METHODS

AOPPs-MSA preparation

AOPPs-MSA were prepared *in vitro* by incubation of MSA (Sigma Chemical, St Louis, MO) with hypochlorous acid (Fluke, Buchs, Switzerland) in the absence of free amino acid/carbohydrates/lipids to exclude the formation of AGEs-like structures as previously described.[9,23] Prepared samples were dialyzed against phosphate-buffered saline to remove free hypochlorous acid and passed through a DetoxiGel column (Pierce, Rockford, IL) to remove contaminated endotoxin. Endotoxin levels in the preparation were determined with the amebocyte lysate assay kit (Sigma, St Louis, MO) and were found to be below 0.025 EU/ml. The components of AGEs, including N^{ε}_(carboxylmethyl) lysine, pentosidine, pyridine, or glyoxal-, glycolaldehyde-, and glyceraldehyde-modified proteins were determined as described previously,[22,40–44] and were found to be undetectable in the prepared samples.

RAGE and AOPPs monoclonal antibody preparation

Monoclonal antibodies against RAGE (Cloen B2-2) and AOPPs (Clone 3F2) were generated as described previously.[45,46] Briefly, the recombinant extracellular domain (amino acid 23–342) of RAGE protein (rhRAE) or hypochlorous acid oxidative-human albumin were intraperitoneally injected to BALB/c mice. Mouse slenocytes were fused with mouse myeloma cells. Only hybridoma cells that secreted antibodies against RAGE and AOPP were cloned by

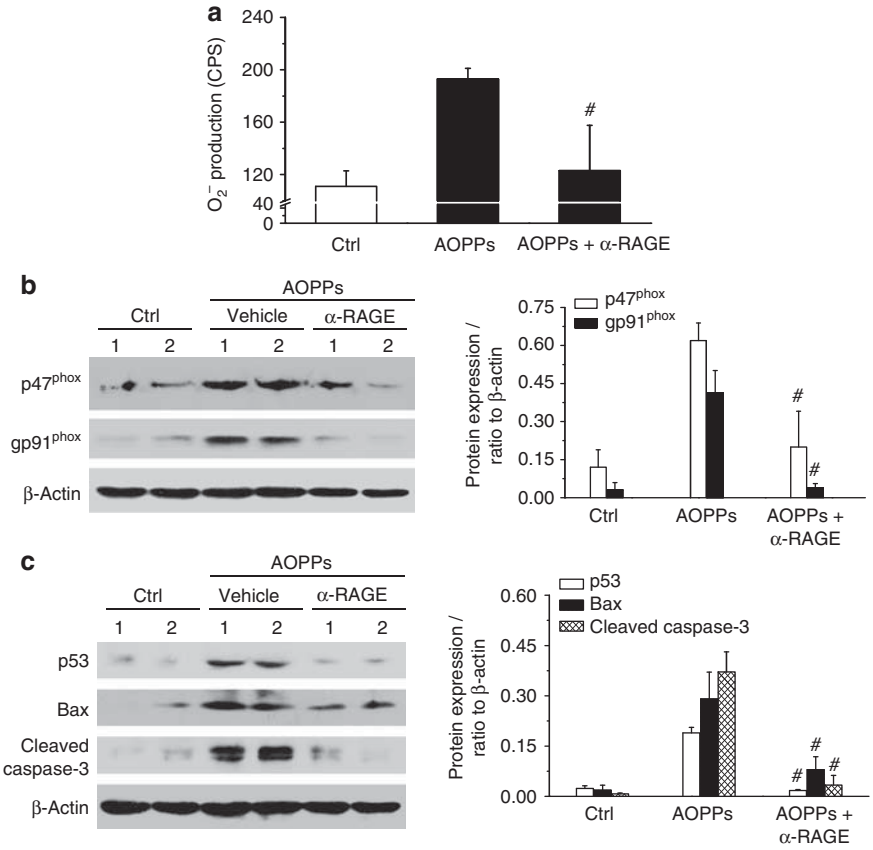

Figure 7 | RAGE neutralizing antibody inhibited AOPPs-induced nicotinamide adenine dinucleotide phosphate oxidase activation, O_2^- generation, and the activation of p53/Bax/caspase pathway *in vivo*. (**a**) O_2^- production in renal cortex homogenates was determined using the lucigenin chemiluminescence method. (**b**) Protein expression of $p47^{phox}$ and $gp91^{phox}$ in renal cortex homogenates was examined by western blot. (**c**) Protein expression of p53, Bax, and cleaved caspase-3 in renal cortex homogenates were examined by western blot. Data are expressed as mean ± s.d. of six animals per group. Analysis of variance, $P<0.001$, $^{\#}P<0.05$ vs. AOPPs. AOPPs, advanced oxidation protein products; Ctrl, control; RAGE, receptor of advanced glycation end products.

successive limiting dilution. The ascites were purified by affinity chromatography using HiTrap protein G Hp (GE Healthcare Bioscience, Piscataway, NJ). The monoclonal antibody isotypes were determined using a Monoclonal Antibody Isotype Kit (Thermo Scientific Pierce, Rockford, IL).

The prepared monoclonal anti-RAGE antibody (B2-2) specifically recognizes recombinant RAGE and natural RAGE expressed on cell surface.[45] We have examined the efficacy of B2-2 on blocking AOPPs-induced reactive oxygen species production on podocytes and macrophages. As shown in Supplementary Figure S4A and C online, B2-2 significantly inhibited AOPPs-induced reactive oxygen species production in a dose-dependent manner, and its efficacy was comparable with the anti-RAGE antibody purchased from R&D System (Minneapolis, MN; Supplementary Figure S4B and D online). As a neutralizing antibody, B2-2 has been shown to be able to block AGE-induced phosphorylation of moesin in endothelial cells[47] and AGE-induced CD83 expression in dendritic cells.[48]

Cell culture and siRNA transfection

Conditionally immortalized murine podocyte cell line was generously provided by Professor Peter Mundel (Sinai School of Medicine, New York) and cultured as described previously.[49] Experiments were performed using passages 10 to 18, growth-restricted, conditionally immortalized podocytes.

Oligonucleotide siRNA duplex was synthesized by Shanghai Gene Pharma (Shanghai, China). The sequence of mouse RAGE siRNA was 5′-GGUCAGAGCUGACAGUGAUTT-3′. The sequence of scramble siRNA was 5′-UUCUCCGAACGUGUCACGUTT-3′. The transfection of siRNA in podocytes was carried out with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction.

Animal experiment

All animal experiments were approved by the Committee on Animal Experimentation of Southern Medical University, and performed in compliance with the university's Guidelines for the Care and Use of Laboratory Animals. Twenty-four male C57BL/6 mice weighting 19–20 g were housed in a standard environment with regular light/dark cycles and free access to water and chow diet. The animals were randomly assigned into three groups ($n=6$ in each group) and received daily intravenous injection of vehicle (endotoxin-free phosphate-buffered saline, pH 7.4), AOPPs-MSA (50 mg/kg/d), or AOPPs-MSA (50 mg/kg/d) together with intraperitoneal injection of mouse anti-RAGE monoclonal antibody (B2-2), respectively. The RAGE antibody was dissolved in sterilized phosphate-buffered saline and injected in a volume of 0.5 ml, and was administered in an initial bolus dose of 300 μg followed by doses of 100 μg three times weekly. The animals were killed at the end of week 5 and

the kidneys were collected after perfusion with 25 ml of ice-cold normal saline.

Western blot and immunoprecipitation

The interaction of AOPPs-MSA with RAGE, $p47^{phox}$ with $p22^{phox}$ and $gp91^{phox}$, and phosphorylated $p47^{phox}$ in cultured podocytes were determined as previously described.[23,50] The goat anti-mouse RAGE antibody was from R&D Systems, rabbit anti-mouse $p22^{phox}$ goat anti-mouse $gp91^{phox}$ antibody, rabbit anti-mouse $p47^{phox}$ antibody, rabbit anti-mouse $p47^{phox}$ antibody, anti-Bax mAb, anti-Bcl-2 mAb, anti-p-PKCβ1 pAb (Thr-642), anti-PKCβ1 pAb, anti-PKCβ2 pAb, polyclonal rabbit anti-$p47^{phox}$ and rabbit anti-$gp91^{phox}$ were all from Santa Cruz (Santa Cruz, CA), rabbit anti-caspase-3 pAb, anti-PARP mAb, anti-PKCα pAb, anti-p-PKCζ pAb (Thr-642), and anti-PKCζ mAb were all from Cell Signaling Technology (Beverly, MA), anti-PKCα (phospho-T638) mAb, anti-p-PKCβ2 pAb (phospho-T641) were from Abcam (Cambridge, MA), and anti-p53 mAb was from Oncogene (Cambridge, MA). Horseradish peroxidase-conjugated rabbit anti-phosphoserine antibody was from Stressgen Bioreagents (Victoria, BC, Canada) and the horseradish peroxidase-conjugated swine anti-rabbit or goat anti-mouse or rabbit anti-goat IgG was from Dako Cytomation (DakoCytomation, Glostrup, Denmark).

Immunofluorescence staining and immunohistochemistry

Podocytes cultured on coverslips were fixed with cold methanol/acetone (1:1) for 10 min at −20 °C, followed by blocking with 20% normal donkey serum in phosphate-buffered saline with anti-AOPPs and goat anti-mouse RAGE antibody (R&D Systems) followed by incubating with Alexa Fluor 546-conjugated donkey anti-goat IgG and Alexa Fluor 488-conjugated chicken anti-mouse IgG. Nuclei were stained with 4,6-diamidino-2-phenylindole (Sigma Chemical) according to manufacturer's instruction. Images were taken by confocal microscopy (Leica TCS SP2 AOBS, Leica Microsystems, Buffalo Grove, IL).

The expression of RAGE in the kidney section was determined as described previously.[17] Briefly, the sections were co-stained by anti-RAGE (Santa Cruz) and Wilms's tumor-1 (ab89901, Abcam, Cambridge, UK) and then detected using the EnVision G|2 Doublestain System (Dako, Carpinteria, CA).

Real-time PCR

Total RNA was prepared using a TRIzol RNA isolation system (Life Technologies, Grand Island, NY) according to the manufacturer's instruction. The first strand of complementary DNA was synthesized using 1 μg of RNA in 20 μl of reaction buffer using MMLV-RT and random primers at 37 °C for 50 min. Real-time PCR was performed using a Plantinum SYBR Green qPCR SuperMix-UDG kit (Invitrogen). The primer sequences are as follows: mouse RAGE, 5′-ACAGGCGAGGGAAGGAGGTC-3′ and 5′-TTTGCCATCGGGAATCAGAAG-3′; mouse glyceraldehyde-3-phosphate dehydrogenase, 5′-CCAATGTGTCCGTCGTGGAT-3′ and 5′-TGCTGTTGAAGTCGCAGGAG-3′.

Assessment of podocyte apoptosis

Annexin V-labeled cells in cultured podocytes. Determination of Annexin V-labeled apoptotic cells was performed as described previously.[23] Podocytes were serum starved for 24 h and pretreated for 2 h at 37 °C with 2.5–20 μg/ml of goat anti-mouse RAGE neutralizing pAb (R&D Systems), 20 μg/ml of mouse anti-mouse CD36 [JC63.1] blocking mAb (Abcam), 20 μg/ml of goat anti-mouse SR class A neutralizing pAb (R&D Systems), 10 μg/ml of goat anti-mouse LOX1 neutralizing pAb (R&D Systems), 20 μg/ml of rat anti-mouse AGE-R3 capturing mAb (clone 202213; R&D Systems), SR-BI (NB400-113, Novus Biologicals, Littleton, CO) or 20 μg/ml of LEAF purified anti-mouse CD284 (TLR4) blocking antibody (Biolegend, San Diego, CA), respectively, before incubating with indicated amount of AOPPs-MSA for 24 h. Cells were trypsined and double stained with fluorescein isothiocyanate-conjugated Annexin V and propidium iodide according to the manufacturer's instructions (Roche, San Francisco, CA). Cells were analyzed using a flow cytometer (BD FACS Calibur System, Franklin Lakes, NJ).

TUNEL assay. DNA strand breaks in cultured podocytes were identified using the *in situ* nick/end-labeling assay (TUNEL) as described previously.[23]

NADPH-dependent superoxide (O_2^-) production

NADPH-dependent O_2^- production by homogenates from cultured podocytes or renal cortex was assessed by lucigenin-enhanced chemiluminescence as described previously.[23]

Intracellular production of reactive oxygen species

Intracellular reactive oxygen species was detected in podocytes and macrophages by analyzing the fluorescence intensity of the intracellular fluoroprobe 5-(6)-chloromethyl-2 0,70-dichlorodihydrofluorescein diacetate (Molecular Probe, Carlsbad, CA) using a fluorescent microplate reader at an excitation wavelength of 480 nm and an emission wavelength of 530 nm. Untreated cells were used to determine the background fluorescence. The final fluorescent intensity was normalized to the protein content. Data were expressed as percentage of controls (untreated cells).

Urine albumin assay

Urine albumin was measured by using a mouse Albumin ELISA quantitation kit, according to the manufacturer's protocol (Bethyl Laboratories, Montgomery, TX).

Statistical analysis

All experiments were performed in triplicate. Continuous variables, expressed as mean ± s.d., were compared using one-way analysis of variance. Pairwise comparisons were evaluated by the Student–Newman–Keuls procedure or Dunnett's T3 procedure when the assumption of equal variances did not hold. The Dunnett procedure was used for comparisons between reference group and other groups. Two-tailed *P*-value of less than 0.05 was considered statistically significant. Statistical analyses were conducted with SPSS 13.0, (SPSS Inc, Chicago, IL).

DISCLOSURE

All the authors declared no competing interests.

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SUPPLEMENTARY MATERIAL

Figure S1. Anti-RAGE antibody blocked AOPPs induced podocyte apoptosis in a dose-dependent manner.

Figure S2. AOPPs-induced podocytes apoptosis was not blocked by the neutralizing antibodies against the other receptors.
Figure S3. Anti-RAGE antibody or RAGE siRNA blocked AOPPs-induced podocytes apoptosis.
Figure S4. The efficacy of anti-RAGE neutralizing antibody (B2-2) on blocking AOPPs-induced ROS production.
Supplementary material is linked to the online version of the paper at http://www.nature.com/ki

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