Advanced oxidation protein products induce mesangial cell perturbation through PKC-dependent activation of NADPH oxidase

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Wei XF, Zhou QG, Hou FF, Liu BY, Liang M. Advanced oxidation protein products induce mesangial cell perturbation through PKC-dependent activation of NADPH oxidase. Am J Physiol Renal Physiol 296: F427–F437, 2009. First published November 19, 2008; doi:10.1152/ajprenal.90536.2008.—Mesangial deposition of extracellular matrix (ECM) is a hallmark of several glomerular diseases including diabetic nephropathy. Accumulation of advanced oxidation protein products (AOPPs) has been found in diabetes and chronic kidney disease and linked to mesangial ECM deposition and progressive glomerulosclerosis in these disorders. Although emerging evidence implicates AOPPs as the renal pathogenic factors, the underlying mechanisms have not been investigated. Here, using cultured rat mesangial cells (MCs) as a model, we identify AOPPs as the important mediators for activation of MC NADPH oxidase. Exposure of MCs to AOPPs, through membrane-associated phosphorylation of PKCα, induced rapid phosphorylation of cytosolic p47phox and its membrane translocation, enhanced interaction of p47phox with the membrane components p22phox and Nox4, and increased expression of these key regulatory subunits of NADPH oxidase. Challenge with AOPPs triggered cytosolic superoxide generation, resulting in upregulation of fibronectin and collagen IV genes and proteins and overexpression of TGF-β1 via a PKC-NADPH oxidase-dependent pathway, as these downstream events were blocked by the inhibitors of PKC, inhibitors of NADPH oxidase, or the cytosolic superoxide scavenger. These data provide new information for understanding the molecular basis underlying AOPP-induced MC perturbation and might be a central step toward development of new interventions.

extracellular matrix

DIABETIC NEPHROPATHY IS A leading cause of end-stage renal disease worldwide. The first and most characteristic glomerular lesion of diabetes is mesangial deposition of extracellular matrix (ECM) (6, 35), which closely correlates with deterioration of renal function and therefore has long been considered as a crucial factor for progressive glomerulosclerosis in diabetes (9, 24, 35). Numerous studies have demonstrated that the exaggerated formation of ECM, a typical cellular perturbation of mesangial cells (MCs) occurring in diabetic nephropathy, plays a central role in the pathogenesis of diabetic nephropathy (9, 24). Although hyperglycemia is the underlying metabolic abnormality, multiple other mechanisms have also been proposed for the altered metabolic activity of MCs, including advanced glycation, glomerular hemodynamic alteration, increased oxidative stress, and abnormal growth factor expression (2, 24, 32).

Diabetes is associated with increased modification of proteins. A typical representation is the formation and accumulation of advanced glycation end products (AGEs), the nonenzymatic glycation protein products that stimulate overexpression of ECM in cultured MCs (3, 43). In addition to AGEs, a family of oxidized protein compounds, termed advanced oxidation protein products (AOPPs), has emerged as a novel class of renal pathogenic mediators. AOPPs are the dityrosine-containing and cross-linking protein products formed during oxidative stress by reaction of plasma protein with chlorinated oxidants (39, 40). Plasma AOPPs are mainly carried by albumin, their concentration closely correlating with the level of dityrosine, a hallmark of oxidized protein. Therefore, AOPPs have been considered as the markers of oxidant-mediated protein damage (39).

Accumulation of AOPPs has been found in diabetic patients with or without microvascular complications (12, 23). An increase in plasma AOPPs has also been demonstrated in patients with nondiabetic kidney diseases such as IgA nephropathy (30, 40), in whom the level of AOPPs was a strong predictor for progression of renal disease. It is interesting to note that both diabetic nephropathy and IgA nephropathy are distinguished by glomerular MC injury and perturbation. Our recent in vivo studies demonstrate that elevation of plasma AOPPs is associated with mesangial expansion and fibronectin (FN) deposition in streptozotocin-induced diabetic rats (34) and progressive glomerulosclerosis in a nondiabetic remnant kidney model (17). Although emerging data recognize AOPPs as renal pathogenic factors, the underlying mechanisms have not been investigated.

We and others have revealed a close relationship between levels of AOPPs and serum markers of oxidative stress (17, 34, 39). Chronic administration of AOPPs increases generation of reactive oxygen species (ROS) in the diabetic kidney (34) and worsens redox imbalance in arteries in a hyperlipidemic model (22). These data suggest that these oxidized proteins, by themselves, may contribute to the persistent oxidative stress in tissues under pathological states.

Intracellular oxidative stress has been implicated in the pathogenesis of diabetic complications, including diabetic nephropathy (5, 16). Generation of cytosolic ROS can result from the activation of various enzymes, including NADPH oxidase, myeloperoxidase, nitric oxide, with increasing evidences that NADPH oxidase appears to be the major cytosolic source of ROS production in diabetes (10). Classically, NADPH oxidase consists of several cytosolic regulatory subunits (p47phox, p40phox, p67phox, and Rac1) and two membrane-associated components, p22phox and gp91phox/Nox2. Mesangial cells express p47phox and p22phox, but not gp91phox/Nox2. However,

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they express the homolog subunit Nox4 (7). Specific upregulation of the subunits of NADPH oxidase, p47\textsuperscript{phox}, p22\textsuperscript{phox}, and Nox4, has been demonstrated in the diabetic kidney (21, 25, 26).

Therefore, this study was conducted to examine how AOPPs might prime glomerular MC perturbation. Here, we identify a potential role for AOPPs in activation of MC NADPH oxidase via membrane-associated phosphorylation of PKC, which, through induction of cytosolic superoxide (O\textsubscript{2}\textsuperscript{-}), upregulates FN and collagen IV genes and proteins and expression of TGF-\beta1. These data provide new information for understanding the molecular basis underlying AOPP-induced MC perturbation.

**MATERIALS AND METHODS**

**AOPPs Preparation**

AOPPs-rat serum albumin (AOPPs-RSA) was prepared as described previously (17). Briefly, RSA (100 mg/ml, Sigma, St. Louis, MO) was exposed to 200 mmol/l of HOCl (Fluke, Buchs, Switzerland) for 30 min and dialyzed overnight against PBS to remove free HOCl. The AOPPs preparation was passed through a Detoxi-Gel column (Pierce, Rockford, IL) to remove any contaminated endotoxin. Endotoxin levels in the preparation were determined with the amebocyte lysate assay kit (Sigma) and were found to be below 0.025 EU/ml. AOPPs content in the preparation was determined as described previously (34). The content of AOPPs was 72.4 ± 9.8 mmol/mg protein in prepared AOPPs-RSA and 0.2 ± 0.02 mmol/mg protein in native RSA.

**Cell Culture**

Established rat glomerular MCs (HBZY-1, Life-Science Academy of Wuhan University, Wuhan, China) were cultured and maintained in DMEM supplemented with 10% fetal bovine serum (GIBCO BRL, Grand Island, NY), 2 mM glutamine, 100 U/ml penicillin and streptomycin at 37°C, at 5% CO\textsubscript{2}. Cells were seeded into 24-well plates at the density of 4 × 10\textsuperscript{4} cells/well. After growing near confluence, the cells were treated with indicated concentrations of AOPPs-RSA for the indicated times. Supernatants of cell cultures were collected, and the protein levels of FN, Collagen IV, and TGF-\beta1 were determined by using ELISA kits (Sigma, St. Louis, MO), as described previously (34). The content of AOPPs was 72.4 ± 9.8 mmol/mg protein in prepared AOPPs-RSA and 0.2 ± 0.02 mmol/mg protein in native RSA.

**FN, Collagen IV, and TGF-\beta1 Expression**

To determine the mRNA expression of collagen IV, FN, and TGF-\beta1, the semiquantitative RT-PCR was performed as described previously (17). Briefly, total RNA was isolated using TRIzol reagent. Two micrograms of each RNA sample was converted into cDNA using a RT-PCR kit (Invitrogen, LaJolla, CA). Equal amounts of cDNA were subsequently amplified by PCR reactions using gene-specific primer pairs. Amplification products were electrophoresed through 1.5% (wt/vol) agarose gels and visualized by ethidium bromide staining. After band intensities were quantified by using densitometry, the relative level of mRNA was calculated after normalization to GAPDH. The sequences of the primer sets were as follows: FN, 5’-GGGCAAGCTG CTGGCTTAC-3’ (sense) and 5’-CCGTTGTAAG GGTCAAGGAT-3’ (anti-sense); collagen IV, 5’-TACTGCGAGGC CTTGAGCC-3’ (sense) and 5’-CATC TTCTGGAATTTAGGAAC-3’ (anti-sense); and TGF-\beta1, 5’-ATACGCTCTCAGTGGCTTAC-3’ (sense) and 5’-GGGACTGAT CCCATTGATT-3’ (antisense).

To determine protein synthesis of collagen IV, FN, and TGF-\beta1, MCs were seeded into 24-well plates at the density of 4 × 10\textsuperscript{4} cells/well. After growing near confluence, the cells were treated with indicated concentrations of AOPPs-RSA for the indicated times. Supernatants of cell cultures were collected, and the protein levels of FN, collagen IV, and TGF-\beta1 were determined by using ELISA kits (TGF-\beta1 ELISA Kit, BioSource; FN and collagen IV ELISA Kit, Uscn Life Science Technology, Wuhan, China) according to the manufacturer’s protocol. The results were normalized by the number of cells.

**PKC Activity**

PKC activity was determined by measurement of membrane-associated phosphorylation using Western blotting. Membrane protein of MCs was prepared by using a Membrane Protein Extraction Kit (Pierce) according to the manufacturer’s protocol. For Western blotting, membrane protein samples were incubated at 100°C for 5 min and separated on 8% SDS-polyacrylamide gels (PAGE) and then transferred to nitrocellulose membranes (Amersham Pharmacia Biotech, Piscataway, NJ). Nonspecific binding was blocked with 5% (wt/vol) nonfat milk powder in TBS followed by overnight incubation of primary antibodies, polyclonal rabbit anti-phospho-PKC\alpha, anti-phospho-PKC\beta1, and anti-phospho-PKC\beta2 antibodies (1:1,000, Upstate, Lake Placid, NY) or with 2% bovine serum albumin in PBS. The membranes were washed with PBS before incubation with horseradish peroxidase (HRP)-conjugated swine-antirabbit IgG (Dako-Cytomation, Glostrup, Denmark) and detected by reaction with ECL (Amersham Biosciences). To determine pan-PKC, the membranes were reacted with polyclonal rabbit anti-PKK\alpha, anti-PK\beta1, and anti-PK\beta2 antibody (1:1,000, Santa Cruz Biotechnology, Santa Cruz, CA) separately and detected as described above. The band intensity was quantified using a microcomputer imaging device and expressed as the density ratio of phospho-PKC to pan-PKC.

**Intracellular O\textsubscript{2} Production**

Intracellular O\textsubscript{2}\textsuperscript{-} production was assessed by lucigenin-enhanced chemiluminescence as described previously (34). MCs were incubated with indicated concentrations of AOPPs for indicated times. Cell homogenates (100 μg/well) were added onto a 96-well microplate. Immediately before recording, dark-adapted lucigenin (5 μmol/l) with or without NADPH (100 μmol/l) was added to cell homogenates. Light emission was recorded every minute for 30 min (Victor V Wallac 1420, PerkinElmer, Turku, Finland). Data were expressed as counts per min.

To verify NADPH oxidase contribution, MC homogenates were pretreated for 60 min at 37°C with NADPH oxidase inhibitors [i.e., diphenyletheniodium (DPI), 10–100 μmol/l; apocynin 5–500 μmol/l; a nitric oxide synthase inhibitor [N\textsuperscript{-}nitro-\text{-}l-arginine methyl ester (l-NAME), 100–1,000 μmol/l], a xanthine oxidase inhibitor (allopurinol, 10–100 μmol/l), a mitochondria complex I inhibitor (rotenone; 10–100 μmol/l), a mitochondria complex II inhibitor (thenoyl-trifluoroacetone; 1–100 μmol/l), a mitochondria complex III inhibitor (myxothiazol; 1–100 μmol/l), a cyclooxygenase inhibitor (indomethacin; 1–100 μmol/l), an O\textsubscript{2} scavenger that can enter the cells (4) (bovine cytosolic Cu/Zn SOD; 2–200 U/ml; all from Sigma), an inhibitor of PKC\alpha (Ro-32-0432; 10 nM), or a broad-spectrum PKC inhibitor (Gö6983; 1 μM). The experiments were then repeated as described above.

**NADPH Oxidase Activity**

p47\textsuperscript{phox} phosphorylation. Phosphorylation of p47\textsuperscript{phox} was detected by immunoprecipitation as described previously (19). Briefly, the cell lysates were preabsorbed with protein A/G agarose beads (Santa Cruz Biotechnology) at 4°C for 30 min. The supernatants were incubated with a rabbit anti-p47\textsuperscript{phox} polyclonal antibody (Upstate-Cell Signaling Solutions, Temecula, CA) overnight at 4°C. The immunocomplexes were resolved with SDS-PAGE and transferred to nitrocellulose membranes. The membranes were then incubated with a HRP-conjugated rabbit anti-phosphoserine antibody (Stressgen Bioreagents Victoria, BC, Canada) and detected with ECL. To determine pan-p47\textsuperscript{phox}, the membranes were washed with an elute buffer, reacted with a mouse anti-p47\textsuperscript{phox} monoclonal antibody (BD Biosciences Pharmingen, San Diego, CA), and subsequently detected with HRP-conjugated rabbit anti-mouse IgG (Dako-Cytomation) and ECL. Bands were quantified by densitometry (Universal Hood 2, Bio-Rad, Milan, Italy).
p47\textsuperscript{phox} membrane translocation. p47\textsuperscript{phox} membrane translocation was analyzed by using immunofluorescence staining. MCs were fixed with 3.7% paraformaldehyde. After washing and blocking, the cells were incubated with a rabbit anti-p47\textsuperscript{phox} antibody overnight at 4°C. After washing, the slides were incubated with FITC-conjugated goat anti-rabbit IgG (Dako) at 37°C. The nuclear were stained with propidium iodide (Sigma) and observed under the LSM-510 laser confocal microscope system (Carl Zeiss, Göttingen, Germany).

Interaction of p47\textsuperscript{phox} with p22\textsuperscript{phox} and Nox4. The interaction of p47\textsuperscript{phox} with p22\textsuperscript{phox} and Nox4 was analyzed by immunoblotting as described previously (20). The immunocomplexes were obtained by incubation of cell lysates with the rabbit anti-p22\textsuperscript{phox} or Nox4 polyclonal antibodies (Santa Cruz Biotechnology) overnight at 4°C separately. Immunoblotting was performed using the mouse anti-p47\textsuperscript{phox} monoclonal antibody or anti-Nox4 antibody as the primary antibody and HRP-conjugated rabbit anti-mouse IgG as the secondary antibody. The membranes were detected by ECL, as mentioned above. To determine the total p22\textsuperscript{phox} and Nox4, the membranes were eluted and incubated with the rabbit anti- p22\textsuperscript{phox} and Nox4 polyclonal antibodies and then detected by reaction with the HRP-linked anti-rabbit IgG (Cell Signaling Technology, Beverly, MA).

Expression of NADPH oxidase subunits. MCs were incubated with or without 200 µg/ml of AOPPs-RSA for 6–24 h. The total protein of the cells was extracted by using a RIPA Lysis Kit (Beyotime Institute of Biotechnology, Haimen, China). Expression of p47\textsuperscript{phox}, p22\textsuperscript{phox}, and Nox4 was determined by Western blotting as described above. The primary antibodies were rabbit anti-p47\textsuperscript{phox} (1:1,000, Upstate-Cell Signaling Solutions), rabbit anti-p22\textsuperscript{phox} (1:1,000), and rabbit anti-Nox4 antibody (1:1,000, Santa Cruz Biotechnology).

Effect of PKC Inhibitors on AOPPs-Induced NADPH Oxidase Activation

To determine the relationship between PKC and NADPH oxidase activation, MCs were pretreated with 10 nM Ro-32-0432 (a selective cell-permeable PKC inhibitor, which display a 10-fold greater selec-

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**Fig. 1.** Effect of advanced oxidation protein products (AOPPs) on expression of fibronectin (FN) and collagen IV in cultured mesangial cells (MCs). MCs serum-deprived overnight were treated with indicated concentration of AOPPs or 200 µg/ml of native rat serum albumin (RSA) for 48 h. To determine the time course of stimulation with AOPPs, MCs were incubated with 200 µg/ml of AOPPs for indicated times. Cells cultured in medium alone served as a control. The total RNA was extracted and subjected to RT-PCR analysis. Supernatants were harvested and subjected to ELISA analysis. AOPPs upregulated expression of FN and collagen IV mRNA in a dose (A) and time (B)-dependent manner. AOPPs also increased secretion of FN and collagen IV protein in a dose (C) and time (D)-dependent manner. Values are means ± SE of 3 independent experiments. *P < 0.05 in A–D by ANOVA. *P < 0.05 vs. control.
tivity for PKCα over other PKC isoforms, Calbiochem, Darmstadt, Germany) or with 1 μM Go6983 (a broad-spectrum PKC inhibitor) for 60 min at 37°C and then incubated with 200 μg/ml of AOPPs-RSA for 30 min. Phosphorylation of p47phox and the interaction of p47phox with p22phox and Nox4 were analyzed as described above.

Role of PKC-NADPH Oxidase Activation in MC Perturbation

To evaluate the role of PKC-NADPH oxidase activation in MC perturbation, MCs were pretreated with the PKC inhibitor Ro-32-0432 or the NADPH oxidase inhibitor (DPI or apocynin) or a cytosolic O2 scavenger c-SOD for 60 min at 37°C followed by incubation of 200 μg/ml of AOPPs-RSA for 48 h. The cells and supernatants were harvested. The expression of FN, collagen IV, and TGF-β1 mRNA and protein were measured as mentioned above.

In another experiment, MCs were treated with 200 μg/ml of AOPPs in the presence or absence of neutralizing antibody against TGF-β1 (the maximal neutralizing activity of the antibody was seen at 1 μg/ml, R&D Systems, Minneapolis, MN) for 48 h and then subjected to analysis of FN and collagen IV expression.

To determine whether the newly produced TGF-β1 was involved in PKC-induced NADPH oxidase activation, MCs were incubated with AOPPs-RSA in the presence of neutralizing antibody against TGF-β1 for 24 h and then subjected to analysis of PKCα and NADPH oxidase activation as described above.

Statistical Analysis

All experiments were performed in triplicate. Continuous variables, expressed as means ± SE, were compared using one-way ANOVA. Pairwise comparisons were evaluated by the a 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.

Fig. 2. Effect of AOPPs on expression of transforming growth factor (TGF)-β1 in cultured MCs. MCs serum-deprived overnight were treated with indicated concentration of AOPPs or 200 μg/ml of native RSA for 48 h. To determine the time course of stimulation with AOPPs, MCs were incubated with 200 μg/ml of AOPPs for indicated times. The total RNA was extracted and subjected to RT-PCR analysis. Supernatants were harvested and subjected to ELISA analysis. AOPPs upregulated expression of TGF-β1 mRNA in a dose (A)- and time (B)-dependent manner. AOPPs also increased secretion of TGF-β1 protein in a dose (C)- and time (D)-dependent manner. Values are means ± SE of 3 independent experiments. P < 0.05 in A–D by ANOVA. *P < 0.05 vs. control.
RESULTS

AOPPs Induced ECM Overproduction in MCs

Increased synthesis of ECM components is one of the major characteristics in diabetic nephropathy (24). Given the fact that expression of type IV and FN are present in normal mesangium and upregulated in diabetic nephropathy, whereas collagen I is only detected in late glomerulosclerosis (3), to determine whether stimulation of AOPPs affects ECM metabolism, we measured FN and collagen IV expression as the representation of ECM synthesis in cultured MCs. As shown in Fig. 1, AOPPs-RSA upregulated mRNA expression and protein synthesis of FN and collagen IV in a dose (Fig. 1, A and C)- and time (Fig. 1, B and D)-dependent manner (P < 0.01). Expression of FN and collagen IV was unchanged in cells cultured with native RSA or medium alone (control), suggesting that upregulated ECM production was associated with advanced oxidation of RSA.

AOPPs Induced Overexpression of TGF-β1

TGF-β1 has been identified as a key mediator for MC fibrogenesis (31). To examine the effect of AOPPs on expression of TGF-β1, MCs were triggered by AOPPs-RSA for indicated times. As shown in Fig. 2, AOPPs-RSA increased expression of TGF-β1 at both mRNA and protein levels in a dose (Fig. 2, A and C)- and time (Fig. 2, B and D)-dependent manner (P < 0.01). Native RSA had no effect on TGF-β1 expression.

AOPPs Induced Activation of PKC in MCs

The conventional PKC isoforms, PKCα, PKCβ1, and PKCβ2, have been shown to be involved in MC perturbation under diabetic conditions (1, 38). To investigate the effect of AOPPs on PKC activity, PKCα, PKCβ1, and PKCβ2, were evaluated by measurement of their membrane-associated phosphorylation. As shown in Fig. 3, a challenge with AOPPs significantly increased membrane-associated phosphorylation of PKCα from 5 to 15 min (P < 0.01). Activity of PKCβ1 was also increased 10 min after exposure to AOPPs. However, the increment was marginal compared with cells cultured in medium alone (P = 0.033). Meanwhile, lack of change in membrane-associated phosphorylation of PKCβ2 was observed. These results suggest that PKCα might be the major isoform of the conventional PKC activated by AOPPs in MCs.

AOPPs Induced NADPH-Dependent O2·− Production Mainly Through Activation of PKCα

To examine the effect of AOPPs on intracellular ROS production, NADPH-dependent O2·− generation in MCs was evaluated by lucigenin-enhanced chemiluminescence. In the presence of exogenous NADPH, O2·− generation was significantly increased in cells treated with AOPPs-RSA compared with unstimulated MCs or cells treated with native RSA (Fig. 4, A and B). O2·− generation was increased by exposure of MCs to AOPPs-RSA in a dose (Fig. 4A)- and time (Fig. 4B)-dependent manner. To verify the enzymatic source of O2·− generation, the response of MCs to AOPPs-RSA was examined in the presence of inhibitors of various enzymatic systems involved in ROS production (Fig. 4C). AOPPs-RSA-induced O2·− production was significantly suppressed by the NADPH oxidase inhibitor DPI (by 86%) or apocynin (by 81%), but not by the nitric oxide synthase inhibitor, the mitochondria complex inhibitors, the cyclooxygenase inhibitor, or the xanthine oxidase inhibitor. Those inhibitors alone, at the concentration used in the experiments, had no effect on O2·− production in MCs cultured in medium alone. These data indicate that NADPH oxidase plays a central role in AOPPs-induced O2·− production.

As shown in Fig. 4C, AOPPs-induced O2·− production could be blocked by the PKCα inhibitor Ro-32-0432 by 83%. Pre-treatment of MCs with G66983, a broad-spectrum inhibitor of PKC, resulted in similar reduction in O2·− generation (by 88%) compared with cells preincubated with Ro-32-0432. This result suggest that activation of PKC, particularly the α-isofrom, might be the necessary upstream event of AOPPs-triggered intracellular oxidative stress.

AOPPsActivated MC NADPH Oxidase

To further examine the mechanism underlying the induction of O2·− production, we investigated NADPH oxidase activity in AOPPs-challenged MCs. As shown in Fig. 5A, AOPPs-RSA treatment induced rapid phosphorylation of p47phox at 5 min, peaking at 30 min. Native RSA had no effect. At 30 min after exposure to AOPPs-RSA, membrane translocation of p47phox was apparent in MCs (Fig. 5B).

To examine the interaction between activated p47phox with membrane subunits p22phox and Nox4, we immunoprecipitated p22phox and Nox4 with specific antibodies and probed for the coexistence of p47phox in MCs. As shown in Fig. 5C, the binding of p47phox to p22phox, a process that served as a switch...
Fig. 4. Intracellular O$_2^-$ production in MCs incubated with or without AOPPs. MCs serum-deprived overnight were treated with indicated concentration of AOPPs for 48 h or 200 µg/ml of AOPPs for indicated times. Cell homogenates were subjected to lucigenin-enhanced chemiluminescence analysis. AOPPs induced intracellular O$_2^-$ overproduction in a dose (A) and time (B)-dependent manner. To verify the source of O$_2^-$ generation, the experiments were repeated in the presence of rotenone, thenoyltrifluoroacetone (TTFA), myxothiazol, indomethacin, N$^\text{ω}$-nitro-ω-arginine methyl ester (L-NAME), allopurinol, diphenyleneiodonium (DPI), apocynin, c-SOD, Ro-32-0432, or Gö6983 (C). Values are means ± SE of 3 independent experiments. $P < 0.05$ in A and B by ANOVA. *$P < 0.05$ vs. control. #$P < 0.05$ vs. AOPPs group.

for activation of Nox homologs (27), rapidly increased in AOPPs-stimulated MCs. The challenge with AOPPs promoted the interaction of the p47phox-p22phox complex with Nox4, a key step for O$_2^-$ generation in nonphagocytic cells (13).

Increased expression of NADPH oxidase subunits might be necessary for its sustained activation. To examine the effect of AOPPs on expression of the enzyme subunits, MCs were incubated with AOPPs-RSA for 6–24 h. Compared with cells treated with native RSA, MCs incubated with AOPPs-RSA showed significantly increased expression of p22phox, p47phox, and Nox4 after 6-h stimulation (Fig. 5D). To examine the interaction among these upregulated subunits, we immunoprecipitated p22phox with anti-p22phox, then probed for the coexistence of p47phox and Nox4. We found that p47phox-p22phox- Nox4 complex formation was increased in AOPPs-stimulated MCs compared with cells treated with native RSA (Fig. 5E).

To further evaluate the relationship between activation of PKCα and NADPH oxidase, MCs were pretreated with a selective and cell-permeable PKCα inhibitor, Ro-32-0432, as well as Gö6983 (a broad-spectrum PKC inhibitor) and then stimulated with AOPPs-RSA for 30 min. AOPPs-induced phosphorylation of p47phox and binding of p47phox with p22phox or Nox4 were almost completely blocked by pretreatment of MCs with Ro-32-0432 ($P < 0.01$), and, to a similar extent, by Gö6983, suggesting that activation of NADPH oxidase in response to AOPPs might be mainly due to PKCα activation.

**AOPPs Induced MC Perturbation via PKC-Dependent Activation of NADPH Oxidase**

To further explore the role of PKC-NADPH oxidase activation in AOPPs-induced MC perturbation, MCs were pretreated with the NADPH oxidase inhibitors DPI or apocynin, PKCα inhibitor Ro-32-0432, or a cytosolic O$_2^-$ scavenger, c-SOD, followed by incubation with AOPPs-RSA. As shown in Fig. 6, A–C, AOPPs-induced overexpression of FN, collagen IV, and TGF-β1 could be significantly blocked by DPI, apocynin, Ro-32-0432, or c-SOD ($P < 0.01$), suggesting that AOPPs-induced MC perturbation was associated with activation of PKC and NADPH oxidase and O$_2^-$ dependent.

**Effect of Anti-TGF-β1 Antibody on AOPPs-Induced ECM Production**

It has been demonstrated that TGF-β1 promotes ECM synthesis in MCs in vitro (31). To investigate whether the newly produced TGF-β1 was involved in mediation of AOPPs-in-
Fig. 5. AOPPs activated NADPH oxidase in cultured MCs. MCs serum-deprived overnight were treated with 200 μg/ml of AOPPs for indicated times. AOPPs triggered phosphorylation of p47phox (A), induced p47phox membrane translocation (B), and enhanced interaction of p47phox with p22phox and Nox4 (C). Exposure of cells with AOPPs for 24 h upregulated expression of p47phox, p22phox, and Nox4 (D) and increased binding of membrane p22phox with p47phox and Nox4 (E). In another experiment, MCs were pretreated with PKC inhibitor Ro-32-0432 for 30 min and then incubated with 200 μg/ml of AOPPs for 30 min. AOPPs-induced phosphorylation of p47phox (F) and binding of p47phox with p22phox and Nox4 (G) were blocked by Ro-32-0432 or Gö6983. Values are means ± SE of 3 independent experiments. *P < 0.05 in A, B, and D by ANOVA. #P < 0.05 vs. control. ##P < 0.05 vs. AOPPs group.
Fig. 6. Inhibition of NADPH oxidase or PKC blocked AOPPs-induced MC perturbation. MCs serum-deprived overnight were pretreated with NADPH oxidase inhibitor DPI or apocynin, PKC inhibitor Ro-32-0432, or c-SOD for 1 h and then incubated with 200 μg/ml of AOPPs for 48 h. Total RNA was extracted and subjected to RT-PCR analysis. The supernatant was subjected to ELISA analysis. Pretreatment of MCs with DPI, apocynin, Ro-32-0432, or c-SOD almost completely inhibited AOPPs-induced overexpression of FN, collagen IV, and TGF-β1 at both mRNA (A) and protein levels (B and C). In another experiment, MCs were treated with 200 μg/ml of AOPPs for 48 h in the presence or absence of TGF-β1-neutralizing antibody (1 μg/ml). The mRNA and protein expression of FN and collagen IV were determined as mentioned above. Neutralizing TGF-β1 reduced AOPPs-induced FN and collagen IV mRNA expression (D) and their protein synthesis (E and F). In a separate experiment, MCs were treated with 200 μg/ml of AOPPs for 24 h in the presence of a neutralizing antibody against TGF-β1. Neutralizing TGF-β1 partly inhibited AOPPs-induced membrane phosphorylation of PKC (G) and upregulation of NADPH oxidase subunit expression (H). Values are means ± SE of 3 independent experiments. #P < 0.05 vs. AOPPs group.
duced ECM generation, MCs were incubated with AOPPs-RSA in the presence of a neutralizing antibody against TGF-β1. As shown in Fig. 6, addition of the anti-TGF-β1 antibody attenuated AOPPs-induced FN and collagen IV mRNA overexpression by 35 and 38% (Fig. 6D) and decreased their protein synthesis by 40 and 36% (Fig. 6, E and F), respectively, suggesting that newly synthesized TGF-β1 was involved in AOPPs-induced ECM overproduction.

To evaluate the relationship between the TGF-β1 pathway and the PKC-induced NADPH oxidase activation, NADPH oxidase and PKC activity were measured after 24-h incubation with AOPPs-RSA in the presence or absence of a neutralizing antibody against TGF-β1. As shown in Fig. 6, G and H, AOPPs-induced membrane phosphorylation of PKC or up-regulation of NADPH oxidase expression was partly inhibited by neutralizing TGF-β1.

DISCUSSION

Increased recognition of AOPPs, as a class of potential renal pathogenic mediators, and the means by which they form in diverse disorders has highlighted the importance of determining the mechanisms by which AOPPs might modulate renal cell properties. This study demonstrated that AOPPs-albumin triggered cytosolic O$_2^-$ generation by activation of MC NADPH oxidase, mainly through PKCα phosphorylation. Activation of the pathway by AOPPs resulted in overproduction of ECM such as FN and collagen IV and overexpression of fibrogenic factor TGF-β1. AOPPs-RSA, but not native RSA, activated the PKC-NADPH oxidase-dependent pathway, suggesting that observed effects were due to the protein oxidative modification and not a property of RSA or other contaminants. To the best of our knowledge, this is the first study clarifying the pathobiological pathway of AOPPs in MCs.

The most important finding in this study is that AOPPs-induced overproduction of ECM is dependent on cytosolic O$_2^-$ generated by a MC NADPH oxidase. Here, we provided several lines of evidence demonstrating that AOPPs triggered MC perturbation through induction of NADPH-dependent O$_2^-$ in MCs. First, AOPPs-triggered O$_2^-$ generation could be blocked by the NADPH oxidase inhibitor apocynin or DPI, but not by inhibitors of other enzymatic systems involved in ROS generation, suggesting NADPH oxidase as the major source of ROS triggered by AOPPs. Second, incubation of MCs with AOPPs induced cytosolic subunit p47phox phosphorylation and its membrane translocation and increased binding of p47phox to the membrane subunit p22phox, a process that served as a switch for activation of NADPH oxidase (13). AOPPs also increased interaction of p47phox with the membrane component Nox4, which is predominately expressed in MCs and plays a critical role in O$_2^-$ generation upon activation (8). Moreover, exposure of MCs to AOPPs for several hours upregulated the expression of the key regulatory subunits of NADPH oxidase: p47phox, p22phox, and Nox4. Increased expression of NADPH oxidase subunits is necessary for sustained enzymatic activity (11, 15). Third, AOPPs-induced ECM overproduction and TGF-β1 overexpression could be blocked by pretreatment of MCs with the NADPH oxidase inhibitor or the cytosolic O$_2^-$ scavenger c-SOD, suggesting that AOPPs-triggered MC perturbation is associated with NADPH oxidase activation; O$_2^-$ dependent. ROS generated during cellular oxidative stress has been demonstrated to activate the signal transduction cascade (mitogen-activated protein kinases, Janus kinase/signaling transducers, and activators of transcription) and transcription factors (nuclear factor-kB, activated protein-1) and upregulate TGF-β1 and ECM genes and proteins (16). Our results are consistent with the previous in vitro study in which HOCl-modified low-density lipoprotein upregulates the fibrogenic genes in human renal tubular epithelial cells (29). Our in vivo study has also demonstrated that AOPPs-induced renal damage can be prevented by intervention of apocynin (34).

An increase of PKC activity has been demonstrated in both diabetic kidney and MCs cultured with high-glucose medium (18). Several isozymes of PKC, including PKCα and PKCβ, have been identified in MCs. It has been reported that MCs ROS generation by NADPH oxidase in high glucose is dependent on conventional PKCα and PKCβ (41), with these isoforms also required for the increased expression of collagen IV (37, 41). We therefore examined particularly the role of AOPPs with regard to the conventional PKCα and β. Our data showed that stimulation with AOPPs mainly triggered PKCα phosphorylation on the cell membrane, although PKCβ was also activated. AOPPs-induced activation of NADPH oxidase could be blocked by a PKC inhibitor with major selectivity for PKCα and, to a similar extent, by a broad-spectrum inhibitor of PKC, suggesting that this process may be mainly mediated by PKCα activation. Furthermore, we found that AOPPs-induced cytosolic O$_2^-$ generation and its downstream events, such as overproduction of ECM and induction of TGF-β1, were effectively blocked by the PKC inhibitor and the cytosolic O$_2^-$ scavenger c-SOD, suggesting that the activation of PKC, predominantly the α-isofrom, may contribute to MC perturbation by induction of NADPH oxidase-dependent signaling. Given the fact that a slight increase in membrane-associated PKCβ1 phosphorylation occurred in AOPPs-treated MC and that the PKCα inhibitor has some selectivity for PKCβ1, it is likely that activation of PKCβ1 may also be involved in the AOPPs-induced MC perturbation. It is interesting to note that, like AOPPs, glycation-modified protein products, AGEs, induce activation of NADPH oxidase through PKCα phosphorylation. Inhibition of PKCα or NADPH oxidase attenuates cytosolic O$_2^-$ generation in both diabetic ro-

![Fig. 7. Schematic illustration depicting signal pathway linked with AOPPs-induced MC perturbation. AOPPs induce NADPH oxidase activation through activation of PKC, leading to overproduction of intracellular O$_2^-$, which results in ECM overproduction and upregulation of TGF-β1. TGF-β1 further activates PKC and NADPH oxidase and forms a positive feedback loop.](http://ajprenal.physiology.org/Downloadedfromhttp://ajprenal.physiology.org/)
dents and AGE-treated MCs (36). The molecular mechanisms underlying the receptor binding of AOPPs and activation of PKC and NADPH oxidase remain unclear. Given the central role of PKC-NADPH oxidase activation for MC perturbation, exploration of the molecular mechanisms by which AOPPs triggered activation of PKC would be important and are currently under active study.

The present study found that stimulation with AOPPs up-regulated expression of TGF-β1, which has been identified as the key cytokine mediating the production of different ECM proteins in kidney cells including MCs (9, 24, 28). To examine whether the newly produced TGF-β1, in response to AOPPs, was involved in mediating AOPPs-induced ECM overproduction, MCs were incubated with AOPPs in the presence of an anti-TGF-β1 antibody. Neutralizing the effect of TGF-β1 attenuated AOPPs-induced expression of FN and collagen IV by 35–38%, suggesting that newly synthesized TGF-β1 is also involved in ECM overproduction in AOPPs-challenged MCs. Previous study has also shown that exposure of preglomerular vascular smooth muscle cells to TGF-β1 triggers a rapid increase in ROS that is inhibited by NADPH oxidase inhibitors (33). Consistent with other studies that were conducted in MCs stimulated by a high concentration of glucose (16, 42), our data showed that AOPPs-induced activation of PKC and NADPH oxidase could be partly inhibited by neutralizing the effect of TGF-β1. Therefore, it seems reasonable to assume that in addition to O2− produced by AOPPs-activated NADPH oxidase, newly synthesized TGF-β1, as a downstream signaling event, can further activate PKCα and NADPH oxidase to produce ROS (14). This positive feedback loop (Fig. 7) may amplify or maintain the intracellular oxidative stress and MC perturbation.

To summarize, we have identified AOPPs as important mediators for activation of MC NADPH oxidase. AOPPs induced MC perturbation through activation of NADPH oxidase, which is dependent on the phosphorylation of PKC, particularly PKCα. AOPPs are prevalent in diverse pathophysiological conditions such as diabetes and chronic kidney diseases, in which enhanced oxidative stress favors oxidative protein damage and, therefore, AOPPs formation. Thus understanding the molecular basis underlying AOPPs-induced MC perturbation, possibly linked to the kidney damage observed in these disorders, might be a central step toward development of new interventions.

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REFERENCES


