Rosiglitazone Prevents Advanced Glycation End Products–Induced Renal Toxicity Likely through Suppression of Plasminogen Activator Inhibitor-1

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In the development of diabetic nephropathy, advanced glycation end products (AGEs) play a causative role via induction of extracellular matrix (ECM) accumulation. Plasminogen activator inhibitor-1 (PAI-1), as a major inhibitor of plasminogen activator that plays an important role in degrading ECM, was found to significantly increase in renal fibrotic diseases. Activation of peroxisome proliferator-activated receptor (PPAR)-γ prevented diabetic nephropathy. The present study, therefore, was to define whether or not AGE-induced renal ECM accumulation and renal dysfunction are mediated by upregulation of PAI-1 expression and whether or not PPAR-γ agonist can attenuate these AGE effects via suppressing PAI-1 expression. Rats were given AGEs alone by iv injection at 100 mg/kg daily with or without oral supplementation of PPAR-γ agonist rosiglitazone (RGZ) at 2 mg/kg daily for 6 weeks. Results showed that AGEs induced a renal ECM accumulation, as shown by increases in periodic acid-Schiff-positive materials, fibronectin, and type IV collagen (Col IV) contents in glomeruli, and a mild renal dysfunction, as shown by an increase in urinary proteins. AGEs also caused an increase in PAI-1 expression and a decrease in plasminogen activator bioactivity in the kidney. Treatment with RGZ significantly ameliorated AGE-induced renal ECM accumulation, proteinuria, and PAI-1 upregulation. Direct exposure of rat mesangial cells to AGEs in vitro induced increases in fibronectin and Col IV syntheses along with an increase in PAI-1 expression, effects significantly attenuated by RGZ. Preincubation of PAI-1 antibody to AGE-treated mesangial cells completely prevented AGE-induced fibronectin and Col IV production. These results suggest that upregulation of PAI-1 expression plays a critical role in AGE-induced renal ECM accumulation. Renal protection of RGZ from AGEs may be associated with the suppression of PAI-1 expression through PPAR-dependent and independent mechanisms.

Key Words: advanced glycation end products; diabetic nephropathy; extracellular matrix accumulation; peroxisome proliferator-activated receptor-γ; plasminogen activator inhibitor-1.

Diabetic nephropathy, characterized by an accumulation of extracellular matrix (ECM), is one of the most important causes of chronic renal failure. Advanced glycation end products (AGEs), the late products of the covalent modification of proteins by glucose, were found to play a critical role in the development of diabetic nephropathy (Ramasamy et al., 2005; Vlassara et al., 1992, 1994; Zhou et al., 2004). AGEs are able to covalently cross-link and biochemically modify protein structure to affect protein function and cause renal ECM accumulation (Ramasamy et al., 2005). ECM accumulation in the kidney, however, is dependent on the balance between synthesis and degradation of ECM. In vivo intravenous infusion of AGEs to healthy rats resulted in glomerular hypertrophy, mesangial sclerosis, and excessive matrix synthesis (Vlassara et al., 1994; Yang et al., 1994; Zhou et al., 2004). In vitro exposure of the cultured human and rat mesangial cells to AGEs increased the synthesis of fibronectin and type IV collagen (Col IV) (Doi et al., 1994; Kim et al., 2001; Zhou et al., 2004).

There are two kinds of protease systems to degrade ECM in the kidney, matrix metalloproteinase (MMP) and plasmin systems. The latter includes plasminogen, plasmin, plasminogen activator and plasminogen activator inhibitor (PAI). As a major inhibitor of plasminogen activator, PAI-1 regulates fibrinolysis and MMP activation. We have demonstrated a significant ECM accumulation in AGE-treated rat kidney (Zhou et al., 2004); however, we do not know whether or not AGE-induced renal ECM accumulation is mediated by alterations of plasminogen activator and PAI-1 expression. Several studies have indicated the upregulated PAI-1 expression in the kidney of diabetic and nondiabetic individuals, probably due to increases in growth factors or cytokines (transforming growth factor [TGF]-β, TGF-α, epidermal growth factor, and tumor necrosis factor-α [TNF-α], renin, angiotensin II) and metabolic factors (glucose, lipoproteins) (Chen et al., 2006; Zhou et al., 2004).

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear hormone receptor superfamily of
ligand-dependent transcription factors and were found to be a therapeutic target in renal diseases. Following activation, PPAR-γ heterodimerizes with retinoic X receptor and binds to peroxisome proliferator response elements in the promoter region of target genes, thereby regulating their transcriptions (Guan and Breyer, 2001). PPARs play important roles in the general transcriptional control of numerous cellular processes, including lipid metabolism, glucose homeostasis, cell-cycle progression and differentiation, inflammation, and ECM remodeling. All three PPAR isoforms, designated PPARα, -β, and -γ, differentially express in the kidney. Mesangial cells were found to express mainly PPAR-γ. Thiazolidinediones including troglitazone and rosiglitazone (RGZ) are specific agonists of PPAR-γ (Fu et al., 2001; Guan and Breyer, 2001; Hong et al., 2003). PPAR-γ activation by these agonists provided a significant prevention of the nephropathy in the diabetic rats induced by streptozotocin (Fujii et al., 1997; Ishiki et al., 2000) and in the Zucker fatty diabetic rats (Buckingham et al., 1998; Shibata et al., 2000; Smith et al., 2000).

Furthermore, the fact that PPAR-γ agonist treatment significantly decreased PAI-1 expression (Hong et al., 2003; Kato et al., 1999; Liu et al., 2005; Nicholas et al., 2001) encouraged us to assume that PPAR-γ activation by its agonists may also prevent AGES-induced renal ECM accumulation and nephropathy through suppression of the PAI-1 upregulation. AGE has been found to activate PAI-1 expression in the cultured mesangial cells (Lee et al., 2004). In the present study, therefore, we have explored the effect of infusion of AGE to rats on the PAI-1 expression and the effect of RGZ-activated PPAR-γ activation on AGE-induced the PAI-1 expressions in the kidney. To further dissect the link of them in vitro primary cultures of rat mesangial cells exposed to AGES with or without the presence of RGZ were investigated.

MATERIALS AND METHODS

AGE synthesis. AGE-bovine serum albumin (AGE-BSA) and AGE-rat serum albumin (AGE-RSA) were prepared by incubating BSA and RSA (fraction V, low endotoxin; Sigma, St Louis, MO) with 500nm D-glucose under aerobic conditions for 10 weeks at 37°C in the presence of protease inhibitors and antibiotics, as described in our previous study (Zhou et al., 2004). AGE content in the preparations was assessed by means of fluorescence photometer (at excitation wavelength of 370 nm and emission of 440 nm with slit width of 10 nm in arbitrary fluorescence units per milligram protein) as 19.8 ± 1.3 for control BSA, 46.8 ± 5.6 for AGE-BSA, 18.6 ± 2.1 for control RSA, and 101.5 ± 12.1 for AGE-RSA. All reagents were prepared under endotoxin-free conditions. Each preparation was tested by Limulus amebocyte lysate assay (Zhanjiang A&C Biological Ltd, China) for endotoxin content (< 0.8 EU endotoxin per milliliter).

Animal treatments. Thirty male Wister rats (6–7 weeks old) were purchased from Jilin University Experimental Animal Center and randomly divided into five groups as follows—control group: given tail vein injection with sterile phosphate-buffered saline (PBS); RSA group: given tail vein injections with RSA (100 mg/kg daily) for 6 weeks; AGE group: given tail injections with AGE-RSA (100 mg/kg daily) for 6 weeks; AGE + AG group: given with AGE-RSA (100 mg/kg daily) followed by abdominal injections of AGE cross-link inhibitor aminguanidine (AG, 40 mg/kg daily) for 6 weeks; and AGE + RGZ group: given with AGE-RSA (100 mg/kg daily) followed by oral administration of PPAR-γ agonist RGZ (2 mg/kg daily) for 6 weeks. The dose of AGE-RSA was based on previous studies. To develop a typical diabetic nephropathy, oral supplementation with AGE-RSA at 25 mg/kg daily for 5 months has been used previously (Vlassara et al., 1994); however, to investigate molecule or gene regulation in response to AGE, subacute supplementation with AGE-RSA at 100 mg/kg daily in rats for 4–8 weeks also has been used (Vlassara et al., 1992; Zhou et al., 2004). AG was used because it has been extensively indicated to react with Amdaori-derived products to form stable compounds to avoid the formation of reactive AGES, consequently preventing AGE-protein cross-linking (Kelly et al., 2001; Vlassara et al., 1992, 1994; Yang et al., 1994). The RGZ dose was based on a published study (Cai et al., 2000). The University Animal Care and Use Committee have approved all these procedures.

Primary cultures of rat mesangial cells and treatments. Rat mesangial cells were isolated from rat glomeruli and cultured in vitro, as described in our previous study (Zhou et al., 2004). Primary cultures were allowed to grow for 2–3 weeks when mesangial cells were confluent. Mesangial cells between 5 and 10 passages were used for experiments. After trypsinization, cells were grown in six-well or 24-well plates for 5 days in their respective medium with 10% fetal bovine serum (FBS) until confluent. Cells were then incubated in Dulbecco’s modified eagle’s medium with 0.5% FBS for 24 h followed by a change of same new medium and treatment with AGE-BSA and BSA with or without PPAR-γ agonist RGZ. The media used for culturing these cells were harvested, and Trizol reagent lysed these cells. For experiments involving use of neutralizing antibody, cells were preincubated with antibody for 2 h under 0.5% FBS medium before adding AGE-BSA or control BSA to the medium. To neutralize PAI-1 in the cultured cells, anti-PAI-1 antibody at10 μg/ml was used.

Assessments of renal function. Animals were placed in individual metabolic cages for 24 h to collect urine samples after giving only accessible to tap water for 1 day before the experimental rats were sacrificed. No difference for total water intake among groups was observed (data not shown). Total urinary volume and urinary protein (grams per liter) were measured, and the urinary total protein excretion was calculated.

Renal histological changes examined under light and electron microscopes. One-fourth kidneys were immersion fixed in 10% buffered formalin and embedded in paraffin for a light microscopic study. Two sections of 4-μm thickness (an interval of 100 μm) per animal were stained with periodic acid-Schiff reagent (PAS). For electron microscope examination, renal cortex was cut into small pieces and prefixed in 2.5% glutaraldehyde (0.2 mol/l cacodylate buffer, pH 7.4) for 1 h, postfixed in 1% buffered sodium tetroxide for 1 h, and embedded in Epon 812. Ultrathin sections were examined using a JEM-1200 EX electron microscope. Mesangial matrix expansion was determined as PAS-positive materials presented in the mesangial region excluding cellular elements. Percentage of PAS-positive area in each glomerulus was analyzed using Leica Q500MC image analysis software, as described in our previous study (Zhou et al., 2004). Ten glomeruli selected randomly in two slides from each rat (total six rats in each group) were evaluated by two investigators without knowledge of the origin of these slides.

Immunohistochemical study. Renal tissue sections at 4 μm were used to perform immunohistochemical staining for fibronectin, Coll IV, PARE-1, and PPAR-γ, as described in our previous study (Zhou et al., 2004), with the following specific antibodies: polyclonal rabbit anti-fibronectin, Coll IV, PPAR-γ antibodies (Santa Cruz Biotechnology, Inc, Santa Cruz, CA), and polyclonal rabbit anti-PAR-1 antibody (Boster Biological Technology Co., Wuhan, China). Color was developed with diaminobenzidine (DAB) and counterstaining with hematoxylin. Negative controls were obtained by replacing the primary antibody with PBS. Computer imaging analysis system was used for semiquantitative analysis of the percentage of positive staining area in the glomerulus.
Western blotting assay. Proteins (40 μg protein/lane) were separated by electrophoreses on a 7.5% polyacrylamide gel and then transferred to nitrocellulose membranes (GE Healthcare, Beijing). The membranes were blocked in 5% skim milk powder in PBS before overnight incubation with a polyclonal rabbit anti-rat PAI-1 (1:200 dilution) or anti-rat PPAR-γ (1:100 dilution) antibody. Proteins were visualized using a horseradish peroxidase–conjugated IgG and an enhanced chemiluminescence kit (GE Healthcare).

Reverse transcription–polymerase chain reaction. Total RNA was extracted from cultured mesangial cells and renal cortical tissues using TRIzol reagent (Gibco Co., Beijing). One microgram of total RNA was reverse transcribed to cDNA with Super II-reverse transcriptase at 37°C for 1 h in a standard buffer. Polymerase chain reaction (PCR) was carried out using Taq DNA Polymerase (DingGuo Biotech., Co., Beijing). The upstream and downstream of PCR primers are: PAI-1, 5'-ATG AGA TCA GTA CTG CGG CAC CCA TCT TTG-3' and 5'-GCA CGG AGA TGG TGC TAC CAT CAG ACT TGT-3' by which a 333 bp of PAI-1 cDNA would be synthesized (Konkle et al., 1992); PPAR-γ, 5'-CTT CGG AAT CAG CTC TGT GGA C-3' and 5'-GCA TCC TTC ACA AGC ATG GAC TC-3' by which a 351 bp of PPAR-γ cDNA would be synthesized (Robert et al., 2002); β-actin, 5'-GTG GGG CGC CCC AGG CAC CA-3' and 5'-CTT CCT TAATGT CAC GCA CGA TTT C-3' by which a 540 bp of β-actin cDNA would be synthesized. The PCR for PPAR-γ used the following cycle condition: denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 45 s; and that for PAI-1 and β-actin used the following cycle condition: denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 45 s. The number of cycles for the PCRs was 30 and the products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining. Bands were digitized by a Tanon-1000 Gel Image System (Shanghai) for the quantitative analysis as our previous study (Zhou et al., 2004).

Measurements of fibronectin and Col IV by enzyme-linked immunosorbent assays. Mouse anti-rat monoclonal fibronectin or Col IV antibody (5 μg/ml, Boster Biological Technology Co.) in coating buffer was absorbed to

FIG. 1. AGE-caused renal dysfunction and pathological changes. Rats were treated with AGE for 6 weeks and then sacrificed for evaluating urinary protein (A). Pathological changes in the kidney of control (B), RSA-treated (C), AGE-RSA–treated (D), AGE + AG–treated (E) and AGE + RGZ–treated (F) rats were examined by electron microscope. Arrows indicate thickening of glomerular basement membranes. AGE-RSA, glycated RSA; AGE + AG, coadministration of AG with AGE-RSA; AGE + RGZ, coadministration of RGZ with AGE-RSA. *p < 0.01 versus RSA.
96-well microplates by a 20-h incubation at 4°C. The unbounded antibody was removed, and the wells were blocked by incubation with 150 µl PBS/Tween 20 containing 0.5% (w/v) BSA for 2 h at 37°C. After three further washes with PBS/Tween 20, collected media (100 µl/ml) from treated mesangial cells were incubated at 37°C for 2 h. Each sample was repeated five times. The plates were washed and then incubated with rabbit anti-rat polyclonal fibronectin or Col IV antibody (1:1000 dilution, 100 µl) for 2 h at 37°C. After washing, the plate was incubated with 100 µl goat anti-rabbit IgG conjugated to horseradish peroxidase (1:1000 dilution; Boster Biological Technology Co.) for 45 min at 37°C. A final wash was followed by color development using the colorimetric reagent (0.6 mg/ml DAB in 0.01M Tris-HCl [pH 7.6] containing 0.3% H2O2). The reaction was stopped by the addition of 2M H2SO4, and the absorbance was measured at 490 nm using a microplate reader. Content of fibronectin or Col IV was represented as optical density units rather than concentrations, as described in our previous study (Zhou et al., 2004).

Measurements of PAI-1 by enzyme-linked immunosorbent assays. Media used for culturing mesangial cells were coated to 96-well microplates with coating buffer (1:15 dilution). Then the wells were successively blocked with 0.5% BSA, incubated with rabbit anti-rat polyclonal PAI-1 (1:1000 dilution) and goat anti-rabbit IgG (conjugating with horseradish peroxidase; 1:1000 dilution). Other procedures are same with fibronectin enzyme-linked immunosorbent assays (ELISAs).

Plasminogen gel zymography. To detect renal plasminogen activator activity, plasminogen gel zymography was used based on a previous study (Oda et al., 2001). Briefly, kidney supernatants (1 µg protein) were separated under nonreducing condition on 10% SDS-polyacrylamide gel containing plasminogen (10 µg/ml) and casein (2 mg/ml). After electrophoresis, the gel was washed with 2.5% Triton X-100 and incubated in 0.1 mol/l glycine (pH 8.3) for 15 h at 37°C. After the gels were stained with Coomassie blue, plasminogen activator activity was revealed as clear lytic zones. The density of each lytic band was quantified using the Tanon-1000 Gel Image System, human u-PA (33 kD) as a standard. To distinguish the plasminogen activator from metalloproteinases, samples were also run on gels without plasminogen.

FIG. 2. AGE-induced renal ECM accumulation. Rats were treated with AGE-RSA for 6 weeks and then sacrificed for evaluation of renal ECM accumulation, by examination of staining for PAS-positive materials and immunohistochemical staining for fibronectin and Col IV. Labels are same as those in Figure 1.
Total plasminogen activator activity assay. Total plasminogen activator activity in conditioned medium was measured in triplicate by plasminogen activator activity assay kit (Shanghai Sun Biotech Co., Shanghai).

Statistical analysis. Data were presented as mean ± SD from six samples (rats) for in vivo study and at least five samples for in vitro experiments. One-way ANOVA and Student’s t-test were used for statistical analysis. Differences were considered to be significant at \( p < 0.05 \).

RESULTS

General Condition of AGE-Treated Rats

The serum glucose, cholesterol, and triglyceride of all animals were in normal range. However, a significantly low cholesterol level was observed in AGE + RGZ group (control: \( 1.48 ± 0.05 \); RSA: \( 1.36 ± 0.07 \); AGE: \( 1.39 ± 0.17 \); AGE + AG: \( 1.17 ± 0.17 \); AGE + RGZ: \( 0.89 ± 0.20 \) mmol/l; AGE + RGZ vs. AGE, \( p < 0.01 \)). No difference in serum glucose and triglyceride was found among three groups.

AGE-Induced Renal Dysfunction, Pathological Change, and ECM Accumulation

All animals treated with AGE-RSA displayed a mild renal dysfunction, as shown by an increase in urinary protein excretion. Coadministration of AGEs with RGZ significantly

<table>
<thead>
<tr>
<th>Group</th>
<th>PAS</th>
<th>Fibronectin</th>
<th>Col IV</th>
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<tbody>
<tr>
<td>Control</td>
<td>9.7 ± 0.8</td>
<td>5.6 ± 0.9</td>
<td>6.1 ± 0.8</td>
</tr>
<tr>
<td>RSA</td>
<td>10.2 ± 1.0</td>
<td>7.2 ± 1.9</td>
<td>8.3 ± 1.1</td>
</tr>
<tr>
<td>AGE-RSA</td>
<td>25.4 ± 2.3 ( ^a )</td>
<td>18.3 ± 4.0 ( ^b )</td>
<td>21.2 ± 5.6 ( ^c )</td>
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<tr>
<td>AGE + AG</td>
<td>15.5 ± 2.6 ( ^a,b )</td>
<td>12.4 ± 1.6 ( ^a,b )</td>
<td>14.6 ± 3.2 ( ^a,b )</td>
</tr>
<tr>
<td>AGE + RGZ</td>
<td>17.8 ± 3.1 ( ^a,b )</td>
<td>13.5 ± 2.5 ( ^a,b )</td>
<td>15.4 ± 3.6 ( ^a,b )</td>
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\( ^a p < 0.05 \) versus control or RSA.

\( ^b p < 0.05 \) versus AGEs.

\( ^c p < 0.05 \) versus AGEs.

TABLE 1

Morphometric analysis of PAS staining and immunohistochemical staining for fibronectin and Col IV (%)
prevented AGE-induced renal dysfunction, as observed in the group of coadministration of AGEs with AGE cross-link breaker (AG, labeled as AGE + AG in Fig. 1A). Morphological changes of glomerulus were assessed by examination under electron microscopy (Figs. 1B–E). Segmental thickening of glomerular basement membranes, widely fused foot processes podocytes, and excessively deposited mesangial matrix were observed in the rats treated with AGE-RSA (Fig. 1D). These ultrastructural abnormalities were significantly prevented by AG (Fig. 1E) or RGZ (Fig. 1F).

PAS-positive materials are markedly increased in the kidneys of rats treated with AGE-RSA (Fig. 2). Quantitative analysis for the percentage of PAS in the glomerulus using computer image system was summarized in Table 1. Immunohistochemical staining indicated that fibronectin or Col IV could be lightly observed in the normal glomeruli: fibronectin staining is observed mainly in the intraglomerular mesangium, and Col IV staining is located mainly in the glomerular basement membrane (Fig. 2). The intensities and areas of fibronectin and Col IV staining were significantly increased in the glomeruli of AGE-treated rats as compared to control and RSA groups. Treatment with either AG or RGZ partially prevented these effects (Table 1).

**AGE-Increased PAI-1 mRNA and Protein Expressions and Decreased Plasminogen Activator Activity**

In the kidney cortex of rats treated with AGE-RSA, PAI-1 mRNA expression, measured by reverse transcription–polymerase chain reaction (RT-PCR), significantly increased relative to control (Fig. 3A). Correspondingly PAI-1 protein content, measured by Western blotting assay, was also significantly increased (Fig. 3B). Increased expression of PAI-1 resulted in a significant decrease in plasminogen activator activity, measured by plasminogen gel zymography (Fig. 4). Coadministration of AG with either AG or RGZ could reduce AGE-induced PAI-1 mRNA and protein expressions to nearly normal levels (Figs. 3 and 4).

Immunohistochemical staining showed that PAI-1 was moderately expressed in the partial tubular epithelial cells but not in glomeruli of the normal rat kidney (Fig. 5). In AGE-treated rat kidneys, however, there were increased stains both in the tubular epithelial cells and glomeruli, an effect significantly attenuated by coadministration of AG or RGZ (Fig. 5).

**AGE-Decreased Renal PPAR-γ mRNA and Protein Expressions**

Expression of PPAR-γ mRNA, detected by RT-PCR using kidney cortex tissues, was decreased in the rats treated with AGE-RSA for 6 weeks (Fig. 3C). Correspondingly, PPAR-γ protein expression was also lower in AGE-treated rat kidneys than that in control rat kidneys (Fig. 3D). Coadministration of AG or RGZ with AG could completely prevent AGE-caused decreases in PPAR-γ mRNA and protein expressions. Immunohistochemical staining provides further evidence that AGEs significantly decreased the PPAR-γ expression that was expressed diffusely in tubules and resident glomerular cells including mesangial cells, but coadministration of AG or RGZ could completely prevent AGE’s effect (Fig. 5).

**RGZ Prevents AGE-Induced ECM Accumulation Likely through Suppression of PAI-1 Upregulation**

To test whether AGEs suppress PPAR-γ expression and consequently upregulates PAI-1 expression that in turn inhibits plasminogen activator activity, leading to the renal ECM accumulation, we have exposed the primary cultures of mesangial cells to AGEs in vitro to investigate the role of PAI-1 in fibronectin and Col IV production. As shown in Figure 6A, ELISA displayed that AGEs significantly induced PAI-1 expression with a slightly dose-dependent manner within the dose range of 50–200 μg/ml. The increased protein level of PAI-1 was coincided with the increase in its mRNA level, measured by RT-PCR (Fig. 6B). The increased PAI-1 expression resulted in a significant decrease in plasminogen activator activity (also without significant dose-dependent manner within the dose range of 50–200 μg/ml, Fig. 6C) and significant increases in fibronectin and Col IV levels (Fig. 6D). Coadition of RGZ with AGEs into the mesangial cells significantly prevented the
increase in PAI-1 expression (Fig. 7A) and decrease in plasminogen activator activity (Fig. 7B) and Col IV production (Fig. 7A).

All results obtained from the in vitro studies (Figs. 6, 7A, and 7B) are similar to those observed in the kidneys of AGE-treated rats with or without coadministration of RGZ (Figs. 1–5). Therefore, our next experiment was to determine whether or not PAI-1 plays the critical role in AGE-induced renal ECM accumulation. To this end, mesangial cells were treated with PAI-1 antibody for 2 h prior to exposure to AGEs. Results showed that pretreatment of PAI-1 antibody almost completely blocked AGE-induced fibronectin and Col IV production (Fig. 7C).

DISCUSSION

AGEs play important roles in the pathogenesis of diabetic nephropathy via stimulating the production of several cytokines and growth factors, leading to renal ECM accumulation.
We have demonstrated that administration of AGEs to rats at 100 mg/kg daily for 6 weeks induced a significant increase in renal ECM accumulation with increased expressions of connective tissue growth factor (CTGF) and TGF-β and significantly pathological changes, which in turn led to a mild renal dysfunction (Zhou et al., 2004). In the present study, we used the same protocol to produce mild renal dysfunction in the rats that displayed a significantly renal ECM accumulation along with pathological changes. Consistent with the previous study (Zhou et al., 2004), these fibrotic effects can be prevented by simultaneous supplementation with the AGE formation inhibitor AG, suggesting the importance of AGEs in the pathogenesis of diabetic nephropathy (Kelly et al., 2001; Vlassara et al., 1992, 1994; Yang et al., 1994). In these AGE-treated rats, we furthermore demonstrated, for the first time, a significant increase in the expression of PAI-1 mRNA and protein along with a decreased expression of PPAR-γ mRNA and protein in the kidney cortex. Furthermore, the direct role of PAI-1 in AGE-induced fibronectin and Col IV production was defined using primary cultures of mesangial cells. Therefore, these results suggest the critical role of PAI-1 upregulation in AGE-induced renal ECM accumulation and renal injury or dysfunction.

PAI-1, as a major inhibitor of u-PA and t-PA, negatively regulates the formation and activity of plasmin, which can cleave most matrix proteins (Huang and Noble, 2005; Kenichi et al., 2004; Lee et al., 2005a). Increased PAI-1 expression leads to decreases in plasminogen activator activity and plasmin formation and consequently renal ECM accumulation. In addition, since PAI-1 also regulates the adhesion and migration of a variety of cells, the elevated PAI-1 may promote collagen deposition through stimulating migration of leukocytes and collagen-producing cells into the damaged tissues (Huang and Noble, 2005; Matsuo et al., 2005). Mice with PAI-1 deficiency were found to be resistant to TGF-β- or diabetes-induced renal fibrosis (Collins et al., 2006; Krag et al., 2005; Nicholas et al., 2005). In consistent with these studies, the present study shows that in vivo infusion of AGEs to rats for 6 weeks caused both PAI-1 mRNA and protein expressions in the kidney cortex, which is coincided with a decreased plasminogen activator activity and a ECM accumulation in the kidney, shown by increases in PAS-positive materials and fibronectin and Col IV contents. This direct role of AGE-upregulated PAI-1 expression in the ECM accumulation was further defined in the in vitro experiments using PAI-1–specific antibody that prevents AGE-induced fibronectin and Col IV production in mesangial cells (Fig. 7C).
Although we do not know how AGEs induce PAI-1 upregulation in the present study, we hypothesize that AGEs induce the production of various inflammatory or growth factors such as TNF-α, TGF-β, and/or CTGF expression with and without reactive oxygen species (ROS) generation in kidney, which in turn cause PAI-1 upregulation. We have previously demonstrated the upregulation of TGF-β and CTGF expression in the kidney of AGE-treated rats (Zhou et al., 2004), a same model as that used in the present study, and other studies have shown the roles of CTGF, TGF-β, TNF-α, and ROS in the induction of PAI-1 in the kidney or other tissues (Lee et al., 2005a; Liu et al., 2005; Zhang et al., 2004). Although both TGF-β and CTGF were found to be upregulated in kidneys of AGE-treated rats, we found that AGE-induced CTGF upregulation was predominantly TGF-β independent and also played the pivotal role in renal ECM accumulation (Zhou et al., 2004). That finding was supported by a recent observation in NRK-49F cells that AGE-induced type I collagen production was found to depend on CTGF pathway instead of TGF-β (Lee et al., 2005b). In addition, another early study also showed that although Smad4 is essential for TGF-β–induced type I collagen expression in mesangial cells, TGF-β–induced PAI-1 expression are predominantly dependent on Smad3 that is a specific upstream mediator for CTGF expression, suggesting the importance of CTGF in the PAI-1 upregulation (Fu et al., 2001; Tsuchida et al., 2003). Although there is no direct information on the role of CTGF in AGE-induced PAI-1 expression, there was a study indicating that inhibition of CTGF expression by CTGF antisense oligodeoxynucleotides significantly blocked expressions of PAI-1 and fibronectin in renal tubular cells in response to TGF-β1 (Zhang et al., 2004). In the present study, we investigated the role of PPAR-γ in AGE-induced renal ECM accumulation. To our knowledge, this study is the first to demonstrate that in vivo infusion of AGE to the rats reduced renal PPAR-γ expression along with an induction of renal dysfunction and also that PPAR-γ agonist alone can ameliorate AGE-induced renal dysfunction completely and pathological changes partially. Pelzer et al. (2005) have demonstrated a reduced expression of PPAR-γ in the hearts of Zucker diabetic fatty rats. Whether the decreased PPAR-γ expression is responsible for the fibrotic pathogenesis of diabetic hearts is unclear; however, supplementation of exogenous PPAR agonists can prevent diabetes-induced renal PAI-1 upregulation and fibrosis (Chen et al., 2006), suggesting the importance of PPAR function in inhibiting PAI-1 expression. Many studies have shown the efficacy of thiazolidinedione family agents in diabetic renal lesions due to its insulin-sensitizing action (Buckingham et al., 1998; Hong et al., 2003; Kato et al., 1999; Shibata et al., 2000; Smith et al., 2000). In the model used in the present study, however, the preventive effects of RGZ on AGE-induced renal upregulation of PAI-1 expression along with the renal dysfunction and ECM accumulation are apparently independent on its insulin-sensitizing (glucose lowering) effect and may be partially due to lipid-lowering effects as AGE treatment did not affect glucose levels but caused a significant decrease in cholesterol level in AGE + RGZ group compared to that in AGE group. This is consistent with the recently emerging evidence to indicate the PPAR-independently beneficial effects of PPAR agonists on diabetic and nondiabetic pathogenesis in different organs (Artwohl et al., 2005; Chana et al., 2006; Galli et al., 2004; Ward et al., 2004). For example, studies have clearly indicated that the suppression of PAI-1 production by PPAR agonists in different organs under various conditions can be mediated by both PPAR-dependent
and -independent mechanisms (Galli et al., 2004; Hong et al., 2003; Liu et al., 2005).

In summary, the present study provides the evidence that PAI-1 may act as a downstream mediator of CTGF profibrotic signaling to play a critical role in AGE-induced renal ECM accumulation, pathological changes, and dysfunction. PPAR-γ agonist RGZ prevents AGE-induced renal fibrosis probably through suppression of PAI-1 expression, which may be mediated either by PPAR-dependent or -independent mechanisms or both together. The detail mechanisms need to be dissected in the future studies.

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