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AGEs activate mesangial TGF-β–Smad signaling via an angiotensin II type I receptor interaction

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AGEs activate mesangial TGF-β–Smad signaling via an angiotensin II type I receptor interaction.

Background. The renin-angiotensin system (RAS) and the accumulation of advanced glycation end products (AGEs) have been implicated in the pathogenesis of diabetic nephropathy. Whether there is a functional interaction between the RAS and AGEs in diabetic nephropathy is not known. In this study, we investigated whether AGEs could activate autocrine angiotensin II (Ang II) signaling and subsequently induce transforming growth factor-β (TGF-β)–Smad signaling in cultured rat mesangial cells.

Methods. The intracellular formation of reactive oxygen species (ROS) was detected using the fluorescent probe CM-H_2DCFDA. Ang II was measured by radioimmunoassay. TGF-β released into media was quantitatively analyzed in an enzyme-linked immunosorbent assay (ELISA). Smad2, $p27^{Kip1}$ (p27), fibronectin, and receptor for AGEs (RAGE) protein expression were determined by Western blot analysis. TGF-β–inducible promoter activity was analyzed by a luciferase assay. DNA synthesis was evaluated by 5-bomo-2′-deoxyuridine (BrdU) incorporation and de novo protein synthesis was determined by [^{3}H]leucine incorporation.

Results. AGEs increased intracellular ROS generation in mesangial cells, and this effect was significantly inhibited by an antiserum against RAGE. AGEs also were found to stimulate Ang II production in a time- and dose-dependent manner, which was completely prevented by an antioxidant, *N*-acetylcysteine (NAC). AGE-induced TGF-β overproduction was completely blocked by candesartan, an Ang II type 1 receptor (AT_1R) antagonist. Both candesartan and neutralizing antibody against TGF-β completely prevented AGEs-induced Smad2 phosphorylation and TGF-β–inducible promoter activity. Furthermore, AGEs were found to inhibit DNA synthesis and to stimulate de novo protein synthesis and fibronectin production in association with up-regulation of p27. All of these phenomena were completely prevented by candesartan or a polyclonal antibody against TGF-β.

Conclusion. The present study suggests that AGE-RAGE–mediated ROS generation activates TGF-β–Smad signaling and subsequently induces mesangial cell hypertrophy and fibronectin synthesis by autocrine production of Ang II. This pathway may provide an important link between metabolic and haemodynamic factors in promoting the development and progression of diabetic nephropathy.

Key words: advanced glycation end products, mesangial cell, diabetic nephropathy, oxidative stress.

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Diabetic nephropathy is a leading cause of end-stage renal disease (ESRD), and accounts for significant morbidity and mortality in patients with diabetes [1, 2]. The development of diabetic nephropathy is characterized by glomerular hyperfiltration, hypertrophy of glomerular and tubuloepithelial components, and thickening of glomerular basement membranes (GBMs), followed by an expansion of extracellular matrix (ECM) in mesangial areas and an increased albumin excretion rate. Diabetic nephropathy ultimately progresses to glomerular sclerosis associated with renal dysfunction [3].

There is a growing body of evidence suggesting that the intrarenal renin-angiotensin system (RAS) plays an important role not only in the regulation of glomerular hemodynamics but also in glomerular hypertrophy and sclerosis [4, 5]. Indeed, blockade of the RAS with drugs such as angiotensin-converting enzyme (ACE) inhibitors and angiotensin II (Ang II) type 1 receptor (AT_1R) antagonists was found to suppress the development and progression of diabetic nephropathy in both type 1 and type 2 diabetic patients [6–8]. Furthermore, several in vitro and in vivo studies have implicated transforming growth factor-β (TGF-β), a fibrogenic cytokine, as a key mediator in advanced diabetic renal disease, and the prosclerotic action of Ang II is mediated, at least in part, by TGF-β [9–13].

Reducing sugars react nonenzymatically with the amino groups of proteins to initiate a complex series of rearrangements and dehydrations to produce a class of irreversibly cross-linked, fluorescent moieties termed advanced glycation end products (AGEs) [14–16]. The formation and accumulation of AGEs are characteristic features of aged or diabetic tissues and these products also have been strongly implicated in the pathogenesis of diabetic micro- and macrovascular complications [16–24]. In fact, there is compelling evidence to suggest that the formation and accumulation of AGEs mediate the progressive alteration in renal architecture and loss of renal function and that inhibitors of advanced glycation prevent the progression of experimental diabetic nephropathy [25–33]. Whether there is a functional interaction between the RAS-TGF-β system and AGE-mediated signaling pathways in diabetic nephropathy is not known. In this study, we investigated whether AGEs could activate autocrine Ang II signaling and subsequently induce TGF-β–Smad signaling in cultured rat mesangial cells. We further examined the involvement of the receptor for AGEs (RAGE) and reactive oxygen species (ROS) generation in the deleterious effects of AGEs.

METHODS

Materials

Bovine serum albumin (BSA) (fraction V) and a monoclonal antibody against mouse β-actin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). D-glyceraldehyde and *N*-acetylcysteine (NAC) were from Nakalai Tesque (Kyoto, Japan). Candesartan, an AT_1R antagonist, was provided by Takeda Chemical Industries, Ltd. (Osaka, Japan). A polyclonal antibody directed against phosphorylated Smad2 was a gift from Dr. Peter ten Dijke (Division of Cellular Biochemistry, The Netherlands Cancer Institute, Amsterdam, The Netherlands). Human recombinant TGF-β and a polyclonal antibody against TGF-β for neutralizing assays were from R&D Systems (Minneapolis, MN, USA). [^{3}H]leucine was from Amersham Bioscience (Buckinghamshire, UK). Goat polyclonal IgG against RAGE (sc-8230) and rabbit polyclonal IgG against $p27^{Kip1}$ (p27) (sc-528) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Antiserum against human RAGE for neutralizing assays, which recognizes the amino acid residues 167 to 180 of human RAGE protein, was prepared as described previously [34]. A polyclonal antibody against rabbit fibronectin was obtained from Chemicon International, Inc. (Temecula, CA, USA). Polyclonal antibody against glyceraldehyde-derived AGEs that did not cross-react with well defined AGE epitopes and antibodies against carboxymethyllysine (CML) for neutralizing assays were prepared as described previously [27, 35].

Preparation of AGE-BSA and CML-BSA and purification of AGEs from human serum

AGE-BSA was prepared as described previously [35]. Briefly, BSA was incubated under sterile conditions with 0.1 mol/L D-glyceraldehyde in 0.2 mol/L phosphate-buffered saline (PBS) (pH 7.4) for 7 days. Unincorporated sugars were then removed by dialysis against PBS. Nonglycated BSA was incubated in the same conditions except for the absence of D-glyceraldehyde as a negative control. Preparations were tested for endotoxin using Endospecy ES-20S system (Seikagaku Co., Tokyo, Japan) and no endotoxin was detected. The extent of chemical modification was determined as described with 2,4,6-trinitrobenzenesulfonic acid as a difference in lysine residues of modified and unmodified protein preparations [36]. The extent of lysine modification (%) of modified BSA preparation was 65% for glyceraldehyde-derived AGE-BSA. The concentration of AGEs used in this experiment was comparable with that of in vivo situation of diabetes [35].

CML-BSA (CML) was prepared as described previously [35]. Briefly, 50 mg/mL protein was incubated at 37°C for 24 hours with 45 mmol/L glyoxylic acid and 150 mmol/L $NaCNBH_3$ in 2 mL of 0.2 mol/L phosphate buffer (pH 7.4), followed by PD-10 column chromatography and dialysis against PBS.

Serum AGEs fractions were obtained from diabetic patients on maintenance hemodialysis and from normal volunteers at Suiyukai Clinic, as described previously [37]. Informed consent was obtained from all the subjects.

Cells

Mesangial cells were obtained by culturing glomeruli isolated from the kidneys of 200 to 250 g male Wistar rats by a conventional sieving method [38]. All surgical interventions and anesthesia were conducted according to institutional guidelines and in compliance with international laws and policies (EEC Council Directive 86/609, OJL 358, December 1987; *Guide for the Care and Use of Laboratory Animals*, NIH Publication No. 85-23, 1985). The cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Nissui Pharmaceutial Co., Tokyo, Japan) supplemented with 10% fetal calf serum (FCS) (Filtron Pty Ltd., Brooklyn, Australia), 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. Mesangial cells at less than 10 passages were used for the experiments. AGE treatments were carried out in a medium containing 0.5% FCS.

Measurement of intracellular ROS generation

The intracellular formation of ROS was detected using the fluorescent probe CM-H_2DCFDA (Molecular Probes, Inc., Eugene, OR, USA) as described previously

[39]. Briefly, cells were plated at 5×10^3 cells/well in 96-well plates. The cells were loaded with 10 μmol/L CM-H_2DCFDA, incubated for 60 minutes at 37°C, and analyzed with an EZS-FL fluorescent plate reader (Asahi Techno Glass, Tokyo, Japan) using EZScan-FL for Windows program.

Measurement of Ang II

Cells were plated at 5×10^5 cells/well in 6-well plates. The next day, the cells were rested in a serum-free medium for 24 hours, and then treated with various concentrations of AGEs in the presence or absence of 1 mmol/L NAC for the various time periods. After incubation, cells were lysed and Ang II levels in the cell lysates were measured by a radioimmunoassay system according to the method of Kashiwagi et al [40].

Measurement of TGF-β

Cells were plated at 5×10^3 cells/well in 96-well plates. The next day, the cells were rested in a serum-free medium for 24 hours, and then treated with 100 μg/mL of AGE-BSA or nonglycated BSA in the presence or absence of 10^{-7} mol/L candesartan for 48 hours. Total TGF-β released into the media was measured by an enzyme-linked immunosorbent assay (ELISA) system (Promega, Madison, WI, USA).

Western blot analysis for phosphorylated Smad2, cyclin-dependent kinase inhibitor, p27, fibronectin, and RAGE proteins

Mesangial cells were treated with the various concentrations of AGEs, 100 μg/mL of nonglycated BSA, 100 μg/mL of CML, 500 μg/mL of purified-AGEs from human plasma, or 10 ng/mL of TGF-β in the presence or absence of 10^{-7} mol/L candesartan or 10 μg/mL of polyclonal antibody against TGF-β for 48 hours. The cells were then lysed and 30 μg/mL of proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidine difluoride (PVDF) membranes (Immobilon™) (Millipore, MA, USA). Membranes were probed with 1:1000 dilution of polyclonal antibodies against phosphorylated Smad2, p27, fibronectin, or RAGE. The immune complexes were visualized with an enhanced chemiluminescence detection system (ECL) (Amersham Bioscience), as described previously [41].

Measurement of TGF-β–inducible promoter activity

Plasmid containing the TGF-β–inducible promoter $(CAGA)_{12}$ attached upstream to the luciferase reporter gene, was the kind gift from Dr. Peter ten Dijke [42]. Mesangial cells were plated at 5×10^4 cells/well in 6-well plates. The next day, cells were rested in a serum-free medium for 24 hours. The cells were subsequently transfected with luciferase-reporter plasmids (2 μg) using FuGENE 6 (Roche Diagnostics Corp., Indianapolis, IN, USA). After transfection, the cells were treated with 100 μg/mL of AGE-BSA, 100 μg/mL of nonglycated BSA, or 10 ng/mL of TGF-β in the presence or absence of 10^{-7} mol/L candesartan or 10 μg/mL of polyclonal antibody against TGF-β for 48 hours. Luciferase activity was then measured with a luminometer (Promega).

Measurement of 5-bromo-2′deoxyuridine (BrdU) incorporation

Cells were plated at 5×10^3 cells/well in 96-well plates. The next day, the cells were rested in a serum-free medium for 24 hours, and then incubated with 100 μg/mL of AGE-BSA or nonglycated BSA in the presence or absence of 10^{-7} mol/L candesartan or 10 μg/mL of polyclonal antibody against TGF-β for 24 hours. Cells were treated with BrdU for the last 16 hours and its incorporation into the cells was determined using a cell proliferation ELISA colorimetric kit (Roche).

Measurement of [^{3}H]leucine incorporation

Cells were plated at 4×10^4 cells/well in 24-well plates. The next day, the cells were rested in serum-free medium for 24 hours, and then incubated with 100 μg/mL of AGE-BSA or nonglycated BSA in the presence or absence of 10^{-7} mol/L candesartan or 10 μg/mL of polyclonal antibodies against TGF-β for 24 hours. Cells were treated with 2 μCi/well [^{3}H]leucine for the last 16 hours and its incorporation into the cells was determined as previous described [43]. The count of [^{3}H]leucine was divided by the cell number, which was counted using a hemocytometer after tripan blue exclusion.

Statistical analysis

All values are presented as mean ± SEM. Experimental groups were compared by analysis of variance (ANOVA), and, when appropriate, with Scheffe's test for multiple comparisons. A level of $P < 0.05$ was accepted as statistically significant.

RESULTS

Effects of AGEs on ROS production

As shown in Figure 1A, glyceraldehyde-derived AGEs, a class of representative ligands for RAGE [23], dose-dependently increased intracellular ROS generation in mesangial cells. Compared with control conditions (treatment with 100 μg/mL of nonglycated BSA), treatment with 100 μg/mL of AGE-BSA significantly increased ROS generation in mesangial cells by twofold. Furthermore, a neutralizing antiserum directed against RAGE completely prevented the increase in ROS generation in

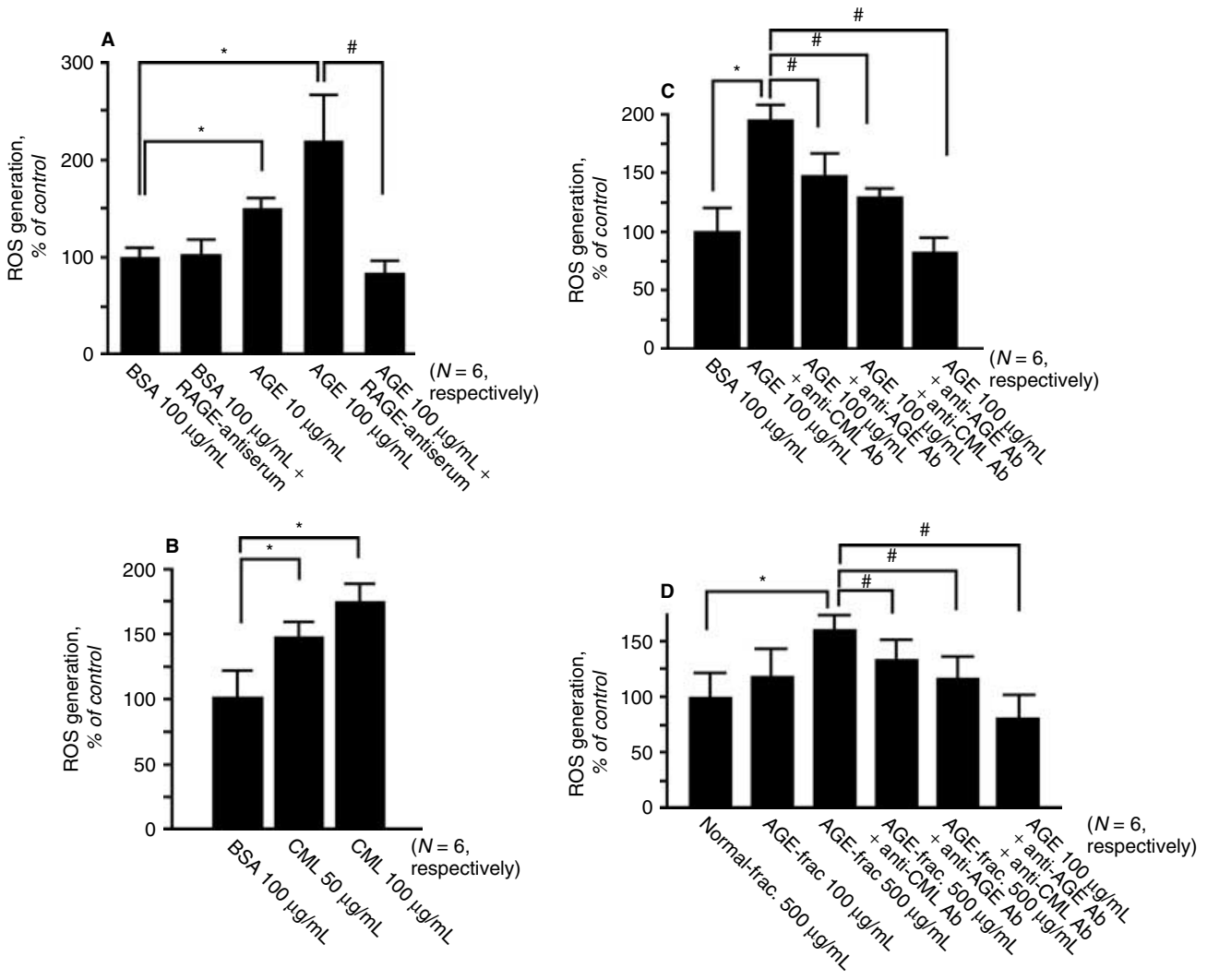

Fig. 1. Effects of advanced glycation end products (AGEs) on reactive oxygen species (ROS) production in mesangial cells. (*A*) Cells were preincubated with or without 0.1% antiserum against receptor of AGE (RAGE) 30 minutes prior to the treatment with 10 or 100 μg/mL of AGE-bovine serum albumin (BSA) or 100 μg/mL of non-glycated BSA for 48 hours. Then ROS were quantitatively analyzed. (*B*) Effects of carboxymethyllysine (CML) on ROS production in mesangial cells. Cells were incubated with 100 μg/mL of nonglycated BSA or 50 to 100 μg/mL of CML-BSA for 48 hours. Then ROS were quantitatively analyzed. (*C*) Effects of neutralizing antibodies (Abs) against CML or non-CML AGEs on ROS generation in mesangial cells. Cells were preincubated with or without 10 μg/mL of antibodies against CML (anti-CML Ab) or glyceraldehyde-derived AGEs (anti-AGE Ab) 60 minutes prior to stimulation with 100 μg/mL of AGEs or 100 μg/mL of nonglycated BSA for 48 hours. Then ROS were quantitatively analyzed. $^{*}P < 0.05$ compared to the value of 100 μg/mL of nonglycated BSA; $^{\#}P < 0.05$ compared to the value of 100 μg/mL of AGE-BSA. (*D*) Effects of AGE proteins purified from diabetic patients on hemodialysis on ROS generation in mesangial cells. Cells were preincubated with or without anti-CML Ab or anti-AGEs Ab 60 minutes prior to the treatment with 100 to 500 μg/mL of purified AGEs from end-stage diabetic nephropathy patient (AGE-frac) or 500 μg/mL of purified AGEs from normal volunteer (normal-frac) for 48 hours. Then ROS were quantitatively analyzed. $^{*}P < 0.05$ compared to the value of 500 μg/mL of normal-frac; $^{\#}P < 0.05$ compared to the value of 500 μg/mL of AGE-frac.

AGE-exposed cells, consistent with AGEs eliciting ROS generation in mesangial cells through an interaction with RAGE.

CML is reported to one of the ligands for RAGE [44]. So, we investigated whether CML is a major component of AGEs used in this experiment that mediated the ROS generation. CML-BSA increased ROS generation in a dose-dependent manner (Fig. 1B). Further, neutralizing antibodies against CML or non-CML AGEs, partially, but significantly, inhibited the AGEs-induced ROS generation in mesangial cells (Fig. 1C). Since the AGEs effects were completely inhibited by the additive treatments of both antibodies, either CML or non-CML AGE component in our AGEs preparations could stimulate the ROS generation in mesangial cells.

We next investigated the effects of AGE proteins, which were purified from diabetic patients on hemodialysis, on ROS generation in mesangial cells. As shown in Figure 1D, AGE proteins purified from diabetic patients significantly increased ROS generation, which was

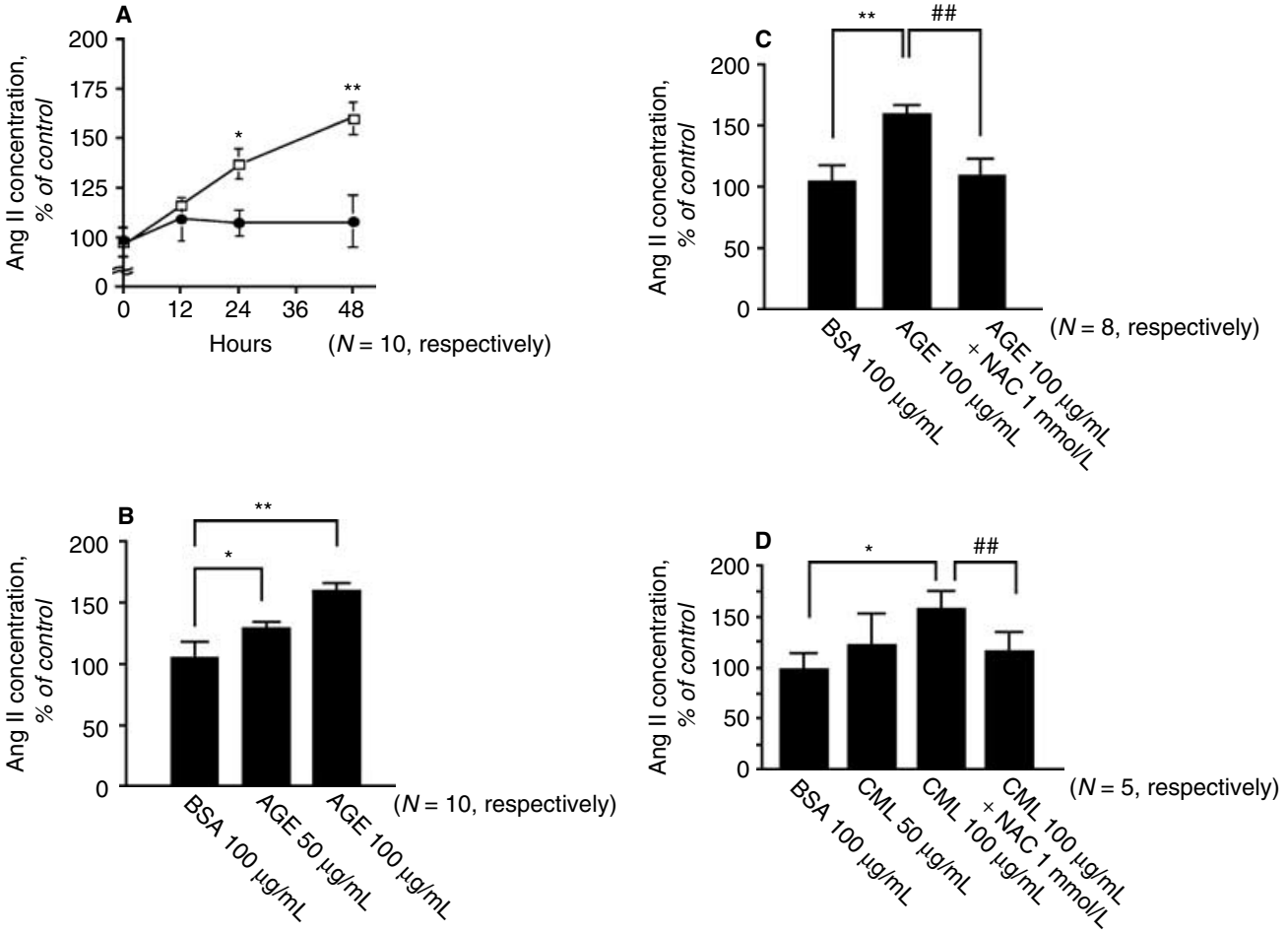

Fig. 2. Time-dependent effects of advanced glycation end products (AGEs) on angiotensin II (Ang II) production. (*A*) Cells were incubated on 6-well plates then stimulated with 100 μg/mL of nonglycated bovine serum albumin (BSA) (•) or 100 μg/mL of AGE-BSA (□) for the indicated periods. (*B*) Dose-dependent effect of AGEs on Ang II production. Cells were treated with 100 μg/mL of nonglycated BSA or 50 to 100 μg/mL of AGE-BSA for 48 hours. (*C*) Effect of an antioxidant on AGE-induced Ang II production. Cells were preincubated with or without 1 mmol/L *N*-acetylcysteine (NAC) 30 minutes prior to the treatment with 100 μg/mL of AGE-BSA or nonglycated BSA for 48 hours. Ang II levels in the cell lysate were measured in the radioimmunoassay system. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to the value of non-glycated BSA; $^{\#\#}P < 0.01$ compared to the value of AGE-BSA. (*D*) Cells were preincubated with or without 1 mmol/L NAC 30 minutes prior to the treatment with 50 to 100 μg/mL of carboxymethyllysine (CML)-BSA or nonglycated BSA for 48 hours. Then Ang II levels were measured. $^{*}P < 0.01$ compared to the value of nonglycated BSA; $^{\#\#}P < 0.05$ compared to the value of CML-BSA.

significantly inhibited by either CML or non-CML AGEs antibody.

Effects of AGEs on Ang II production

As shown in Figure 2A and B, AGEs increased Ang II production in a time- and dose-dependent manner. To examine the functional involvement of ROS generation in AGE-elicited Ang II production, we studied the effect of an antioxidant, NAC, on Ang II generation in AGE-exposed mesangial cells. As shown in Figure 2C, NAC completely inhibited the increase in Ang II generation in AGE-exposed mesangial cells. CML-BSA also increased Ang II production in mesangial cells, which was completely inhibited by NAC (Fig. 2D).

Effects of AGEs on TGF-β expression and its downstream signaling

Ang II has been shown to stimulate expression of TGF-β in various types of cells, including mesangial cells [9, 10, 45]. Therefore, we investigated whether AGEs could stimulate TGF-β expression through autocrine production of Ang II. As shown in Figure 3A, AGEs increased TGF-β production by mesangial cells, which was completely prevented by 10^{-7} mol/L of an AT_1R antagonist candesartan.

In order to confirm that TGF-β actually activated the downstream signaling pathway in AGE-exposed mesangial cells, the effects of AGEs on Smad2 phosphorylation and TGF-β–inducible promoter activity were assessed. As shown in Figure 3B and E, AGEs increased

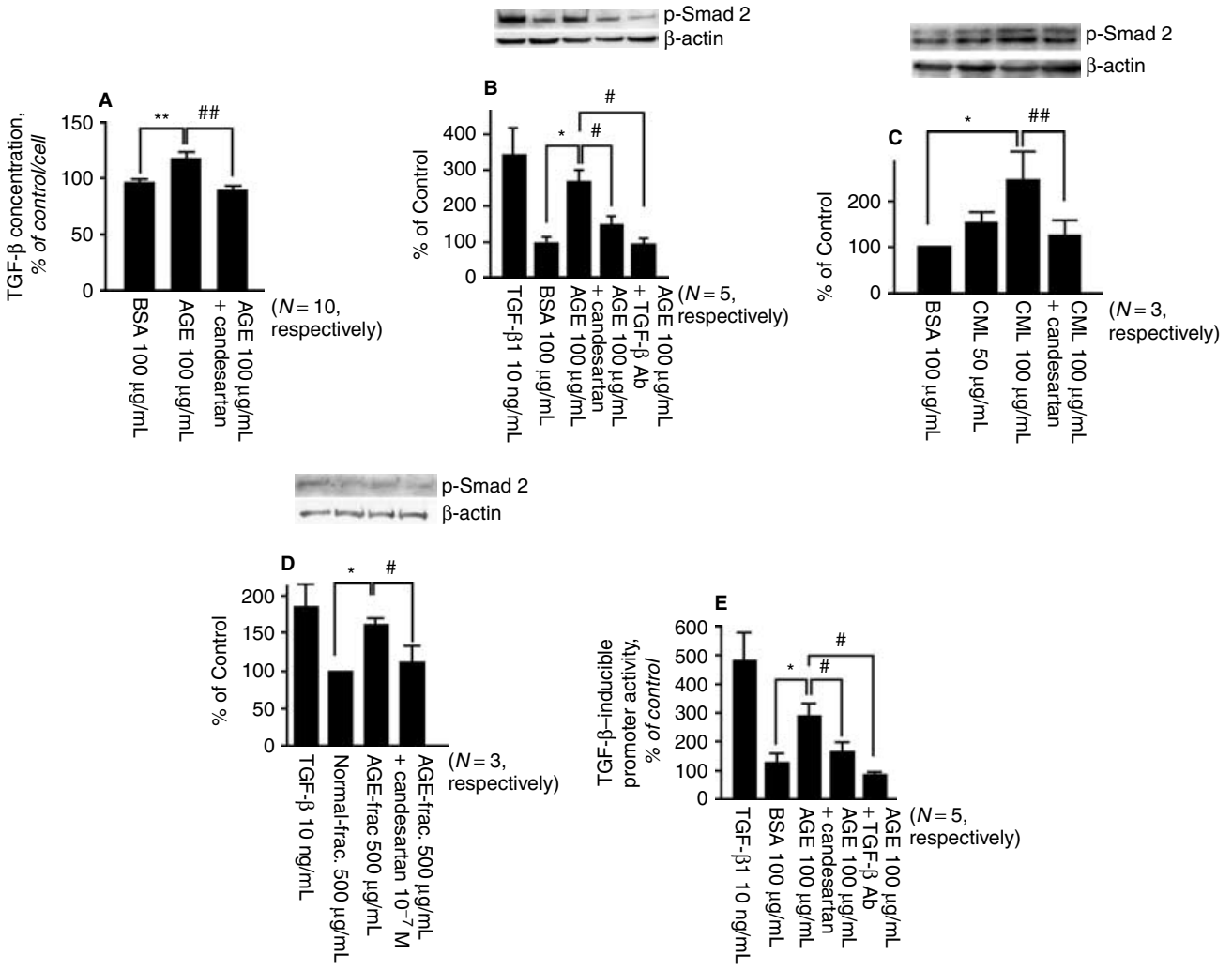

Fig. 3. Effects of advanced glycation end products (AGEs) on transforming growth factor-β (TGF-β) expression and its downstream signaling in mesangial cells. (*A*) Total TGF-β expression. Mesangial cells were incubated with 100 μg/mL of AGE-bovine serum albumin (BSA) or 100 μg/mL of nonglycated BSA in the presence or absence of 10^{-7} mol/L candesartan for 48 hours. Then total TGF-β production in mesangial cells were determined. (*B* to *D*) Phosphorylation of Smad2 protein expression. Mesangial cells were incubated with 100 μg/mL of nonglycated BSA, 100 μg/mL of AGE-BSA (B), 50 to 100 μg/mL of carboxymethyllysine (CML)-BSA (C), 500 μg/mL of purified AGEs from end-stage diabetic nephropathy patient (AGE-frac) or 500 μg/mL of purified AGEs from normal volunteer (normal-frac) (D) in the presence or absence of 10^{-7} mol/L candesartan or 10 μg/mL of polyclonal antibody (Ab) against TGF-β (TGF-β Ab) for 48 hours. Then phosphorylated Smad2 (p-Smad2) protein expression was determined and the relative ratio to the β-actin expression was shown. (*E*) Mesangial cells were transfected with a luciferase-reporter plasmid containing the TGF-β–inducible promoter $(CAGA)_{12}$, and then treated with 100 μg/mL of AGE-BSA, 100 μg/mL of nonglycated BSA, or 10 ng/mL of TGF-β in the presence or absence of 10^{-7} mol/L candesartan or 10 μg/mL of TGF-β Ab for 48 hours. Then luciferase activity was measured. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to the value of nonglycated BSA; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared to the value of AGE-BSA, CML-BSA, or AGE-frac.

Smad2 phosphorylation and TGF-β–inducible promoter activity in mesangial cells, these effects were completely blocked by treatment with either candesartan or the polyclonal antibody against TGF-β. Both CML-BSA (Fig. 3C) and AGE proteins purified from diabetic patients on hemodialysis (Fig. 3D) also stimulated Smad2 phosphorylation, which were also blocked by candesartan.

Effects of AGEs on DNA and protein synthesis

As shown in Figure 4, AGEs significantly inhibited DNA synthesis and stimulated de novo protein synthesis in mesangial cells. Furthermore, AGEs up-regulated the expression of the cyclin-dependent kinase inhibitor, p27, in mesangial cells (Fig. 5). Both candesartan and the polyclonal antibody against TGF-β completely prevented the AGE-induced decrease in DNA synthesis and increase in protein synthesis associated with p27 overexpression.

Effects of AGEs on fibronectin production

AGEs significantly increased fibronectin production by mesangial cells, and this was completely inhibited by candesartan or the polyclonal antibody against TGF-β (Fig. 6).

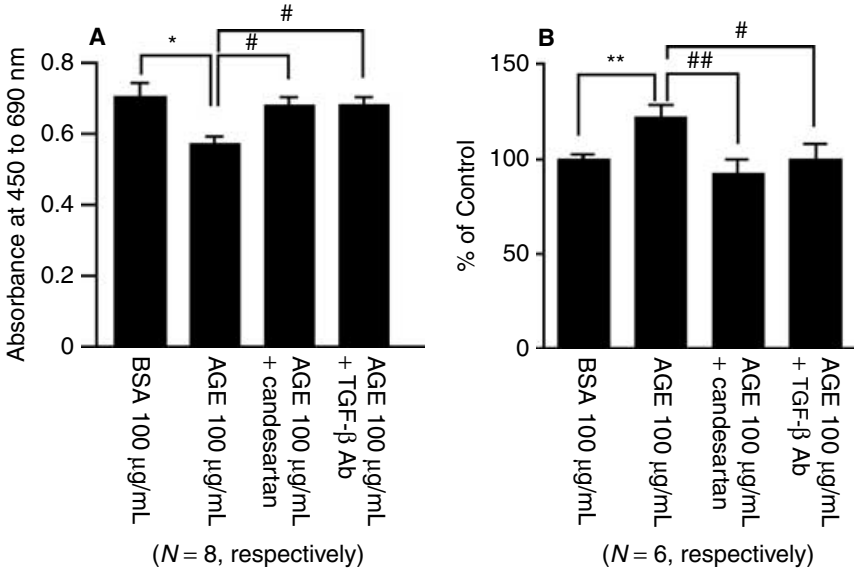

Fig. 4. Effects of advanced glycation end products (AGEs) on DNA and protein synthesis in mesangial cells. Mesangial cells were incubated with 100 μg/mL of AGE-bovine serum albumin (BSA) or nonglycated BSA in the presence or absence of 10^{-7} mol/L candesartan or 10 μg/mL of polyclonal antibody (Ab) against transforming growth factor-β (TGF-β) (TGF-β Ab) for 24 hours. Cells were treated with 5-bomo-2′-deoxyuridine (BrdU) (*A*) or [^{3}H]leucine (*B*) for the last 16 hours of incubation, and their incorporations into the cells were measured. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to the value of nonglycated BSA. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared to the value of AGE-BSA.

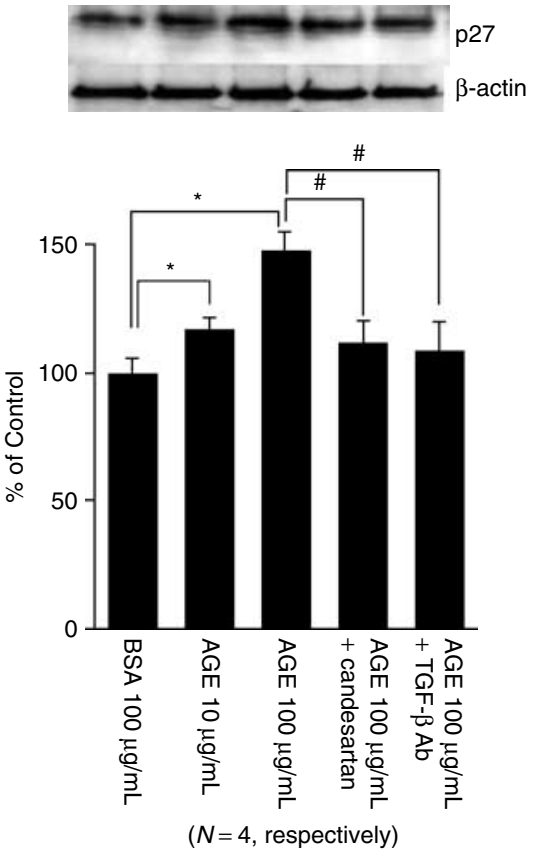

Fig. 5. Effects of advanced glycation end products (AGEs) on p27 expression in mesangial cells. Mesangial cells were treated with the indicated concentrations of AGE-bovine serum albumin (BSA) or nonglycated BSA in the presence or absence of 10^{-7} mol/L candesartan or 10 μg/mL of polyclonal antibody (Ab) against transforming growth factor-β (TGF-β) (TGF-β Ab) for 48 hours. Then p27 protein expression was determined and the relative ratio to the β-actin expression was shown. $^{*}P < 0.05$ compared to the value of nonglycated BSA; $^{\#}P < 0.05$ compared to the value of AGE-BSA.

Effects of AGEs on RAGE expression

We previously have shown that AGEs increase RAGE expression in microvascular endothelial cells and pericytes [46, 47]. As shown in Figure 7, AGEs significantly increased RAGE expression in mesangial cells. Furthermore, this increased RAGE expression was completely inhibited by candesartan or the polyclonal antibody against TGF-β. Moreover, exogenously applied TGF-β also was found to significantly up-regulate RAGE expression in mesangial cells.

DISCUSSION

In the present study, we have demonstrated for the first time that AGEs increase intracellular ROS generation in cultured mesangial cells by an interaction with RAGE and the subsequent stimulation of Ang II production. Since candesartan, an AT_1R antagonist, completely inhibited TGF-β overexpression, Smad2 phosphorylation and TGF-β–inducible promoter activity in AGE-exposed mesangial cells, AGE-induced activation in TGF-β-Smad signaling pathways could be mainly mediated by autocrine production of Ang II. New and original findings in the present study are that the AGE-RAGE interaction in mesangial cells activates TGF-β–Smad signaling pathways and subsequently induces mesangial cell hypertrophy and fibronection synthesis through autocrine Ang II generation via ROS overproduction. So, our present study suggests that AT_1R antagonist could act as a blocker of the AGE-RAGE signaling as well, thus providing a novel mechanistic explanation for understanding why this type of drugs has beneficial effects on diabetic nephropathy.

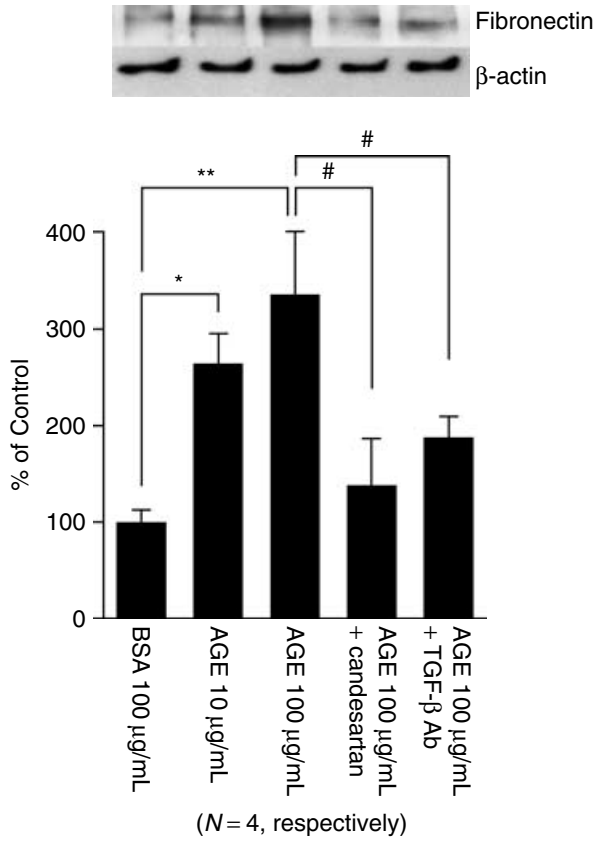

Fig. 6. Effects of advanced glycation end products (AGEs) on fibronectin synthesis in mesangial cells. Mesangial cells were treated with the indicated concentrations of AGE-bovine serum albumin (BSA) or nonglycated BSA in the presence or absence of 10^{-7} mol/L candesartan or 10 μg/mL of polyclonal antibody (Ab) against transforming growth factor-β (TGF-β) (TGF-β Ab) for 48 hours. Then fibronectin expression in mesangial cells was measured and the relative ratio to the β-actin expression was shown. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to the value of nonglycated BSA; $^{\#}P < 0.05$ compared to the value of AGE-BSA.

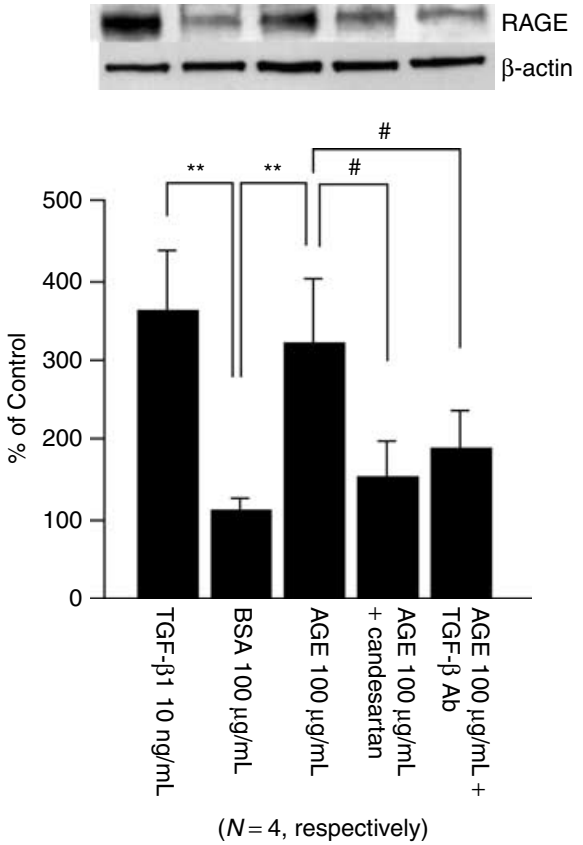

Fig. 7. Effects of advanced glycation end products (AGEs) on receptor for AGE (RAGE) expression in mesangial cells. Mesangial cells were treated with 100 μg/mL of AGE-bovine serum albumin (BSA), 100 μg/mL of nonglycated BSA, or 10 ng/mL of transforming growth factor-β (TGF-β) in the presence or absence of 10^{-7} mol/L candesartan or 10 μg/mL of polyclonal antibody (Ab) against TGF-β (TGF-β Ab) for 48 hours. Then RAGE expression in mesangial cells was determined and the relative ratio to the β-actin expression was shown. $^{**}P < 0.01$ compared to the value of nonglycated BSA; $^{\#}P < 0.05$ compared to the value of AGE-BSA.

Previously it has been shown that RAGE overexpressing diabetic mice exhibit progressive glomerulosclerosis with renal dysfunction, when compared with diabetic littermates lacking the RAGE transgene [48]. The present study has extended our previous works; the AGE-RAGE interaction in mesangial cells could also be involved in the development and progression of diabetic glomerulosclerosis by activating the Ang II-TGF-β–Smad signaling pathway.

Recent studies in human and experimental models of diabetes suggest that oxidative stress plays an important role in the pathogenesis of diabetic vascular complications, including diabetic nephropathy [49]. Indeed, high glucose up-regulates TGF-β and fibronectin mRNA levels and protein synthesis in cultured mesangial cells through nuclear factor-κB (NF-κB) and activator protein-1 via ROS generation [50]. Further, antioxidants have been shown to prevent glomerular hypertrophy, albuminuria, and renal expression of ECM proteins in experimental diabetes [51–53]. Since ACE is known to be up-regulated by redox-sensitive mechanisms in vascular wall cells [54], ROS generation elicited by AGE-RAGE interaction may stimulate Ang II production by stimulating ACE activity in mesangial cells.

Early diabetic nephropathy is characterized by glomerular hypertrophy, which is associated with mesangial cell cycle arrest in the late G_1 phase [55–57]. Among the cyclin-dependent kinase inhibitors that cause G_1 arrest, p27 has been reported to be necessary for high glucose-induced mesangial cell hypertrophy [55–57]. In the present study, we identified that AGEs induced mesangial cell hypertrophy through an up-regulation of p27 expression. Since a decrease in DNA synthesis and an increase in de novo protein synthesis, which is an index of cell hypertrophy, is associated with p27 overexpression were completely prevented by candesartan or polyclonal antibody against TGF-β, AGE-induced mesangial

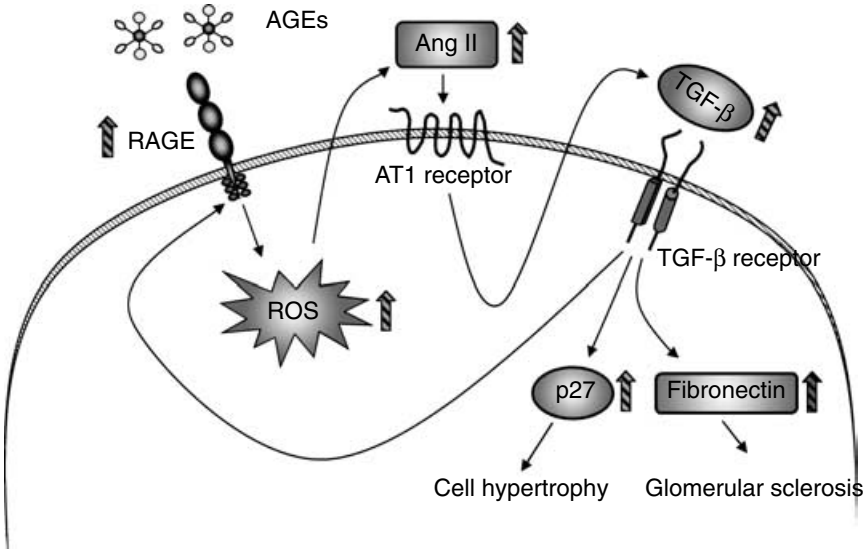

Fig. 8. Hypothetic pathways of advanced glycation end products (AGE)-mediated mesangial alterations in diabetic nephropathy. The picture depicts the functional relationship among the AGE-receptor for AGE (RAGE) interaction, reactive oxygen species (ROS) generation, local renin-angiotensin system, and transforming growth factor-β (TGF-β) signaling in mesangial cells, implicating the deleterious effects of the AGEs in the pathogenesis of diabetic nephropathy, which may form a vicious circuit.

cell hypertrophy may be primarily mediated by autocrine Ang II production and subsequent activation of TGF-β signaling. In support of this hypothesis, ACE inhibitor treatment reduces glomerular p27 expression and hypertrophy in diabetic rats [57], and TGF-β–induced cellular hypertrophy is attenuated in p27 null cells [58]. Recently, the lack of p27 has been shown to ameliorate glomerular hypertrophy and albuminuria in diabetic mice despite overexpression of TGF-β, further supporting the concept that p27 is a key downstream mediator of mesangial hypertrophy elicited by TGF-β [58].

Advanced diabetic nephropathy is characterized by a progressive accumulation of ECM protein within the glomerular mesangium and the tubulointerstitium [59]. There is a growing body of evidence suggesting that the RAS and TGF-β systems are involved in the pathogenesis of diabetic glomerulosclerosis [60]. In the present study, AGEs stimulated fibronectin production in cultured mesangial cells, and this effect was completely prevented by candesartan or a polyclonal antibody against TGF-β. Blockade of the RAS by ACE inhibitors ameliorates structural and functional abnormalities in association with inhibition of TGF-β overexpression [61–63]. Furthermore, it has been demonstrated that chronic treatment with a monoclonal antibody directed against TGF-β prevents glomerulosclerosis and renal insufficiency in diabetic mice [11]. Therefore, it is postulated that AGE-RAGE–mediated ROS generation participates in the development of glomerulosclerosis by activating Ang II-TGF-β signaling pathways with subsequent effects in inducing increased ECM expression.

In our study, AGEs per se were noted to enhance RAGE expression in cultured mesangial cells. Furthermore, this effect on RAGE was completely prevented by candesartan or a polyclonal antibody against TGF-β. Consistent with these findings, we have previously reported that AGEs up-regulated RAGE gene expression in microvascular endothelial cells through the redox-sensitive transcriptional factor, NF-κB [46]. Since we also observed that exogenous TGF-β stimulates RAGE expression, it is predicted that AGE-RAGE–mediated ROS generation followed by TGF-β activation forms a mutually amplifying circuit (Fig. 8). Recently, the ACE inhibitor, ramipril, and the AT_1R antagonist, lomesartan, were both reported to inhibit the accumulation of AGEs in experimental diabetic nephropathy, possibly via reducing the generation of oxidation products [64, 65], providing further support for this pathway.

The present study provides a novel functional relationship among the AGE-RAGE interaction, ROS generation, the local RAS, and TGF-β signaling in the pathogenesis of diabetic nephropathy. It is hypothesized that deleterious effects of the AGE-RAGE interaction in diabetic nephropathy can be ascribed to intra-renal activation of the RAS-TGF-β signaling pathways, which is possibly mediated via increased oxidative stress.

We have previously shown that the structural epitope of in vitro-modified AGE-BSA actually existed in vivo in serum of diabetic patients, and that the concentration of AGEs used in this experiment was comparable with that of in vivo situation of diabetes [35]. Further, the ROS generation elicited by diabetic patients-derived AGEs was completely inhibited by the combination treatments of both neutralizing antibodies against CML and non-CML AGEs. These results suggest that AGE proteins purified from diabetic patients show the same biologic effects on mesangial cells, thus supporting the physiologic relevance of in vitro-prepared AGEs in vivo. Two types of AGEs (i.e., CML and non-CML AGEs) have been identified in diffuse and nodular lesion in human diabetic nephropathy [66, 67]. Since CML is one of ligands for RAGE [44], we also found that either CML or non-CML AGE

components in our AGE preparations have the biologic activities on mesangial cells on the basis of the following evidence. First, CML-BSA increased ROS generation in a dose-dependent manner. Second, CML-BSA increased Ang II production in mesangial cells, which was completely inhibited by an antioxidant, NAC. Third, CML-BSA induced Smad2 phosphorylation, which was also blocked by an AT_1R, candesartan. Fourth, neutralizing antibodies against CML or non-CML AGEs, partially, but significantly, inhibited the ROS generation in mesangial cells. Finally, the effects of combination of both antibodies on the ROS generation were additive. It remains to be determined if these in vitro findings in mesangial cells ultimately occur in the diabetic kidney. However, these results emphasize the important interrelationships that occur between metabolic and hemodynamic pathways and provide important targets to optimize renoprotection in diabetes.

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