Journal of Cellular Biochemistry 95:281–292 (2005)

Advanced Glycation End-Product-Induced Mitogenesis and Collagen Production Are Dependent on Angiotensin II and Connective Tissue Growth Factor in NRK-49F Cells

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Abstract Diabetic nephropathy (DN) is characterized by glomerulopathy and tubulointerstitial expansion followed by renal fibrosis. Angiotensin II (Ang II) and connective tissue growth factor (CTGF) are involved in the pathogenesis of DN, while Janus kinase 2 (JAK2) is important in advanced glycation end-product (AGE)-induced effects in renal interstitial (NRK-49F) fibroblasts. Thus, we studied the role of Ang II, CTGF, and JAK2 in AGE-induced effects in NRK-49F cells. We found that AGE (150 μg/ml) increased mitogenesis and type I collagen production at 7 days while Ang II (10^{-7}M) increased mitogenesis and type I collagen production at 3 days. We also found that AGE (150 μg/ml) increased angiotensinogen protein at 2 days, which was attenuated by AG-490 (a JAK2 inhibitor). AGE (150 μg/ml) increased CTGF mRNA and protein expression at 3 and 5 days, respectively. Ang II (10^{-7}M) increased CTGF mRNA and protein expression at 1 and 2 days, respectively, which were attenuated by AG-490. Moreover, losartan (a type I angiotensin receptor blocker) and captopril (an angiotensin converting enzyme inhibitor) attenuated AGE-induced CTGF mRNA/protein expression while attenuating AGE-induced mitogenesis and type I collagen production. AG-490 and CTGF antisense (but not sense) oligodeoxynucleotide (ODN) attenuated Ang II (10^{-7}M) and AGE-induced mitogenesis and type I collagen production at 3 and 7 days, respectively. We concluded that AGE (150 μg/ml)-induced mitogenesis and type I collagen production are dependent on the Ang II-JAK2-CTGF pathway in NRK-49F cells. Moreover, Ang II-induced mitogenesis and type I collagen production are dependent on the JAK2-CTGF pathway. J. Cell. Biochem. 95: 281–292, 2005.

Key words: advanced glycation end-product; angiotensin; connective tissue growth factor; mitogenesis; collagen

Diabetic nephropathy (DN) is characterized by extracellular matrix expansion, glomerulopathy, and tubulointerstitial damage followed by renal fibrosis (glomerulosclerosis and tubulointerstitial fibrosis) [Chuang and Guh, 2001]. Advanced glycation end-product (AGE), transforming growth factor-β (TGF-β), and angiotensin II (Ang II) are three of the important mediators of DN [Chuang and Guh, 2001; Forbes et al., 2003a].

We have been studying the role of renal interstitial fibroblast (NRK-49F cell) proliferation and collagen production in models of DN in vitro [Chuang and Guh, 2001]. For example, we have shown that AGE-induced mitogenesis and collagen production are dependent on the JAK2 pathway in NRK-49F cells [Huang et al., 1999, 2001]. JAK2 is a signaling molecule used by many cytokines/growth factors, including Ang II [Chuang and Guh, 2001], whereas the

Abbreviations used: ACEI, angiotensin converting enzyme inhibitor; ARB, type I angiotensin receptor blocker; DN, diabetic nephropathy; AGE, advanced glycation end-product; Ang II, angiotensin II; CTGF, connective tissue growth factor; JAK2, Janus kinase 2; TGF-β, transforming growth factor-β; BSA, bovine serum albumin; ODN, oligodeoxynucleotide.

Grant sponsor: National Science Council, Taiwan (to C.L.Y.); Grant number: NSC-91-2320-B-037-044; Grant sponsor: National Science Council, Taiwan (to G.J.Y.); Grant number: NSC-89-2314-B-037-008.

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Received 17 July 2004; Accepted 13 October 2004
DOI 10.1002/jcb.20380

pathogenesis of DN is dependent on the intrarenal renin-angiotensin system [Carey and Siragy, 2003]. However, the role of JAK2 in Ang II-induced effects in NRK-49F cells remains to be studied.

Connective tissue growth factor (CTGF) is a protein of 36–38 kDa, depending on its degree of glycosylation [Brigstock, 1999]. It is a profibrotic cytokine recently implicated in DN [Wahab et al., 2001; Wang et al., 2001; Wada et al., 2002]. For example, we have shown that AGE increased CTGF expression in NRK-49F cells [Lee et al., 2004].

Angiotensin-converting enzyme inhibitor (ACEI, e.g., captopril) and type 1 angiotensin receptor blocker (AT1 blocker [ARB], e.g., losartan) inhibit the action of Ang II and is effective in the treatment of DN [Chuang and Guh, 2001]. In fact, a complete renin-angiotensin system exists in renal interstitial fibroblasts [Okada et al., 2002]. Moreover, Ang II induces mitogenesis and collagen production in renal fibroblasts [Ruiz-Ortega and Egido, 1997; Schuttert et al., 2003]. Interestingly, two recent studies found that Ang II induces CTGF in the kidney and fibroblasts [Finckenberg et al., 2003; Iwanciw et al., 2003]. However, the role the angiotensin system in AGE-induced effects in renal fibroblasts remains unknown. Additionally, the role of the Ang II-CTGF axis in AGE-induced effects in fibroblasts also remains unknown.

Therefore, AGE-cultured NRK-49F cells were used to address six questions: First, what were the effects of AGE or Ang II on cellular mitogenesis and collagen production? Second, what was the effect of AGE on angiotensinogen protein expression? Third, what was the effect of AGE or Ang II on CTGF mRNA and protein expression? Fourth, can AGE-induced CTGF, mitogenesis, and collagen production be attenuated by captopril or losartan? Fifth, can AGE or Ang II-induced mitogenesis and collagen production be attenuated by CTGF antisense oligodeoxynucleotide (ODN)? Finally, can Ang II-induced CTGF, mitogenesis, and collagen production be attenuated by AG-490 (a specific JAK2 inhibitor)?

MATERIALS AND METHODS

Reagents

AG-490 and Ang II were purchased from Calbiochem Corp. (San Diego, CA). According to a previous study [Kondo et al., 1999] and our previous study [Lee et al., 2004], CTGF antibody was produced by immunizing rabbit with the peptide RPC EAD LEE NIK KGK KCI RT (corresponds to amino acids +238 to +257 of rat CTGF peptide sequence from Genbank Accession No. NM_022266) by Cashmere Biotech Co. (Taipei, Taiwan). Angiotensinogen antibody, actin (C-11) antibody, anti-rabbit IgG HRP, and anti-goat IgG HRP were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Restore^{TM} Western Blot Stripping Buffer, SuperSignal West PicoChemiluminescent Substrate were purchased from Pierce Co. (Rockford, IL). [α-^{32}P] dCTP, (methyl-) thymidine and [^{3}H] proline were purchased from Amersham Corp. (Amersham, UK). Captopril, losartan, LipofectAmine plus^{TM} reagent, Dulbecco's modified Eagle's medium (DMEM), collagenase VII, sodium ascorbate, β-aminoproprionitrile, and all other chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO).

Cell Culture

NRK-49F cells were obtained from the American Type Culture Collection (Manassas, VA). NRK-49F cells were grown in culture flasks and maintained in DMEM (5.5 mM D-glucose). Medium supplemented with 100 IU/ml penicillin, 100 mg/ml streptomycin, and 5% (V/V) fetal calf serum (FCS) in a humidified incubator under air/CO_2 (19:1) at 37°C. Cells were exposed to serum-free (0.5% FCS) medium for 2 days before adding fresh DMEM (5% FCS) containing 150 μg/ml AGE, reagents (5 μM AG-490, 0.5–1 mM captopril, 5–10 μM losartan) or 10^{-7}M Ang II. Note that after starvation for 2 days, 75%–80% of NRK-49F cells were synchronized in G_0/G_1 phase of the cell cycle by flow cytometry. This result is similar to our previous study [Guh et al., 2001]. In contrast, some previous studies have starved NRK-49F cells for 1 day before studying the effects of CTGF [Frazier et al., 1996; Wang et al., 2001].

AGE or Ang II was added with 5% FCS as our previous studies [Huang et al., 1999, 2001; Lee et al., 2004] because the concentration of epidermal growth factor in 5% FCS is 0.024 ng/ml [Philippoussis et al., 2004] while it has been shown that CTGF is only mitogenic with epidermal growth factor concentration of greater than 0.02 ng/ml [Grotendorst et al., 2004]. Moreover, the concentration of Ang II in 5% FCS is 1.8 pM [Bluher et al., 2001], which is

considerably lower than 10^{-7}M used in this study. Medium and all reagents were refreshed every 3 days. Cell viability was assessed by the Trypan blue exclusion test. To count cells, cells were dissociated by trypsinization and viable cells were counted after Trypan blue staining.

Preparation of AGE

Briefly, bovine serum albumin (BSA) Fraction V (1 mM) was glycated by incubation with 0.5M glucose in 50 mM potassium phosphate (pH 7.3)/1 mM EDTA under sterile conditions at 37°C for 8 weeks as previously described [Huang et al., 1999]. Fluorescence of AGE was determined with a Perkin–Elmer fluorescence spectrometer; the average fluorescence for AGE was 66 arbitrary units compared with 1 arbitrary unit/mg for BSA. Then AGE was stored at −20°C until used. Control, non-glycated BSA consisted of the same initial preparations of albumin incubated at 37°C in the same manner, except that no glucose was added.

DNA Synthesis

This was assessed by ^{3}H-thymidine incorporation as described in our previous study [Huang et al., 1999]. Briefly, 8.0×10^3 cells were transferred to 24-well microplates and maintained in 5% FCS for 1 day. After a fast (0.5% FCS) for 2 days, fresh medium containing BSA or AGE (with 5% FCS-containing DMEM) was added for various times. ^{3}H-thymidine (4 μCi) (Amersham, Arlington Heights, IL) was added during the last 5 h. Cells were washed and protein was precipitated, cell lysate was harvested to 2 ml of scintillation fluid and counted on a β-counter (Beckman, Palo Alto, CA). Results were plotted as cpm per well. Each experimental datum point represents the mean of triplicate wells from three independent experiments.

Synthesis of ODN

Phosphorothioated single-stranded ODNs used in this study were synthesized with a DNA/RNA synthesizer (Applied Biosystems Division Perkin Elmer) as described in our previous study [Lee et al., 2004]. Thus, CTGF antisense ODN (5′-AAG GCG AGG CTA ACG GGA-3′) and CTGF sense ODN (5′-TCC CGT TAG CCT CGC CTT-3′) correspond to the region of bp +245 to +262 derived from the cDNA sequence for rat CTGF (Genbank accession no. NM_022266).

Transfection of ODN Into Cells

This was performed as described in our previous study [Huang et al., 1999]. Thus, cationic liposomes (Lipofectin) were used to facilitate the transfection of ODNs into NRK-49F cells. In brief, 5 μM ODNs dissolved in medium were mixed with Lipofectin, which was also dissolved in medium as a ratio of 1 nmol/μg, and incubated for 20 min at room temperature. The ODN liposome complexes were added to flasks or 24-well plates. We had determined the specific conditions under which cationic liposomes could successfully transfect without causing cytotoxicity. Thus, cell viability was not statistically different between control (92% ± 2%) and ODN-transfected (90% ± 1.5%) cells in six independent experiments.

Immunoblotting

This was performed as described in our previous study [Huang et al., 1999]. Briefly, 30 μl conditioned media or cell lysates of 25–50 μg proteins (for CTGF protein analysis), or 100 μg proteins (for angiotensinogen protein analysis) were subjected to SDS/PAGE using 12% acrylamide gel and transferred to polyvinylidene difluoride membrane, blocked overnight at 4°C with 5% defatted milk and incubated with primary antibody (anti-CTGF or anti-angiotensinogen antibody) at room temperature and the membrane was incubated in horseradish peroxidase-conjugated anti-rabbit secondary antibody for 1 h. The protein bands were detected with the enhanced chemiluminescence system. The membrane was stripped with stripping buffer and then incubated with β-actin antibody diluted 1:500 as an internal control.

Northern Blotting of CTGF mRNA

Total RNA was extracted as described in our previous study [Yang et al., 1997]. As described in our previous study [Lee et al., 2004], CTGF cDNA probe was obtained from a PCR fragment (558 bp, derived from forward primer: 5′-GAG TGG GTG TGT GAC GAG CCC AAG G-3′ and reverse primer: 5′-ATG TCT CCG TAC ATC TTC CTG TAG T-3′) of HK-2 cDNA. GAPDH cDNA probe was obtained from American Type Culture Collection (Manassas, VA). CTGF mRNA was determined by Northern blotting modified from our previous study [Yang et al., 1997]. Briefly, 100 μg of RNA were separated on 1% agarose, 1M formaldehyde gel, transferred

onto nylon filters (Hybond-N, Amersham Co., Buckinghamshier, UK) and fixed by ultraviolet irradiation. Hybridization was carried for 24 h using ^{32}P-labeled CTGF and GAPDH probes. GAPDH was used as a control to normalize RNA loading. After wash, filters were exposed to X-ray film at −80°C for 1–5 days. Relative density of the autoradiograms was then determined by scanning densitometry.

Electrophoretic Analysis of Collagen Protein Expression

Electrophoretic analysis of collagen was performed as described in our previous study [Huang et al., 2001]. Briefly, cells were labeled and the [^{3}H]proline-labeled proteins were precipitated by cold absolute ethanol and 60–120 μg of protein was applied to each lane of a 7.5% sodium dodecyl sulfate polyacylamide gel with a 4% stacking gel. Gels were fluorographed at −70°C.

Statistics

The results were expressed as the mean ± standard errors of the mean. Unpaired Student's *t*-tests were used for the comparison between two groups. One-way analysis of variance followed by unpaired *t*-test was used for comparison between more than three groups. A *P*-value of less than 0.05 was considered statistically significant.

RESULTS

Dose- and Time-Dependent Effects of AGE or Ang II on ^{3}H-Thymidine Incorporation in NRK-49F Cells

Cell viability was not statistically different between control (90% ± 2%) and AGE-treated (89% ± 3%) cells at 7 days in six independent experiments. ^{3}H-thymidine incorporation was used to study the effects of AGE and Ang II on mitogenesis in NRK-49F cells. In a previous study, we have already shown that AGE dose-dependently (10–200 μg/ml) increased DNA synthesis and cell numbers in these cells at 7 days [Huang et al., 1999]. As shown in Figure 1A, Ang II dose-dependently (10^{-8}–10^{-6}M) increased DNA synthesis at 3 days. As shown in Figure 1B, Ang II (10^{-7}M) time-dependently increased DNA synthesis at 2–3 days.

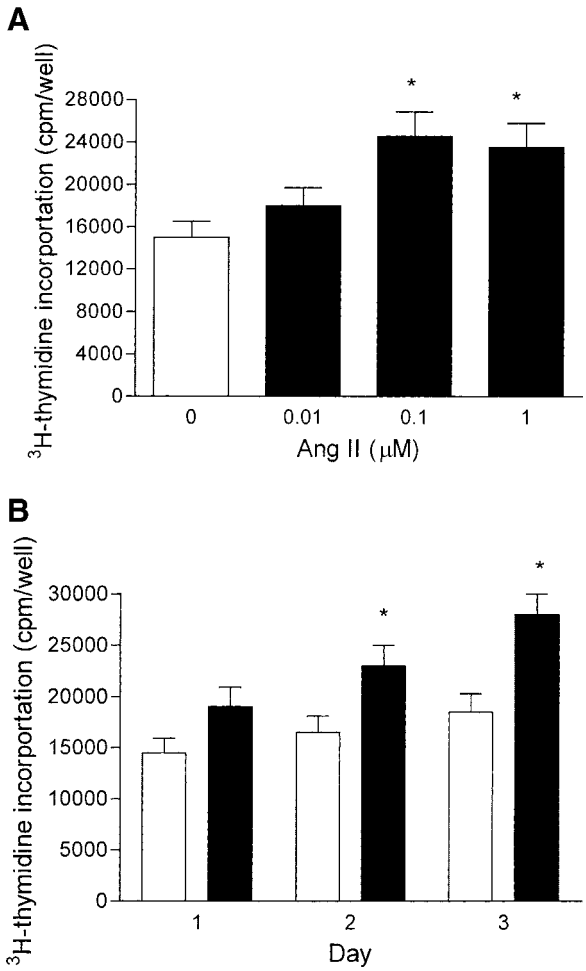

Fig. 1. Dose- and time-dependent effects of Ang II on ^{3}H-thymidine incorporation in NRK-49F cells. **A**: NRK-49F cells were cultured with control medium (medium alone, empty bar) or Ang II (10^{-8}–10^{-6}M, closed bars) for 3 days. **B**: NRK-49F cells were cultured with control medium (medium alone, empty bars) or Ang II (10^{-7}M, closed bars) for 1–3 days. Results are shown as the mean ± standard errors of the mean of three independent experiments. Note that Ang II dose-dependently increased DNA synthesis at 3 days, while Ang II (10^{-7}M) time-dependently increased DNA synthesis at 2–3 days. *, $P < 0.05$ versus control.

Effects of AGE or Ang II on Collagen Production in NRK-49F Cells

^{3}H-proline-labeled proteins followed by electrophoresis were used to study the effects of AGE and Ang II on collagen protein production in NRK-49F cells. As shown in Figures 2 and 3, AGE (150 μg/ml) increased type I collagen production at 7 days while Ang II (10^{-7}M) increased type I collagen production at 3 days.

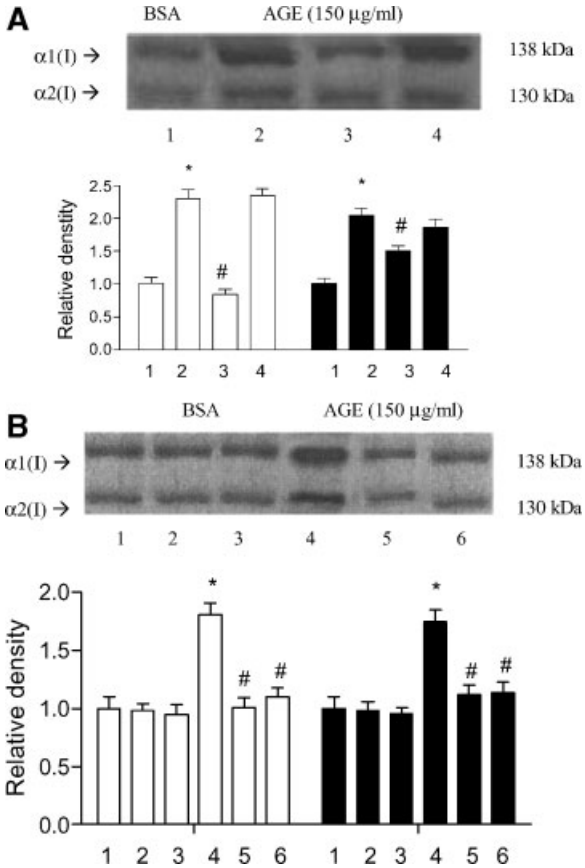

Fig. 2. Effects of AGE (150 μg/ml) on collagen protein expression in NRK-49F cells. **A**: Cells were cultured with BSA (150 μg/ml, **lane 1**) or AGE (**lanes 2–4**) for 7 days. AGE-treated cells were treated with CTGF antisense ODN (lane 3) or CTGF sense ODN (lane 4) for 7 days. **B**: Cells were cultured with BSA (150 μg/ml, **lanes 1–3**) or AGE (**lanes 4–6**) for 7 days. BSA-treated cells were treated with captopril (0.5 mM, lane 2) or losartan (5 μM, lane 3) for 7 days. AGE-treated cells were treated with captopril (0.5 mM, lane 5) or losartan (5 μM, lane 6) for 7 days. ^{3}H-proline-labeled proteins were precipitated and electrophoresis was performed as described in ''Materials and Methods.'' Relative density of the bands, where the density of lane 1 was assumed to be one, was determined by scanning densitometry. Note that captopril, losartan, and CTGF antisense (but not CTGF sense) ODN attenuated AGE-induced type I collagen protein expression in these cells. However, captopril and losartan had no effect on collagen expression in the absence of AGE. α1 (I) and α2 (I), type I collagen. This was a representative result of three independent experiments (empty bars, α1 (I); closed bars, α2 (I)). *, $P < 0.05$ versus BSA; #, $P < 0.05$ versus AGE.

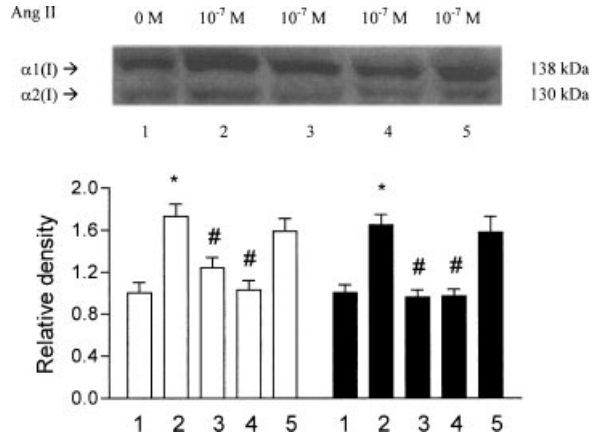

Fig. 3. Effects of Ang II (10^{-7}M) on collagen protein expression in NRK-49F cells. Cells were cultured with medium alone (**lane 1**) or Ang II (**lanes 2–5**) for 3 days. Ang II-treated cells were treated with AG-490 (5 μM, pretreated for 16 h before adding Ang II, lane 3), CTGF antisense ODN (lane 4) or CTGF sense ODN (lane 5) for 3 days. [^{3}H]proline-labeled proteins were precipitated and electrophoresis was performed as described in ''Materials and Methods.'' Relative density of the bands, where the density of lane 1 was assumed to be one, was determined by scanning densitometry. Note that that AG-490 and CTGF antisense (but not CTGF sense) ODN attenuated Ang II-induced type I collagen protein expression in these cells. α1 (I) and α2 (I), type I collagen. This was a representative result of three independent experiments (empty bars, α1 (I); closed bars, α2 (I)). *, $P < 0.05$ versus medium; #, $P < 0.05$ versus Ang II.

Effects of AGE on Angiotensinogen Protein Expression in NRK-49F cells

Western blotting of angiotensinogen (upper panel) and β-actin (lower panel) was performed to study the effects of AGE (150 μg/ml) on angiotensinogen protein expression in NRK-49F cells. Thus, AGE (150 μg/ml) increased angiotensinogen protein expression at 2 days (Fig. 4). Moreover, AG-490 or captopril attenuated AGE-induced angiotensinogen protein expression at 2 days (Fig. 4).

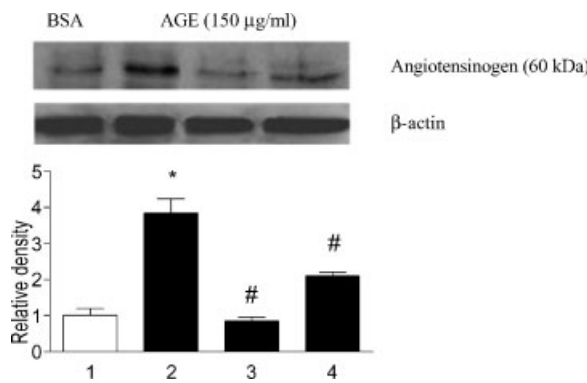

Fig. 4. Effects of AGE (150 μg/ml) on angiotensinogen protein expression in NRK-49F cells. Cells were cultured with BSA (150 μg/ml, empty bars) or AGE (closed bars) for 2 days. Western blotting of angiotensinogen (**upper panel**) and β-actin (**lower panel**) was performed as described in ''Materials and Methods.'' AGE-treated (**lanes 2–4**) cells were treated with AG-490 (5 μM, pretreated for 16 h before adding AGE, lane 3) or captopril (1 mM, lane 4) for 2 days. Note that AG-490 or captopril attenuated AGE-induced angiotensinogen protein expression at 2 days. *, $P < 0.05$ versus BSA; #, $P < 0.05$ versus AGE alone.

Effects of AGE on CTGF mRNA and Protein Expression in NRK-49F Cells

Northern or Western blotting was performed to study the effects of AGE (150 μg/ml) on CTGF mRNA and protein expression in NRK-49F cells. Thus, AGE (150 μg/ml) increased CTGF mRNA (Fig. 5A) at 3 days and increased CTGF protein (Fig. 5B) expression at 5 days. Note that there was only an authentic 38-kDa CTGF protein, but not the low molecular weight CTGF proteins. This observation is similar to a previous study [Twigg et al., 2001] and our previous study [Lee et al., 2004].

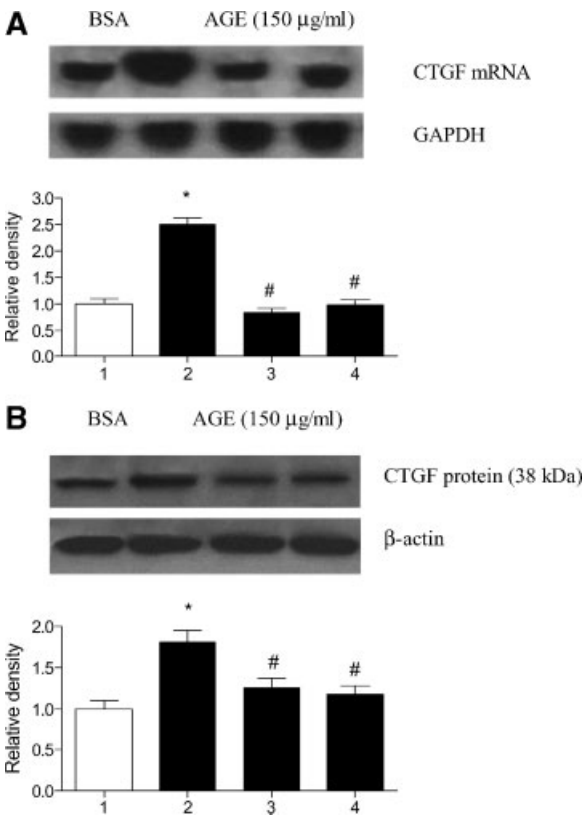

Fig. 5. Effects of AGE (150 μg/ml) on CTGF mRNA (**A**) and protein (**B**) expression in NRK-49F cells. Cells were cultured with BSA (150 μg/ml, empty bars) or AGE (closed bars) for 3 or 5 days. Northern blotting of CTGF (**upper panel**) and GAPDH (**lower panel**) or Western blotting of CTGF (upper panel) and β-actin (lower panel) were performed as described in ''Materials and Methods.'' AGE-treated cells were treated with losartan (5 μM, **lane 3**) or captopril (0.5 mM, **lane 4**) for 3 days. Note that AGE increased CTGF mRNA at 3 days and increased CTGF protein expression at 5 days. Moreover, losartan or captopril attenuated AGE-induced CTGF mRNA and protein expression at 3 and 5 days, respectively. *, $P < 0.05$ versus BSA; #, $P < 0.05$ versus AGE alone.

Effects of Ang II on CTGF mRNA and Protein Expression in NRK-49F cells

Northern blotting of CTGF (upper panel) and GAPDH (lower panel) was performed to study the effects of Ang II (10^{-7}M) on CTGF mRNA expression in NRK-49F cells. Thus, Ang II (10^{-7}M) increased CTGF mRNA expression at 1 day (Fig. 6A). Western blotting of CTGF (upper panel) and β-actin (lower panel) was performed to study the time and dose-dependent effects of Ang II on CTGF protein expression in NRK-49F cells. Thus, Ang II dose-dependently (10^{-8}–10^{-6}M) increased CTGF protein expression at 2 days (Fig. 6B). Moreover, Ang II (10^{-7}M) increased CTGF protein expression only at 2 days (Fig. 6C).

Role of Ang II in AGE-Induced Effects

Captopril (an ACEI, 0.5–1 mM) and losartan (an ARB, 5–10 μM) were used to elucidate the role of Ang II in AGE-induced CTGF, DNA synthesis, and collagen production. Because our preliminary studies showed that captopril (1 mM) and losartan (10 μM) also attenuated type I collagen production and CTGF mRNA/protein expression in the absence of AGE (data not shown), captopril (0.5 mM), and losartan (5 μM) were used thereafter. Thus, captopril (0.5 mM) and losartan (5 μM) attenuated AGE (150 μg/ml)-induced DNA synthesis at 7 days (Fig. 7). Additionally, captopril (0.5 mM) and losartan (5 μM) attenuated AGE (150 μg/ml)-induced type I collagen production at 7 days (Fig. 2B). Moreover, captopril (0.5 mM) and losartan (5 μM) attenuated AGE-induced CTGF mRNA and protein expression at 3 and 5 days, respectively (Fig. 5). Thus, AGE-induced DNA synthesis, collagen production, and CTGF expression are dependent on Ang II and AT1.

Role of CTGF in AGE or Ang II-Induced Effects

CTGF antisense ODN was used to elucidate the role of CTGF in AGE or Ang II-induced DNA synthesis and collagen production. As shown in Figures 7 and 8, CTGF antisense (but not sense) ODN attenuated AGE (150 μg/ml)-induced DNA synthesis at 7 days and attenuated Ang II (10^{-7}M)-induced DNA synthesis at 3 days. As shown in Figure 2A, CTGF antisense (but not sense) ODN attenuated AGE (150 μg/ml)-induced type I collagen production at 7 days. As shown in Figure 3, CTGF antisense (but not

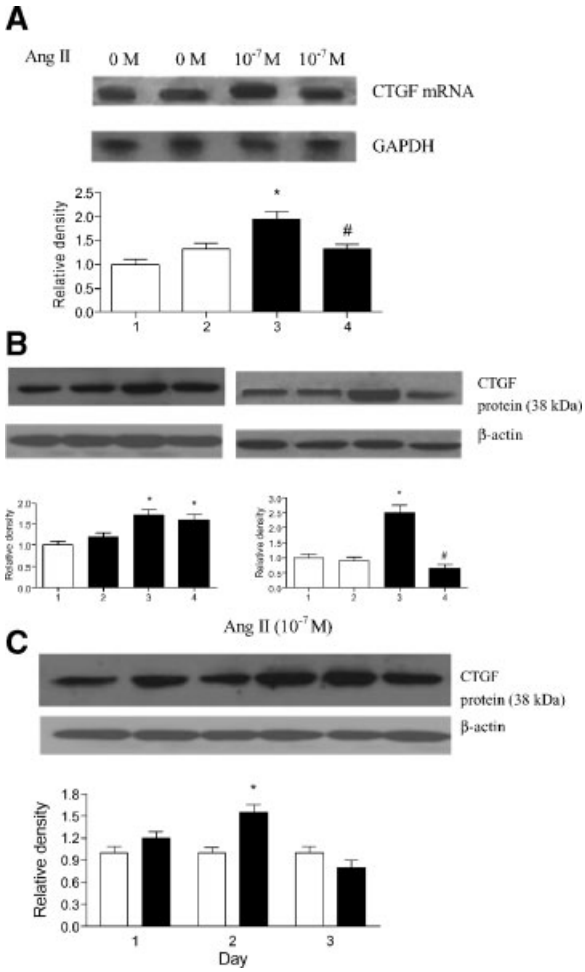

Fig. 6. Effects of Ang II (10^{-7}M) on CTGF mRNA (**A**) and dose-dependent (**B**) or time-dependent (**C**) effects of Ang II on CTGF protein expression in NRK-49F cells. Cells were cultured with medium (empty bars) or Ang II (closed bars) for 1 day (Northern blotting) or 1–3 days (Western blotting). Northern blotting of CTGF (**upper panel**) and GAPDH (**lower panel**) or Western blotting of CTGF (upper panel) and β-actin (lower panel) were performed as described in ''Materials and Methods.'' Ang II-treated cells were treated with AG-490 (5 μM, pretreated for 16 h before adding Ang II) for 1–2 days. Note that Ang II (10^{-7}M) increased CTGF mRNA at 1 day (A, **lane 3**) while Ang II dose-dependently (10^{-8}–10^{-6}M) increased CTGF protein expression at 2 days (B, **left panel**). Moreover, AG-490 attenuated Ang II-induced CTGF mRNA (A, **lane 4**) and protein (B, **right panel**, **lane 4**) expression. However, AG-490 had no effect on either CTGF mRNA (A, **lane 2**) or protein (B, right panel, **lane 2**) in the absence of Ang II. As shown in (C), Ang II (10^{-7}M) time-dependently increased CTGF protein expression only at 2 days (**lanes 3–4**), but not at day 1 (**lanes 1–2**) and day 3 (**lanes 5–6**). *, $P < 0.05$ versus medium; #, $P < 0.05$ versus Ang II.

sense) ODN attenuated Ang II (10^{-7}M)-induced type I collagen production at 3 days.

Role of JAK2 in AGE or Ang II-Induced Effects

By immunoblotting, tyrosine phosphorylation study, and electrophoretic mobility shift assay (DNA binding activity) of STAT, we have already shown that AGE (via the receptor for AGE, RAGE) activates the JAK2-STAT (STAT1, STAT3, and STAT5) pathway [Huang et al., 1999, 2001; Guh et al., 2001] while AG-490 (a specific JAK2 inhibitor) attenuates

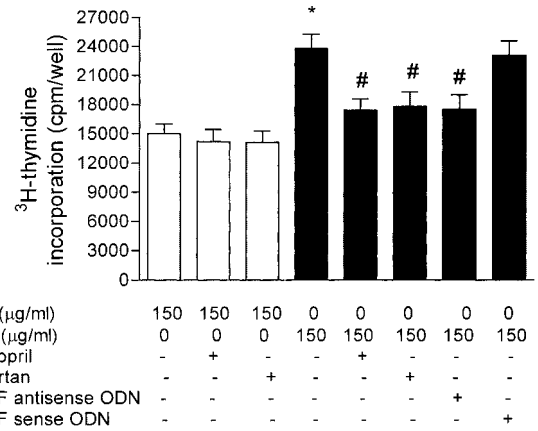

Fig. 7. Effects of AGE (150 μg/ml) on ^{3}H-thymidine incorporation in NRK-49F cells. NRK-49F cells were cultured with BSA (150 μg/ml, empty bars) or AGE (closed bars) for 7 days. AGE-treated cells were treated with captopril (0.5 mM), losartan (5 μM), or CTGF sense/anti-sense ODN. Results are shown as the mean ± standard errors of the mean of three independent experiments. Note that AGE-induced DNA synthesis at 7 days was attenuated by captopril, losartan, and CTGF antisense ODN (but not sense ODN). However, captopril and losartan had no effects on DNA synthesis in the absence of AGE. *, $P < 0.05$ versus BSA alone; #, $P < 0.05$ versus AGE alone.

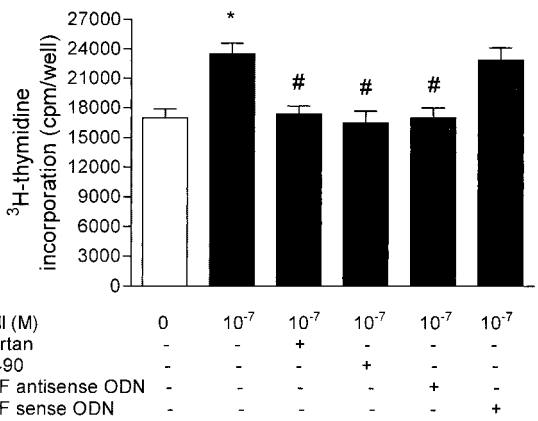

Fig. 8. Effects of Ang II (10^{-7}M) on ^{3}H-thymidine incorporation in NRK-49F cells. NRK-49F cells were cultured with medium (medium alone, empty bar) or Ang II (closed bars) for 3 days. Ang II-treated cells were treated with AG-490 (5 μM, pretreated for 16 h before adding Ang II), losartan (10 μM), or CTGF sense/anti-sense ODN. Results are shown as the mean ± standard errors of the mean of three independent experiments. Note that Ang II-induced DNA synthesis at 3 days was attenuated by losartan, AG-490, and CTGF antisense ODN (but not sense ODN). *, $P < 0.05$ versus medium alone; #, $P < 0.05$ versus Ang II alone.

AGE-induced CTGF expression, DNA synthesis, and collagen production [Lee et al., 2004] in NRK-49F cells in our previous studies. In this study, AG-490 was also used to elucidate the role of JAK2 in Ang II-induced CTGF, DNA synthesis, and collagen production. As shown in Figure 8, AG-490 attenuated Ang II (10^{-7}M)-induced DNA synthesis at 3 days. As shown in Figure 3, AG-490 attenuated Ang II (10^{-7}M)-induced type I collagen production at 3 days. As shown in Figure 6A,B, AG-490 attenuated Ang II (10^{-7}M)-induced CTGF mRNA expression at 1 day and attenuated Ang II (10^{-7}M)-induced CTGF protein expression at 2 days. These findings corroborate with a previous study showing that Ang II (via AT1) activates the JAK2-STAT (STAT1 and STAT2) pathway [Marrero et al., 1995].

Hypothetical Scheme of CTGF Gene Regulation by TGF-β, Ang II, and RAGE

Based on the above findings and previous studies by us and others [Chuang and Guh, 2001], a hypothetical scheme of CTGF gene regulation by the interactions among TGF-β, Ang II, leptin, and AGE (via RAGE) is shown in Figure 9. Thus, Smad and Ras/mitogen-activated protein kinase (MAPK)/AP-1 (Jun/Fos) cascade have been shown to be necessary for the transcription of CTGF gene [Leask et al., 2003; Moritani et al., 2003]. In contrast, although JAK2 has been shown to be necessary

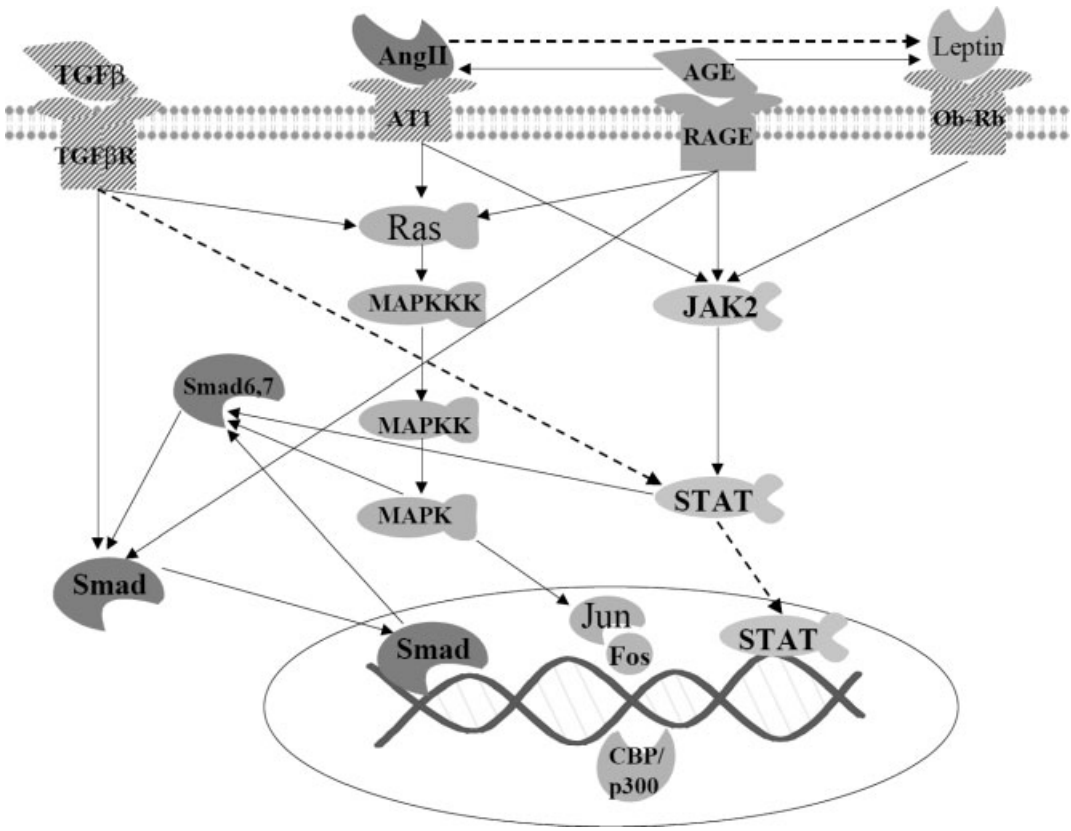

Fig. 9. Hypothetical scheme of connective tissue growth factor (CTGF) gene regulation by TGF-β, angiotensin II (Ang II), leptin, and receptor for advanced glycation end-product (RAGE) in NRK-49F cells. Solid arrows are established links while broken arrows are hypothetical links. AGE induces Ang II (via angiotensinogen) and leptin, but not TGF-β. Moreover, Ang II may induce leptin or potentiate the effects of leptin. TGF-β binds to TGF-β receptor (TGFβR) and activates Smad, which in turn activates the inhibitory Smads (Smad6 and Smad7). Smad6 and Smad7 can also be activated by mitogen-activated protein kinase (MAPK) and signal transducers and activators of transcription (STAT). Ang II binds to type I angiotensin receptor (AT1), which activates the Ras/MAPK kinase kinase (MAPKKK)/MAPK kinase (MAPKK)/MAPK and Janus kinase 2 (JAK2)/STAT cascades. AGE binds to RAGE, which activates TGF-β-independent Smad and the Ras/MAPK and JAK2/STAT cascades. Leptin binds to the long form of leptin receptor (Ob-Rb) and activates JAK2/STAT. TGF-β also activates the Ras/MAPK cascade and STAT5. Finally, nuclear Smad, Jun/Fos (AP-1), and STAT bind to the promoter of CTGF gene and modulate the transcription of CTGF gene. Note that Smad, AP-1, and STAT can also interact with each other by interacting with the transcriptional coactivator CBP/p300.

[Blom et al., 2002; Lee et al., 2004], STAT (STAT1, STAT3, and STAT5 downstream of JAK2) has never been shown to be necessary for the transcription of CTGF gene. Note that TGF-β can also activate the Ras/ MAPK cascade and STAT5 [Derynck and Zhang, 2003; Brizzi et al., 2004]. AGE induces Ang II (via angiotensinogen) while both AGE and Ang II can activate the Ras/MAPK and JAK2/STAT1, 3, 5 cascades [Marrero et al., 1995; Chuang and Guh, 2001]. AGE also activates TGF-β-independent Smad [Li et al., 2004] while Smad, MAPK, and STAT can activate the inhibitory Smads (Smad6 and Smad7) [Derynck and Zhang, 2003]. Moreover, AGE induces leptin [Lee et al., 2004] while leptin binds to the long form of leptin receptor (Ob-Rb) and activates JAK2/STAT [Sweeney, 2002]. Finally, Smad, AP-1, and STAT bind to the promoter of CTGF gene and interact with each other by interacting with the transcriptional coactivator CBP/p300 [Chuang and Guh, 2001].

DISCUSSION

Our previous study and the current study found that AGE-induced DNA synthesis and collagen production in NRK-49F cells are dependent on the leptin-CTGF pathway [Lee et al., 2004] and the Ang II-CTGF pathway. However, it should be noted that these findings have not been performed in human renal interstitial fibroblasts. Interestingly, Ang II has been shown to increase leptin [Correia and Haynes, 2004] or potentiate the action of leptin [Wolf et al., 1999] in some cells. Thus, we speculate that AGE may induce Ang II, thereby inducing leptin and CTGF (Fig. 9). This speculation is compatible with our findings that AGE increased angiotensinogen at 2 days (this study) while increasing leptin at 3 days [Lee et al., 2004].

AGE has been implicated in renal fibrosis [Cooper, 2001]. This concept is strengthened by two recent studies showing that inhibition of AGE attenuated diabetic renal fibrosis [Wilkinson-Berka et al., 2002; Forbes et al., 2003b]. One of the mechanisms whereby AGE could induce fibrosis is through the induction of cytokines/growth factors. Thus, we found that AGE induced Ang II and CTGF in NRK-49F cells in this study.

Our findings that AGE and Ang II induced CTGF are similar to two previous studies in fibroblasts [Twigg et al., 2001; Iwanciw et al., 2003]. In this regard, CTGF is upregulated in experimental and human DN [Riser and Cortes, 2001; Twigg et al., 2002; Riser et al., 2003]. Additionally, inhibition of AGE ameliorated renal expression of CTGF in experimental DN [Twigg et al., 2002; Forbes et al., 2003b]. Our present study further showed that AGE-induced CTGF was attenuated by captopril and losartan, that is, by inhibiting the actions of Ang II. Interestingly, a recent study found that ACEI ameliorated CTGF overexpression in experimental diabetic retinopathy [Tikellis et al., 2004].

The significance of AGE or Ang II-induced CTGF was studied by CTGF antisense ODN. Thus, we found that CTGF antisense ODN attenuated AGE or Ang II-induced mitogenesis and collagen production in NRK-49F cells. Interestingly, a recent study also found that CTGF antisense ODN attenuates renal tubulointerstitial fibrosis in rats [Yokoi et al., 2004]. Thus, the pro-fibrotic actions of both AGE and Ang II may be mediated by the induction of endogenous CTGF in renal interstitial fibroblasts.

A recent study found that tubulointerstitial expression of Ang II was increased in human DN [Mezzano et al., 2003]. Similarly, we found that AGE induced angiotensinogen in renal interstitial fibroblasts. Moreover, we found that AGE-induced angiotensinogen was attenuated by AG-490, that is, dependent on JAK2. Thus, our current finding complements our previous studies, which showed that AGE-induced CTGF expression, mitogenesis, and collagen production were also dependent on JAK2 signaling in NRK-49F cells [Huang et al., 1999, 2001].

The role of Ang II in mitogenesis and collagen production in NRK-49F cells was studied with a dose of 10^{-7}M in this study, which is similar to that used in previous studies in fibroblasts [Iwanciw et al., 2003; Schuttert et al., 2003]. Moreover, we found that captopril and losartan attenuated AGE-induced mitogenesis and collagen production. This observation is similar to two previous studies showing that ACEI and ARB inhibit collagen production in skin fibroblasts [Lijnen et al., 2004; Min et al., 2004]. Thus, AGE-induced mitogenesis and collagen production in NRK-49F cells are dependent on Ang II. These findings are compatible with the notion that ACEI and ARB exert their therapeutic effects in DN by both hemodynamic and

non-hemodynamic mechanisms [Chuang and Guh, 2001].

Captopril was used at a dose of 0.5–1 mM in this study and our previous studies [Guh et al., 1996; Huang et al., 2001] based on two considerations. First, renal tissue concentration of captopril has been found to be 5 and 0.87 mM at 15 and 30 min after an intravenous and oral dose in the rat [Heald et al., 1985]. Second, urinary level of captopril can reach 0.5–1 mM [Guh et al., 1996]. In contrast, losartan was used at a dose of 5–10 μM in this study, which is similar to a previous study in renal fibroblasts [Schuttert et al., 2003].

We found that Ang II-induced mitogenesis and collagen production were dependent on JAK2 in NRK-49F cells. This finding is compatible with the notion that Ang II induces JAK2 in many cells [Chuang and Guh, 2001; Luo and Laaja, 2004]. In a previous study, we found that AGE-induced JAK2 activation in NRK-49F cells is attenuated by captopril [Huang et al., 2001]. In this study, we found that captopril attenuates AGE-induced angiotensinogen protein expression. Thus, captopril may attenuate Ang II-induced angiotensinogen by inhibiting JAK2 in this study. Interestingly, a recent study also found that the autoinduction of angiotensinogen by Ang II is mediated by JAK2 [Guo et al., 2004].

TGF-β is a major mediator of DN [Ziyadeh, 2004]. For example, many of AGE-induced effects are mediated by endogenous TGF-β [Chuang and Guh, 2001; Forbes et al., 2003a]. In this regard, TGF-β stimulates CTGF production via Smad binding element in the CTGF gene promoter in fibroblasts [Jerums et al., 2003; Leask et al., 2003; Leask and Abraham, 2004]. Moreover, CTGF may act as a downstream effecter of some the profibrotic effects of TGF-β (e.g., cell proliferation and collagen production) in fibroblasts [Leask and Abraham, 2004]. Thus, one may speculate that TGF-β may mediate AGE-induced effects in this study. However, others and we have shown that AGE-induced effects in NRK-49F cells are not dependent on TGF-β [Huang et al., 2001; Twigg et al., 2001].

In conclusion, AG-490, captopril, and losartan attenuate AGE-induced mitogenesis and type I collagen production by inhibiting the JAK2-CTGF pathway in NRK-49F cells. Namely, AGE (150 μg/ml)-induced mitogenesis and type I collagen production are dependent on the Ang II-JAK2-CTGF pathway. Moreover, Ang II (10^{-7}M)-induced mitogenesis and type I collagen production are dependent on the JAK2-CTGF pathway.

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