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Leptin and Connective Tissue Growth Factor in Advanced Glycation End-Product-Induced Effects in NRK-49F Cells

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Abstract Previously, we showed that Janus kinase 2 (JAK2) is important in advanced glycation end-product (AGE)-induced effects in renal interstitial (NRK-49F) fibroblasts. Leptin is a JAK2-activating cytokine via the long form leptin receptor (Ob-Rb). Leptin and connective tissue growth factor (CTGF) may be involved in renal fibrosis. However, the relationship between leptin and CTGF in terms of AGE-induced effects remains unknown. Thus, the effects of AGE (150 μg/ml) and leptin on mitogenesis, CTGF and collagen expression in NRK-49F cells were determined. We found that leptin and AGE increased mitogenesis and type I collagen protein expression at 3 and 7 days, respectively. AGE increased leptin mRNA and protein expression at 2–3 days. AGE increased CTGF mRNA and protein expression at 3–5 days. AG-490 (JAK2 inhibitor) abrogated AGE-induced leptin mRNA and protein expression at 2–3 days. AG-490 and Ob-Rb anti-sense oligodeoxynucleotides (ODN) abrogated AGE-induced CTGF mRNA and protein expression at 3–5 days. AG-490 and CTGF anti-sense ODN abrogated AGE-induced mitogenesis and collagen protein expression at 7 days. Additionally, leptin dose (0.2–1 μg/ml) and time (1–2 days)-dependently increased CTGF protein expression. AG-490 abrogated leptin (1 μg/ml)-induced CTGF protein expression at 2 days. AG-490 and CTGF anti-sense ODN abrogated leptin-induced mitogenesis and collagen protein expression at 3 days. We concluded that AGE induced JAK2 to increase leptin while leptin induced JAK2 to increase CTGF-induced mitogenesis and type I collagen protein expression in NRK-49F cells. Additionally, AGE-induced mitogenesis and type I collagen protein expression were dependent on leptin-induced CTGF. J. Cell. Biochem. 93: 940–950, 2004.

Key words: diabetic nephropathy; JAK2; leptin; connective tissue growth factor; collagen

Diabetic nephropathy (DN) is characterized by glomerulosclerosis and tubulointerstitial disease culminating in renal fibrosis [Chuang, 2001; Phillips and Steadman, 2002]. Transforming growth factor-β (TGF-β) and connective tissue growth factor (CTGF) are two of the key factors in DN and renal fibrosis [Gupta et al., 2000; Chuang, 2001; Sakharova et al., 2001; Twigg et al., 2001; Forbes et al., 2003]. Moreover, advanced glycation end-product (AGE) and interstitial fibroblast are important in the pathogenesis of diabetic renal fibrosis [Chuang, 2001; Forbes et al., 2003; Kikkawa et al., 2003].

Leptin is produced mainly in the adipose tissue and regulates food intake and energy expenditure [Wolf et al., 2002]. There are two

Abbreviations used: DN, diabetic nephropathy; TGF-β, transforming growth factor-β; CTGF, connective tissue growth factor; Ob-Rb, long form of leptin receptor; AGE, advanced glycation end-product; JAK2, Janus kinase 2; BSA, bovine serum albumin; FCS, fetal calf serum; ODN, oligodeoxynucleotide; SEM, standard errors of the mean.

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forms of leptin receptors: short form (Ob-Ra) and long-form (Ob-Rb). Only Ob-Rb has signaling capabilities by activating Janus kinase 2 (JAK2) [Sweeney, 2002; Wolf et al., 2002]. Interestingly, serum leptin level is increased in patients with obesity, obese type 2, diabetes and chronic renal failure [Sharma and Considine, 1998; Mantzoros, 1999; Stenvinkel, 1999; Verrotti et al., 2001; Adelman, 2002].

Leptin is cleared from the circulation primarily by the kidneys [Cumin et al., 1996]. Both the kidney and the fibroblast express leptin and leptin receptors (both short and long forms) [Glasow et al., 2001; Wolf et al., 2002]. Leptin has been implicated in some renal diseases [Ballermann, 1999; Adelman, 2002; Wolf et al., 2002]. Three recent studies also suggested a role for leptin in DN [Fruehwald-Schultes et al., 1999; Han et al., 2001; Verrotti et al., 2001]. However, the effects of AGE on the expression of leptin in renal cells remain unknown and the biologic effects of leptin in interstitial fibroblasts also remain unknown.

CTGF is a protein of 36–38 kDa, depending on its degree of glycosylation [Brigstock, 1999]. It is a pro-fibrotic cytokine recently implicated in DN [Wahab et al., 2001; Wang et al., 2001; Wada et al., 2002]. However, there have been no studies concerning the effects of leptin on CTGF protein expression. There were also no studies concerning the relationship among AGE, leptin, and CTGF in terms of mitogenesis and collagen production in cells.

Among the intracellular pathways in DN [Chuang, 2001], we were the first to show that JAK2 is necessary for AGE-induced mitogenesis and collagen production in NRK-49F (normal rat kidney interstitial fibroblast) cells [Huang et al., 1999b, 2001; Guh et al., 2001]. Because leptin is a JAK2-activating cytokine, it would be interesting to know whether AGE-induced effects in NRK-49F cells are dependent on leptin. However, the role of JAK2 in the AGE, leptin, and CTGF network in terms of mitogenesis and collagen production remains unexplored.

Therefore, seven questions were addressed in the present study of AGE-cultured NRK-49F cells. First, what were the effects of AGE and leptin on mitogenesis and collagen protein expression? Second, did AGE increase leptin and CTGF mRNA and protein expression? Third, what were the effects of leptin on CTGF protein expression, mitogenesis, and collagen protein expression? Fourth, what were the roles of CTGF in AGE-induced mitogenesis and collagen protein expression? Fifth, what were the roles of JAK2 and Ob-Rb in AGE-induced CTGF mRNA and protein expression? Sixth, what is the role of JAK2 in leptin-induced CTGF protein expression? Finally, what were the roles of JAK2 and CTGF in leptin-induced mitogenesis and collagen protein expression?

MATERIALS AND METHODS

Reagents

AG-490 (JAK2 inhibitor) was purchased from Calbiochem Corp. (San Diego, CA). Leptin was purchased from R&D Systems, Inc. (Minneapolis, MN). According to a previous study [Kondo et al., 1999], 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/Ab Production, Inc. (Taipei, Taiwan). Leptin antibody, Ob-Rb antibody, actin antibody, anti-rabbit IgG HRP, and anti-goat IgG HRP were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Restore™ Western Blot Stripping Buffer, SuperSignal West PicoChemiluminescent Substrate were purchased from Pierce Corp. [α-^{32}P]dCTP (methy-^{3}H)thymidine and [^{3}H]proline were purchased from Amersham Corp. (Amersham, UK). LipofectAmine plus™ 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 (ATCC, Manassas, VA). NRK-49F cells were grown in culture flasks and maintained in DMEM (5.5 mM D-glucose) supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin, and 5% fetal calf serum (FCS) in a humidified incubator under air/CO2 (19:1) at 37°C. In all experiments, cells were exposed to 0.5% FCS medium for 2 days before experiments. Additionally, medium and all reagents were refreshed every 48 h. Note that serum-free medium (DMEM supplemented with 10 μg/ml insulin, 10 μg/ml transferring, and 10 ng/ml selenium) was used in our preliminary studies with inconsistent results

regarding leptin. This observation can be explained by the fact that leptin and/or Ob-Rb can be modulated by 0.012–0.12 μg/ml [Glasow et al., 2001] or 6 μg/ml insulin [Meissner et al., 2003]. Therefore, we chose to use low concentrations of FCS (0.5%) in later experiments. 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 0.5 M glucose in 50 mM potassium phosphate (pH 7.3)/1 mM EDTA under sterile conditions at 37°C for 8 weeks as described in our previous study [Huang et al., 1999a]. Fluorescence of the AGE was determined with a Perkin-Elmer fluorescence spectrometer; the average fluorescence for AGE was 56 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.

Cellular Mitogenesis

This was performed as described in our previous study [Huang et al., 1999b]. 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 day, fresh medium (5% FCS) containing different concentrations of BSA, AGE, or leptin was added for various times. Four microCurie of [^{3}H]thymidine (Amersham, Arlington Heights, IL) was during the last 5 h. Cell 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 Oligodeoxynucleotide (ODN)

Phosphorothioated single-stranded ODNs used in this study were synthesized with a DNA/RNA synthesizer (Applied Biosystems Division Perkin Elmer), respectively. CTGF anti-sense 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 bp +262 derived from the cDNA sequence for rat CTGF (GenBank accession no. NM_022266). Ob-Rb anti-sense ODN (5′-AGA ATT TCT GAC ACG TCA T-3′) and Ob-Rb sense ODN (5′-ATG ACG TGT CAG AAA TTC T-3′) correspond to the region of bp +1 to bp +19 derived from the cDNA sequence for rat Ob-Rb (GenBank accession no. AF287268).

Transfection of ODN Into Cells

This was performed as described in our previous study [Huang et al., 1999b]. 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 ($92 \pm 2\%$) and ODN-transfected ($90 \pm 1.5\%$) cells in six independent experiments.

Immunoblotting

This was performed as described in our previous study [Huang et al., 1999b]. Briefly, 30 μl conditioned media or cell lysates of 25–50 μg proteins (for CTGF protein analysis), or 100 μg proteins (for leptin 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, anti-leptin, or anti-Ob-Rb 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 (ECL) 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]. 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 ATCC. CTGF mRNA was determined by Northern blotting modified from our previous study [Yang et al., 1997]. Briefly, one hundred micrograms of RNA were separated on 1% agarose, 1 M 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 intensity of autoradiograms was then determined by scanning densitometry.

Real-Time Reverse Transcription-Polymerase Chain Reaction (RT-PCR) of Leptin and Ob-Rb mRNA

This was performed by fluorogenic 5′ nuclease assay-based TaqMan real-time RT-PCR by using the ABI PRISM 7900 Sequence Detection System (Perkin-Elmer, Inc., Boston, MA) as described in our previous study [Guh et al., 2003]. Briefly, primers and probes were purchased (ABI Assays-on-Demand Gene Expression Products, leptin: Rn00565158_m1, Ob-Rb: Rn00561465_m1) (Perkin-Elmer, Inc.). After RT, 1 μg of total RNA was used to generate cDNA using a random primer. Equal amounts of cDNA were used in triplicate and amplified. Products were quantified by using the relative standard curve method and normalized to GAPDH mRNA.

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 and 60–120 μg of protein was applied to each lane of a 7.5% sodium dodecyl sulfate (SDS)–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 (SEM). 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 consdered statistically significant.

RESULTS

Effect of AGE or Leptin on Mitogenesis in NRK-49F Cells

Cellular mitogenesis was performed by [^{3}H]thymidine incorporation into cells. As shown in Figure 1, AGE (150 μg/ml) increased mitogenesis at 7 days. As shown in Figure 2, leptin (1 μg/ml) also increased mitogenesis at 3 days.

Role of JAK2 and CTGF in AGE-Induced Mitogenesis in NRK-49F Cells

Specific inhibitors of JAK2 (AG-490) and CTGF (anti-sense ODN) were used to investigate the roles of JAK2 and CTGF in AGE-induced mitogenesis. As shown in Figure 1, AG-490 and CTGF anti-sense (but not CTGF sense) ODN abrogated AGE-induced mitogenesis at 7 days. Thus, AGE-induced mitogenesis in NRK-49F cells was dependent on JAK2 and CTGF.

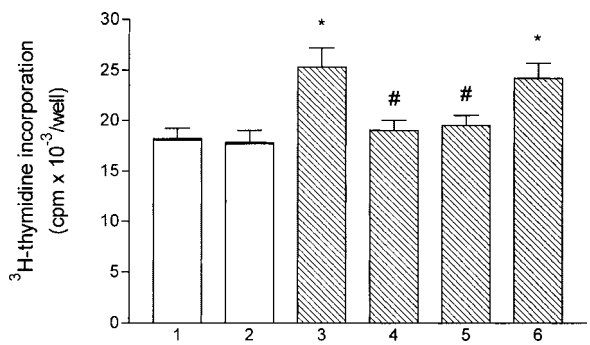

Fig. 1. Effects of advanced glycation end-product (AGE) (150 μg/ml) on cellular mitogenesis in NRK-49F cells. Cells were cultured with bovine serum albumin (BSA) (150 μg/ml, empty bars) or AGE (striped bars) for 7 days. BSA-treated (**lane 2**) or AGE-treated cells (**lane 4**) were treated with AG-490 (5 μM, pretreated for 16 h before adding BSA or AGE). AGE-treated cells were treated with connective tissue growth factor (CTGF) anti-sense oligodeoxynucleotide (ODN) (**lane 5**) or CTGF sense ODN (**lane 6**) for 7 days and [^{3}H]thymidine incorporation was performed. Note that AGE alone (**lane 3**) increased mitogenesis while AG-490 and CTGF anti-sense (but not CTGF sense) ODN abrogated AGE-induced mitogenesis. Results were expressed as the mean ± standard errors of the mean (SEM) of three independent experiments performed in triplicate. *, $P < 0.05$ versus BSA. $^{\#}$, $P < 0.05$ versus AGE alone.

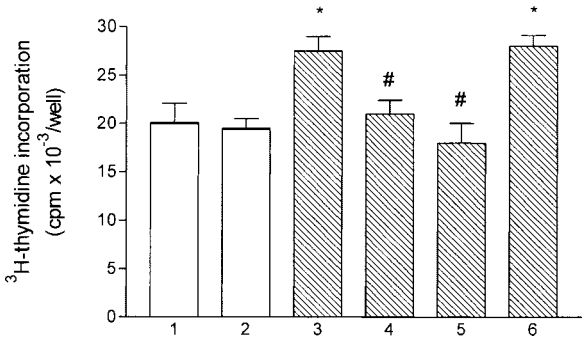

Fig. 2. Effects of leptin (1 μg/ml) on cellular mitogenesis in NRK-49F cells. Cells were cultured with (striped bars) or without (empty bars) leptin for 3 days. Medium-treated (**lane 2**) or leptin-treated (**lane 4**) cells were treated with AG-490 (5 μM, pretreated for 16 h before adding medium or leptin). Leptin-treated cells were treated with CTGF anti-sense ODN (**lane 5**) or CTGF sense ODN (**lane 6**) for 3 days and [^{3}H]thymidine incorporation was performed. Note that leptin alone (**lane 3**) increased mitogenesis while AG-490 and CTGF anti-sense (but not CTGF sense) ODN abrogated leptin-induced mitogenesis. Results were expressed as the mean ± SEM of three independent experiments performed in triplicate. *, $P < 0.05$ versus medium alone. #, $P < 0.05$ versus leptin alone.

Role of JAK2 and CTGF in Leptin-Induced Mitogenesis in NRK-49F Cells

Specific inhibitors of JAK2 (AG-490) and CTGF (anti-sense ODN) were used to investigate the roles of JAK2 and CTGF in leptin-induced mitogenesis. As shown in Figure 2, AG-490 and CTGF anti-sense (but not CTGF sense) ODN abrogated leptin (1 μg/ml)-induced mitogenesis at 3 days. Thus, leptin-induced mitogenesis was dependent on JAK2 and CTGF.

Effect of AGE or Leptin on Collagen Protein Expression in NRK-49F Cells

Collagen production was assessed by electrophoretic analysis of collagen followed by fluorograph. As shown in Figure 3, AGE (150 μg/ml) increased type I collagen protein expression at 7 days. As shown in Figure 4, leptin (1 μg/ml) increased type I collagen protein expression at 3 days.

Role of JAK2 and CTGF in AGE-Induced Collagen Protein Expression in NRK-49F Cells

Specific inhibitors of JAK2 (AG-490) and CTGF (anti-sense ODN) were used to investigate the roles of JAK2 and CTGF in AGE-induced collagen protein expression by electrophoretic analysis of collagen. As shown in Figure 3, AG-490 and CTGF anti-sense ODN abrogated AGE-

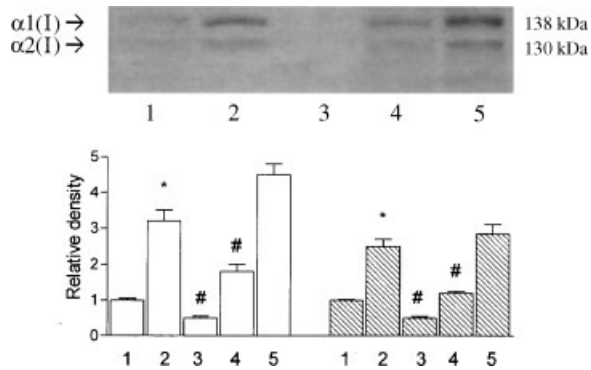

Fig. 3. Effects of AGE (150 μg/ml) on collagen protein expression in NRK-49F cells. Cells were cultured with BSA (150 μg/ml, **lane 1**) or AGE (**lanes 2–5**) for 7 days. AGE-treated cells were treated with AG-490 (5 μM, pretreated for 16 h before adding AGE, lane 3), CTGF anti-sense ODN (lane 4), and CTGF sense ODN (lane 5) for 7 days. [^{3}H]proline-labeled proteins were precipitated and electrophoresis was performed. Note that AG-490 and CTGF anti-sense (but not CTGF sense) ODN abrogated AGE-induced type I collagen protein expression in these cells. α1 (I) and α2 (I), type I collagen. Results were expressed as the mean ± SEM of four independent experiments for α1 (empty bars) and α2 (striped bars) type I collagen. Note that lane 2 (AGE-treated alone) was compared to lane 1 (BSA-treated), whereas lanes 3–5 (chemical plus AGE-treated) were compared to lane 2 (AGE-treated alone). *, $P < 0.05$ versus BSA. #, $P < 0.05$ versus AGE alone.

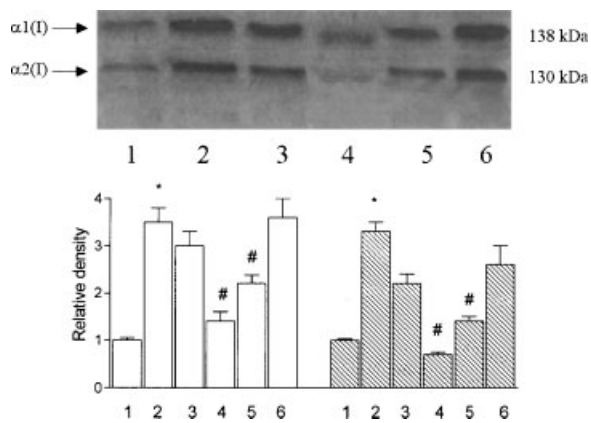

Fig. 4. Effects of leptin (1 μg/ml) on collagen protein expression in NRK-49F cells. Cells were cultured with (**lanes 2–6**) or without (**lane 1**) leptin for 3 days. Leptin-treated cells were treated with AG-490 (1 and 5 μM, pretreated for 16 h before adding leptin, lanes 3 and 4), CTGF anti-sense ODN (lane 5) and CTGF sense ODN (lane 6) for 3 days. [^{3}H]Proline-labeled proteins were precipitated and electrophoresis was performed. Note that AG-490 (5 μM) and CTGF anti-sense (but not CTGF sense) ODN abrogated leptin-induced type I collagen protein expression in these cells. α1 (I) and α2 (I), type I collagen. Results were expressed as the mean ± SEM of four independent experiments for α1 (empty bars) and α2 (striped bars) type I collagen. Note that lane 2 (leptin-treated alone) was compared to lane 1 (control), whereas lanes 3–6 (chemical plus leptin-treated) were compared to lane 2 (leptin-treated alone). *, $P < 0.05$ versus control. #, $P < 0.05$ versus leptin alone.

induced type I collagen protein expression at 7 days. Thus, AGE-induced type I collagen protein expression was dependent on JAK2 and CTGF.

Role of JAK2 and CTGF in Leptin-Induced Collagen Protein Expression in NRK-49F Cells

Specific inhibitors of JAK2 (AG-490) and CTGF (anti-sense ODN) were used to investigate the roles of JAK2 and CTGF in leptin-induced collagen protein expression by electrophoretic analysis of collagen. As shown in Figure 4, AG-490 and CTGF anti-sense (but not CTGF sense) ODN abrogated leptin (1 μg/ml)-induced type I collagen protein expression at 3 days. Thus, leptin-induced type I collagen protein expression was dependent on JAK2 and CTGF.

Effects of AGE on Leptin and CTGF mRNA Expression

Effects of AGE (150 μg/ml) on leptin and CTGF mRNA expression were assessed by real-time RT-PCR and Northern blotting. As shown in Figure 5, AGE (150 μg/ml) induced leptin mRNA expression at 2 days and induced CTGF mRNA expression at 3 days. In contrast, a previous study found that AGE increased CTGF mRNA at 8 h or 2 days in various primarily cultured human dermal fibroblasts [Twigg et al., 2001]. They also found that AGE increased CTGF protein in conditioned medium and cell lysates at 1 and 3 days, respectively.

Dose-Dependent Effects of AGE on Leptin and CTGF Protein Expression

Dose-dependent effects of AGE (50–250 μg/ml) on leptin and CTGF protein expression were assessed by immunoblotting. As shown in Figure 6A, AGE dose-dependently increased leptin protein expression at 3 days. As shown in Figure 6B, AGE dose-dependently increased CTGF protein expression in cell lysates at 5 days. Additionally, AGE (150 μg/ml) also increased CTGF protein expression in conditioned medium at 4 days. Note that in addition to the authentic 38 kDa CTGF, there was a CTGF fragment of approximately 20 kDa in molecular weight in conditioned medium. In contrast, there were no low-molecular weight CTGF fragments in cell lysates. These observations are similar to a previous study in AGE-treated fibroblasts [Twigg et al., 2001].

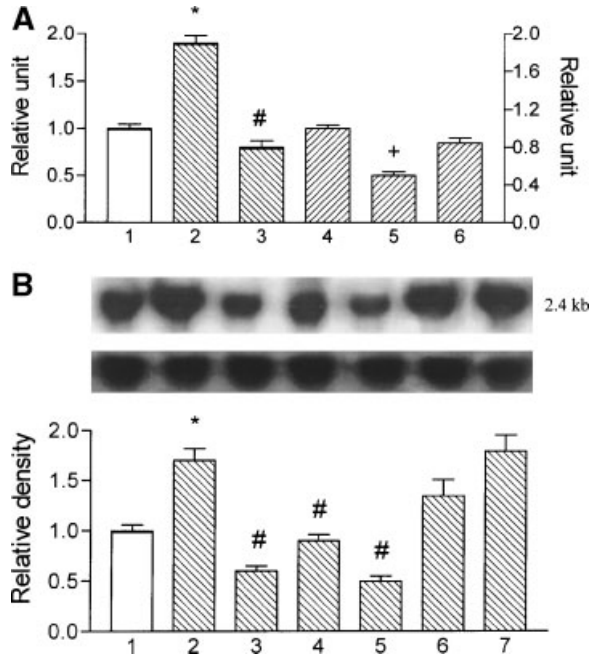

Fig. 5. Effects of AGE (150 μg/ml) on leptin, long form of leptin receptor (Ob-Rb) (**A**) and CTGF (**B**) mRNA expression in NRK-49F cells. Cells were cultured with BSA (150 μg/ml, empty bars) or AGE (striped bars) for 2–3 days. A: Results of real-time RT-PCR for leptin (**lanes 1–3**) and Ob-Rb (**lanes 4–6**) mRNA. Note that AG-490 (5 μM, pretreated for 16 h before adding AGE, lane 3) abrogated AGE (lane 2)-induced leptin mRNA expression at 2 days. Also note that Ob-Rb anti-sense ODN (lane 5), but not Ob-Rb sense ODN (lane 6), abrogated AGE-associated (lane 4) Ob-Rb mRNA expression at 2 days. B: AG-490 (5 μM, pretreated for 16 h before adding AGE, **lane 3**), CTGF anti-sense ODN (**lane 4**) and Ob-Rb anti-sense ODN (**lane 5**), but not CTGF sense ODN (**lane 6**) or Ob-Rb sense ODN (**lane 7**) abrogated AGE (**lane 2**)-induced CTGF mRNA expression at 3 days. The amount of mRNA was expressed as the ratio between CTGF (**upper panel**) and β-actin (**lower panel**). Results were expressed as the mean ± SEM of three independent experiments. *, $P < 0.05$ versus lane 1. #, $P < 0.05$ versus lane 2. +, $P < 0.05$ versus lane 4.

Time-Dependent Effects of AGE on Leptin and CTGF Protein Expression

Time-dependent effects of AGE (150 μg/ml) on leptin and CTGF protein expression were assessed by immunoblotting. As shown in Figure 7A, AGE time-dependently increased leptin protein expression at 1–3 days and then decreased at 4 days. As shown in Figure 8A, AGE time-dependently increased CTGF protein expression at 3–5 days.

Role of JAK2 in AGE-Induced Leptin mRNA and Protein Expression

Specific inhibitor of JAK2 (AG-490) was used to investigate the role of JAK2 in AGE-induced

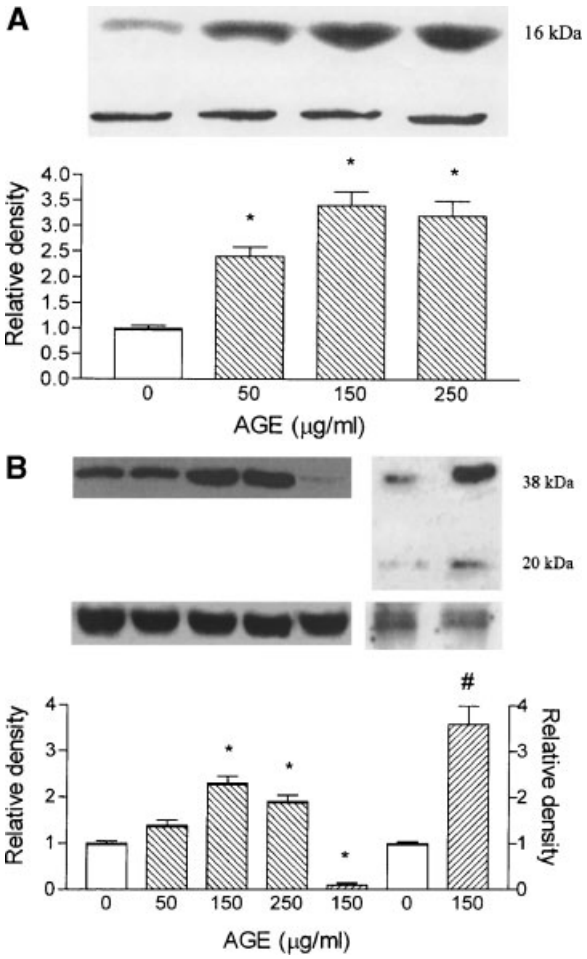

Fig. 6. Dose-dependent effects of AGE (50–250 μg/ml) on leptin (**A**) and CTGF (**B**) protein expression in NRK-49F cells. Cells were cultured with BSA (150 μg/ml, empty bars) or AGE (striped bars) and Western blotting was performed. A: AGE dose-dependently increased leptin protein expression at 3 days. *, $P < 0.05$ versus BSA. B: AGE dose-dependently increased CTGF protein expression in cell lysates at 5 days (**left panel**: **lanes 1–5**). Specificity of CTGF antibody was proven by the near absence of the CTGF band when the antibody was previously absorbed with 50 μg/ml of the synthetic peptide used for immunization for 24 h at 4°C (lane 5). Relative protein amount was expressed as the ratio between CTGF (**upper panel**) and β-actin (**lower panel**). AGE (150 μg/ml) also increased CTGF protein expression in conditioned medium at 4 days (**right panel**: **lanes 6–7**). Relative protein amount was expressed as the ratio between 38 kDa CTGF (upper panel) and β-actin (lower panel). Results were expressed as the mean ± SEM of three independent experiments. *, $P < 0.05$ versus lane 1 (cell lysate of BSA-treated cells). #, $P < 0.05$ versus lane 6 (conditioned medium of BSA-treated cells).

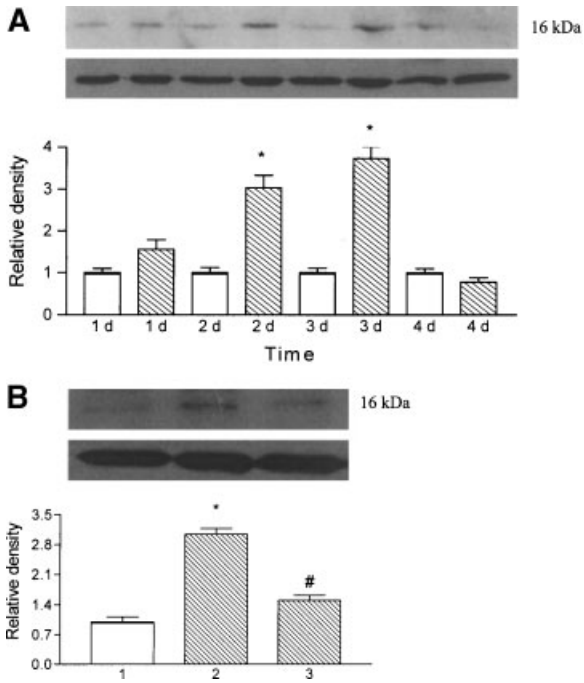

Fig. 7. Time course of the effects of AGE (150 μg/ml) on leptin protein expression in NRK-49F cells. Cells were cultured with BSA (150 μg/ml, empty bars) or AGE (striped bars) for 1–4 days and Western blotting was performed. **A**: AGE time-dependently increased leptin protein expression at 1–3 days and then decreased at 4 days. **B**: AGE alone (**lane 2**) increased leptin protein expression at 3 days. AG-490 (5 μM, pretreated for 16 h before adding leptin, **lane 3**) abrogated AGE-induced leptin protein expression at 3 days. Relative protein amount was expressed as the ratio between leptin (**upper panel**) and β-actin (**lower panel**). Results were expressed as the mean ± SEM of three independent experiments. *, $P < 0.05$ versus BSA. #, $P < 0.05$ versus AGE alone.

leptin mRNA and protein expression by real-time RT-PCR and immunoblotting. Thus, AG-490 abrogated AGE (150 μg/ml)-induced leptin mRNA expression at 2 days (Fig. 5A) and leptin protein expression at 3 days (Fig. 7B). Thus, AGE-induced leptin mRNA and protein expression was dependent on JAK2.

Role of JAK2 and Ob-Rb in AGE-Induced CTGF mRNA and Protein Expression

As shown in Figure 5A, Ob-Rb anti-sense ODN abrogated AGE-associated Ob-Rb mRNA expression at 2 days. Thus, specific inhibitors of JAK2 (AG-490) and Ob-Rb (anti-sense ODN) were used to investigate the roles of JAK2 and Ob-Rb in AGE-induced CTGF mRNA expression by Northern blotting and protein expression by immunoblotting. Thus, AG-490 and Ob-Rb anti-sense (but not Ob-Rb sense) ODN abrogated AGE (150 μg/ml)-induced CTGF mRNA expression at 3 days (Fig. 5B) and CTGF protein expression at 5 days (Fig. 8B). Thus, AGE-induced CTGF protein expression was dependent on JAK2 and Ob-Rb.

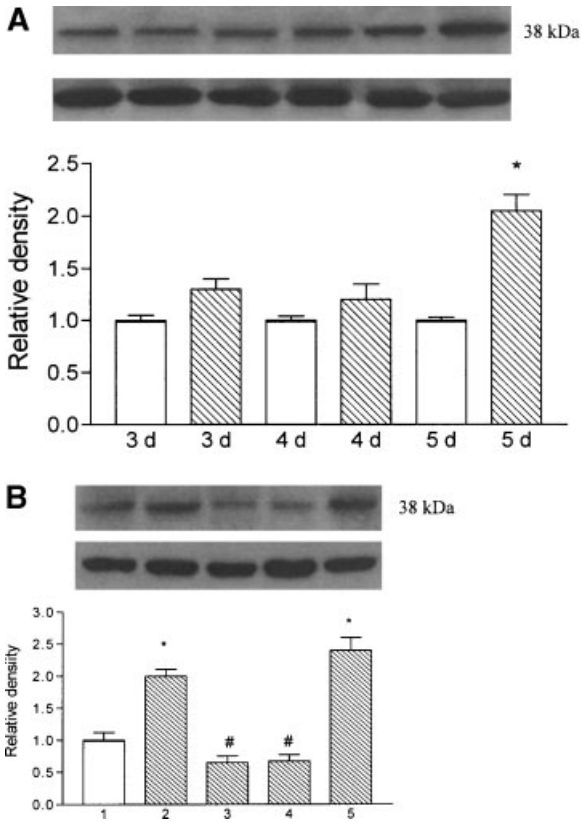

Fig. 8. Time course of the effects of AGE (150 μg/ml) on CTGF protein expression in NRK-49F cells. Cells were cultured with BSA (150 μg/ml, empty bars) or AGE (striped bars) for 3–5 days and Western blotting was performed. **A**: AGE time-dependently increased CTGF protein expression at 3–5 days. **B**: Cells were treated with BSA (150 μg/ml, empty bar) or AGE (striped bars) for 5 days. AGE alone (**lane 2**) increased CTGF protein expression at 5 days. Note that AG-490 (5 μM, pretreated for 16 h before adding AGE, **lane 3**) and Ob-Rb anti-sense ODN (**lane 4**), but not Ob-Rb sense ODN (**lane 5**) abrogated AGE-induced CTGF protein expression at 5 days. Relative protein amount was expressed as the ratio between CTGF (**upper panel**) and β-actin (**lower panel**). Results were expressed as the mean ± SEM of three independent experiments. *, $P < 0.05$ versus BSA. #, $P < 0.05$ versus AGE alone.

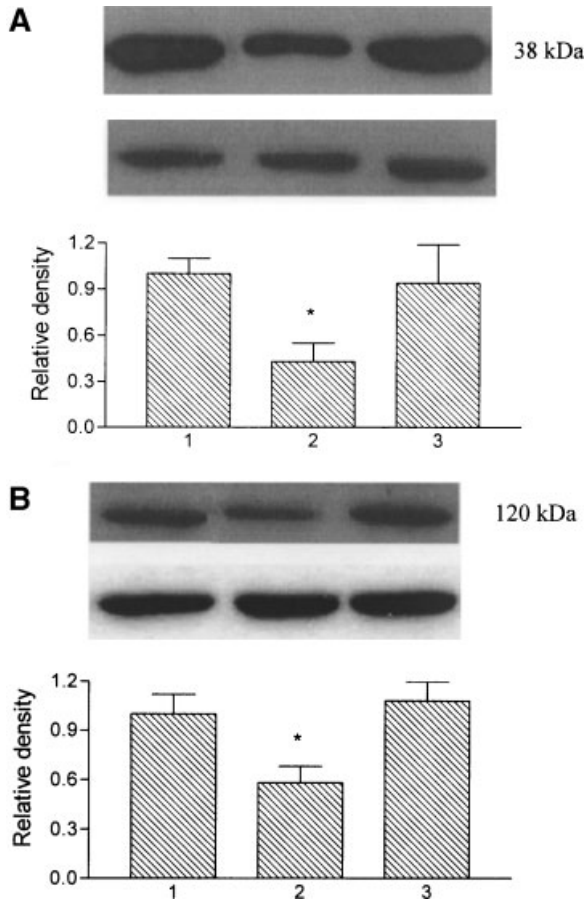

Fig. 9. Effect of anti-sense ODN on AGE-induced CTGF (**A**) or Ob-Rb (**B**) protein expression in NRK-49F cells. Cells were cultured with AGE for 5 days and Western blotting was performed. A: CTGF anti-sense ODN (**lane 2**), but not CTGF sense ODN (**lane 3**) decreased CTGF protein expression. B: Ob-Rb anti-sense ODN (**lane 2**), but not Ob-Rb sense ODN (**lane 3**) decreased Ob-Rb protein expression. Relative protein amount was expressed as the ratio between CTGF or leptin (**upper panel**) and β-actin (**lower panel**). Results were expressed as the mean ± SEM of three independent experiments. *, $P < 0.05$ versus AGE alone.

Effect of Anti-Sense ODN on AGE-Induced CTGF/Ob-Rb mRNA and Protein Expression

CTGF anti-sense ODN, but not CTGF sense ODN, abrogated AGE-induced CTGF mRNA (Fig. 5B) and protein (Fig. 9A) expression. Similarly, Ob-Rb anti-sense ODN, but not Ob-Rb sense ODN abrogated AGE-induced Ob-Rb mRNA (Fig. 5A) and protein (Fig. 9B) expression.

Dose- and Time-Dependent Effects of Leptin on CTGF Protein Expression

As shown in Figure 10A, leptin dose-dependently (0.2–5 μg/ml) increased CTGF protein expression at 2 days. As shown in Figure 10B, leptin (1 μg/ml) time-dependently (0.5–2 days) increased CTGF protein expression.

Role of JAK2 in Leptin-Induced CTGF Protein Expression

Specific inhibitor of JAK2 (AG-490) was used to investigate the role of JAK2 in leptin-induced CTGF protein expression by immunoblotting. As shown in Figure 11, AG-490 abrogated leptin (1 μg/ml)-induced CTGF protein expression at 2 days. Thus, leptin-induced CTGF protein expression was dependent on JAK2.

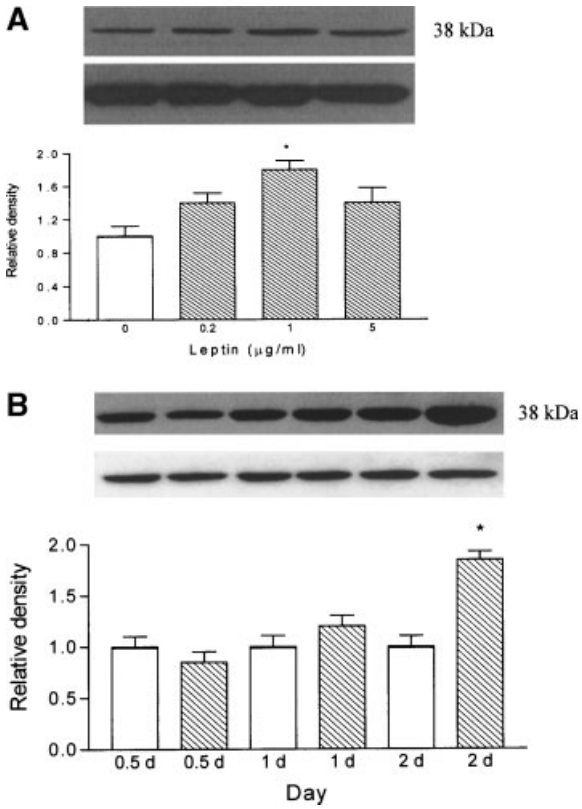

Fig. 10. Dose (**A**) and time-dependent (**B**) effects of leptin on CTGF protein expression in NRK-49F cells. Cells were cultured with (striped bars) or without (empty bars) leptin and Western blotting was performed. A: Leptin dose-dependently increased CTGF protein expression at 0.2–1 μg/ml and then decreased at 5 μg/ml at 2 days. B: Leptin (1 μg/ml) time-dependently increased CTGF protein expression at 0.5–2 days. Relative protein amount was expressed as the ratio between CTGF (**upper panel**) and β-actin (**lower panel**). Results were expressed as the mean ± SEM of three independent experiments. *, $P < 0.05$ versus medium alone.

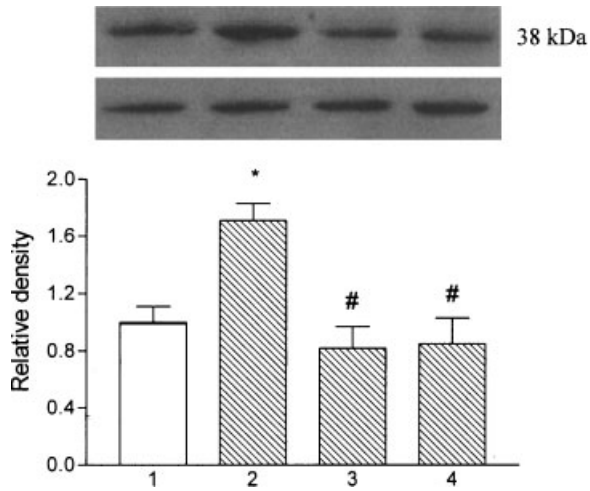

Fig. 11. Role of Janus kinase 2 (JAK2) in leptin (1 μg/ml)-induced CTGF protein expression in NRK-49F cells. Cells were cultured with medium alone (empty bar) or leptin (striped bars) for 2 days. Cells were treated with AG-490 (1 and 5 μM, pretreated for 16 h before adding leptin, **lanes 3** and **4**) for 2 days and Western blotting was performed. Note that AG-490 abrogated leptin-induced CTGF protein expression. Relative protein amount was expressed as the ratio between CTGF (**upper panel**) and β-actin (**lower panel**). Results were expressed as the mean ± SEM of three independent experiments. *, $P < 0.05$ versus medium alone. #, $P < 0.05$ versus leptin.

DISCUSSION

This is the first study showing the relationship among AGE, leptin, and CTGF in terms of mitogenesis and collagen protein expression in renal interstitial fibroblasts. In accordance with our previous finding that AGE increased mitogenesis and collagen protein expression in NRK-49F cells [Huang et al., 1999b, 2001; Guh et al., 2001], this study elucidates a novel pathway whereby AGE, JAK2, leptin, and CTGF jointly contribute to mitogenesis and collagen protein expression in renal interstitial fibroblasts. Because interstitial fibroblasts are important in renal fibrosis [Zeisberg et al., 2001], our findings may be relevant in the pathogenesis of diabetic renal fibrosis. However, it should be noted that similar studies have not been performed in human renal interstitial fibroblasts.

The role of leptin in renal diseases has only been noticed recently [Wolf et al., 2002]. For example, leptin may be involved in obesity-related renal diseases [Adelman, 2002]. Leptin also induced an overproduction of collagen in glomeruli and enhanced mitogenesis in glomerular endothelial cells [Wolf et al., 1999]. Thus, leptin has been suggested to foster the development of focal glomerulosclerosis [Ballermann, 1999].

The role of leptin in DN was suggested by three recent studies. Two studies found elevated serum leptin level in type 2 diabetic patients with microalbuminuria or proteinuria [Fruehwald-Schultes et al., 1999; Verrotti et al., 2001]. Another study found that exogenous leptin induced collagen production in diabetic mesangial cells [Han et al., 2001]. Interestingly, a recent study found that fibroblasts expressed leptin, with unknown functions [Glasow et al., 2001]. Our present study extends this observation by showing that AGE increased leptin expression while leptin increased mitogenesis and collagen protein expression in NRK-49F cells. Thus, leptin may be involved in diabetic

tubulointerstitial fibrosis. Note that we have used leptin at a dose of 1 μg/ml because we found that leptin increased CTGF protein expression at 1 μg/ml. Similarly, a previous study has also used leptin at a dose of 1 μg/ml in glomerular endothelial cells [Wolf et al., 1999].

CTGF is another factor implicated in the pathogenesis of diabetic renal fibrosis [Wahab et al., 2001; Wang et al., 2001; Wada et al., 2002]. Thus, CTGF was overexpressed in the diabetic kidneys [Wada et al., 2002]. AGE also upregulated CTGF expression in mesangial cells [Twigg et al., 2002] and fibroblasts [Twigg et al., 2001]. Our present study extends these previous studies by showing that AGE-induced mitogenesis and type I collagen protein expression were dependent on CTGF in NRK-49F cells. This observation is compatible with a previous study showing that CTGF induced mitogenesis and type I collagen protein expression in NRK-49F cells [Frazier et al., 1996].

This is the first study elucidating the relationship between leptin and CTGF. Thus, we found that leptin enhanced CTGF protein expression while AGE-induced CTGF was dependent on Ob-Rb. These observations are unique in that TGF-β is necessary for the induction of CTGF in many cells [Gupta et al., 2000; Riser and Cortes, 2001; Sakharova et al., 2001]. Moreover, CTGF mediates the pro-fibrotic actions of TGF-β in NRK-49F cells [Yokoi et al., 2002]. However, we and others have shown that AGE did not induce TGF-β in fibroblasts [Huang et al., 2001; Twigg et al., 2001]. Additionally, a recent study found that AGE-induced CTGF in fibroblasts was independent of TGF-β [Twigg et al., 2001]. Thus, CTGF can also be induced by TGF-β-independent pathways (e.g., leptin and Ob-Rb) in fibroblasts.

We have been studying intracellular pathways in models of DN in vitro [Chuang, 2001; Chuang et al., 2003; Guh et al., 2003]. For example, the JAK2 pathway is one of the relevant pathways first discovered by us [Huang et al., 1999b, 2001; Guh et al., 2001]. JAK2 is induced by many cytokines and growth factors related to DN [Huang et al., 1999b; Chuang, 2001]. Thus, our previous studies showed that AGE-induced mitogenesis and collagen protein expression in NRK-49F cells were dependent on the receptor for AGE–JAK2 pathway [Huang et al., 1999b, 2001; Guh et al., 2001]. This study further showed that AGE-induced leptin and CTGF protein expression were dependent on JAK2. Interestingly, a recent study also found that hydrogen peroxide-induced CTGF expression was dependent on JAK2 [Park et al., 2001]. Thus, we have established the link whereby AGE induced JAK2 while JAK2 increased leptin to increase CTGF.

A previous study found that fibroblasts expressed Ob-Rb, albeit with unknown functions [Glasow et al., 2001]. Leptin is a cytokine that activates JAK2 by way of Ob-Rb [Sweeney, 2002; Wolf et al., 2002]. In the present study, we found that AGE-induced CTGF was dependent on Ob-Rb while leptin-induced CTGF protein expression, mitognesis and type I collagen protein expression were dependent on JAK2 in NRK-49F cells. Thus, Ob-Rb is functional in NRK-49F cells.

In conclusion, AGE increased leptin and CTGF proteins while leptin increased CTGF protein in NRK-49F cells. AGE-induced CTGF protein expression in these cells was dependent on JAK2 and Ob-Rb while AGE-induced mitogenesis was dependent on JAK2-induced CTGF. AGE-induced type I collagen protein expression in these cells was dependent on the AGE/JAK2/leptin/JAK2/CTGF pathway. Finally, leptin-induced mitogenesis and type I collagen production in these cells were dependent the JAK2/CTGF pathway.

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