Connective Tissue Growth Factor and IGF-I Are Produced by Human Renal Fibroblasts and Cooperate in the Induction of Collagen Production by High Glucose

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Tubulointerstitial fibrosis is an important component in the development of diabetic nephropathy. Various renal cell types, including fibroblasts, contribute to the excessive matrix deposition in the kidney. Although transforming growth factor-β (TGF-β) has been thought to play a major role during fibrosis, other growth factors are also involved. Here we examined the effects of connective tissue growth factor (CTGF) and IGF-I on collagen type I and III production by human renal fibroblasts and their involvement in glucose-induced matrix accumulation. We have demonstrated that both CTGF and IGF-I expressions were increased in renal fibroblasts under hyperglycemic conditions, also in the absence of TGF-β signaling. Although CTGF alone had no effect on collagen secretion, combined stimulation with IGF-I enhanced collagen accumulation. Furthermore, IGF-I also had a synergistic effect with glucose on the induction of collagens. Moreover, we observed a partial inhibition in glucose-induced collagen secretion with neutralizing anti-CTGF antibodies, thereby demonstrating for the first time the involvement of endogenous CTGF in glucose-induced effects in human renal fibroblasts. Therefore, the cooperation between CTGF and IGF-I might be involved in glucose-induced matrix accumulation in tubulointerstitial fibrosis and might contribute to the pathogenesis of diabetic nephropathy. *Diabetes* 52:2975–2983, 2003

Diabetic nephropathy develops in 30–40% of patients with diabetes. It is characterized by thickening of the basement membranes, mesangial expansion and proliferation, and tubulointerstitial fibrosis (1–3). The histologic characteristics are caused by excessive deposition of extracellular matrix (ECM) proteins, which are induced by the hyperglycemic milieu (4–6) via the induction of various growth factors.

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DMEM, Dulbecco's modified Eagle's medium; ECM, extracellular matrix; ELISA, enzyme-linked immunosorbent assay; IGFBP, IGF-binding protein; mAb, monoclonal antibody; OD, optical density; TBP, TATA-box binding protein; TGF-β, transforming growth factor-β.

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Transforming growth factor-β (TGF-β) and its role in the fibrotic process have been studied extensively, and it is thought to play a central role in the development of diabetic nephropathy (7). Recently, we demonstrated in vitro that TGF-β1 was not required for glucose-induced ECM accumulation in renal fibroblasts, in a process that might involve alternative growth factors (8). Moreover, other growth factors have been implicated in vivo to contribute to the pathogenesis of diabetic nephropathy (9–11).

Connective tissue growth factor (CTGF) has been described as a growth factor that acts downstream of TGF-β in the fibrotic process (10). However, TGF-β–independent induction of CTGF has also been described (12–14). It has been demonstrated that CTGF is induced in mesangial cells under hyperglycemic conditions, whereby it mediates the production of ECM components (14–16). The role of CTGF in vivo has been demonstrated in various experimental models: in diabetic NOD mice, diabetic *db/db* mice, and streptozotocin-induced diabetic rats, CTGF expression was increased in the cortex (15–17). Moreover, overexpression of CTGF was observed in human renal fibrosis in various renal diseases, including diabetic nephropathy (18).

IGF-I is a mitogenic factor and promotes ECM accumulation in various cell types, including fibroblasts (17,19–22). In experimental diabetic kidney diseases, increased renal IGF-I levels were correlated with pathological alterations. However, increases in renal IGF-I levels were not accompanied by an increase in serum IGF-I (23–26). Therefore, it is thought that local overproduction of IGF-I is pathophysiologically more relevant.

Recently, we demonstrated that TGF-β1 is not required for glucose-induced ECM accumulation in human renal fibroblasts, a process that might involve alternative growth factors (8). Moreover, collagen type III was not affected by TGF-β1, suggesting that the growth factor requirement for the induction of collagen type III is different from other ECM components. Furthermore, it has been demonstrated that the expression of interstitial collagens, including collagen type III, is increased during tubulointerstitial fibrosis (27). Therefore, we have examined the role of CTGF and IGF-I in the induction of interstitial collagen secretion in renal fibroblasts under hyperglycemic conditions. Our data show that CTGF and IGF-I are upregulated by high glucose in human renal fibroblasts and act synergistically in the increase of collagen type I and III secre-

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TABLE 1
Primer sequences and PCR conditions

Gene	Primer	[$MgCl_2$]	Annealing	Cycles	Product size
GAPDH					
Forward	5′-ACC ACA GTC CAT GCC ATC AC-3′	1.5 mmol/l	55°C	35	433 bp
Reverse	5′-TCC ACC ACC CTG TTG CTG TA-3′				
COL1A1					
Forward	5′-ACG AAG ACA TCC CAC CAA TC-3′	1.5 mmol/l	57°C	35	733 bp
Reverse	5′-GCA CCA TCC AAA CCA CTG A-3′				
COL3A1					
Forward	5′-TGA AAG GAC ACA GAG GCT TCG-3′	2.0 mmol/l	57°C	35	532 bp
Reverse	5′-GCA CCA TTC TTA CCA GGC TC-3′				
IGF-I					
Forward	5′-TCG CAT CTC TTC TAC CTG GC-3′	1.0 mmol/l	55°C	40	202 bp
Reverse	5′-AGC AGC ACT CAT CCA CGA T-3′				
TBP*					
Forward	5′-CAG GAG CCA AGA GTG AAG AAC-3′	3.0 mmol/l	64°C	40	204 bp
Reverse	5′-AGG AAA TAA CTC TGG CTC ATA ACT ACT-3′				
CTGF*					
Forward	5′-CAC AGA GTG GAG CGC CTG TTC-3′	3.0 mmol/l	64°C	40	163 bp
Reverse	5′-GAT GCA CTT TTT GCC CTT CTT AAT G-3′				

*Primer sets used in real-time PCR.

tion. We propose that this mechanism might contribute to the hyperglycemia-induced tubulointerstitial ECM expansion in diabetic nephropathy.

RESEARCH DESIGN AND METHODS

Cell culture. The human renal fibroblast cell line TK173 (28) was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated FCS (ΔFCS), 100 IU/ml penicillin, and 100 μg/ml streptomycin (all purchased from Gibco/Invitrogen, Paisley, Scotland). TK173 cells expressing a truncated form of the TGF-β type II receptor (TK173-ΔTβRII) were previously described (8) and were maintained under the same conditions with the addition of hygromycin B (0.1 mg/ml; Boehringer Mannheim, Mannheim, Germany).

For experimental purposes, cells were seeded at a density of 50,000 cells/well in 48-well plates (Costar, Corning, NY), grown until confluence, and starved in DMEM with 0.5% ΔFCS. After 24 h, cells were cultured in 0.3 ml of DMEM containing 5.5 mmol/l D-glucose supplemented with 0.5% ΔFCS, 50 μg/ml ascorbic acid, and 100 μg/ml β-aminopropionitrile to promote collagen synthesis and prevent cross-links, respectively (both purchased at Sigma Chemical Co., St. Louis, MO). Cells were exposed to 25 mmol/l D-glucose (Merck, Darmstadt, Germany), 25 mmol/l L-glucose (Sigma), recombinant human TGF-β1 (R&D Systems, Abingdon, U.K.), recombinant human IGF-I (Roche Diagnostics, Mannheim, Germany), or recombinant human CTGF (FibroGen, South San Francisco, CA) at concentrations indicated. Blocking antibody studies were performed by the addition of a human neutralizing anti-CTGF monoclonal antibody (mAb; FibroGen) (29), a neutralizing pan-specific anti–TGF-β mAb (30), or a control IgG at the start of the culture. After 4 days, culture supernatants were harvested and measured for secreted proteins. Cells were trypsinized, and viable cells were counted using trypan blue exclusion.

CTGF enzyme-linked immunosorbent assay. CTGF was measured using a sandwich enzyme-linked immunosorbent assay (ELISA) specific for the NH_2-terminal part of CTGF (FibroGen). Microtiter plates were coated overnight at 4°C with capture antibody (10 μg/ml) in 100 μl of coating buffer (0.05 mol/l sodium bicarbonate, pH 9.6). After blocking the plates with 1% BSA in PBS for 1 h at room temperature and washing with PBS/0.05% Tween, a 50-μl sample was added to each well together with 50 μl of biotinylated monoclonal anti-human CTGF detection antibody diluted in assay buffer (0.05 mol/l Tris [pH 7.8], 0.1% BSA, 4 mmol/l $MgCl_2$, 0.2 mol/l $ZnCl_2$, 0.1% Na Azide, 50 mg/l sodium heparin, and 0.1% Triton X-100). After 2 h of incubation at 37°C, plates were washed and incubated with 100 μl of Streptavidin-conjugated alkaline phosphatase for 1 h at room temperature. The ELISA was developed with *p*-nitrophenyl phosphate (1.5 mg/ml; Sigma) in diethanolamine buffer (1 mol/l diethanolamine, 0.5 mmol/l $MgCl_2$, 0.02% Na azide) and read at an optical density (OD) of 405 nm. Purified recombinant human CTGF (FibroGen) was used as a standard.

Collagen type I and III inhibition ELISA. Collagen type I and III accumulation in supernatants was measured using a specific inhibition ELISA as previously described (8). Briefly, 96-well Nunc Maxisorb microtiter plates (Gibco/Invitrogen) were coated with 0.5 μg/ml human collagen type I or III (both purchased from Sigma) in PBS (100 μl/well) overnight at room temperature. Collagen type I or III standard or samples were preincubated overnight at 4°C with goat–anti-human collagen type I or goat–anti-human collagen type III, respectively (both purchased from Immunologicals Direct, Oxfordshire, U.K.) in PBS/0.05% Tween/2% casein (PTC). After a blocking step with PTC for 1 h at 37°C, 100 μl of preincubated standard or sample was added onto the plates and incubated at 37°C for 1 h. After washing the plates, a peroxidase-conjugated secondary rabbit–anti-goat IgG (Nordic Immunology, Tilburg, the Netherlands) was added and incubated for 1 h at 37°C. The ELISA was developed with 2,2′-amino-*bis*-3-ethylbenzthiazoline-6-sulfonic acid/H_2O_2 and read at OD 415 nm.

RNA isolation and semiquantitative RT-PCR. Total cellular RNA was extracted using RNAzolB (Campro Scientific, Veenendaal, Netherlands) according to the manufacturer's description. The quantity and the purity of the isolated RNA were measured at OD_{260} and OD_{280} and analyzed on a 0.5× TBE (0.045 mol/l Tris borate, 0.001 mol/l EDTA) 1% agarose gel to check the integrity of the RNA.

For RT-PCR, 1 μg of total RNA was reverse-transcribed into cDNA by oligo dT priming using Moloney Murine Leukemia Virus reverse transcriptase (all purchased from Invitrogen). The cDNA was amplified by PCR using primers as described in Table 1. Each PCR reaction was performed in a total volume of 40 μl, containing 10 mmol/l Tris.Cl (pH 8.3), 50 mmol/l KCl, $MgCl_2$ (see Table 1 for concentrations), 0.06 mg/ml BSA, 0.25 mmol/l dNTPs, 25 pmol forward primer, 25 pmol reverse primer (all primers were purchased from Invitrogen), and 0.8 units AmpliTaq (Perkin Elmer, Foster City, CA). Amplification of cDNA started with 5 min of denaturation at 95°C, followed by PCR cycles of 95°C for 1 min, subsequent annealing for 1 min at a primer-specific temperature, and 72°C for 1 min (the number of cycles and the annealing temperatures are summarized in Table 1). The final extension was performed at 72°C for 5 min. All PCR reactions were performed in a PTC-200 DNA engine (MJ Research, Waltham, MA) thermal cycler. PCR products were analyzed on a 0.5× TBE 1% agarose gel. The intensity of bands was determined by densitometry, using EagleSight software (Stratagene, La Jolla, CA).

Real-time PCR. Real-time PCR was performed in a Roche LightCycler using 1 μl of undiluted cDNA as template in a total reaction volume of 10 μl, containing 0.5 μmol/l of each forward and reverse primer (Table 1), 3 mmol/l $MgCl_2$, and 1 μl of FastStart DNA Master SYBR Green I reagent (all purchased from Roche Diagnostics). The temperature profile was as follows: an initial denaturation step for 6 min at 95°C, followed by 40 amplification cycles, each consisting of 15 s at 95°C, annealing for 5 s at 64°C, and elongation for 10 s at 72°C, each of these steps with ramp rates of 20°C/s. SybrGreen fluorescence was measured at the end of each elongation phase. Immediately after the last amplification cycle, a melting curve was recorded as follows: after denatur-

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ation at 95°C and rehybridization during 15 s at 65°C, the samples were slowly heated to 95°C with a ramp rate of 0.1°C/s with continuous acquisition of SybrGreen fluorescence. One sample was used in each PCR experiment as a standard, of which the cDNA was used at 2-, 4-, 8-, and 16-fold dilutions. The mRNA levels of TBP, encoding for TATA-box binding protein, were measured as a reference to correct for variable input.

Immunoprecipitation and Western blot analysis. Anti-CTGF mAb-coated Sepharose beads were incubated with CTGF for 1 h at 37°C. After three washing steps with PBS, IGF-I was added for 2 h at 37°C. As controls, either CTGF or IGF-I was incubated with anti-CTGF mAB-coated Sepharose beads for 1 h at 37°C only, without the secondary incubation step. After the last incubation step, Sepharose beads were washed again three times with PBS. The beads were resuspended in 20 μl of PBS, and bound proteins were denatured in 3× SDS sample buffer (New England Biolabs, Beverly, MA) by boiling and separated under reducing conditions by SDS-PAGE. The proteins were semidry electroblotted on polyvinylidene fluoride membranes (Immobilon-p; Millipore, Bedford, MA). Membranes were blocked with PTC and then probed with a biotinylated anti-human IGF-I antibody (R&D Systems). After incubation with peroxidase-conjugated streptavidin (Zymed Laboratories, San Francisco, CA), detection was performed with Supersignal (Pierce) and the blots were exposed to Hyperfilm films (Amersham Pharmacia Biotech, Buckinghamshire, U.K.).

Statistical analysis. The data are presented as means ± SD of triplicate cultures and are representative of at least three independent experiments. Differences between various culture conditions were evaluated by ANOVA with a Bonferroni correction for multiple comparison. In some experiments, relative differences were tested with a one-sample t test. A value of $P < 0.05$ was considered to represent a significant difference.

RESULTS

Induction of collagens in human renal fibroblasts by high glucose and TGF-β1. Exposure of human renal fibroblasts to hyperglycemic conditions resulted in the upregulation of the mRNA expression of both the α1 subunit of collagen type I (COL1A1) and type III (COL3A1; Fig. 1*A*). This increase was not due to the higher osmolarity to which the cells were exposed because there were no effects on the COL1A1 and COL3A1 mRNA expression when cells were exposed to 25 mmol/l L-glucose. Exposure to the profibrotic cytokine TGF-β1 also increased COL1A1 mRNA expression. In contrast, COL3A1 mRNA expression was not upregulated by TGF-β1.

The increased transcription of COL1A1 mRNA by high glucose and exogenous TGF-β1 was reflected by an increase in collagen type I protein secretion (Fig. 1*B*, left). Collagen type III protein secretion was increased by high glucose but not by TGF-β1 (Fig. 1*B*, right), thus following the same pattern as the induction of COL3A1 mRNA expression. Because collagen type III can be induced by glucose independent of TGF-β1, we also investigated whether TGF-β was involved in glucose-induced collagen type I secretion by renal fibroblasts. For this purpose, neutralizing anti–TGF-β antibodies were added to fibroblast cultures, which completely inhibited the stimulatory effect of TGF-β1 on collagen type I secretion. In contrast, glucose-induced collagen type I was not attenuated (Fig. 1*C*). In addition, TK173-ΔTβRII was exposed to either TGF-β1 or high glucose. We recently demonstrated that glucose-induced collagen type III was not affected in these transfected cells (8). Now we also show that collagen type I secretion induced by high glucose was not affected in TK173-ΔTβRII, whereas the effect of TGF-β1 was completely abolished (Fig. 1*D*). Thus, glucose-induced upregulation of collagens type I and III in human renal fibroblasts seems to be independent of endogenous TGF-β1. This observation prompts the question of which alternative growth factors might be involved.

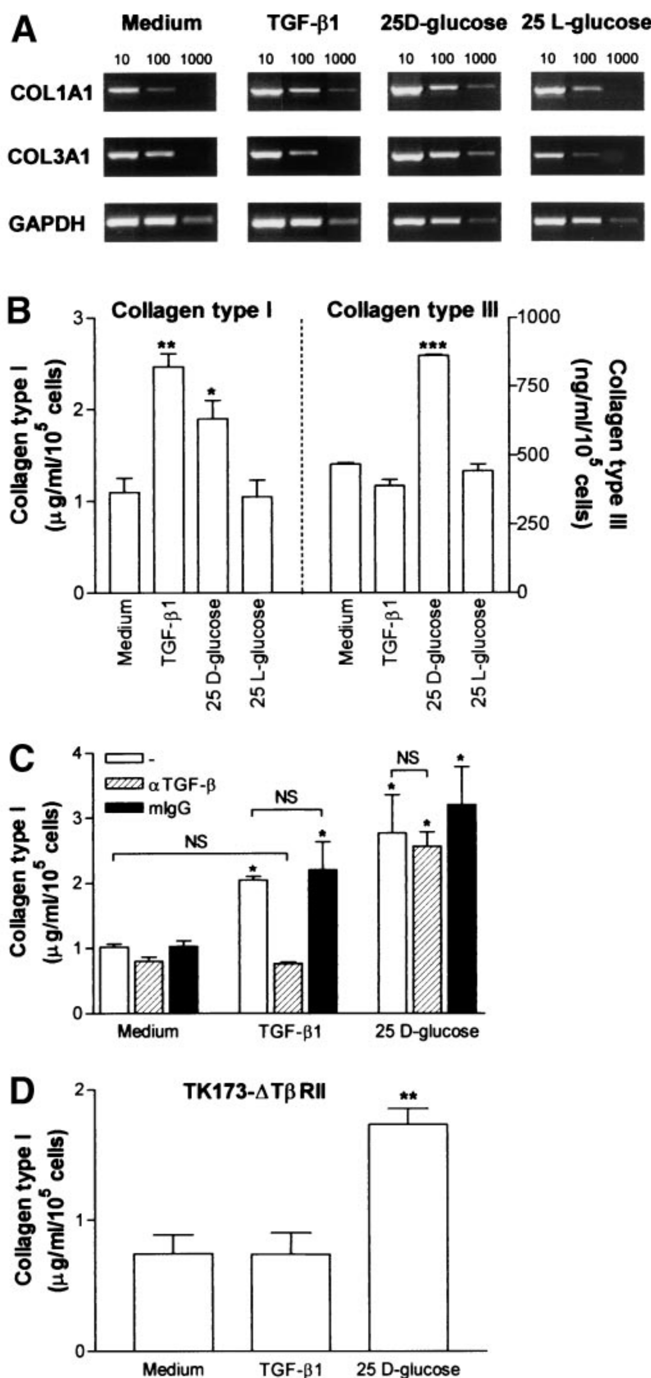

FIG. 1. Glucose-induced collagens type I and III in human renal fibroblasts. Cells were cultured with medium containing 5.5 mmol/l, 25 mmol/l D-, or 25 mmol/l L-glucose, or TGF-β1 (1 ng/ml). *A*: After 3 days of stimulation, total RNA was isolated from TK173 cells and a semi-quantitative RT-PCR was performed for the α1 subunit of collagen type I (COL1A1) and III (COL3A1) and GAPDH using cDNA titration (10-, 100-, and 1,000-fold dilutions). Gels shown are representative of four independent experiments. For the measurement of collagen type I and III protein in a specific inhibition ELISA, culture supernatants were harvested after 4 days of stimulation. *B*: Secretion of collagen type I and III protein, respectively, by TK173 wild-type cells. *C*: Collagen type I protein secretion by TK173 wild-type cells in the presence of a neutralizing pan-specific anti–TGF-β antibody (20 μg/ml) or a control IgG (20 μg/ml). *D*: Collagen type I protein secretion by human renal fibroblasts expressing a dominant negative TβRII. Results are expressed as mean ± SD of triplicate cultures and representative of five independent experiments. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ compared with medium control.

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High glucose increases CTGF levels in renal fibroblasts. CTGF has been demonstrated to be an important protein in mediating fibrotic processes (18,31,32). Because it has been demonstrated that CTGF is induced in mesangial cells under hyperglycemic conditions (14–16), we investigated the regulation of CTGF expression in renal fibroblasts. Stimulation with TGF-β1, a known inducer of CTGF, resulted in an increase of CTGF mRNA expression as shown by real-time PCR (Fig. 2*A*, left). Also, exposure to high glucose resulted in an increased CTGF expression, however, with altered kinetics. Although TGF-β1 showed an early response at 24 h, glucose-induced CTGF expression became apparent only after 72 h of stimulation (Fig. 2*A*, right).

We next investigated the regulation of CTGF at the protein level. Stimulation with either TGF-β1 or high glucose resulted in increased secretion of CTGF protein (Fig. 2*B*). Furthermore, when fibroblasts were incubated with TGF-β1 in medium containing high glucose, an additive increase in CTGF protein secretion was observed. This suggestion that TGF-β1 and glucose are independent stimulators of CTGF expression was confirmed by the induction of CTGF in TK173-ΔTβRII upon exposure to glucose (Fig. 2*C*).

The role of CTGF in glucose-induced collagen secretion by renal fibroblasts. Because CTGF was increased in renal fibroblasts exposed to high glucose, the role of endogenous CTGF in glucose-induced collagen secretion was investigated by blocking the effect of CTGF. Addition of a neutralizing anti-CTGF mAb to fibroblast cultures had no effect on the basal levels of collagen secretion. However, when this neutralizing antibody was added to high-glucose cultures, the effect of high ambient glucose on collagen type I secretion was partially inhibited (49.6 ± 23.0%; Fig. 3, left). In addition, glucose-induced collagen type III secretion by renal fibroblasts was partially blocked (53.5 ± 1.8% inhibition; Fig. 3, right). These data demonstrate that endogenous CTGF partially mediates glucose-induced collagen type I and III secretion in human renal fibroblasts.

It is interesting that addition of exogenous recombinant CTGF at a concentration of 50 ng/ml to normoglycemic cultures had no effect on collagen type I and III levels (Fig. 3). Moreover, exposure of renal fibroblasts to CTGF at concentrations ranging from 12.5 to 200 ng/ml had no effect on either collagen (Fig. 4). These results suggest that CTGF might exert its effects on collagen type I and III accumulation only in combination with another factor that seems to be absent in normoglycemic cultures of fibroblasts.

Effects of IGF-I on collagen secretion and its induction by glucose. IGF-I seems to be an interesting candidate because it can be induced by hyperglycemia (33), it can function independent of TGF-β1 (34), and a functional collaboration with CTGF has been shown in rat fibroblasts (17). In nonstimulated fibroblasts, there were no detectable levels of IGF-I mRNA. However, IGF-I mRNA expression was induced in fibroblasts upon stimulation with either high glucose or TGF-β1 (Fig. 5*A*). In TK173-ΔTβRII cells, the TGF-β1–induced upregulation of IGF-I mRNA expression was completely inhibited, whereas the glucose-induced increase in IGF-I was still intact. Therefore, glucose-induced IGF-I seemed to be independent of TGF-β signaling.

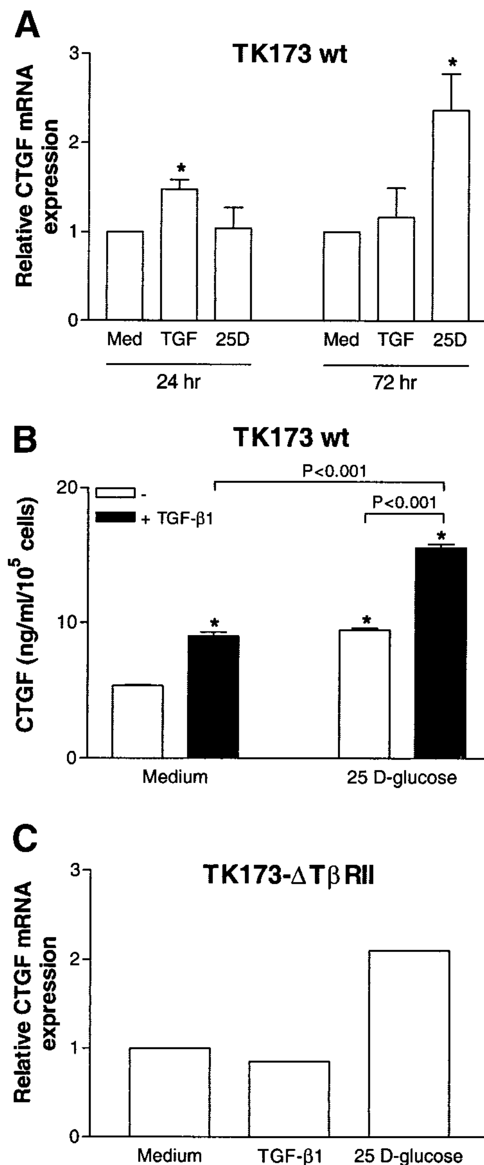

FIG. 2. CTGF mRNA and protein expression by human renal fibroblasts. Cells were cultured with medium containing 5.5 mmol/l (Med), 25 mmol/l D-glucose (25D), or TGF-β1 (1 ng/ml) (TGF). *A*: After 24 and 72 h of stimulation, total RNA was isolated from TK173 wild-type cells and reverse-transcribed into cDNA for the measurement of CTGF in a real-time PCR, using TBP as a reference to correct for variable input. CTGF mRNA expression levels were calculated relative to those of TBP. Measurements of ratios in normal glucose media without supplements were assigned a relative value of 1, representing control values. Results are expressed as mean ± SE of four independent experiments; $*P < 0.05$ compared with the medium control of the same time point. *B*: After 4 days of stimulation, culture supernatants were harvested and CTGF protein levels in TK173 wild-type cells were measured in a specific ELISA. Results are expressed as mean ± SD of triplicate cultures and are representative of three independent experiments; $*P < 0.001$ compared with medium control. *C*: TK173-ΔTβRII were stimulated with normal medium, TGF-β1 (1 ng/ml), or 25 mmol/l D-glucose for 72 h; total RNA was isolated and reverse-transcribed into cDNA for the measurement of CTGF in a real-time PCR, using TBP as a reference.

Next, fibroblasts were stimulated with recombinant IGF-I to investigate the effect on collagen type I and III secretion. Collagen type I secretion was not affected by IGF-I (Fig. 5*B*). In contrast, collagen type III was already

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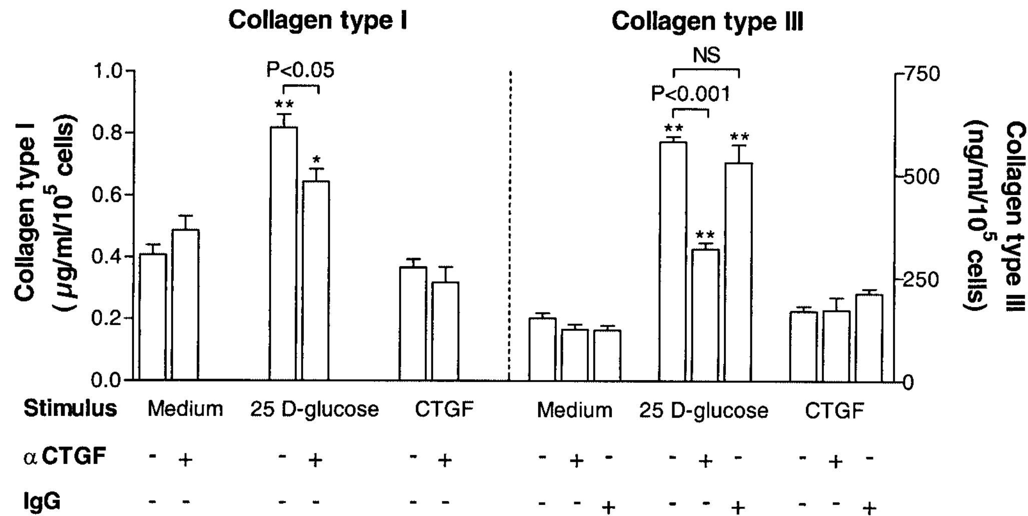

FIG. 3. Blockade of CTGF using a neutralizing antibody. TK173 cells were incubated with 5.5 mmol/l glucose (normal medium), 25 mmol/l D-glucose, or CTGF (50 ng/ml), with or without the addition of a neutralizing anti-CTGF mAb (10 μg/ml) or a control IgG (10 μg/ml). After 4 days of culture, supernatants were harvested and collagens type I and III were measured in a specific inhibition ELISA. Results are expressed as mean ± SD of triplicate cultures and are representative of three independent experiments. *$P < 0.01$ and **$P < 0.001$ compared with medium control.

significantly and strongly upregulated by IGF-I at a concentration of 6.25 ng/ml, reaching a maximum at 25 ng/ml (Fig. 5*C*).

CTGF and IGF-I act synergistically in the induction of collagens in renal fibroblasts. Next, fibroblasts were stimulated with a combination of CTGF and IGF-I. Although both CTGF and IGF-I individually had no effects on collagen type I accumulation, the combination of both factors resulted in a synergistic induction (Fig. 6*A*, left). Moreover, collagen type III secretion was further enhanced by the addition of CTGF and IGF-I (Fig. 6*B*, left).

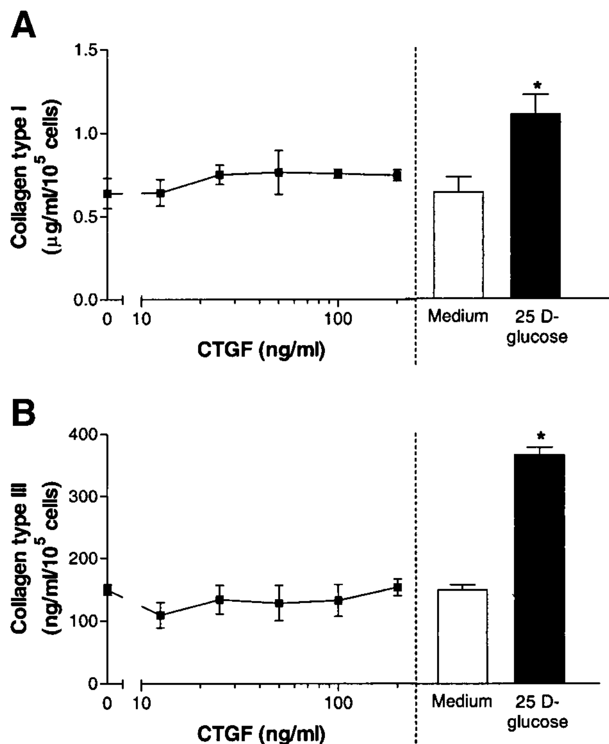

FIG. 4. Effect of exogenous CTGF on collagen secretion by renal fibroblasts. TK173 cells were stimulated with increasing doses of CTGF in the concentrations 12.5–200.0 ng/ml or with 25 mmol/l D-glucose. After 4 days, culture supernatants were harvested, and collagen type I (*A*) and type III (*B*) were measured in a specific inhibition ELISA. Results are expressed as mean ± SD of triplicate cultures and are representative of three independent experiments. *$P < 0.001$ compared with medium control.

Because CTGF and IGF-I have synergistic effects on collagen secretion in normoglycemic fibroblast cultures and both growth factors are induced upon glucose stimulation, the effects of CTGF and IGF-I were also investigated under hyperglycemic conditions. Stimulation with CTGF in high-glucose medium did not result in a further increase in collagen type I and III secretion compared with incubations with high glucose alone (Fig. 6*A* and *B*, right). In contrast, exposure to combinations of IGF-I and high glucose resulted in enhanced collagen type I and III secretion when compared with the individual stimuli. The synergistic effects of CTGF and IGF-I on collagen secretion were not observed under hyperglycemic conditions. This was probably because plateau levels were reached in these conditions. When lower concentrations of growth factor were used, which have only marginal effects under normoglycemic conditions, there was indeed an enhanced secretion of collagens type I and III under hyperglycemic conditions (data not shown).

Mechanism of synergy between CTGF and IGF-I. To try to elucidate the mechanism of the synergy between CTGF and IGF-I, we examined the effect of these growth factors on each other's expression. CTGF mRNA expression by renal fibroblasts was decreased by IGF-I, even in the presence of exogenous CTGF (Fig. 7*A*). The downregulation in CTGF mRNA was reflected by a decreased secretion of CTGF protein by renal fibroblasts (Fig. 7*B*). Although CTGF protein secretion was slightly decreased by exogenous CTGF (Fig. 7*B*), this was not reflected by a negative feedback on its own mRNA expression (Fig. 7*A*). In addition, IGF-I mRNA expression was measured in renal fibroblasts after CTGF and IGF-I stimulation. Stimulation with either growth factor did not have any clear effects on IGF-I mRNA expression (data not shown).

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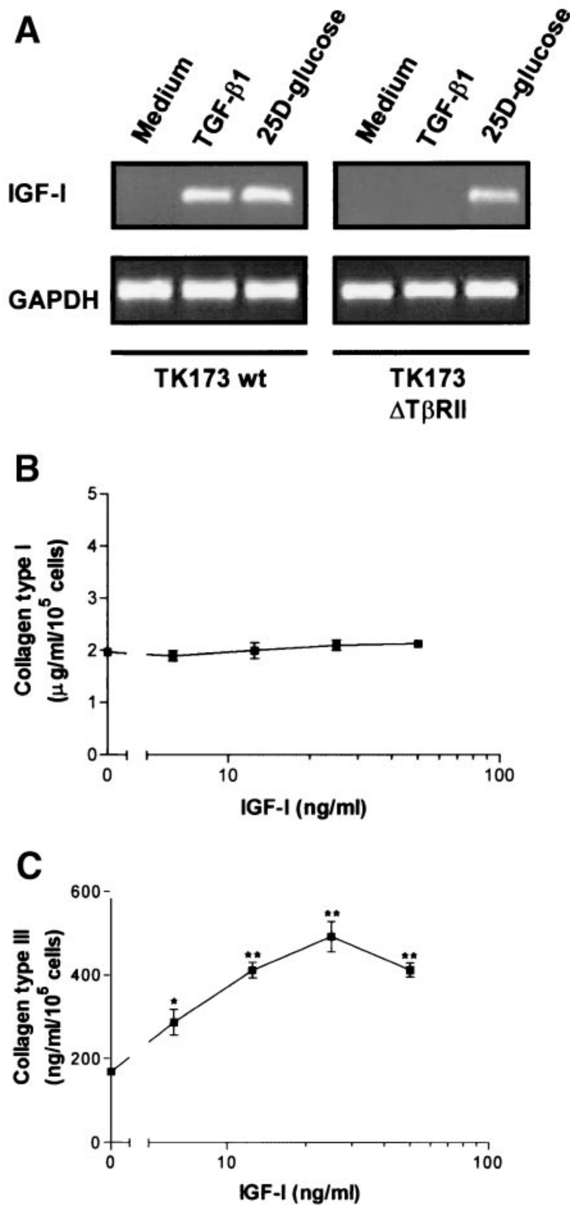

FIG. 5. IGF-I mRNA expression in renal fibroblasts and its effect on collagen accumulation. *A*: TK173 wild-type and TK173 expressing a dominant negative TβRII were stimulated with normal medium, TGF-β1 (1 ng/ml), or 25 mmol/l D-glucose. After 3 days of stimulation, total RNA was isolated and reverse-transcribed and analyzed for IGF-I (undiluted cDNA) and GAPDH (1,000-fold diluted cDNA) mRNA expression. Gels shown are representative of three independent experiments. *B* and *C*: TK173 cells were stimulated with increasing doses of IGF-I in the concentrations 6.25–50.0 ng/ml. After 4 days, culture supernatants were harvested and collagen type I and type III were measured in a specific inhibition ELISA. Results are expressed as mean ± SD of triplicate cultures and are representative of three independent experiments. *$P < 0.05$; **$P < 0.001$ compared with medium control.

These results suggest that the synergistic effects of CTGF and IGF-I are not mediated by the increase in endogenous CTGF and IGF-I.

Another possible mechanism of the synergy between CTGF and IGF-I might be the direct binding between the two growth factors. With the use of anti-CTGF mAb-coated Sepharose beads, the captured CTGF was able to bind IGF-I, which was demonstrated in an IGF-I Western blot (Fig. 7*C*). When only CTGF or IGF-I was incubated with the Sepharose beads, no IGF-I bands could be detected. These results confirm that CTGF has the ability to bind IGF-I.

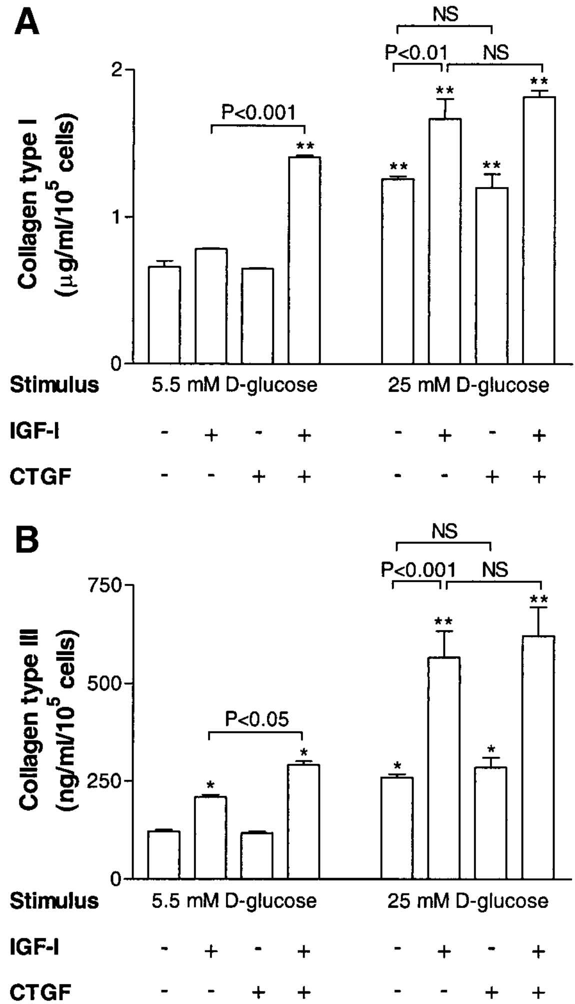

FIG. 6. CTGF and IGF-I are synergistic in the induction of collagens in renal fibroblasts. TK173 cells were stimulated with normal medium or IGF-I (25 ng/ml) in combination with or without CTGF (50 ng/ml) under normo- and hyperglycemic conditions. After 4 days, culture supernatants were harvested and collagen type I (*A*) and type III (*B*) were measured in a specific inhibition ELISA. Results are expressed as mean ± SD of triplicate cultures and are representative of three independent experiments. *$P < 0.05$; **$P < 0.001$ compared with medium control.

DISCUSSION

In the present study, we investigated the role of CTGF and IGF-I in the induction of collagen type I and III secretion by high glucose in human renal fibroblasts. We have demonstrated that both CTGF and IGF-I expression are increased in human renal fibroblasts cultured under hyperglycemic conditions, also in the absence of TGF-β signaling. Exogenous IGF-I had a pronounced effect on the secretion of collagen type III but not on collagen type I. Although CTGF alone had no effect on the expression of either collagen type I or type III, when combined with IGF-I, an enhanced induction of both matrix components was observed. Furthermore, glucose-induced collagen accumulation was partially blocked by neutralizing anti-CTGF antibodies and further enhanced by exogenous

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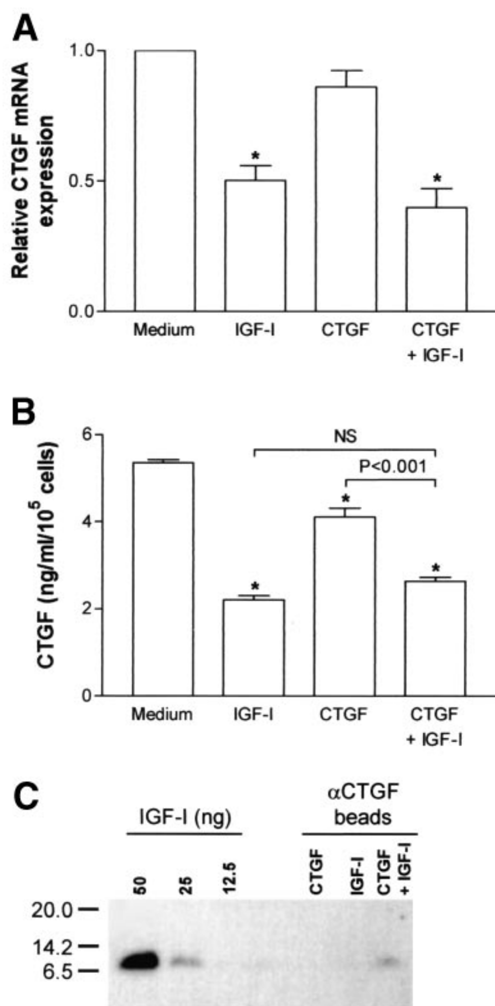

FIG. 7. Mechanism of synergy between CTGF and IGF-I. Cells were cultured with normal medium containing CTGF (50 ng/ml) and/or IGF-I (25 ng/ml). *A*: After 24 h of stimulation, total RNA was isolated from TK173 wild-type cells and reverse-transcribed into cDNA for the measurement of CTGF in a real-time PCR, using TBP as a reference to correct for variable input. CTGF mRNA expression levels were calculated relative to those of TBP. Measurements of ratios in normal glucose media without supplements were assigned a relative value of 1, representing control values. Results are expressed as mean ± SE of three independent experiments. **$P < 0.001$ **compared with the medium control of the same time point. *B*: After 4 days of stimulation, culture supernatants were harvested and CTGF protein levels in TK173 wild-type cells were measured in a specific ELISA. Results are expressed as mean ± SD of triplicate cultures and are representative of three independent experiments. **$P < 0.02$ **compared with medium control. *C*: Anti-CTGF mAb-coated Sepharose beads were incubated with CTGF (0.4 μg) and/or IGF-I (2 μg). Bound proteins were separated by SDS-PAGE and probed with biotinylated anti–IGF-I. As controls, recombinant IGF-I (12.5, 25, 50 ng) was run.

IGF-I. These results indicate a role for both CTGF and IGF-I in glucose-induced matrix accumulation by human renal fibroblasts, which might involve the cooperation between CTGF and IGF-I, a mechanism that might contribute to the pathogenesis of diabetic nephropathy.

One of the most potent mediators of fibrosis is undoubtedly TGF-β, which has been studied extensively. It has been shown that TGF-β plays a central role in the development of diabetic nephropathy (7). In the present study, we have shown that glucose-induced collagens type I and III can occur independent of endogenous TGF-β1. Our finding seems to be in contrast to the findings by Han et al. (35), who demonstrated that glucose-induced collagen type I was TGF-β–mediated in murine fibroblasts. These results were obtained using fibroblasts from mice, whereas we used fibroblasts from human origin, which might contribute to the observed differences. More recently, other growth factors, such as CTGF and IGF-I, have also been implicated to contribute to the pathogenesis of diabetic nephropathy (9–11). Previous studies have shown that CTGF expression was increased in various models of experimental diabetic kidney disease (15–17), as well as in human diabetic nephropathy (18). Concerning IGF-I, a correlation between increased renal IGF-I levels and diabetic nephropathy has been found (23–26).

Recently, we demonstrated a TGF-β1–independent mechanism of fibronectin and collagen type III upregulation in human renal fibroblasts by high glucose, in a process that might involve alternative growth factors (8). In the present study, we have shown that high glucose increased CTGF expression in renal fibroblasts. Moreover, using fibroblasts expressing a dominant negative TGF-β type II receptor, we could demonstrate TGF-β–independent CTGF induction. Several groups have reported the involvement of CTGF in glucose-induced ECM expression in mesangial cells (14–16). We now demonstrate for the first time that endogenous CTGF is also involved in glucose-induced collagen accumulation by human renal fibroblasts because this effect can be partially blocked by neutralizing anti-CTGF antibodies. However, surprisingly, we could not observe any effects of recombinant CTGF on collagen production by renal fibroblasts. This finding is in agreement with a previous study that showed no effect of recombinant CTGF on collagen type III mRNA expression in NRK cells (17), although others have found direct effects of CTGF on ECM accumulation by fibroblasts from other origins (31,36,37). Next to CTGF, we have identified IGF-I as a growth factor involved in the regulation of matrix accumulation by renal fibroblasts. We have demonstrated that IGF-I mRNA expression was increased in renal fibroblasts by high glucose, independent of endogenous TGF-β. In addition, IGF-I is a potent inducer of collagen type III secretion, whereas collagen type I was not affected. The production of fibronectin and collagen type IV was also not affected by IGF-I (data not shown). In addition, in these cells, TGF-β1 could not induce collagen type III under conditions in which collagens type I and IV and fibronectin were increased. This suggests that the requirement of growth factors for the induction of collagen type III is different from other ECM components. Whether there is a relation between the increase in collagen type III in response to IGF-I and the absence of a stimulatory effect of TGF-β is at present unknown.

Recently, it was demonstrated that collagen type III mRNA expression was synergistically upregulated by CTGF and IGF-I in NRK fibroblasts (17). We now demonstrate that CTGF and IGF-I functionally cooperate in the secretion of both collagens type I and III in human renal fibroblasts, although the mechanism is at present unknown. One possible mechanism is via the induction of autocrine growth factors. However, we have found that neither CTGF nor IGF-I was increased in renal fibroblasts upon stimulation with CTGF and/or IGF-I. On the contrary, IGF-I stimulation resulted in a decreased secretion of

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CTGF, even in the presence of exogenous CTGF. Therefore, we conclude that it is unlikely that the induction of either growth factor is involved in the enhanced effects of CTGF and IGF-I. Nevertheless, at present, we cannot exclude the involvement of alternative growth factors. Another possible mechanism for the functional collaboration between CTGF and IGF-I might be via a direct binding between both growth factors. CTGF has also been designated as IGF-binding protein (IGFBP)-related protein 2 (38) or IGFBP-8 (39) because it has the capacity to bind IGF-I via its IGF-binding domain, albeit with relatively low affinity compared with classical IGFBPs (39,40). In the present study, we were able to demonstrate this interaction between IGF-I and CTGF by immunoprecipitation followed by Western blotting. Although the potential of IGF-I to bind to CTGF has been discovered for some years now, there is still little information about the biological consequence of this interaction. It is known that the bioavailability of IGF-I is modulated by classical IGFBPs, which can either potentiate or inhibit IGF-I (41,42). Therefore, it is possible that the underlying mechanism of the functional cooperation between the two growth factors might be through increased bioavailability of IGF-I when it is bound to CTGF. The modulating action of CTGF on growth factors is not limited to IGF-I because it has been demonstrated recently that it can also promote TGF-β1 signaling but inhibits the action of BMP4 (43). In addition, CTGF can inhibit vascular endothelial growth factor–induced angiogenesis (44). Taken together, CTGF might function as a cofactor for other growth factors, thereby modulating the biological activity of these growth factors. However, more study on the molecular and biological level is needed to unravel the precise pathways involved in the functional collaboration between CTGF and other growth factors.

Apart from the effect of hyperglycemic conditions on CTGF and IGF-I production and the cooperation between CTGF and IGF-I in collagen type I and III accumulation, we have also investigated the effect of these growth factors under hyperglycemic conditions. We have demonstrated that IGF-I and not CTGF was able to further enhance glucose-induced collagen secretion. These data suggest that induction of IGF-I, not CTGF, is the rate-limiting step in the glucose-induced matrix accumulation. Therefore, it will be important to delineate further the molecular mechanism of the CTGF-IGF-I interactions.

In conclusion, this study demonstrates that CTGF and IGF-I cooperate in their upregulation of collagen type I and III expression in human renal fibroblasts. The synergy between CTGF and IGF-I might be involved in glucose-induced matrix accumulation, because both factors are induced by hyperglycemia.

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