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The Roles of Connective Tissue Growth Factor and Integrin-Linked Kinase in High Glucose-Induced Phenotypic Alterations of Podocytes

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ABSTRACT

Emerging evidence has suggested that podocytes undergo epithelial–mesenchymal transition (EMT) in diabetic nephropathy (DN). Connective tissue growth factor (CTGF) and integrin-linked kinase (ILK) are involved in the progression of DN. However, the underlying mechanisms of EMT are not well understood. The study aimed to investigate the roles of CTGF and ILK in high glucose-induced phenotypic alterations of podocytes and determine whether ILK signaling is downstream of CTGF. The epithelial marker of nephrin and the mesenchymal marker of desmin were investigated by real-time RT-PCR and Western blotting. The results demonstrated that podocytes displayed a spreading, arborized morphology in normal glucose, whereas they had a cobblestone morphology in high glucose conditions, accompanied by decreased nephrin expression and increased desmin expression, suggesting podocytes underwent EMT. In response to high glucose, CTGF and ILK expression in podocytes were increased in a dose- and time-dependent manner, whereas the increase did not occur in the osmotic control. Furthermore, the inhibition of CTGF with anti-CTGF antibody prevented the phenotypic transition, as demonstrated by the preservation of epithelial morphology, the suppression of high glucose-induced desmin overexpression and the restoration of nephrin. Of note, the upregulation of ILK induced by high glucose was partially blocked by the inhibition of CTGF. In summary, these findings suggested that CTGF and ILK were involved in high glucose-induced phenotypic alterations of podocytes. ILK acted as a downstream kinase of CTGF and high glucose-induced ILK expression might occur through CTGF-dependent and -independent pathways. J. Cell. Biochem. 113: 293–301, 2012. © 2011 Wiley Periodicals, Inc.

KEY WORDS: CONNECTIVE TISSUE GROWTH FACTOR; INTEGRIN-LINKED KINASE; PHENOTYPIC ALTERATIONS; PODOCYTE; HIGH GLUCOSE

Diabetic nephropathy (DN) is the leading cause of end-stage renal disease worldwide. However, the mechanisms underlying its pathogenesis are not completely understood. It is well known that the podocyte plays a critical role in the progression of DN [Wolf et al., 2005; Shankland, 2006; Li et al., 2007; Jefferson et al., 2008; Reddy et al., 2008]. Recently, accumulating evidence has suggested that podocytes undergo epithelial–mesenchymal transition (EMT) in many diseases including DN, which causes proteinuria and ultimately results in kidney fibrosis [Li et al., 2008; Reidy and Susztak, 2009; Yamaguchi et al., 2009; Liu, 2010]. EMT is the phenotypic transition from polarized epithelial cells to cells with spindle-shaped mesenchymal morphology [Kalluri and Neilson, 2003; Burns and Thomas, 2010]. During EMT, podocytes lose epithelial markers such as nephrin, P-cadherin and zonula occludens-1, a process that is accompanied by the development of mesenchymal features, such as desmin, fibroblast-specific protein-1, and matrix metalloproteinase-9 [Li et al., 2008].

Several molecules are thought to participate in the progression of glomerular injury in DN. Among them, TGF-β has been most extensively studied. Both TGF-β and connective tissue growth factor (CTGF) play key roles in the pathogenesis of DN. However, it is important to note that blockade of TGF-β did not attenuate proteinuria in db/db mice despite its beneficial effects on mesangial matrix expansion and renal function [Ziyadeh et al., 2000]. Because TGF-β also has important anti-proliferative and anti-inflammatory effects on other systems, CTGF has been suggested to be an alternative target for therapeutic intervention. Generally, CTGF is considered to be downstream of TGF-β, playing an important role

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via TGF-β-dependent and -independent pathways in the progression of DN [Murphy et al., 1999; Ziyadeh et al., 2000; Li et al., 2004; Zhou et al., 2004; Guha et al., 2007; Schmidt-Ott, 2008; Mason, 2009; Chung et al., 2010]. Moreover, data from experimental models have suggested that the inhibition of CTGF could attenuate proteinuria [Guha et al., 2007]. Of note, increasing evidence has suggested that CTGF is also involved in EMT of renal tubular epithelial cells [Chen et al., 2006; Liu et al., 2006, 2008; Burns and Thomas, 2010]. In murine models of DN, CTGF overexpression was predominantly localized in visceral podocytes within the glomeruli, which suggested a potential role for CTGF in the development of podocyte injury in diabetes [Roestenberg et al., 2006]. Again, a study in podocyte-specific CTGF-transgenic mice provided further in vivo evidence for the role of CTGF in the development of podocyte injury and mesangial expansion in diabetes [Yokoi et al., 2008]. However, the role of CTGF in the EMT of podocytes remains unclear.

Integrin-linked kinase (ILK) is an intracellular serine/threonine kinase that interacts with integrins and is involved in the regulation of a number of integrin-mediated processes, including cell adhesion, cell shape changes, gene expression and deposition of the extracellular matrix [Wu and Dedhar, 2001], which is essential to the normal function of glomerular basement membrane (GBM) and podocytes [Blattner and Kretzler, 2005; Teixeira Vde et al., 2005; Dai et al., 2006; El-Aouni et al., 2006]. It has been reported that ILK overexpression is involved in diabetes [Guo et al., 2001; Han et al., 2006; Chen et al., 2008]. Additionally, ILK induces EMT in DN, and the inhibition of ILK ameliorates proteinuria or attenuates EMT [Kang et al., 2010].

Our previous studies showed that CTGF directly induced the expression of ILK in HK-2 cells through ERK1/2 and PI3-K-dependent signaling pathways. In addition, the inhibition of ILK by siRNA attenuated CTGF-induced EMT [Liu et al., 2007, 2008]. However, in diabetes, the role of CTGF in mediating ILK expression and high glucose-induced phenotypic alterations of podocytes has not been well elucidated. In this study, we aimed to investigate the role of CTGF and ILK in high glucose-induced phenotypic alterations of podocytes and determine whether ILK signaling is downstream of CTGF.

MATERIALS AND METHODS

CELL CULTURES

Conditionally immortalized mouse podocytes were kindly provided by Dr. Peter Mundel (Albert Einstein College of Medicine, Bronx, NY, USA) and were cultured as described previously [Mundel et al., 1997]. Briefly, to propagate the podocytes, cells were cultured at 33°C in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS, Gibco, USA), 10 U/ml mouse recombinant IFN-γ (Sigma, USA) and 100 U/ml of penicillin/streptomycin in type I collagen-coated flasks. To induce differentiation, podocytes were grown under non-permissive conditions at 37°C in the absence of IFN-γ for 10 days.

EXPERIMENTAL DESIGN

Differentiated podocytes were cultured for 24 h in a medium containing 5 mmol/L D-glucose and 1% FBS before being exposed to the various experimental conditions. To study the effect of different concentrations, the podocytes were cultured in a medium containing 5, 15, and 30 mmol/L D-glucose for 24 h. To study the effect of time, podocytes were exposed to high glucose levels (30 mmol/L) for 0, 6, 12, 24, and 48 h. Some of the cells were preincubated with anti-CTGF antibody (G-14, Santa Cruz, USA, 10 μg/ml) for 2 h and then incubated under high glucose conditions for another 24 h, as described in a previous study [Zhou et al., 2004]. As an osmotic control, podocytes were cultured in medium containing 5 mmol/L D-glucose, and 25 mmol/L D-mannitol. Cells were then collected for subsequent analyses.

IMMUNOCYTOCHEMISTRY

After being fixed in 4% paraformaldehyde for 30 min, differentiated podocytes were permeabilized with 0.5% Triton X-100 in PBS for 30 min and blocked with 1% BSA for 1 h. The cells were then incubated with rabbit anti-WT-1 (1:100) or rabbit anti-desmin (1:100, both from Santa Cruz) overnight at 4°C. After washing with PBS, the cells were incubated with FITC-conjugated goat anti-rabbit IgG (1:100, Santa Cruz) for 1 h at 37°C. The cell nuclei were counterstained with DAPI. A negative control was performed using the same concentration of normal IgG instead of the primary antibody, and no staining occurred. The pictures were captured with a Zeiss confocal microscope (Carl Zeiss, Gena, Germany).

REAL-TIME RT-PCR

Total RNA was extracted using the RNAiso plus reagent, and cDNA was synthesized using a reverse transcription system kit according to the manufacturer's instructions (Takara, Japan). Real-time RT-PCR was performed using an ABI PRISM 7300 real-time PCR System (Applied Biosystems, USA). The following primer pairs were used: CTGF, forward 5′-CTG CCT ACC GAC TGG AAG-3′, reverse 5′-GAA GGT ATT GTC ATT GGT AAC TC-3′; ILK, forward 5′-CAT CAA TGC AGT GAA TGA GC-3′, reverse 5′-GAC ATT CCT CAT TGA AGT CC-3′; nephrin, forward 5′-CCC AGG TAC ACA GAG CAC AA-3′, reverse 5′-CTC ACG CTC ACA ACC TTC AG-3′; desmin, forward 5′-TGC AGC CAC TCT AGC TCG TA-3′, reverse 5′-GAC ATG TCC ATC TCC ACC TG-3′; α3 integrin, forward 5′-CTC ATC ATC CTC CTC TTG TG-3′, reverse 5′-TCA GTA GTC GTC GGT CAG-3′; β1 integrin, forward 5′-AGT AGA GGT CGT TCT TCAG-3′, reverse 5′-TCT TCA CTG TTC ACT TCA TC-3′; β-actin, forward 5′-CCTCTATGCCAACACAGTGC-3′, reverse 5′-GTA CTC CTG CTT GCT GAT CC-3′. The cycling condition was performed using a two-step process: 95°C for 30 s, 40 cycles at 95°C for 5 s and 60°C for 31 s. PCR without the cDNA template was used as a negative control. All measurements were performed in duplicate. The results were analyzed using the [Delta][Delta]Ct technique, as previously described [Fink et al., 1998].

WESTERN BLOTTING

At the end of the experimental period, the podocytes were lysed in RIPA buffer. The protein concentration was assayed by the Bradford method, and equal amounts of proteins were loaded onto a 10% polyacrylamide gel containing 0.2% SDS for separation. The separated proteins were transferred onto PVDF membranes, which were blocked with 5% nonfat milk in Tris-buffered saline with 0.5% Tween-20 overnight at 4°C. Next, they were incubated with primary

antibodies of goat anti-CTGF (1:200), mouse anti-ILK (1:200), rabbit anti-desmin (1:100), or goat anti-nephrin (1:100, all from Santa Cruz) for 1 h at room temperature. After washing, the membranes were incubated with the appropriate HRP-conjugated secondary antibodies of donkey anti-goat, goat anti-mouse or goat anti-rabbit (each 1:1,000, Santa Cruz) for another 1 h at room temperature. Finally, the signals were detected by the ECL advanced system (GE Healthcare, UK). β-actin was used as a control.

STATISTICAL ANALYSIS

All data were expressed as the mean ± SD. Statistical analyses of the data were performed using SPSS13.0 statistical software. Comparisons between the groups were analyzed by a paired Student's *t*-test or one-way ANOVA followed by post hoc tests for either selected or multiple comparisons. A value of $P < 0.05$ was considered to be significant.

RESULTS

BOTH CTGF AND ILK EXPRESSION WERE UPREGULATED BY HIGH GLUCOSE LEVELS IN PODOCYTES

To determine the effects of high glucose levels on CTGF and ILK expression, mouse podocytes were incubated in mediums containing 5, 15, or 30 mM glucose for 24 h had increased CTGF and ILK expression in a dose-dependent manner (Fig. 1A,B). Podocytes exposed to 25 mM D-mannitol which was used as the osmotic control showed no difference in the expression of CTGF and ILK compared to the normal glucose group (data not shown). Again, in response to high glucose levels (30 mM) for 6, 12, 24, or 48 h, CTGF and ILK expression in podocytes were significantly increased in a time-dependent manner (Fig. 1C,D). Furthermore, ILK mRNA expression was increased subsequent to CTGF, which was shown by a higher level of CTGF compared to ILK when podocytes were cultured under high glucose conditions for 6 h (Fig. 1C).

THE EFFECTS OF ANTI-CTGF ANTIBODY ON HIGH GLUCOSE-INDUCED ILK EXPRESSION IN PODOCYTES

To determine whether high glucose-induced ILK expression was mediated by CTGF, podocytes were preincubated with anti-CTGF antibody (10 μg/ml) for 2 h and then were incubated under high glucose conditions (HG) for 24 h. As shown in Figure 2, treatment with neutralizing anti-CTGF antibody could partially attenuate the upregulation of ILK mRNA (Fig. 2A) and protein expression (Fig. 2B) induced by high glucose levels, demonstrating that high glucose levels induced ILK expression in podocytes through a CTGF-dependent pathway.

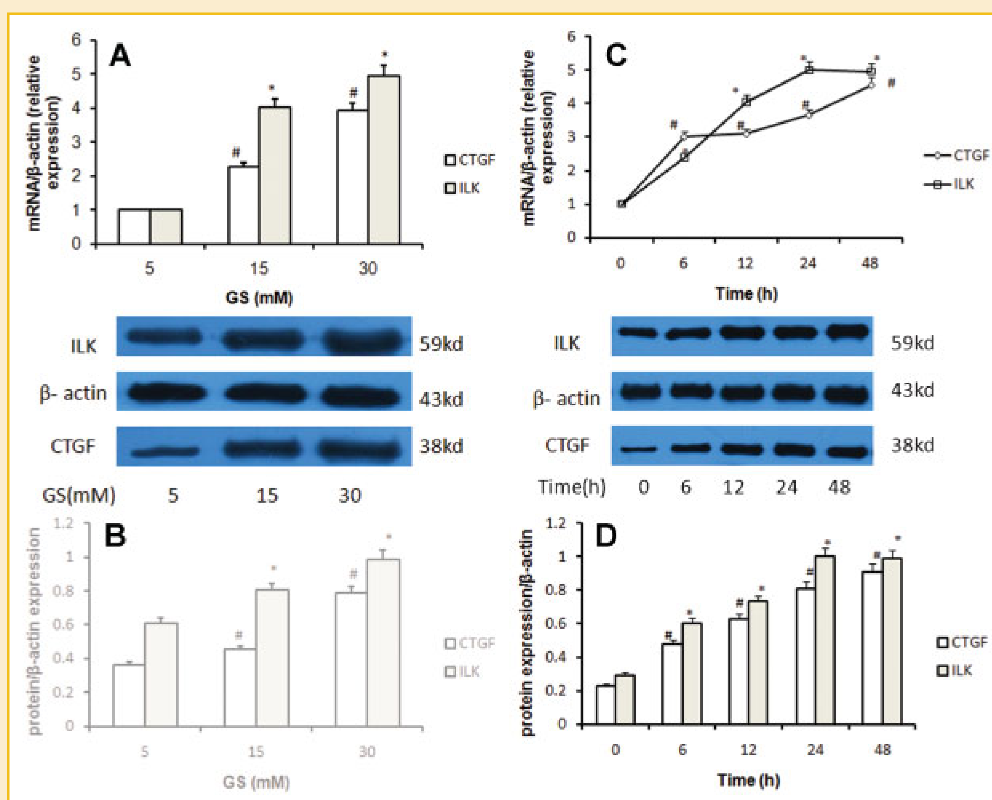

Fig. 1. The effect of high glucose levels on CTGF and ILK expression in podocytes. A,B: The dose response to glucose concentrations of 5, 15 and 30 mM for 24 h. C,D: The time-response to high glucose levels (HG, 30 mM) for 0, 6, 12, 24, and 48 h. The mRNA expression was examined by real-time RT-PCR (A,C), and the protein expression was detected by Western blotting (B,D). Data are presented as the mean ± SD. The results are representative of three independent experiments (#$P < 0.05$ vs. control in CTGF expression, *$P < 0.05$ vs. control in ILK expression).

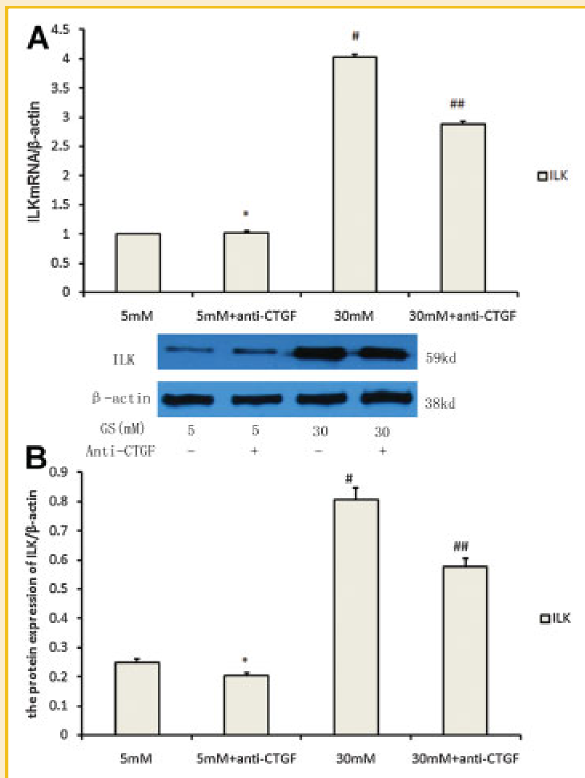

Fig. 2. The effect of anti-CTGF antibody on high glucose-induced ILK expression in mouse podocytes. The ILK mRNA (A) and protein expression (B) in podocytes cultured under high glucose conditions for 24 h with or without anti-CTGF antibody (10 μg/ml) for 2 h before exposure to high glucose levels. Gene and protein expression were detected by real-time RT-PCR and Western blotting, respectively. β-actin was used as an internal control. Data are presented as the mean ± SD. The results are representative of three independent experiments ($^{*}P > 0.05$ vs. normal glucose (5 mM), $^{\#}P < 0.05$ vs. normal glucose, $^{\#\#}P < 0.05$ vs. high glucose (30 mM)).

THE EFFECTS OF CTGF-ANTIBODY ON α3 AND β1 INTEGRIN GENE EXPRESSION IN PODOCYTES

The influence of CTGF on ILK expression induced by high glucose in podocytes may be related to α3β1 integrin. Additionally, α3 and β1 integrin mRNA expression was measured by real-time PCR in the absence or presence of anti-CTGF antibody (10 μg/ml), which was preincubated with podocytes for 2 h and then cultured under normal glucose or high glucose conditions for another 24 h. As shown in Figure 3, compared to the normal condition (5 mM glucose), high glucose reduced α3 and β1 integrin mRNA expression. Inhibition of CTGF significantly prevented these changes. Of note, the anti-CTGF antibody had no significant influence on α3 and β1 integrin gene expression under normal conditions.

THE EFFECTS OF ANTI-CTGF ANTIBODY ON HIGH GLUCOSE-INDUCED PHENOTYPIC ALTERATIONS OF PODOCYTES

In normal glucose conditions, differentiated podocytes exhibited a typical spreading arborized morphology with processes (Fig. 4B and C) and expressed WT-1 in the nucleus (Fig. 4B). Morphological analysis using phase contrast microscopy demonstrated that podocytes developed a series of phenotypic changes, including a cobblestone, elongating morphology. In high glucose conditions (Fig. 4D). However, anti-CTGF antibody prevented these phenotypic alterations, demonstrated by the largely preserved epithelial morphology (Fig. 4E).

THE EFFECTS OF ANTI-CTGF ANTIBODY ON NEPHRIN EXPRESSION IN MOUSE PODOCYTES

We further assess whether high glucose could induce podocyte EMT. Nephrin, an epithelial marker was determined by real-time RT-PCR and Western blotting. The results showed that high glucose downregulated nephrin expression compared to normal glucose. Anti-CTGF antibody treatment partially restored nephrin expression, demonstrating that CTGF mediated podocyte EMT in high glucose via the loss of the epithelial marker (Fig. 5).

THE EFFECTS OF ANTI-CTGF ANTIBODY ON DESMIN EXPRESSION IN MOUSE PODOCYTES

Furthermore, the mesenchymal marker of desmin was evaluated in high glucose conditions. As shown by immunofluorescence staining, the desmin staining of podocytes was almost negative in normal conditions (Fig. 6A). In contrast, the desmin staining markedly increased in response to high glucose (Fig. 6B) and was inhibited by anti-CTGF antibody (Fig. 6C). In addition, the staining further confirmed the phenotypic transition of podocytes from a spreading arborized morphology with processes (Fig. 6A) to a cobblestone appearance (Fig. 6B) in a high glucose environment. Inhibition of CTGF largely preserved the epithelial morphology (Fig. 6C). As shown in Figure 6D, the desmin mRNA of podocytes cultured under high glucose was significantly upregulated compared to normal conditions; however, the upregulation of desmin mRNA was blocked by anti-CTGF antibody treatment. In line with the real-time RT PCR result, the protein expression of desmin was increased in high glucose conditions and was inhibited by anti-CTGF antibody (Fig. 6E), demonstrating that CTGF mediated the EMT of podocytes by expressing the mesenchymal marker in a high glucose condition.

DISCUSSION

In this study, we found that high glucose enhanced CTGF and ILK expression in podocytes (Fig. 1). In a normal glucose environment, podocytes displayed a spreading arborized morphology, whereas they had a cobblestone morphology in a high glucose condition (Figs. 4 and 6A). Inhibition of CTGF prevented the phenotypic transition, as demonstrated by largely preserved epithelial morphology, prevented high glucose levels induced desmin overexpression and restored nephrin expression (Figs. 5 and 6). Furthermore, the upregulation of ILK under high glucose conditions was partially blocked by the inhibition of CTGF (Fig. 2). High glucose decreased α3β1 integrin expression, which was prevented by anti-CTGF antibody (Fig. 3). This finding demonstrated that CTGF mediated the EMT of podocytes and high glucose-induced ILK expression in diabetes.

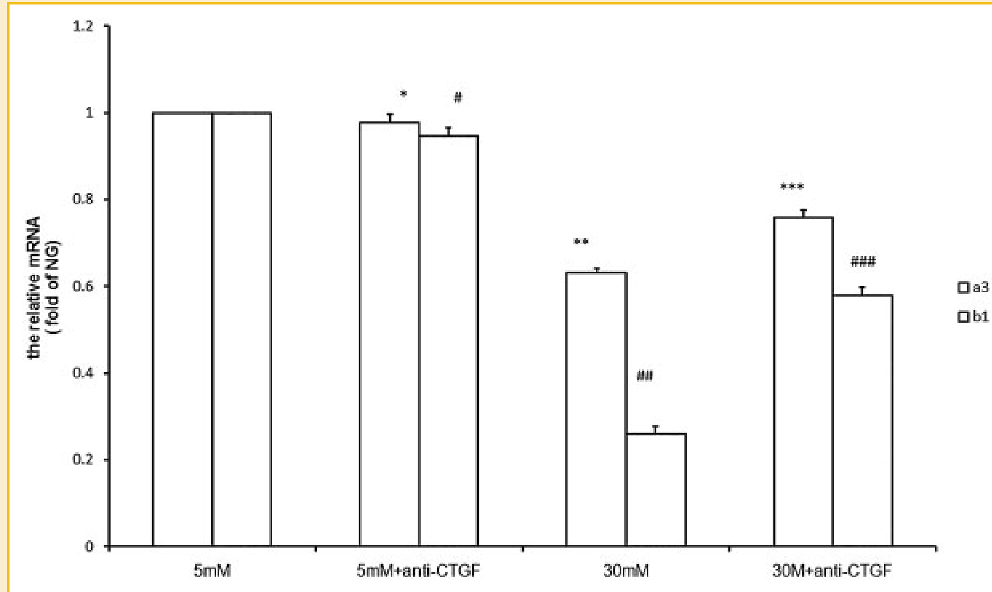

Fig. 3. Effect of CTGF inhibition on α3 and β1 integrin mRNA expression measured by real-time PCR in podocytes. Podocytes were treated with normal glucose, normal glucose + anti-CTGF antibody, high glucose and high glucose + anti-CTGF antibody, respectively. Podocytes were pretreated with anti-CTGF antibody (10 μg/ml) for 2 h and then cultured under normal glucose or high glucose conditions for another 24 h. The results showed that both α3 and β1 mRNA expression were reduced in high glucose conditions compared to normal glucose conditions, but these reductions could be ameliorated by CTGF inhibition. Data are presented as the mean $\pm$ SD. The results are representative of three independent experiments ($^{*}P > 0.05$ vs. normal glucose (5 mM), $^{**}P < 0.05$ vs. normal glucose, $^{***}P < 0.05$ vs. high glucose (30 mM), $^{\#}P > 0.05$ vs. normal glucose (5 mM), $^{\#\#}P < 0.05$ vs. normal glucose, $^{\#\#\#}P < 0.05$ vs. high glucose (30 mM)).

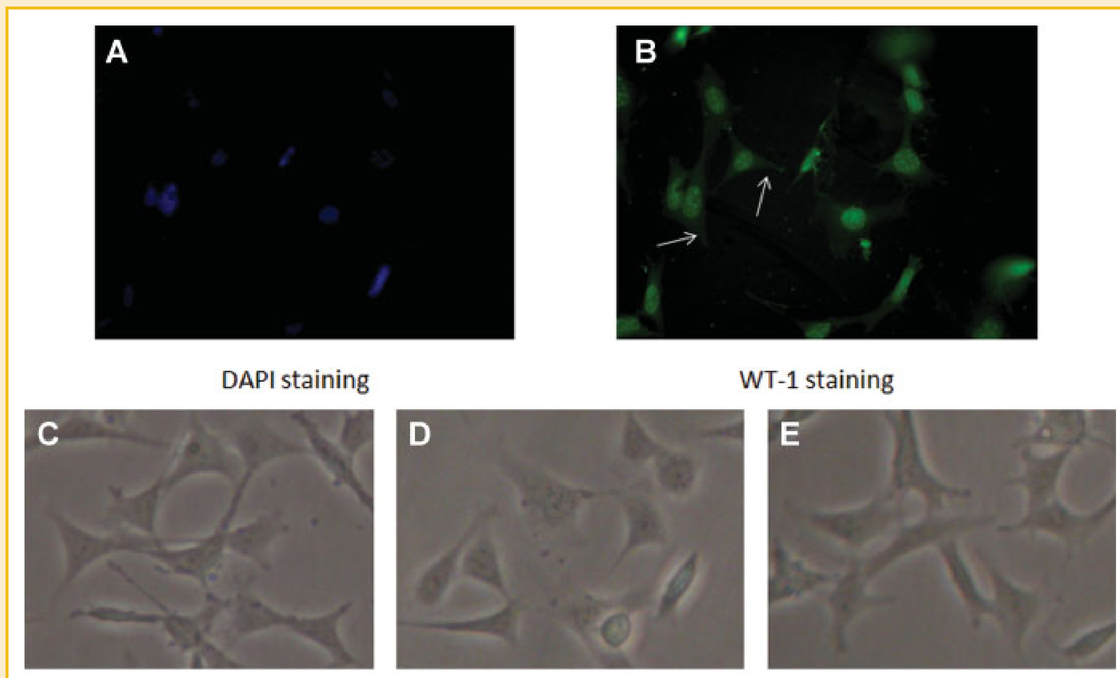

Fig. 4. The effect of CTGF inhibition on high glucose-induced phenotypic alterations of podocytes. Differentiated podocytes cultured under normal glucose conditions detected by confocal microscope (A,B). A: DAPI staining of the nucleus. B: Immunofluorescence staining for WT-1 with the nucleus (green) and spreading arborized morphology with processes (arrowhead). Differentiated podocytes detected by phase contrast microscopy (C through E). Differentiated podocytes cultured under normal glucose conditions (C). Podocytes showed a spreading arborized morphology with processes. Differentiated podocytes cultured under high glucose conditions (D). Podocytes displayed a cobblestone or elongating morphology. Differentiated podocytes cultured under high glucose conditions supplemented with anti-CTGF antibody (E). An arborized morphology was again observed. Original magnification, 200×.

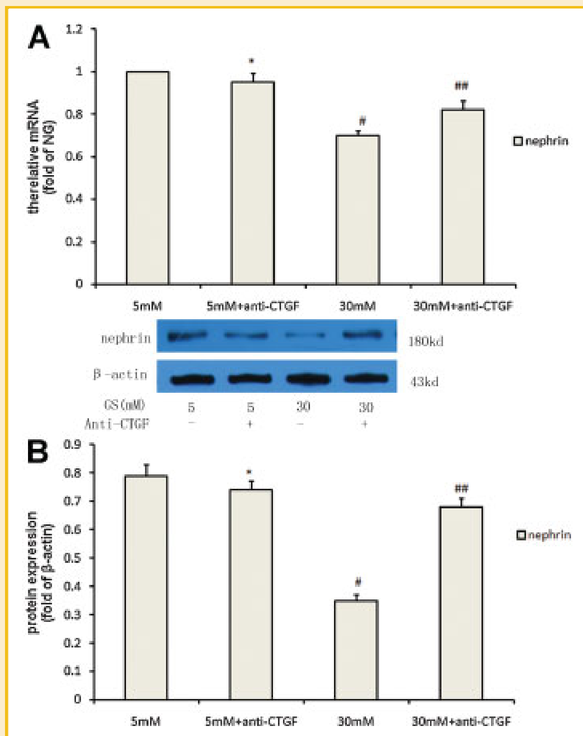

Fig. 5. The effect of CTGF inhibition on high glucose-induced nephrin expression in podocytes. Both mRNA (A) and protein expression of nephrin (B) in podocytes induced by high glucose were downregulated compared to normal glucose conditions. CTGF inhibition with anti-CTGF antibody significantly prevented the downregulation of nephrin. β-actin was used as an internal control. Data are presented as the mean ± SD. The results are representative of three independent experiments ($^{*}P > 0.05$ vs. normal glucose (5 mM), $^{\#}P < 0.05$ vs. normal glucose, $^{\#\#}P < 0.05$ vs. high glucose (30 mM)).

CTGF is well documented as a key matricellular adaptor protein. It not only regulates crucial biological processes, including cell differentiation, proliferation, adhesion, migration, apoptosis, extracellular matrix accumulation, chondrogenesis and angiogenesis, but also promotes fibrosis [Leask and Abraham, 2006]. Increased CTGF expression has already been found in various cell types of human and rodent DN [Ito et al., 1998; Murphy et al., 1999; Roestenberg et al., 2006; Guha et al., 2007], which was further confirmed by our finding in podocytes (Fig. 1). In 1999, CTGF was firstly identified as a potential key mediator in the progression of glomerular injury in diabetes. Its expression was upregulated in mesangial cells exposed to high levels of glucose [Murphy et al., 1999]. In normal conditions, Roestenberg et al. [2006] found that renal CTGF expression was mainly localized in podocytes and parietal glomerular epithelial cells and was less prominent in mesangial cells. Interestingly, in murine models of DN, CTGF overexpressed initially in podocytes and then in mesangial cells. However, the most definitive in vivo evidence for a role of CTGF in mediating DN was not shown until recently. Yokoi et al. [2008] generated podocyte-specific CTGF-transgenic mice to test whether upregulation of CTGF in podocytes can mediate glomerular injury in vivo. Interestingly, they found that CTGF itself was not sufficient to induce glomerular pathology, which was shown by non-diabetic transgenic mice that had normal renal histology even though their glomerular CTGF protein levels were fivefold higher compared to non-transgenic mice. However, after STZ-induced diabetes, they showed an enhanced mesangial matrix expansion and podocyte structural changes compared to diabetic wild-type mice. Together with the data from other CTGF transgenic animals in different organs (e.g., liver, heart, and lung), it was suggested that CTGF plays a modifying rather than a causative role in fibrosis. Overexpression of CTGF might, in concert with other signaling pathways, initiate or exacerbate fibrosis [Brigstock, 2010]. In agreement with these findings, the CTGF overexpression induced by high glucose, together with ILK signaling was complicated in the EMT of podocytes. Furthermore, the inhibition of CTGF could attenuate EMT (Figs. 5 and 6) and inhibit ILK expression (Fig. 2), suggesting that CTGF is involved in the EMT of podocytes and plays a pivotal modifying role in the modulation of ILK. CTGF and ILK have been regarded as an essential matricellular adaptor protein and a key intracellular hub protein, respectively [Leask and Abraham, 2006; McDonald et al., 2008; Schmidt-Ott, 2008; Brigstock, 2010]. Based on these findings and the results showing that ILK mRNA expression was increased subsequent to CTGF (Fig. 1C) and inhibition of CTGF only partially suppressed ILK overexpression (Fig. 2), it is conceivable to speculate that ILK act as downstream of CTGF signaling and high glucose levels induced podocyte ILK expression may through CTGF-dependent and independent pathways.

It is well accepted that ILK is a key player at the interface between the extracellular matrix, integrins, actin-based cytoskeleton, and cellular phenotype in kidney diseases [Blattner and Kretzler, 2005]. Further studies have demonstrated that ILK plays an essential role in cell–matrix integrin signaling and slit diaphragm signaling, which contribute to normal podocyte biology [Dai et al., 2006; El-Aouni et al., 2006]. In addition, it acts as an adaptor protein that interacts with nephrin and α-actinin-4 to form a ternary complex, which is essential for the maintenance of podocyte function and glomerular filter integrity [Dai et al., 2006]. Recently, emerging evidence has suggested that podocytes could undergo EMT induced by TGF-β1 and high glucose levels [Li et al., 2008; Yamaguchi et al., 2009; Kang et al., 2010] and that ILK was a key intracellular mediator in this cellular event, which was demonstrated by the fact that ILK inhibition with small-molecular inhibitor QLT-0267 prevented podocyte EMT and ameliorated proteinuria. Of note, the inhibitor had no adverse effect on normal kidney structure and function [Kang et al., 2010]. In line with these results, our study showed that high glucose could induce ILK overexpression and podocyte EMT. The upregulation of ILK and downregulation of nephrin disrupted the balance of the ternary complex, which might be responsible for EMT [Dai et al., 2006]. Moreover, the inhibition of CTGF could prevent the overexpression of ILK and ameliorate the EMT of podocytes.

It was also demonstrated that ILK activation decreased integrin binding affinity and avidity, which might be responsible for podocyte loss [Kretzler, 2002]. The integrity of podocyte structure and function depends on its attachment to the GBM by α3β1 integrin [Adler, 1992; Shankland, 2006]. In support of this hypothesis, the upregulation of ILK by high glucose was accompanied by α3β1 integrin decrease (Fig. 3), which is in

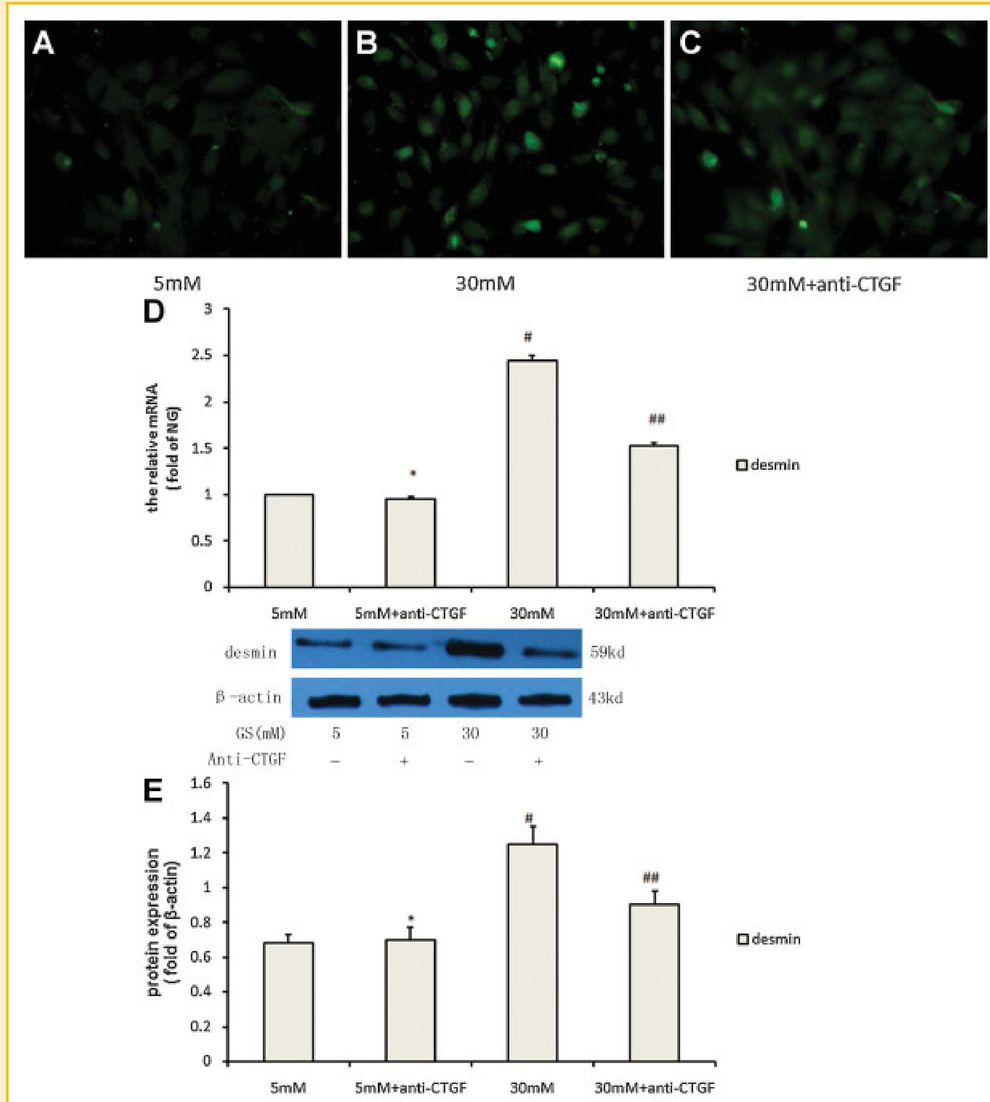

Fig. 6. The effect of CTGF inhibition on high glucose-induced desmin expression in podocytes. Immunofluorescence staining for desmin in normal glucose (A), high glucose (B), and high glucose + anti-CTGF antibody (C). Compared to normal glucose conditions, the desmin staining increased markedly in response to high glucose, whereas CTGF inhibition prevented this upregulation. Original magnification 200×. High glucose levels induced the upregulation of desmin mRNA (D) and protein expression (E), whereas treatment with anti-CTGF antibody abrogated its overexpression. β-actin was used as an internal control. Data are presented as the mean ± SD. The results are representative of three independent experiments ($^{*}P > 0.05$ vs. normal glucose (5 mM), $^{\#}P < 0.05$ vs. normal glucose, $^{\#\#}P < 0.05$ vs. high glucose (30 mM)).

agreement with other studies [Chen et al., 2000, 2008]. The disruption of the integrin interaction with ILK may contribute to podocyte dysfunction, including EMT [Han et al., 2006]. Again, the inhibition of CTGF prevented the decrease in α3β1 integrin. Interestingly, another study indicated that β1 integrin was induced in podocytes incubated with high levels of glucose [Han et al., 2006]. This difference is unclear at present, and further study is needed.

In conclusion, our study demonstrated that the CTGF-ILK pathway played an important role in the high glucose-induced EMT of podocytes. In addition, CTGF inhibition attenuated the EMT induced by diabetes. Based on evidence that local modulation of signaling activity by different domains or modules of CTGF in diabetes, some of these pathways were harmful, whereas others were protective [van Nieuwenhoven et al., 2005; Schmidt-Ott, 2008; de Winter et al., 2008]. Thus, the elucidation of the individual contributions of the various potential molecular targets of CTGF to modulate ILK is required. In the future, a better understanding of EMT and its signaling mediators in diabetes will provide an effective means for the prevention and treatment of DN and other chronic kidney diseases.

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