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IGF-1 Increases Laminin, Cyclin D1, and P21^{Cip1} Expression in Glomerular Mesangial Cells: An Investigation of the Intracellular Signaling Pathway and Cell-Cycle Progression

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Abstract Insulin-like growth factor (IGF)-1 is accumulated in the diabetic kidney and is considered to be involved in the development of glomerular sclerosis. Here, we investigate IGF-1 regulation of laminin, an extracellular matrix (ECM) component, and cyclin D1 and p21^{Cip1}, cell-cycle progression factor, expressions in glomerular mesangial cells. We show that IGF-1 increases the level of laminin γ1 and β1 subunits ~1.5- and 2.5-fold, respectively, in a time-dependent manner. IGF-1 also stimulates protein kinase Akt/PKB phosphorylation at Thr 308, which correlates with its activity, up to 24 h. The Akt activation is coupled with Ser 9 phosphorylation of its downstream target, glycogen synthase kinase-3beta (GSK-3β), which inhibits its kinase activity. Laminin β1 is reduced significantly ($P < 0.03$) by inhibitors of Akt and p38MAPK whereas laminin γ1 is not affected. Surprisingly, IGF-1 activates the expression of both cyclin D1 and cell-cycle arrest factor, p21^{Cip1} parallely. Pharmacological inhibition of calcineurin by cyclosporin A blocks IGF-1-induced cyclin D1 and p21^{Cip1}expression significantly ($P < 0.05$). IGF-1 enhances cellular metabolic activity and viability of rat mesangial cells; however, they are arrested at the G_1 phase of cell cycle as revealed by the FACS analysis. These results indicate that IGF-1 mediates mesangial cell-cycle progression, hypertrophy, and ECM protein synthesis. The Akt/GSK-3β, p38MAPK, and calcineurin pathways may play an important role in IGF-1 signaling, cell-cycle regulation, and matrix gene expression in mesangial cells leading to the development of diabetic glomerulopathy. J. Cell. Biochem. 98: 208–220, 2006.
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Diabetic glomerular disease, like all forms of glomerular sclerosis, is characterized by deranged glomerular remodeling consisting of enhanced extracellular matrix (ECM) accumulation and altered cell growth leading to an enlargement of the mesangial region [Osterby et al., 1983; Mauer et al., 2002]. Recent studies indicate that insulin-like growth factor-1 (IGF-1) is accumulated in the diabetic kidney and involves in the expansion of glomerular mesangium and the development of glomerular sclerosis. IGF-1 is a multifunctional growth factor produced in a variety of tissues including the kidney [Flyvbjerg et al., 1992; Hammerman and Miller, 1993]. The principal actions of IGF-1 in kidney include enhancement of tubular phosphate transport, renal blood flow, glomerular filtration rates, and organ growth [Hirschberg and Kopple, 1989]. Indeed, IGF-1 is considered to participate in several pathological conditions including diabetes mellitus, compensatory renal growth, and hypokalemia [Heilig et al., 1997; Horney et al., 1998]. Increased expression and accumulation of IGF-1 peptide

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and mRNA, and the binding of IGF-1 to its receptor are observed in diabetic glomerulopathy [Bach et al., 1991; Marshall et al., 1991; Flyvbjerg, 1997; Cummings et al., 1998; Verrecchia et al., 2001]. Human mesangial cells increase their proliferative rate when exposed to IGF-1 and the steady-state levels of protein and mRNA for fibronectin and collagen IV are elevated [Feld et al., 1995; Pricci et al., 1996; Pugazhenthi et al., 1999; Poncelet and Schnaper, 2001]. The regulation of the αβγ heterotrimeric laminin, another ECM component, in mesangial cells is still unclear although IGF-1 has been shown to stimulate the laminin-γ1 promoter reporter activity [Phillips et al., 1999]. Mesangial cells express laminin β1/β2, γ1 and a modified form of the α subunit [Hansen et al., 1998; Ekblom et al., 2003]. The expression of laminin 8/9 bearing laminin α4, β1/β2, and γ1 subunits was reported in rat glomerular mesangial cells, which is required for PDGF-mediated mesangial cell migration [Hansen and Abrass, 2003].

The signaling pathways by which IGF-1 promotes cell growth, proliferation, and hypertrophy remain undefined with roles suggested for the phosphatidyl-inositol-3-OH kinase (PI3-K)/Akt [Mehrhof et al., 2001; Kang et al., 2003], p42/44ERKs/p38MAPK [Wu et al., 2000], and the Ca^{2+}/Calmodulin-dependent protein phosphatase 2B, calcineurin, pathways [Schreiber et al., 1995; Delling et al., 2000]. Tack et al. [2002] demonstrated that the IGF-1 signaling pathway in mesangial cells isolated from diabetic nonobese mice is activated. Specifically, phosphorylation of the IGF-1 receptor beta-subunit and insulin receptor substrate (IRS)-1, and the association of the p85 subunit of PI3-K with the IGF-1 receptor and IRS-1 are enhanced. In addition, IGF-I treatment of rat mesangial cells increases the synthesis of calmodulin and protein phosphatase PP2B (calcineurin) activity [Gooch et al., 2001].

Both insulin and IGF-1 signaling involves activation of PI3-K and the downstream Akt/PKB kinase, which in turn phosphorylates GSK-3α at Ser21 and GSK-3β at Ser9, and inhibits its kinase activity [Woodgett, 1990]. GSK-3 is, therefore, considered a negative regulator of cell growth and an inhibition of GSK-3β will lead to cell-cycle progression. Both GSK-3 isoforms have a similar range of substrate specificities and are regulated in parallel in response to growth factor stimulation. However, disruption of *GSK-3β* gene in mice results in embryonic lethality, indicating that GSK-3α cannot completely substitute for a loss of GSK-3β [Hoeflich et al., 2000]. GSK-3β has been studied more extensively than the α-form, and we also examined this isoform in our studies. IGF-1 signaling and the role of GSK-3β in mesangial cell function and renal pathology is still not well understood. In this study, we demonstrate that IGF-1 increases the expression of laminin β1, γ1, and cell-cycle regulation factors cyclin D1 and p21^{Cip1} in mesangial cells. The Akt/GSK-3β signaling pathway together with p38 MAPK and calcineurin play a part in IGF-1 regulation of mesangial cell-cycle progression, ECM expression, and survival.

MATERIALS AND METHODS

Materials

IGF-1, LY294002, and Wortmannin (PI3-K inhibitors) were purchased from Upstate Biotechnology Cell Signaling Solutions (Lake Placid, NY). Antibodies for cyclin D1, p21^{Cip1}, IGF-1, phosphorylated GSK-3β(S9), α-tubulin, and actin were also obtained from Cell Signaling Solutions. Anti-GSK-3α/β antibodies were purchased from Biosource (Camarillo, CA). Chain specific antibodies for laminin γ1 and β1 and Akt (total and Thr308 phosphorylated) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). MTT (3-[4,5-dimethylthiazol-2-yl]-2-5-diphenyltratrazolium bromide) and chicken anti-fibronectin antibodies were purchased from Molecular Probes (Eugene, OR). SH-5 (Akt inhibitor) was from Alexis (San Diego, CA) while SB216763 (GSK-3β inhibitor), SB202190 (p38MAPK inhibitor), and cyclosporin A (calcineurin inhibitor) were obtained from Calbiochem (San Diego, CA). The enhanced chemiluminescence (ECL) system was purchased from Amersham (Arlington Heights, IL). DNase 1 (RNase-free, 10 U/μl) was purchased from Roche Applied Science (Indianapolis, IN). Propidium iodide and DNAse-free ribonuclease A (76 Kunitz U/mg protein) were obtained from Sigma (St. Louis, MO). DMEM and F-12 nutrient mixture (Ham's) were from GIBCO (Grand Island, NY).

Cell Culture

An immortalized rat mesangial cell line (MES) was cultured in medium containing

DMEM and Ham's F-12 (3:1 ratio) supplemented with 2.25% fetal calf serum (FCS) and 0.5 mg/ml gentamicin at 37°C in a humidified chamber with a 5% CO2-95% air mixture [Singh and Crook, 2000; Singh et al., 2004]. Initially, we performed dose-dependent experiments with 25, 50, 100, and 150 ng/ml IGF-1 to examine its effect on mesangial laminin and cyclin D1 expression, and observed that 100 ng/ml gave the optimal effect. Therefore, we chose this concentration of IGF-1 in subsequent experiments. Monolayers at ~70% confluence were serum-starved overnight and IGF-1 (100 ng/ml) was added for different time intervals (0, 2, 6, and 24 h). For this time course, IGF-1 with 24-h treatment was added first, then on the second day IGF-1 was added at 6, 2, or 0 h to the respective cultures before harvesting the cells. Thus, all cells maintain a similar condition and length of serum-starvation during the entire experimental procedure. Proteins were extracted in appropriate buffers and their concentrations were determined using a Coomassie-based reagent from Pierce with BSA as the standard. When inhibitors are included in the culture, they were added 30 min before adding IGF-1 and were present throughout the period of incubation.

SDS–PAGE and Western Blotting

Sodium dodecyl sulfate polyacrylamide gel electrophoresis and Western blot analysis of proteins were performed as described [Singh and Crook, 2000; Singh et al., 2001]. The primary antibodies for cylcin D1, $p21^{Cip1}$, GSK-3β(S9P), GSK-3α/β, and IGF-1 were used at a 1:1,000 dilution in Tris-buffered saline pH 7.4 containing 5% non-fat dry milk while that of laminin β1 and γ1 were 1:2,000 dilution and fibronectin (1:3,000). The dilution for HRP-conjugated secondary antibodies was 1:3,000 proportions. ECL was used to detect the immunoreactive bands. Images were captured in a Kodak Gel Logic 200 Imaging system equipped with a CCD camera, and band intensities were analyzed with the Kodak 1D Analysis software.

MTT Assay for Cell Viability

MTT assay for cellular metabolic activity and viability was performed in 96-well plate with 0.5 mg/ml MTT as recently described [Jiang et al., 2005]. Briefly, MES cells (1×10^3) were cultured in each well overnight and serum-starved for 24 h. IGF-1 (100 ng/ml) and different pharmacological inhibitors for protein kinases and phosphatase PP2B (calcineurin) were added and incubated for 24 h. MTT was added for 3 h, media was removed, and cells were kept in DMSO for 10 min. The resulting color was detected at 570 nm using a Gemini absorbance microplate reader.

Flow Cytometry Analysis

To investigate the IGF-1-induced cell-cycle regulation of mesangial cells, we analyzed the percentage of cells in G_0–G_1, S and G_2/M phase. MES cells were grown to 60% confluence and serum-starved overnight. Cells were washed twice with sterile PBS, and fresh media without serum was added. After 24-h treatment with IGF-1 (100 ng/ml), cells were processed for flow cytometric analysis [Wang and Shi, 2002]. Briefly, cells were trypsinized and collected by centrifugation at 1,000 rpm for 2 min and washed three times with PBS. Cells were resuspended in 20 μl of sterile PBS and fixed by the addition of 80 μl of absolute alcohol. The samples were then kept at 4°C until use (30 min to few days). Just before the flow cytometric analysis, 400 μl of staining buffer (50 μg/ml propidium iodide, PI, in PBS with freshly added RNase A, 10 μg/ml) was added to the sample. Following 30-min incubation at room temperature, the samples were directly subjected to flow cytometry using a Facscalibur flow cytometer for data acquisition. The cell-cycle phases were determined by utilizing Modfit software following acquisition of the data. Values were expressed as mean ± SE of four different experiments.

Measurement of RNA and DNA Content

RNA and DNA contents were determined by the CyQUANT kit from Molecular Probes based on a fluorescence enhancement of dye binding to cellular nucleic acids according to manufacturer's instructions. MES cells were grown in 12-well plates, serum-starved overnight, and IGF-1 (100 ng/ml) was added for different time periods (0, 2, 6, and 24 h). After the treatment, cells were washed with cold PBS thrice and stored at −80°C for 1 h. Plates were taken out of the freezer and incubated with DNAse-free RNAse (1.35 U/ml) for 1 h at room temperature to measure DNA content in a 50 μl reaction volume of CyQUANT lysis buffer containing

180 mM NaCl and 1 mM EDTA. Similarly, to measure the amount of RNA, cells were treated with RNAse-free DNAse (9.0 U/ml) containing 180 mM NaCl, 1 mM $CaCl_2$, and 1 mM $MgCl_2$ for 1 h at room temperature. An equal volume of the 2× CyQuant Dye was added to each well and incubated for another 5 min in the dark. Fluorescence was measured at Ex480/Em520 with the bottom read and scanning device of a Spectra MAX Gemini EM Fluorescence microplate reader using the auto cut off mode.

Diabetes Induction of Rats

Diabetes of adult male Sprague–Dawley rats (275–300 g) was induced by injection of a single dose of streptozotocin, (STZ, 55 mg/kg iv, Sigma) dissolved in 0.01 M citrate buffer, pH 4.5 as described previously [Marliszewska-Scislo et al., 2003]. Normal rats received a similar volume of vehicle alone (n = 4 for diabetic and normal rats). The rats were treated in accordance with the principles of NIH guidelines for the Care and Use of Laboratory Animals and approved by the Institutional Animal Investigation Committee. After 4 weeks of diabetes, which achieved a blood-glucose level 400–450 mg/dl compare to ~117 mg/dl in control rats, they were sacrificed with an overdose of pentobarbital. The kidneys were removed, frozen immediately in liquid N_2, and stored at −70°C until used. The frozen kidneys were thawed, capsule removed, and the outer cortex was separated from the medulla. The cortex was cut into small pieces and cellular extracts were prepared in RIPA buffer containing protease inhibitors by homogenization with a Teflon piston. Cell debris was removed by centrifugation and the protein amount in the supernatant was measured by the Coomassie-based method. IGF-1, cyclin D1, and p21^{Cip1} expressions were examined directly on tissue extracts by Western blotting while that of laminin and fibronectin were determined after glycoprotein fractionation on Agarose-bound Wheat Germ Agglutinin columns as described before in our laboratory [Jiang et al., 2005].

Statistical Analysis

Results are expressed as means ± SE of indicated number of experiments. Student's *t*-test was used to compare differences between cultures. A value of $P < 0.05$ was consider statistically significant.

RESULTS

IGF-1 Increases Laminin β1 and γ1 Protein Expression in Rat Mesangial Cells

IGF-1 is a regulator of ECM protein expression in glomerular mesangial cells [Feld et al., 1995; Pricci et al., 1996; Pugazhenthi et al., 1999; Poncelet and Schnaper, 2001]. We have previously shown that hexosamines and TGF-β1 stimulate laminin and fibronectin expression in rat mesangial cells [Singh et al., 2001, 2004; Jiang et al., 2005]. In this study, we examined whether IGF-1 has an effect on the expression of laminin, a component of ECM, in these cells. As shown in Figure 1, IGF-1 (100 ng/ml) increases the expression of laminin β1 and γ1 levels ~1.5- to 2.5-fold in a time-dependent manner (0, 2, 6, and 24 h; $P < 0.05$ for all time points except for laminin β1 at 2 h, $P = 0.057$). The extent of

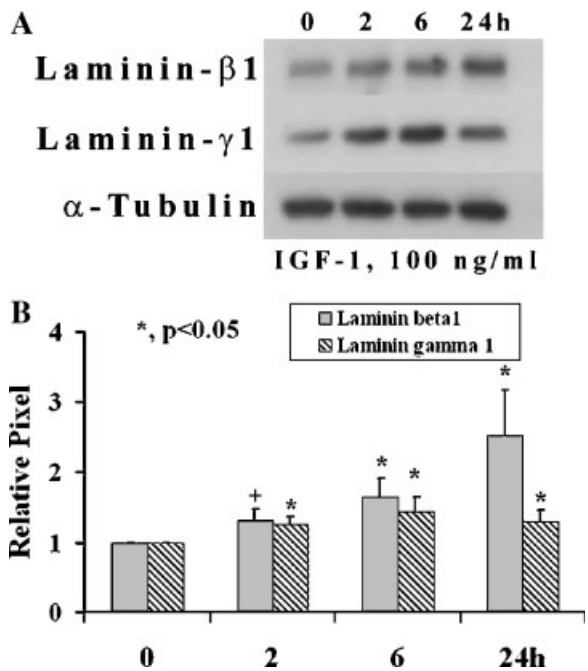

Fig. 1. Time-dependent stimulation of laminin β1 and γ1 expression by IGF-1 in rat mesangial cells. IGF-1 (100 ng/ml) was added for different time periods (0, 2, 6, and 24 h, respectively) to serum-starved mesangial cells as described in Materials and Methods. Cellular extracts (30 μg protein) were applied on to SDS–PAGE, transferred to PVDF membrane and probed with anti-laminin β1 or γ1 antibodies. Enhanced chemiluminescence (ECL) was used to detect the bands. Afterwards, the membranes were stripped off and reprobed for α-tubulin. **A**: Representative blots for laminin β1 (upper panel, n = 3) and γ1 (middle panel, n = 5) and (**B**) their normalized densitometric data are shown (open bar, laminin β1 and cross bar, laminin γ1). A symbol * represents a significant value ($P < 0.05$) versus control at zero and (+) represents $P = 0.057$ (not significant). α-tubulin level is not altered by IGF-1 (A, lower panel).

laminin β1 expression is higher at 24 h (2.52 ± 0.71) than other time periods while that of the laminin γ1 is highest at 6 h (1.48 ± 0.22). The level of α-tubulin is not altered under these experimental conditions.

IGF-1 Activates Akt and GSK-3β Phosphorylation in Mesangial Cells

IGF-1 signaling involves the activation of PI3-K and phosphorylation of downstream Akt/PKB kinase. The activation of Akt leads to the phosphorylation of its target GSK-3β at Ser 9 and the suppression of GSK-3β activity [Woodgett, 1990]. We investigated whether IGF-1 regulates Akt and GSK-3β phosphorylation in mesangial cells. As shown in Figure 2A (top panel), IGF-1 increases the phosphorylation of Akt at Thr 308, which is known to correlate with its elevated activity, up to 24 h significantly ($P < 0.05$ at all time points examined at 2, 6, and 24 h versus the control at 0 h). The representative graph is shown in Figure 2B (open bar). Similarly, the phosphorylation of GSK-3β, therefore, an inactivation of its kinase function, continues in parallel with Akt phosphorylation (Fig. 2A, third panel). Their representative graphs are shown in Figure 2B (cross bar, $P < 0.05$ at 2 and 24 h and $P < 0.001$ for 6 h vs. 0 h). Neither Akt nor GSK-3β level is changed (Fig. 2A, second and fourth panels, respectively).

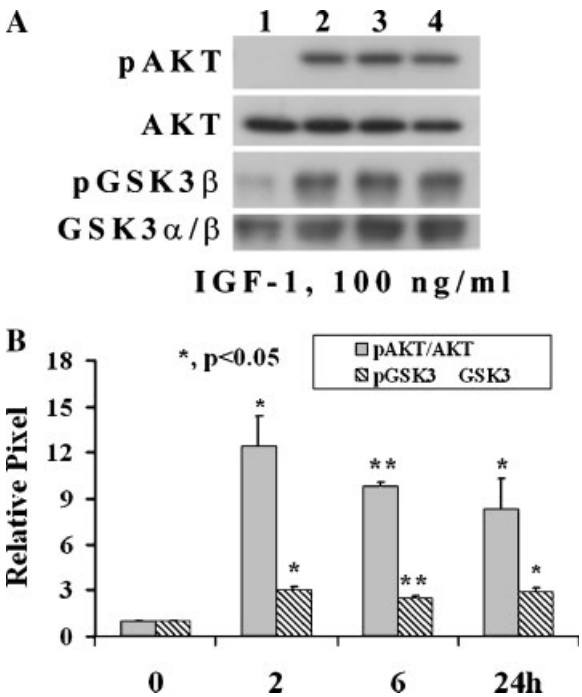

Fig. 2. IGF-1 increases Akt and GSK-3β phosphorylation in mesangial cells. The activation of Akt is associated with an increased phosphorylation at Thr308 and phosphorylation of its downstream substrate GSK-3β (Ser 9). Therefore, the extent of phosphorylation of Akt (Thr308) and GSK-3β (Ser9) was determined when mesangial cells were treated with IGF-1 (100 ng/ml) for 0, 2, 6, and 24 h similar to conditions described in Figure 1. After detecting the phosphorylated Akt and GSK-3β, the membranes were reprobed for total Akt and GSK-α/β for normalization. Representative blots are shown in A and their densitometric data are presented in B (n = 3). A symbol * represents a statistically significant value of $P < 0.05$; and ** denotes *P*-value of <0.001.

Laminin β1 and γ1 are Regulated by Different Signals

The signaling mechanism by which IGF-1 regulates laminin expression in mesangial cells is still unclear. To address this issue, we used various pharmacological inhibitors to block individual signaling pathways, and examined the expression of laminin β1 and γ1 subunits on Western blots. As shown in Figure 3A,B (A, first panel, lanes 1 and 2), the expression of laminin β1 is increased 1.89 ± 0.21-fold by the addition of IGF-1 (100 ng/ml, 24 h), which is reduced significantly to 1.16 ± 0.07 ($P < 0.05$, n = 3) by SB202190 (p38MAPK inhibitor). SH-5 (Akt inhibitor) also reduces IGF-1-mediated laminin β1 expression from 1.89 ± 0.39 to 1.33 ± 0.12-fold significantly ($P < 0.05$, n = 3). On the other hand, the presence of SB216763 (GSK-3β inhibitor) or cyclosporin A (calcineurin inhibitor) does not produce a significant effect on IGF-1-induced laminin β1 expression. Surprisingly, IGF-1 induced laminin γ1 expression is not altered by any of these inhibitors (Fig. 3A, second panel and the corresponding graph at Fig. 3C).

IGF-1 Elevates Cyclin D1 and $p21^{Cip1}$ Expression in Mesangial Cells

IGF-1 is a cell-cycle progression and hypertrophic factor for a number cell types including renal mesangial cells. Therefore, we examined if IGF-1 has an effect on the expression of cell-cycle progression factor cyclin D1 and cyclin-dependent kinase inhibitor $p21^{Cip1}$ in mesangial cells. As shown in Figure 4A,B (first panel (A) and cross bar (B)), IGF-1 increases cyclin D1 expression significantly at 6 (1.58 ± 0.29-fold) and 24 h (1.7 ± 0.3-fold) ($P < 0.05$ versus 0 h, n = 4). Surprisingly, the expression of another cell-cycle inhibitor, $p21^{Cip1}$, is significantly increased at all time points examined (2, 6, and 24 h) against the control (1.55 ± 0.09,

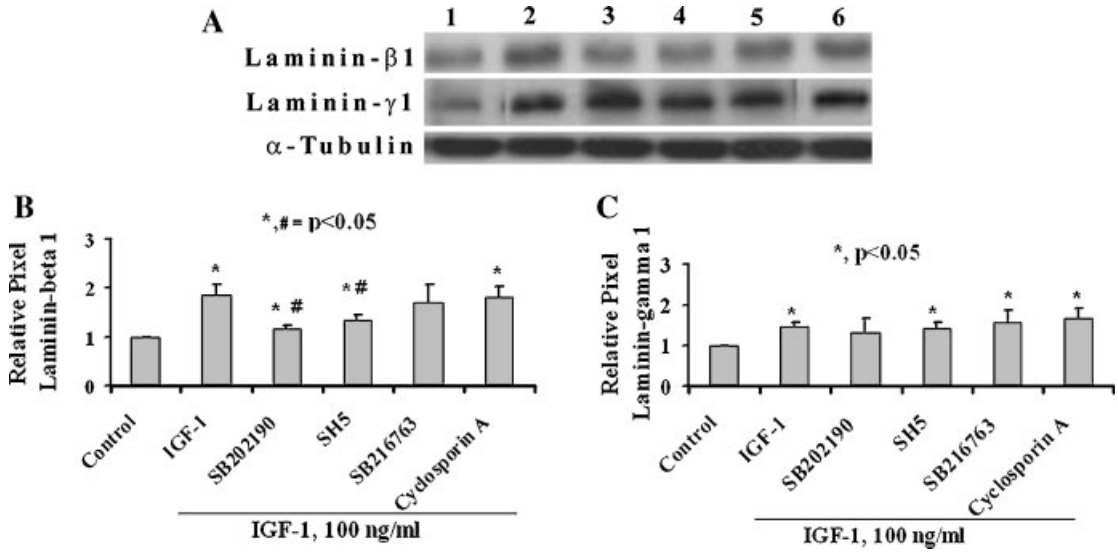

Fig. 3. Effect of pharmacological inhibitors on IGF-1-induced laminin β1 and γ1 expression. Pharmacological inhibitors for protein kinases and phosphatase (see below the names) were pretreated for 30 min before adding IGF-1 (100 ng/ml) and incubated for 24 h as described in Materials and Methods. Cellular extracts (30 μg protein) were applied on to SDS–PAGE, transferred to PVDF membrane and probed with anti-laminin-β1 or γ1 antibodies. ECL detected the immunoreactive bands. Representative blots (n = 3) are shown in **A**: **lane 1,** Control; **lane 2**, IGF-1 alone; **lane 3**, IGF-1 + SB202190 (p38MAPK inhibitor, 10 μM); **lane 4**, IGF-1 + SH-5 (Akt inhibitor, 2 μM); **lane 5**, IGF-1 + SB216763 (GSK-β inhibitor, 100 nM); and **lane 6**, IGF-1 + cyclosporin A (calcineurin inhibitor, 2 μM). The densitometric data for laminin β1 and γ1 are presented in **B** and **C**, respectively. A symbol * represents a significant value of $P < 0.05$ compared to the control. The symbol # represents a significant decrease in laminin β1 level ($P < 0.05$) in the presence of SB202190 or SH-5 versus IGF-1 alone.

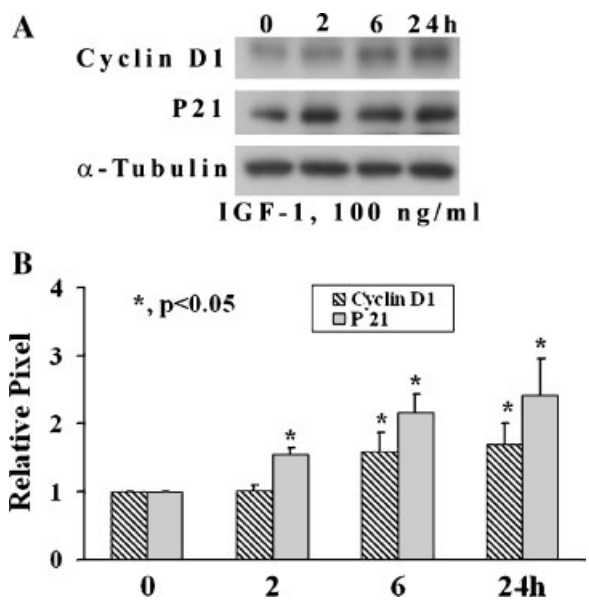

Fig. 4. IGF-1 regulates cyclin D1 and p21^{Cip1} protein expression. Serum-starved MES cells were treated with IGF-1 (100 ng/ml) for 0, 2, 6, and 24 h and subjected to Western blotting for cyclin D1, p21^{Cip1}, and α-tubulin. Representative blots of three different experiments are shown in **A** and their relative densitometric data are plotted in **B**. A symbol * represents a significant change in protein expression of $P < 0.05$ versus their respective controls at time 0. α-tubulin expression is not altered under the above conditions.

$P = 0.002$; 2.17 ± 0.26, $P = 0.006$; and 2.42 ± 0.53-fold, $P = 0.027$, respectively, n = 3) as shown in Figure 4A (third panel) and 4B (vertical cross bar). α-tubulin expression is not changed under these conditions. In addition, IGF-1 does not produce an increase in Rb phosphorylation or the expression of PCNA, markers of cell proliferation in these cells (data not shown).

We further investigated the signaling pathway(s), which may involve in IGF-1-induced increases in the cyclin D1, and p21^{Cip1} expression. As shown above, the expression of cyclin D1 is elevated by IGF-1 after 24 h (Fig. 5A first panel, lanes 1 and 2, and Graph B). The addition of an inhibitor for calcineurin (cyclosporin A) reduces cyclin D1 expression (lanes 2 and 6; 2.22 ± 0.52-fold for IGF-1 alone versus 1.16 ± 0.11-fold for IGF-1 + cyclosporine A, $P < 0.02$). An inhibition of p38MAPK (SB202190) or Akt (SH-5) marginally reduces IGF-1's effect on cyclin D1, but not significant ($P = 0.27$, n = 3 and 0.15, n = 5, respectively, vs. IGF-1). SB216763 (GSK-3β inhibitor) produces no additional change on IGF-1-induced cyclin D1 level. Again, shown in Figure 5A (second panel, lanes 1 and 2, and Graph C), the expression of p21^{Cip1} is increased by IGF-1 and

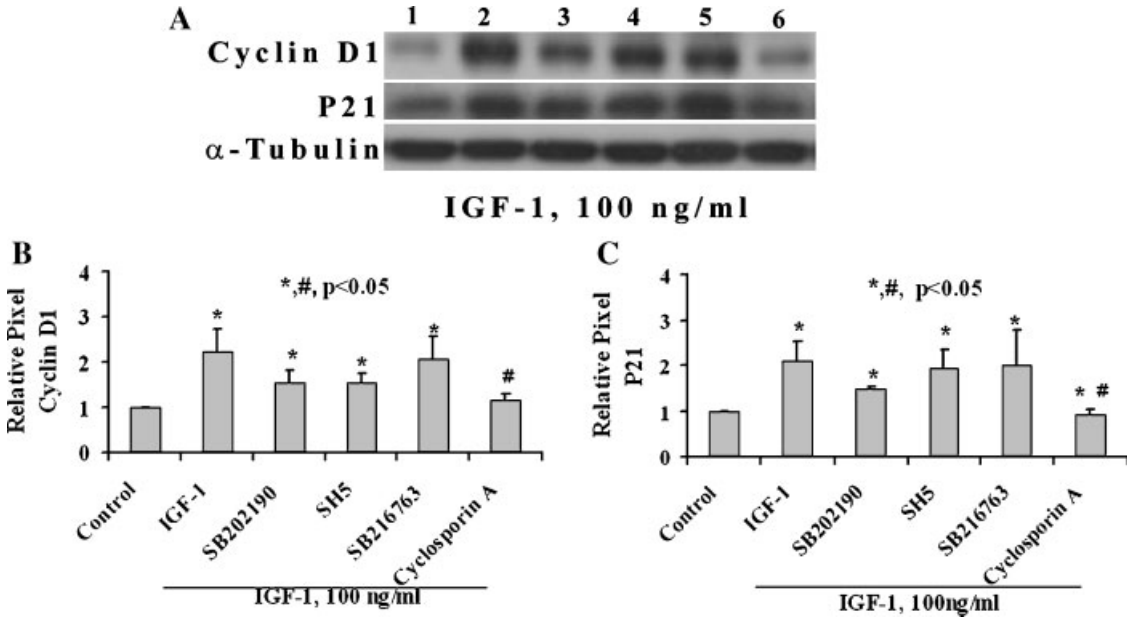

Fig. 5. Effect of pharmacological inhibitors on IGF-1-induced cyclin D1 and $p21^{Cip1}$ expression. Mesangial cell extracts (30 μg protein) were applied on to SDS–PAGE, transferred to PVDF membrane, and probed with anti-cyclin D1 and $p21^{Cip1}$ antibodies. **Lane 1**, Control; **lane 2**, IGF-1 alone; **lane 3**, IGF-1 + SB202190 (p38MAPK inhibitor, 10 μM); **lane 4**, IGF-1 + SH-5 (Akt inhibitor, 2 μM); **lane 5**, IGF-1 + SB216763 (GSK-β inhibitor, 100 nM); and **lane 6**, IGF-1 + cyclosporin A (calcineurin inhibitor, 2 μM). Representative blots for each protein from three different experiments are shown in (**A**), and their corresponding graphs are presented individually in (**B**) for cyclin D1, and (**C**) for $p21^{Cip1}$. The symbol $*P < 0.05$ represents a significant value compared to the control whereas # represents a statistically significant value ($P < 0.05$) versus IGF-1 alone. None of the inhibitors has an effect on α-tubulin level (A, last panel).

blocked significantly by calcineurin inhibition (cyclosporin A, $P < 0.05$) and partially by p38MAPK inhibition (SB202190) but did not reach a significant value ($P = 0.06$, $n = 3$). Akt (SH-5) or GSK-3β (SB216763) inhibition does not have an effect on $p21^{Cip1}$ activation by IGF-1 (Fig. 5A, second panel, lane 2 vs. 4 and 5, and Graph C).

Effect of Pharmacological Inhibitors on IGF-1-Induced Akt and GSK-3β Phosphorylation in Mesangial Cells

As described above in Figure 2, IGF-1 regulates Akt and GSK-3β phosphorylation in mesangial cells and the phosphorylation are sustained up to 24-h period. We also observed that inhibitors of Akt, p38MAPK, and calcineurin have varying effects on laminin, cyclin D1, and $p21^{Cip1}$ levels. Therefore, we investigated whether there exists a crosstalk between Akt/GSK-3β and that of p38MAPK and calcineurin pathways in mesangial cells. As shown in Figure 6A (first panel, lanes 1 and 2) and Graph B, IGF-1 induces Akt phosphorylation (2.99 ± 0.45-fold vs. Control, $P < 0.001$), which is impeded significantly by inhibiting p38MAPK (SB202190) ($P = 0.03$ vs. IGF-1) or by the presence of LY294002, an inhibitor of PI3-K ($P = 0.02$). SH-5, an inhibitor of the Akt activity itself, blocks Akt phosphorylation at Thr 308; but SB216763 (GSK-3β inhibitor) does not have a significant effect on IGF-1-induced Akt phosphorylation. Cyclosporin A (calcineurin inhibitor) increases IGF-1-induced Akt phosphorylation significantly ($P = 0.04$ against IGF-1) (Fig. 6B).

Similarly, GSK-3β Ser9 phosphorylation is increased by IGF-1 (1.88 ± 0.3 vs. Control, $P < 0.05$) and blocked significantly by p38 MAPK inhibition (0.31 ± 0.003, $P = 0.02$ vs. IGF-1; Fig. 6A third panel, lane 3, and Graph C). Cyclosporin A further elevates IGF-1's effect on GSK-3β phosphorylation (5.63 ± 1.2-fold, $P < 0.05$, IGF-1+cyclosporin A vs. IGF-1; Fig. 6A, third panel, lane 2 versus 6, and Fig. 6C). The amount of total GSK-3β is not significantly changed by the presence of the inhibitors (Fig. 6A, last panel).

IGF-1 Increases Mesangial Cell Metabolic Activity and Viability

We have demonstrated above that IGF-1 activates pro-survival factors such as protein

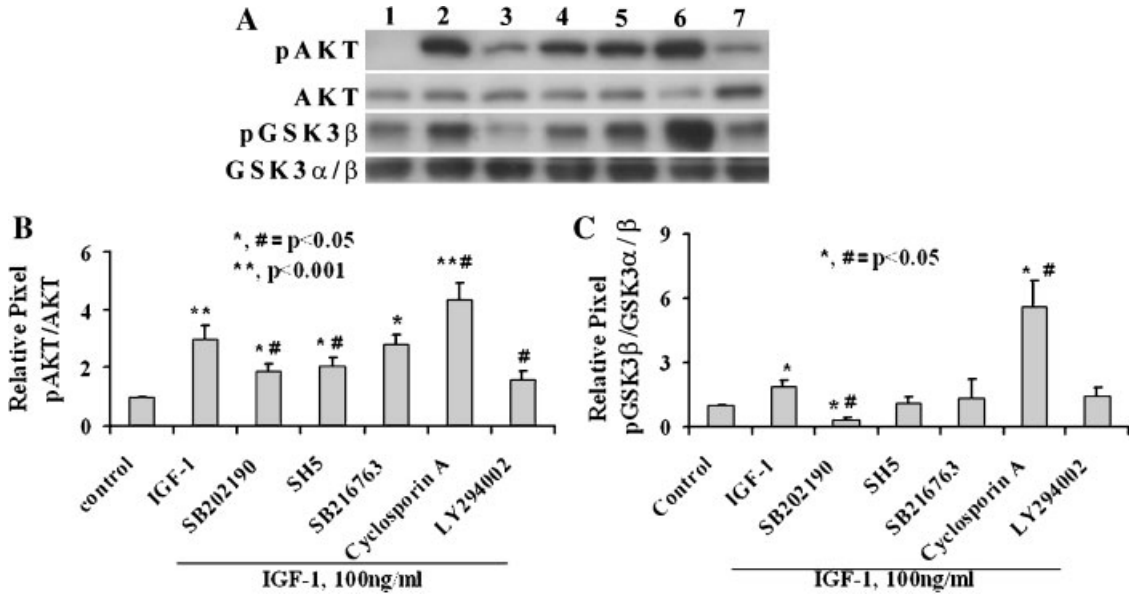

Fig. 6. Effect of pharmacological inhibitors on IGF-1-mediated Akt and GSK-3β phosphorylation. Pharmacological inhibitors for protein kinases and calcineurin were pre-treated for 30 min before adding IGF-1 (100 ng/ml) and incubation was continued for 24 h. Thirty micrograms of protein was used to probe for anti-phospho-Akt (Thr308) or phospho-GSK-3β(S9) antibodies on Western blots. Membranes were stripped off and reprobed with respective antibodies for total Akt and GSK-3α/β. **Lane 1**, Control; **lane 2**, IGF-1 alone; **lane 3**, IGF-1 + SB202190 (p38MAPK inhibitor, 10 μM); **lane 4**, IGF-1 + SH-5 (Akt inhibitor, 2 μM); **lane 5**, IGF-1 + SB216763 (GSK-3β inhibitor, 100 nM); **lane 6**, IGF-1 + cyclosporin A (calcineurin inhibitor, 2 μM); and **lane 7**, IGF-1 + LY294002 (PI3-K inhibitor, 10 μM). Representative blots for each protein from three different experiments are shown in **A**. Their normalized densitometric data are presented in **B** for phospho-Akt/Akt and in **C** for phospho-GSK-3β/GSK-3α/β ratios. A symbol * represents a significant value ($P < 0.05$); and ** represents $P < 0.001$ compared to the control, while # denotes $P < 0.05$ versus IGF-1 alone.

kinase Akt/PKB activity and cyclin D1 expression in mesangial cells (Figs. 2 and 4). Therefore, we examined the effect of IGF-1 on mesangial cell viability using MTT (absorbance) assay. As shown in Figure 7A, IGF-1 increases MTT activity in mesangial cell in a time-dependent manner from 0, 2, 6, and 24 h and significantly at 24 h ($P < 0.05$, n = 7). An inhibition of Akt (SH-5) or calcineurin (cyclosporin A) significantly reduces IGF-1-mediated mesangial cell viability (0.68 ± 0.05, $P = 0.01$ for SH-5 and 0.71 ± 0.09, $P = 0.02$, for cyclosporin A vs. IGF-1) as revealed by a reduction in MTT absorbance (Fig. 7B). Other inhibitors do not produce a significant change in mesangial cell viability.

IGF-1 Does not Induce Mesangial Cell-Cycle Progression from G_1-S Phase

IGF-1 increases mesangial cell metabolic activity and viability as shown above. Furthermore, IGF-1 also increases cyclin D1 and p21^{Cip1} expression simultaneously as shown in Figures 4 and 5. Therefore, flow cytometry was performed with propidium idodide to analyze IGF-1-mediated cell-cycle regulation in these cells. As shown in Figure 8, after 24 h incubation of MES cells with 100 ng/ml IGF1, the average distribution of cells in control cells is G_1 ($54.22 \pm 0.25\%$), S ($19.25 \pm 0.63\%$), and G_2/M phase ($24.75 \pm 0.48\%$) while that of the IGF-1 treated cells is G_1 ($51.75 \pm 1.49\%$, $P = 0.2$), S ($18.75 \pm 1.37\%$, $P = 0.7$), and G_2/M phase ($26.5 \pm 1.32\%$, $P = 0.2$), respectively. Therefore, there is no significant difference in the percentage of cells in the G_1, S, and G_2/M phase of cell cycle between IGF-1-treated and untreated cells. On the other hand, IGF-1 (100 ng/ml) increases the ratio of RNA to DNA content from 0–24 h significantly ($P < 0.05$ at 2 and 24 h vs. time 0 and $P < 0.01$ at 6 h) as shown in Figure 9, suggesting that IGF-1 induces mesangial cell growth and hypertrophy.

Diabetes Increases IGF-1, Cyclin D1, and p21^{Cip1} Levels in the Renal Cortex

IGF-1 accumulates in the diabetic kidney and is considered to involve in the expansion of the glomerular mesangium. We, therefore, examined whether the expression of IGF-1 in the

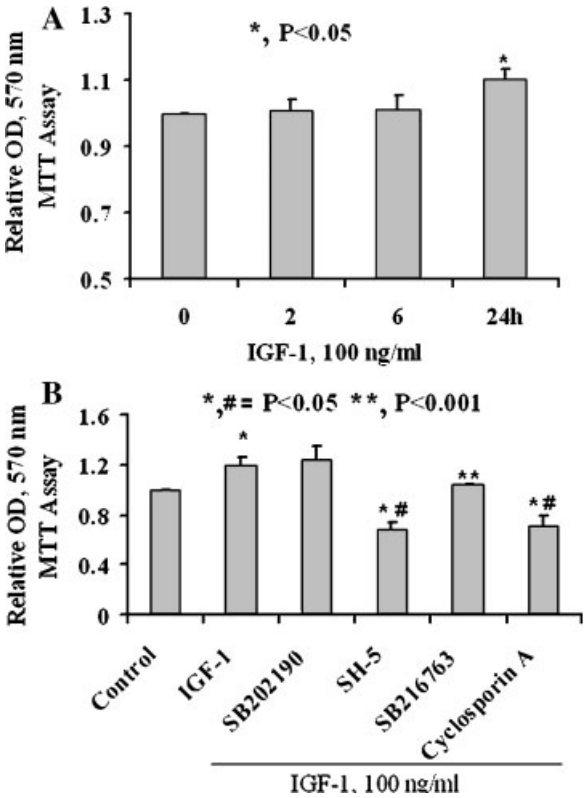

Fig. 7. Effect of IGF-1 on mesangial cell viability. **A**: MTT assay: A test for mesangial cell viability and cellular metabolic activity was performed with 0.5 mg/ml MTT as described in Materials and Methods. MES cells (1×10^3) were cultured in 96-well plates overnight and serum-starved for 24 h. IGF-1 (100 ng/ml) was added for different time periods, namely, 0, 2, 6, and 24 h. At 24 h, IGF-1 induces a significant increase in MTT absorbance (1.13 ± 0.03, $P = 0.02$, vs. time 0, $n = 7$). **B**: Effect of pharmacological inhibitors on MTT: IGF-1 and different pharmacological inhibitors for p38MAPK (SB202190, 10 μM), Akt (SH-5, 2 μM), GSK-3β (SB216763, 100 nM), and calcineurin (cyclosporin A, 10 μM) were added and incubated for 24 h ($n = 3$). MTT absorbance was measured at 570 nm. An asterisk (*) or double asterisks (**) indicate a statistically significant change in absorbance ($P < 0.05$ and $P < 0.001$, respectively) compared to the Control whereas the symbol # denotes a significant change versus IGF-1 ($P < 0.05$). The variation between the results in different experiments falls within 5%–10% for the controls.

renal cortex correlates with the level of cyclin D1 and $p21^{Cip1}$ in diabetic rats. As shown in Figure 10A, 4 weeks induction of diabetes in rats with STZ increases the level of IGF-1, cyclin D1 and $p21^{Cip1}$ expression in the cortex as compared to normal rats. Furthermore, the expression levels of laminin γ1 subunit and fibronectin are also increased in the diabetic rat cortex (Fig. 10B) while that of laminin β1 is not altered at 4 weeks of diabetes. However, laminin β1 expression is elevated after 8 weeks of diabetes (data not shown).

DISCUSSION

IGF-1 is one of the several growth factors that are implicated in renal growth, hypertrophy, and the progression of diabetic glomerulopathy because of its persistent accumulation in the diabetic kidney. In this study, we demonstrate that IGF-1 increases laminin β1 and γ1 expression as well as cell-cycle regulation factors cyclin D1 and $p21^{Cip1}$ in rat glomerular mesangial cells. In chronic kidney diseases such as diabetic nephropathy, the effect of IGF-1 on ECM and cell-cycle control gene expression in glomerular mesangial cells is a cumulative effect of several years of prolonged exposure of renal cells to high ambient glucose and growth factors. Therefore, in due course of time, the alterations in ECM, cyclin D1, and p21Cip1 expressions, although minimal and typically ~2-folds, may result in significant alterations in mesangial cell biology and disease progression. Of particular interest is the demonstration that IGF-1 induces the expression of cyclin D1 and $p21^{Cip1}$ in concert, which is impeded by cyclosporine A, an inhibitor of protein phosphatase 2B calcineurin activity. The fact that cyclosporin A also increases IGF-1-induced Akt and GSK-3β phosphorylation indicates the existence of a signal crosstalk between Akt/GSK-3β and calcineurin (Fig. 6). Calcineurin has been implicated in mediating IGF-1-induced mesangial cell hypertrophy and ECM protein expression, such as fibronectin and collagen IV [Gooch et al., 2001]. One target of calcineurin is the nuclear factors of activating T-cells (NFATc), which is sequestered in the cytosol upon phosphorylation with GSK-3β and also inhibits DNA binding activity in the nucleus [Neal and Clipstone, 1991]. Activation of calcineurin by IGF-1 in rat mesangial cells leads to nuclear accumulation of NFATc1, which is blocked by pretreatment with cyclosporin A [Gooch et al., 2001].

We further demonstrate that IGF-1 stimulates laminin β1 significantly and the effect is blocked by p38MAPK and Akt inhibition. On the other hand, laminin γ1 expression does involve neither p38MAPK nor Akt; in fact, none of the pharmacological inhibitors tested here had a significant effect. Hence, the regulation of laminin expression in mesangial cells is different from those presented by Gooch et al. [2001] for fibronectin and collagen IV expression. In that study, IGF-1 regulation of both fibronectin and collagen IV expression is inhibited signifi-

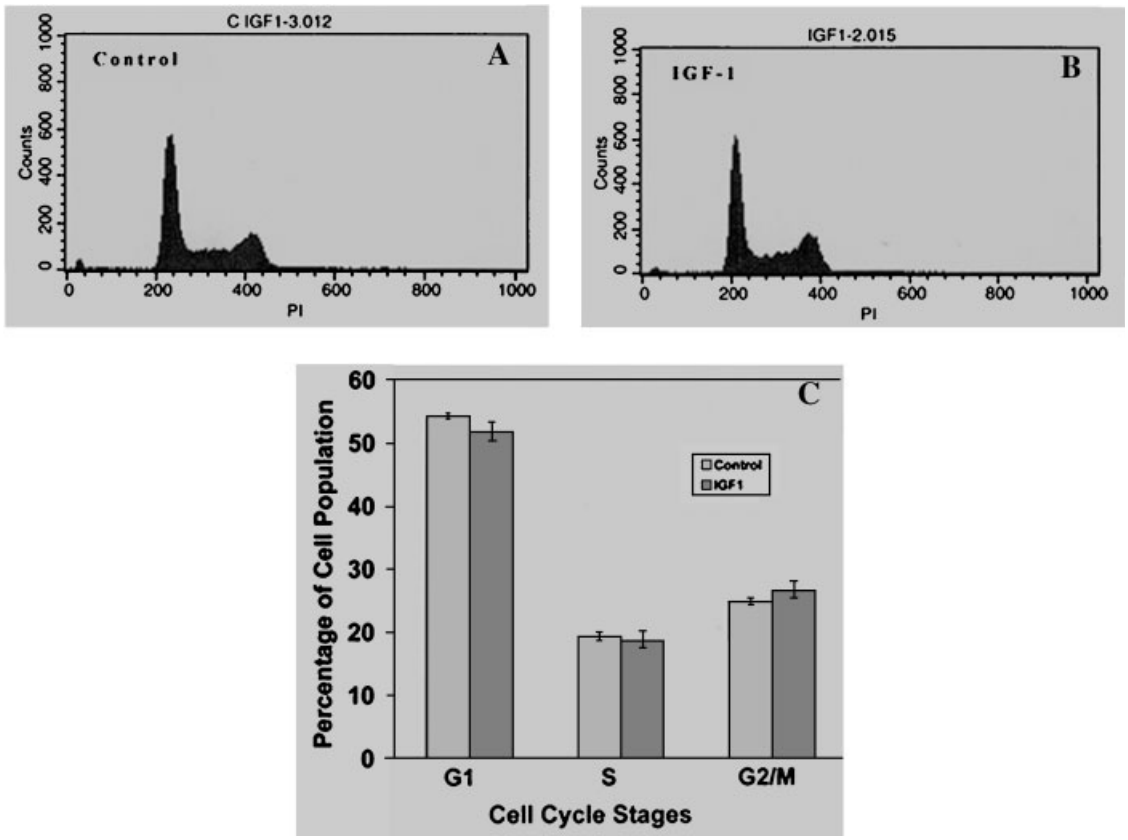

Fig. 8. IGF-1 does not induce mesangial cell-cycle progression from G_1 to S phase. Flow cytometry analysis of MES cells treated for 24 h with IGF-1 (100 ng/ml) or control cells were performed as described in Materials and Methods. The portion of cells in G_1, S, or G_2/M phase does not change significantly between IGF-1 treated and Control mesangial cells (*P*-values were greater than 0.2, not significant). Values are mean ± SE of four different experiments.

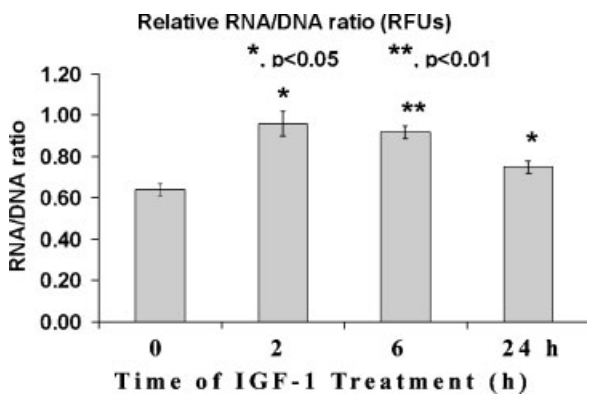

Fig. 9. IGF-1 treatment increases RNA/DNA ratio in mesangial cells: rat mesangial cells were treated with IGF-1 (100 ng/ml) for different time periods and RNA and DNA contents were determined as described in Materials and Methods using the CyQuant Dye binding at Ex480/Em520 nm. The ratio of RNA to DNA was determined at 0, 2, 6, and 24 h and their values were plotted (n = 5) as relative RFUs. The *t*-test values at 2, 6, and 24 h were $P < 0.05$, $P < 0.01$, and $P < 0.05$ (significant), respectively, as compared to the control at time 0.

cantly by cyclosporin A thereby blocking the calcineurin signaling, and does not involve PI3-K or Erk1/2 MAPK pathways. As indicated above, our results demonstrate that laminin β1 or γ1 subunit expression is not inhibited by cyclosporin A. These results suggest that the expression of ECM proteins under various pathological conditions where different intracellular signaling pathways are altered will produce abnormal or different ECM isoforms that do not exist in the normal kidney [Chai et al., 2003]. Such an alteration in the expression of laminin isoform(s) and/or other ECM proteins will certainly lead to changes in mesangial cell function including cell–cell and/or cell–matrix interaction and β-integrin signaling.

IGF-1 is known to increase PI3-K-dependent Akt phosphorylation at Thr 308 and/or Ser 473 and increase its kinase activity. Akt then phosphorylates one of its downstream substrates GSK-3β at Ser 9 and inhibits its kinase

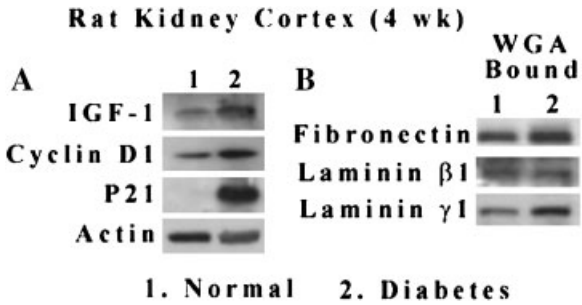

Fig. 10. **A**: Diabetes increases IGF-1, cyclin D1, and $p21^{Cip1}$ levels in the rat kidney. Renal cortex from normal and diabetic rats was homogenized in RIPA buffer in the presence of protease inhibitors, and proteins were extracted. Thirty μg proteins were loaded on to SDS–PAGE and Western blot analysis for IGF-1, cyclin D1, $p21^{Cip1}$, and actin were performed as described in Materials and Methods. The figures are representatives of n = 4 for both normal and diabetic kidneys. The expression of IGF-1, cyclin D1, and $p21^{Clp1}$ was enhanced in the diabetic renal cortex. **B**: Diabetes increases laminin γ1 expression in the renal cortex: 500 μg proteins were loaded on to a WGA-Agarose column and bound glycoproteins were eluted with 250 mM *N*-acetylglucosamine in RIPA buffer containing 0.3 M NaCl. Twenty-five microliters of the supernatant was subjected to Western blotting for laminin γ1, β1, and fibronectin. Laminin γ1 and fibronectin expressions are increased while that of laminin β1 is not changed. A representative blot of n = 3 is shown here.

function in various cell types including mesangial cells. Our findings that IGF-1-mediated intracellular signal transduction involves Akt activation and GSK-3β phosphorylation may correlate with an increase in the expression of G_0/G_1 cell-cycle progression factor cyclin D1 and the activation of cyclin D1-Cdk4 kinase and Cdk-4-directed phosphorylation of retinoblastoma (Rb) protein in diabetic renal hypertrophy [Feliers et al., 2002]. Surprisingly, the expression of the cyclin-dependent protein kinase inhibitor $p21^{Cip1}$ is significantly increased by IGF-1 in parallel with cyclin D1 expression (Fig. 4), and IGF-1 does not induce G_1 to S phase transition in these cells (Fig. 8). Hence, we interpret that IGF-1 increases cyclin D1 to proceed to G_0/G_1 progression but results in cell-cycle arrest at G_1 to S transition due to an increased expression of $p21^{Cip1}$, leading to cell volume increase. This, in junction with the absence of an increase in Rb phosphorylation and the expression of PCNA, markers of cell proliferation, in mesangial cells observed in the present study further support the hypothesis that IGF-1 is a hypertrophic factor for renal glomerular mesangial cells [Fig. 9 and Horney et al., 1998; Gooch et al., 2001, 2003]. The fact that, in 4 weeks diabetic rats, the expression of IGF-1, cyclin D1, and $p21^{Cip1}$ are also increased in the renal cortex (Fig. 10A) further suggests these changes may contribute to diabetic renal growth and mesangial hypertrophy. In addition, IGF-1 expression correlates with an increased expression of ECM proteins, such as laminin γ1 and fibronectin, hallmarks of diabetic nephropathy, in the cortex of diabetic rat kidney (Fig. 10B). Fan and Weiss [2004] have demonstrated that renal mesangial cell hypertrophy induced by hyperglycemia and IGF-1 is attenuated by antisense oligodeoxynucleotide to $p21^{Cip1}$. Our results further reinforce the hypothesis that IGF-1 is a renal hypertrophic factor and is an inducer of ECM expression in glomerular mesangial cells. Although IGF-1 signaling mechanisms in various cell types have been examined to some extent, IGF-1 signaling pathway(s) and crosstalk mechanisms in renal mesangial cells have not been investigated so far. This study represents one of the first such attempts to examine the various signaling mechanisms operating in mesangial cells, and they will form the basis for further studies to dissect out the role of each signaling pathway in IGF-1-mediated mesangial cell hypertrophy, ECM gene expression and cell-cycle control.

In summary, we have demonstrated that IGF-1 induces laminin β1 and γ1 expression in glomerular mesangial cells and that different signaling pathway(s), such as Akt and p38MAPK, may regulate laminin β1, but not laminin γ1, expression. IGF-1 increases Akt/PKB phosphorylation and its kinase activity resulting in the phosphorylation of its downstream substrate GSK-3β. Furthermore, an inhibition of calcineruin by cyclosporine A produces additional increases in the IGF-1-mediated Akt and GSK-3β phopshorylation. An elevated expression of IGF-1 and cell-cycle regulators cyclin D1 and $p21^{Cip1}$ is seen in the diabetic kidney and mesangial cells resulting in cell-cycle restriction at G_1 phase. The results presented in this study demonstrate that IGF-1 contributes to mesangial cell-cycle hypertrophy and matrix gene expression leading to mesangial expansion and the development of diabetic glomerulopathy.

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