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Effects of high glucose and TGF-β1 on the expression of collagen IV and vascular endothelial growth factor in mouse podocytes

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Effects of high glucose and TGF-β1 on the expression of collagen IV and vascular endothelial growth factor in mouse podocytes.

Background. The podocyte takes center stage in the pathogenesis of glomerular basement membrane (GBM) thickening and proteinuria in diabetic glomerulopathy. In part, GBM thickening may occur when the podocyte synthesizes increased amounts of collagen IV. Proteinuria may develop if the podocyte secretes excessive amounts of vascular endothelial growth factor (VEGF), which may increase the glomerular permeability to macromolecules. The augmented production of collagen IV and VEGF may be caused by metabolic mediators of diabetes such as hyperglycemia and transforming growth factor-β (TGF-β).

Methods. The effects of high glucose and exogenous TGF-β1 were examined on a mouse podocyte cell line that retains its differentiated phenotype. The gene expression and protein production of certain alpha chains of collagen IV, the major isoforms of VEGF, and components of the TGF-β system were assayed. An inhibitor of TGF-β signaling was used to determine whether some of the high glucose effects might be mediated by the TGF-β system.

Results. Compared with normal glucose (5.5 mmol/L), high glucose (HG, 25 mmol/L) for 14 days stimulated [^{3}H]-proline incorporation, a measure of collagen production, by 1.8-fold, and exogenous TGF-β1 (2 ng/mL) for 24 hours stimulated proline incorporation by 2.4-fold. Northern analysis showed that exposure to HG for 14 days increased the mRNA level of α1(IV) collagen by 51% and α5(IV) by 90%, whereas treatment with TGF-β1 (2 ng/mL) for 24 hours decreased the mRNA level of α1(IV) by 36% and α5(IV) by 40%. Consistent with these effects on mRNA expression, Western blotting showed that HG increased α1(IV) protein by 44% and α5(IV) by 28%, while TGF-β1 decreased α1(IV) protein by 29% and α5(IV) by 7%. In contrast to their opposing actions on α1 and α5(IV), both HG and exogenous TGF-β1 increased α3(IV) collagen and VEGF, with TGF-β1 having the greater effect. An inhibitor of the TGF-β type I receptor (ALK5) was able to prevent the stimulation of α3(IV) and VEGF proteins by HG. Unlike in other renal cell types, HG did not increase TGF-β1 mRNA or protein in the podocyte, but HG did induce the expression of the ligand-binding TGF-β type II receptor (TβRII). Because HG had up-regulated TβRII after two weeks, the addition of physiological-dose TGF-β1 (0.010 ng/mL) for 24 hours stimulated the production of α3(IV) and VEGF proteins to a greater extent in high than in normal glucose. Up-regulation of TβRII in the podocyte was corroborated by immunohistochemistry of the kidney cortex in the *db/db* mouse, a model of type 2 diabetes.

Conclusions. High glucose and exogenous TGF-β1 exert disparate effects on the expression of α1 and α5(IV) collagen. However, high glucose and TGF-β1 coordinately induce the production of α3(IV) collagen and VEGF in the podocyte. The HG-induced increases in α3(IV) collagen and VEGF proteins are mediated by the TGF-β system. By increasing the expression of TβRII, high glucose may augment the response of the podocyte to ambient levels of TGF-β1.

Key words: diabetic nephropathy, glomerular basement membrane, epithelial cell, proteinuria, hypertrophy, foot process.

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Diabetic nephropathy involves structural alterations that are characterized by early hypertrophy of glomerular and tubular components, the subsequent development of thickened glomerular and tubular basement membranes, and the progressive accumulation of extracellular matrix proteins in the glomerular mesangium and tubulointerstitium. Functionally, the alterations in the diabetic kidney include the early development of glomerular hyperfiltration, the increased urinary excretion of albumin, and the subsequent development of renal insufficiency [1].

Three layers constitute the glomerular filtration barrier: the fenestrated vascular endothelial cells, the glomerular basement membrane (GBM), and the visceral

epithelial cells or podocytes. The podocyte is a highly differentiated cell type that synthesizes components of the GBM, such as type IV collagen [2], and forms an interdigitating network of foot processes, which are spanned by slit diaphragms. The GBM and the slit diaphragm make up the bulk of the glomerular filtration barrier [3]. The GBM in the adult mammal is composed of two different networks of type IV collagen. The ubiquitous α1(IV) and α2(IV) chains are found in virtually all basement membranes and are present in the subendothelial aspect of the GBM. On the other hand, the novel α3(IV), α4(IV), and α5(IV) chains are much more restricted in their tissue distribution and are present throughout the width of the glomerular and alveolar basement membranes [4, 5].

Alterations in the morphology of the podocyte, from the disruption of foot process architecture to the loss of entire podocytes, are associated with significant proteinuria in many glomerular diseases [3, 6, 7]. In diabetic nephropathy, the podocyte number is markedly reduced (with associated podocyturia), the foot process width is significantly widened, and the slit diaphragm becomes narrower as the glomerular filtration rate declines [7–11].

Several pieces of evidence link the effects of hyperglycemia to activation of the intrarenal transforming growth factor-β (TGF-β) system [12]. We and others have shown that TGF-β1 mRNA and protein are elevated in the kidneys of diabetic animals [13–16]. The ligand-binding TGF-β type II receptor (TβRII) is up-regulated in the diabetic kidney as well [17, 18]. Hyperglycemia appears to be a cause of the increased TGF-β1 and TβRII in the kidney because high glucose media reproduce these changes in cultured renal cells [17, 19–22]. Furthermore, the hypertrophic and profibrotic effects of high glucose are duplicated by treatment with TGF-β1 alone [12]. Finally, neutralization of TGF-β activity in cell culture and in diabetic animals prevented cellular and renal hypertrophy, overexpression of extracellular matrix proteins, and diabetic glomerulosclerosis, proving that the TGF-β system mediates many of the nephropathic effects of high glucose and diabetes [16, 23, 24].

Vascular endothelial growth factor (VEGF) stimulates new blood vessel formation (angiogenesis) and dramatically increases the permeability of the microvasculature to plasma proteins, hence its original name vascular permeability factor (VPF) [25–28]. In diabetes, VEGF plays a major role in the neovascularization of proliferative retinopathy [29] and in the breakdown of the blood-retinal barrier, characterized by hyperpermeability of the retinal vessels [30]. In as much as retinopathy and nephropathy are both microvascular complications of diabetes, VEGF also may enhance the permeability of the glomeruli to macromolecules, leading to albuminuria [31]. The expression of VEGF and its receptor is significantly increased in the kidneys of streptozotocin (STZ)-induced diabetic rats [32]. Neutralizing the excess VEGF activity with an anti-VEGF antibody largely prevents the development of albuminuria in STZ-diabetic rats [33]. Because glomerular VEGF expression is strongest in the podocyte [34, 35], investigators have theorized that an increased production of VEGF by the podocyte may promote the development of proteinuria in diabetic nephropathy.

Studying the in vitro podocyte in its differentiated state is desirable, but achieving this has been troublesome because standard cell culture methods induce cellular proliferation but provoke the loss of differentiation [36]. However, with the recent establishment of a mouse podocyte cell line that is conditionally immortalized, the study of fully differentiated podocytes in culture has become feasible [37]. These cells divide under one set of culture conditions (that is, permissive) but then differentiate under another set of conditions (that is, non-permissive). Thus, by manipulating the cell culture environment, the investigator can propagate podocytes to maintain the cell line or induce them to differentiate for the purposes of experimentation [37].

Our current study investigates the effects of high glucose and TGF-β1 on the cultured differentiated podocyte [37] with respect to the expression of type IV collagen and VEGF because these proteins are likely involved in the pathogenesis of GBM thickening and proteinuria. We also examine whether high glucose modulates the expression of components of the TGF-β system in podocytes, both in vitro and in vivo. Finally, an inhibitor of the kinase activity of TGF-β type I receptor (ALK5 inhibitor), SB-431542 [38], was used to ascertain whether some of the high glucose effects on podocytes may be mediated by the TGF-β system.

METHODS

Cell culture

All media and reagents used for cell culture were purchased from Gibco BRL (Gaithersburg, MD, USA). Cultivation of mouse podocytes that were conditionally immortalized with a temperature-sensitive variant of the SV40 large T antigen (tsA58) was performed as described [37]. Briefly, podocytes were maintained in RPMI 1640 that was supplemented with 10% fetal calf serum (FCS), 100 U/mL penicillin, and 100 μg/mL streptomycin. To propagate podocytes, cells were cultivated at 33°C and treated with 10 U/mL of mouse recombinant γ-interferon (permissive conditions) to enhance expression of the large T antigen [39]. To induce differentiation, podocytes were maintained on a bed of type I collagen at 37°C and deprived of γ-interferon (non-permissive conditions) for 14 days in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% FCS, penicillin, and streptomycin.

Experimental design

During the differentiation period, podocytes in culture were exposed to DMEM with 5% FCS containing either a normal D-glucose concentration (5.5 mmol/L) or a high D-glucose concentration (25 mmol/L) for 14 days. At day 7, the culture media were changed to fresh normal glucose or high glucose media. In some experiments, a third group of podocytes was exposed to 5.5 mmol/L D-glucose plus 19.5 mmol/L D-mannitol to control for the osmotic effects of high glucose. Exogenous TGF-β1 (R&D Systems, Minneapolis, MN, USA), ranging from 0.010 to 2.0 ng/mL, was added for the last 24 hours of the 14-day incubation as indicated. In the experiments that block the TGF-β signaling pathway, 1 μmol/L of an ALK5 inhibitor, SB-431542 (gift of Dr. N. Laping; GlaxoSmithKline, King of Prussia, PA, USA), was added for the last 7 days of the 14-day incubation. Total RNA or cell lysate protein or cell culture supernatants were then harvested.

Measurement of [^{3}H]-proline incorporation

To study the kinetics of high glucose-induced collagen production in podocytes, we studied [^{3}H]-proline incorporation into proteins. Podocytes were seeded onto 24-well plates (Nunclon, Roskide, Denmark) and exposed to normal or high glucose for up to 14 days. Exogenous TGF-β1 (2 ng/mL) was added for the last 24 hours of the incubation. In the last 16 hours, the cells were pulsed with 1 μCi of L-[2,3,4,5-^{3}H] proline (Amersham Pharmacia, Piscataway, NJ, USA). Radiolabeled podocytes were washed twice in ice-cold phosphate-buffered saline (PBS) and then dissolved in 0.5 N NaOH with 0.1% Triton X-100. After neutralization with 0.5 N HCl, the incorporated radioactivity was measured in a liquid scintillation counter. Proline incorporation was corrected for the cellular protein content.

Northern analysis

Murine α1(IV), TGF-β1, and TβRII cDNA probes were synthesized by reverse transcription-polymerase chain reaction of mouse kidney RNA and cloned into the pCRII TA cloning vector (Invitrogen, La Jolla, CA, USA) as described previously [16]. An 800-bp BamHI-HindIII fragment encoding α3(IV) and an 800-bp cDNA for α5(IV) were used [40, 41]. The cDNA probe for VEGF recognizes the 523- to 973-bp portion of the mouse VEGF transcript, which is common to the major VEGF isoforms, and was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Total RNA was isolated using TRIzol reagent (Gibco BRL). Northern blot and hybridization were performed as previously described [42]. Exposed films and stained membranes were scanned and analyzed with the NIH Image 1.62c program, and mRNA levels relative to 28S rRNA were calculated.

Isolation of membrane proteins for TβRII

Podocytes were washed twice with ice-cold PBS and then collected by scraping in a detergent-free lysis buffer [50 mmol/L Tris (pH 8.0), 150 mmol/L NaCl, 1 mmol/L ethylenediaminetetraacetic acid (EDTA), 0.5 mmol/L dithiothreitol (DTT)] with protease inhibitor cocktail (Sigma, St. Louis, MO, USA). The cells were passed through a 23-gauge needle 30 times for mechanical disruption. After centrifugation at 1000 × *g* for five minutes at 4°C, the supernatant was collected and subjected to ultracentrifugation at 100,000 × *g* for 30 minutes at 4°C. The supernatant was discarded and the pellet, containing the membrane fraction, was dissolved in lysis buffer with detergent [above plus 0.1% sodium dodecyl sulfate (SDS) and 1% NP-40] to release the proteins from the membrane lipid bilayer. After a final centrifugation at 10,000 × *g* for 15 minutes at 4°C, the supernatant, containing the membrane proteins, was processed for Western blotting.

Immunoblot (Western) analysis

Cell lysates were prepared in Laemmli sample buffer and boiled for five minutes. Aliquots of cellular protein (20 to 25 μg) were subjected to SDS-10% polyacrylamide gel electrophoresis (PAGE) and then transferred to a nitrocellulose membrane (Micron Separations Inc., Westborough, MA, USA). The membrane was blocked with 5% nonfat milk in Tris buffered saline-0.1% Tween-20 at room temperature for one hour and then probed with rabbit anti-α1(IV) collagen [43], human anti-α3(IV) collagen (gift of Dr. M. Madaio, Philadelphia, PA, USA) [44], rabbit anti-α5(IV) collagen [43], mouse anti-VEGF (NeoMarkers, Fremont, CA, USA), or rabbit anti-TβRII (Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibody at 4°C for 16 hours. After several washes, a horseradish peroxidase (HRP)-conjugated secondary antibody was used to detect the immunoreactive bands with an enhanced chemiluminescence system, ECL Plus (Amersham Pharmacia).

Enzyme-linked immunosorbent assay

Supernatants from each cell culture condition were frozen at −20°C until assayed by a TGF-β1 or VEGF enzyme-linked immunosorbent assay (ELISA) kit, performed according to the manufacturer's instructions (R&D Systems). In brief, for the TGF-β1 ELISA, the supernatants of the podocyte cultures were acid-activated with 1 N HCl followed by neutralization with 1.2 N NaOH/0.5 mol/L HEPES to measure total TGF-β1 (latent plus active) or left untreated with acid to measure active TGF-β1 only. Samples were applied to microtiter plates that had been pre-coated with the TGF-β type II receptor. After an incubation of three hours at room temperature, the wells were washed and horseradish per-

oxidase (HRP)-conjugated anti-TGF-β1 antibody was added to the wells for 1.5 hours at room temperature. After another wash, a solution containing substrate for HRP was added. The resulting chromogenic reaction was halted with a stop solution. The absorbance at 450 nm was measured in a microplate reader. TGF-β1 concentrations were determined from the standard curve and corrected for the amount of total cell protein.

For the VEGF ELISA, the cell culture supernatants were dispensed onto a microtiter plate coated with an anti-VEGF antibody that recognizes mouse $VEGF_{120, 164}$. After a two hour incubation, a secondary anti-VEGF antibody, conjugated to HRP, was added for an additional two hours. The chromogenic reaction was developed with the substrate solution as above. After addition of a stop solution, the absorbance at 450 nm was measured. The total amount of secreted VEGF was extrapolated from the standard curve and then corrected for the amount of total cell protein.

In vivo studies

Diabetic *db/db* and non-diabetic *db/m* mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and killed at 16 weeks of age when the *db/db* mice had been frankly hyperglycemic for approximately eight weeks and had developed lesions of diabetic nephropathy [24]. The left kidney was excised, bisected, and processed in fixative solutions. The immunohistochemical staining for TGF-β1 and TβRII was performed as previously described [18]. Kidney sections were incubated overnight at 4°C with one of two primary antibodies at a 1:100 dilution: a polyclonal rabbit antibody against mouse TGF-β1 or a polyclonal rabbit antibody against mouse TβRII (both from Santa Cruz Biotechnology). The same concentration of an isotype-matched antibody of irrelevant specificity was used as a negative control. After three five-minute washes in phosphate-buffered saline (PBS), a biotinylated anti-rabbit antibody and then HRP-conjugated streptavidin (Vector Laboratories, Burlingame, CA, USA) were applied to the kidney sections for 45 minutes. The color reaction was developed with the diaminobenzidine detection kit (Vector Laboratories), and the sections were counterstained with hematoxylin.

Statistical analysis

Results are shown as the mean ± SEM, and *N* represents the number of experiments. In each case, the paired Student *t* test was used to compare the control with the experimental group. $P < 0.05$ was considered statistically significant.

RESULTS

High glucose and exogenous TGF-β1 increase proline incorporation

Because collagen is rich in proline, the incorporation of radiolabeled proline into cells serves as a rough estimate of collagen production. We first examined whether high glucose or exogenous TGF-β1 stimulates collagen production. Exposure to high glucose for the last 24 to 96 hours of the incubation period did not change the proline incorporation (data not shown). However, exposure to high glucose for 14 days significantly stimulated proline incorporation by 84% ($P < 0.05$, $N = 3$, Fig. 1). The stimulatory effect of high glucose was not due to its osmolarity since the addition of D-mannitol to raise the total sugar concentration to 25 mmol/L did not stimulate proline incorporation (NG+MAN, 84 ± 11% of normal glucose, $N = 3$; Fig. 1). Similar to high glucose, TGF-β1 significantly increased proline incorporation into podocytes by more than twofold (NG+T, 242 ± 39% of normal glucose, $P < 0.05$, $N = 3$; Fig. 1).

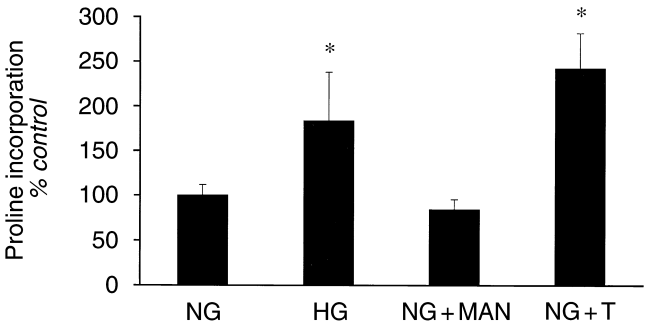

Fig. 1. High glucose and exogenous transforming growth factor β1 (TGF-β1) increase proline incorporation in cultured differentiated mouse podocytes. Podocytes were seeded onto 24-well plates and then exposed to normal glucose (NG), high glucose (HG), or mannitol (NG+MAN) for 14 days; or exposed to 2 ng/mL of TGF-β1 (NG+T) for 24 hours. In the last 16 hours, the podocytes were pulsed with 1 μCi of L-[2,3,4,5-^{3}H] proline (Amersham Pharmacia). The amount of [^{3}H]-proline incorporation, measured in a scintillation counter, was corrected for the cellular protein content. The results are expressed as percentages of the control (NG) and are the mean ± SEM of three independent experiments. *$P < 0.05$ vs. NG.

Effects of high glucose and exogenous TGF-β1 on the expression of collagen IV chains

Having found that both high glucose and TGF-β1 stimulate collagen production, we focused on collagen IV, the predominant type of collagen in the GBM. Specifically, the gene expression and protein production of three of the alpha chains of collagen IV were examined in response to high glucose and exogenous TGF-β1. The α1, α3, and α5 chains were studied because each is half of a pair of genes that encode the six alpha chains of collagen IV. In the mouse, the genes for α1 and α2 are paired head-to-head on chromosome 8 [45], those for α3 and α4 are head-to-head on chromosome 1 [46], and those for α5 and α6 are head-to-head on chromosome X [47].

The relative changes in the mRNA expression and protein levels of the α1 and α5 chains of collagen IV were examined first under normal versus high glucose conditions. By Northern analysis of podocytes under nor-

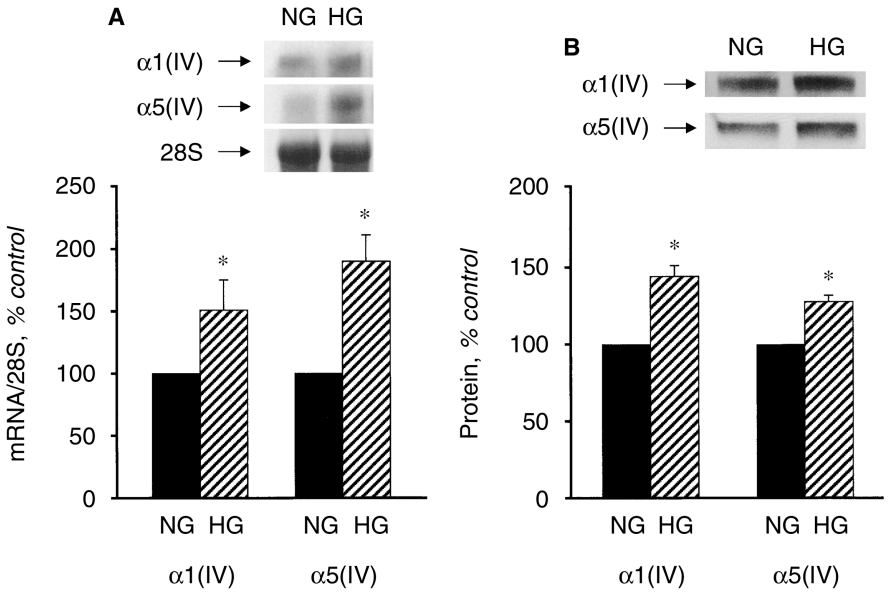

Fig. 2. High glucose increases the expression of α1 and α5(IV) collagen in podocytes. Podocytes were incubated for 14 days in normal glucose (NG) or high glucose (HG), and total RNA or cell lysate protein was harvested as described in the **Methods**. The gene expression and protein production of the α1 and α5 chains of collagen IV were analyzed by Northern blotting and Western blotting, respectively. The densitometric ratios of the α1 and α5(IV) mRNA bands to 28S rRNA (*A*), and the densitometric values of the α1 and α5(IV) protein bands (*B*) are shown as percentages of the control (NG). Results are expressed as the mean ± SEM of five independent experiments. *$P < 0.05$ vs. NG.

mal glucose, the α1 and α5 chains were constitutively expressed. Exposing the podocyte to high glucose for 14 days significantly increased the mRNA levels of both the α1 and α5(IV) chains (151 ± 24% and 190 ± 21% of normal glucose, respectively, $P < 0.05$, $N = 5$; Fig 2A). By Western blotting, high glucose significantly increased the protein production of the α1 and α5(IV) chains as well (144 ± 7% and 128 ± 4% of normal glucose, respectively, $P < 0.05$, $N = 5$; Fig. 2B), consistent with the changes in mRNA expression.

We next focused on the effects of exogenous TGF-β1 on α1 and α5(IV) collagen. Treatment with 2 ng/mL of exogenous TGF-β1 for 24 hours significantly inhibited the gene expression of the α1 chain by 36% and that of the α5 chain by 41% ($P < 0.05$, $N = 4$; Fig. 3A), contrary to the stimulatory effects of high glucose (Fig. 2A). Western blotting showed that TGF-β1 also decreased the amount of α1(IV) protein by 29% ($P < 0.05$, $N = 4$; Fig. 3B) but had little inhibitory effect on the quantity of α5(IV) protein (P = NS, $N = 8$; Fig. 3B).

Finally, the effects of high glucose and TGF-β1 on the gene expression and protein levels of α3(IV) collagen were studied. By Northern analysis, the bands representing the mRNA expression of α3(IV) were weak under normal glucose and high glucose. However, TGF-β1 treatment made the band easily visible (Fig. 4A). It seems that podocytes under basal or high glucose conditions do not express perceivable bands of α3(IV) mRNA by Northern analysis. Nevertheless, the α3(IV) cDNA probe functions well and is probably detecting authentic levels of α3(IV) gene expression since the TGF-β1–induced α3(IV) band is quite evident (Fig. 4A). Interestingly, high glucose was still able to increase the amount of α3(IV) protein by Western blotting (143 ± 5% of normal glucose, $P < 0.05$, $N = 9$; Fig. 4B). Perhaps high glucose causes α3(IV) collagen to accumulate by a post-transcriptional mechanism without affecting its transcript levels in the steady-state. Even more striking than high glucose, exogenous TGF-β1 elevated the level of α3(IV) protein by 3.3-fold ($P < 0.05$, $N = 5$; Fig. 4C).

Effects of high glucose and exogenous TGF-β1 on the expression of VEGF

Compared with incubation in normal glucose, exposure to high glucose for 14 days significantly increased the level of VEGF transcripts in differentiated podocytes by 70% ($P < 0.05$, $N = 5$; Fig. 5A). Likewise, by Western blotting on whole cell lysate, high glucose raised the protein production of VEGF by 75% ($P < 0.05$, $N = 4$; Fig. 5B). The particular VEGF isoform that was increased could not be determined, because the primary antibody used in Western blotting recognizes all three of the major isoforms ($VEGF_{120, 164, 188}$). An ELISA for $VEGF_{120, 164}$ (R&D Systems), however, revealed that cell culture supernatants are not enriched in these secreted isoforms of VEGF under high glucose conditions (Fig. 5C). This suggests that the increase in VEGF by high glucose is due to a rise in $VEGF_{188}$, which is contained in the cell lysate but not secreted into the supernatant.

The addition of 2 ng/mL of TGF-β1 for 24 hours significantly elevated the mRNA expression of VEGF in differentiated podocytes by 2.5-fold ($P < 0.05$, $N = 5$, Fig. 6A). This stimulation was both time- and dose-dependent, becoming evident in as little as two hours and at a dose as low as 0.010 ng/mL (data not shown). The increase in VEGF mRNA due to exogenous TGF-β1 correlated with an increase in the protein production of VEGF, measured by Western blotting of cell lysate

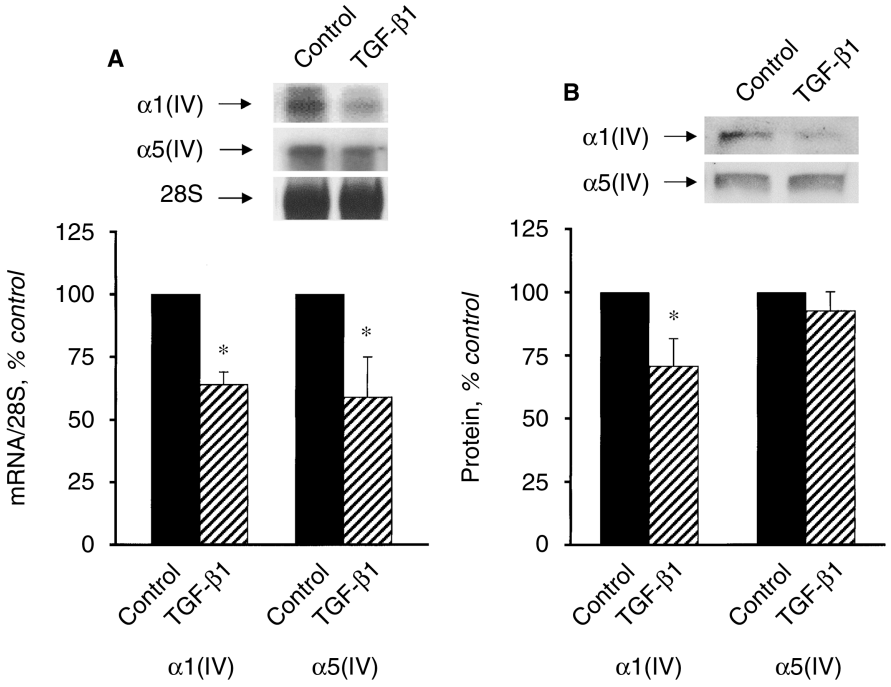

Fig. 3. Effects of exogenous TGF-β1 on the expression of α1 and α5(IV) collagen in podocytes. After incubation in normal glucose for 14 days, podocytes were treated with or without 2 ng/mL of TGF-β1 for 24 hours. The gene expression and protein production of the α1 and α5 chains of collagen IV were analyzed by Northern blotting and Western blotting, respectively. The densitometric ratios of the α1 and α5(IV) mRNA bands to 28S rRNA (*A*), and the densitometric values of the α1 and α5(IV) protein bands (*B*) are shown as percentages of the control (NG). Results are expressed as the mean ± SEM of four to eight independent experiments. $^*P < 0.05$ vs. Control.

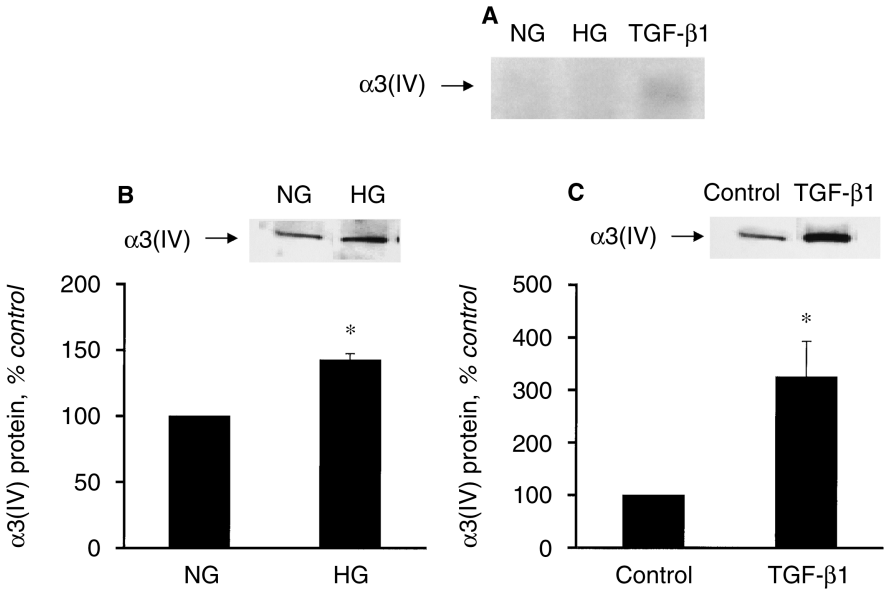

Fig. 4. Both high glucose and exogenous TGF-β1 increase levels of α3(IV) collagen. Podocytes were exposed to high glucose or treated with 2 ng/mL of exogenous TGF-β1, as previously described. Representative bands of α3(IV) collagen mRNA are shown (*A*). Note that the percentage increase in mRNA expression due to high glucose or TGF-β1 could not be quantified, because the control α3(IV) band was not detectable above background. By Western blotting, the protein production of α3(IV) collagen is shown under high glucose conditions (*B*) or with TGF-β1 treatment (*C*). The densitometric values of the α3(IV) protein bands are expressed as percentages of the control (NG in both cases). Results are the mean ± SEM of nine (panel B) or five (panel C) independent experiments. $^*P < 0.05$ vs. NG or Control.

(TGF-β1, 227 ± 44% of control, $P < 0.05$, $N = 4$; Fig. 6B) and ELISA (R&D Systems) of cell culture supernatant (TGF-β1, 216 ± 4% of control, $P < 0.05$, $N = 4$; Fig. 6C). The data suggest that, unlike high glucose, TGF-β1 stimulates the production of secreted VEGF isoforms ($VEGF_{120, 164}$). However, a stimulatory effect of TGF-β1 on $VEGF_{188}$ cannot be ruled out.

Certain high glucose effects mediated by the TGF-β system

Because high glucose and exogenous TGF-β1 both stimulate the expression of α3(IV) collagen and VEGF, these high glucose effects are possibly mediated by the TGF-β system. The TGF-β ligand ultimately signals through the TGF-β type I receptor or activin-like kinase (ALK5), which phosphorylates the Smad proteins that carry the TGF-β signal to the nucleus [48]. Therefore, an ALK5 inhibitor (SB-431542) was used to determine whether the effect of high glucose would be prevented by TGF-β blockade. We found that the increase in α3(IV) protein secondary to high glucose was completely prevented by SB-431542 (HG: 136 ± 3% of normal glucose vs. HG+SB-431542, 77 ± 8%, $P < 0.05$, $N = 6$; Fig. 7A). The high glucose-induced increase in VEGF protein

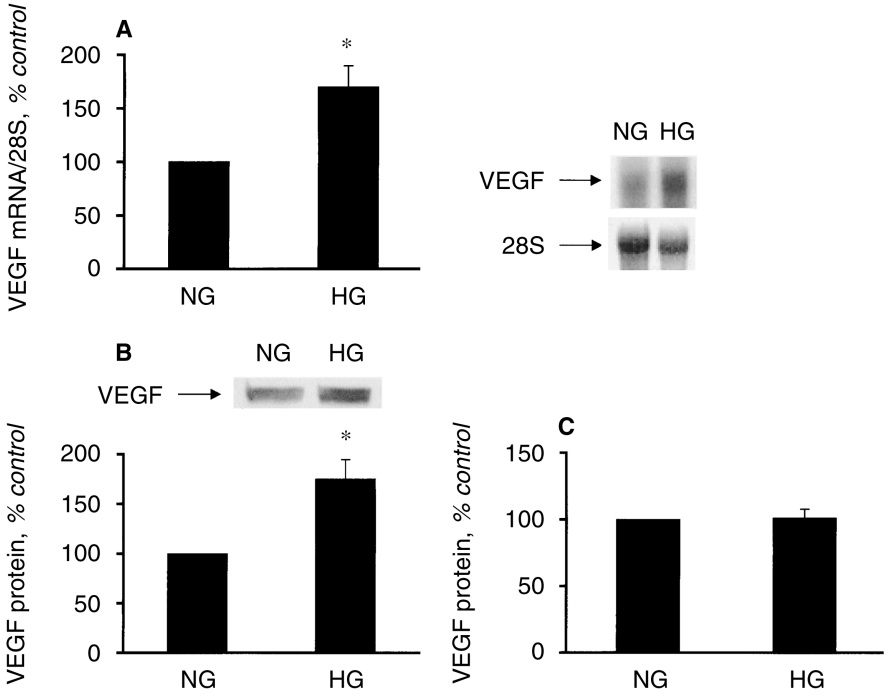

Fig. 5. Effects of high glucose on mRNA expression and protein production of VEGF. Podocytes were incubated for 14 days in normal glucose (NG) or high glucose (HG). Northern analysis using a cDNA probe for VEGF (*A*), Western blotting with an anti-VEGF antibody (*B*), and ELISA for $VEGF_{120, 164}$ on cell culture supernatants (*C*) were performed as described. Representative Northern and Western bands of VEGF mRNA and protein, respectively, are shown. Results are expressed as percentages of the control (NG) and are the mean ± SEM of five (panel A) or four (panels B and C) independent experiments. $^*P < 0.05$ vs. NG.

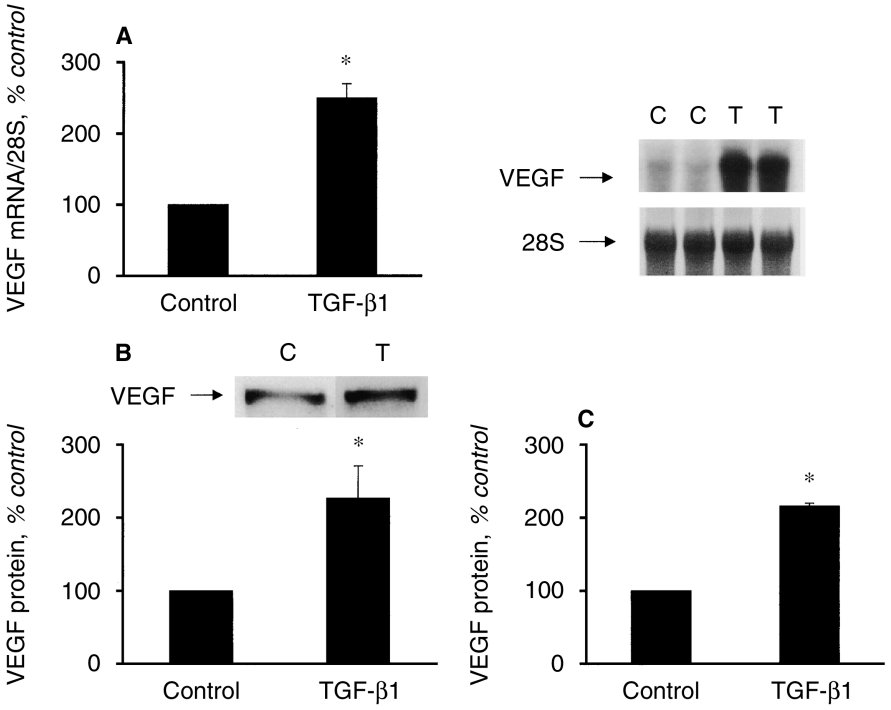

Fig. 6. Exogenous TGF-β1 increases mRNA expression and protein production of VEGF. Podocytes were incubated in normal glucose for 14 days and then treated with or without 2 ng/mL of exogenous TGF-β1 (T) for 24 hours. Northern analysis for VEGF mRNA (*A*) and Western blotting for VEGF protein (*B*) were performed as described. ELISA for mouse $VEGF_{120, 164}$ was performed on cell culture supernatants (*C*). The quantity of VEGF (pg) was corrected for the total protein content (mg), and the results are expressed as percentages of the control (normal glucose). Results are shown as the mean ± SEM of five (panel A) or four (panels B and C) independent experiments. $^*P < 0.05$ vs. Control.

was partially prevented by SB-431542 (HG, 145 ± 14% of normal glucose vs. HG+SB-431542, 109 ± 14%, $P < 0.05$, $N = 7$; Fig. 7B). Of note, the ALK5 inhibitor was probably not toxic to the podocyte, since the individual and total protein levels were not significantly different from normal glucose to normal glucose+SB-431542.

Effects of high glucose and exogenous TGF-β1 on the expression of TGF-β1 and TβRII

Since high glucose stimulates the production of TGF-β1 in almost all renal cell types [19–21, 23], we investigated whether high glucose would stimulate the mRNA expression and protein production of TGF-β1

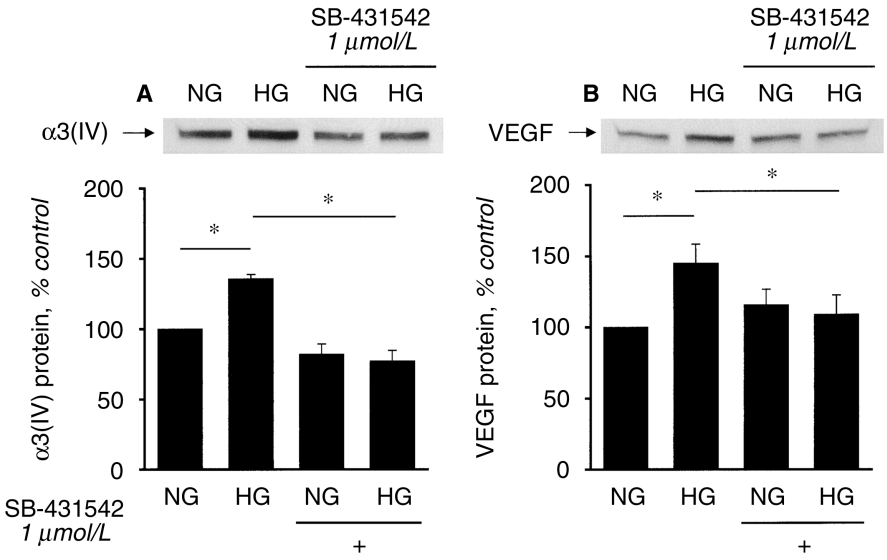

Fig. 7. Inhibition of TGF-β type I receptor (ALK5) prevents high glucose-induced changes in α3(IV) collagen and VEGF. Podocytes were incubated in normal glucose (NG) or high glucose (HG) for 14 days. In the last 7 days, cells were treated with or without an ALK5 inhibitor, SB-431542, at a concentration of 1 μmol/L. The protein production of α3(IV) collagen (*A*) and VEGF (*B*) was analyzed by Western blotting. Representative groups of α3(IV) collagen and VEGF bands, each from a single experiment, are shown. The densitometric values of the protein bands are reported as percentages of the control (NG) and are the mean ± SEM of six (panel A) or seven (panel B) independent experiments. *$P < 0.05$ vs. NG or HG as indicated.

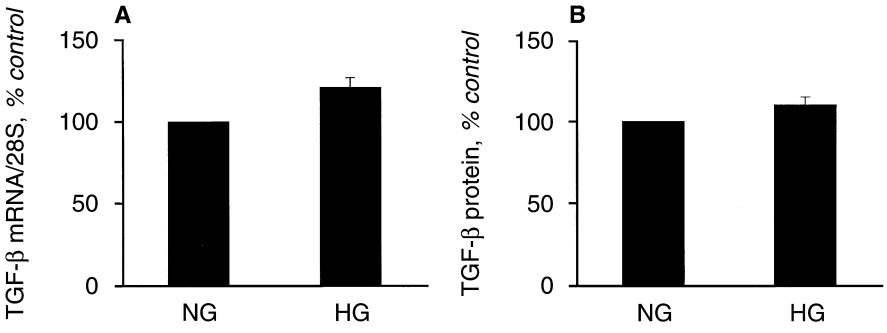

Fig. 8. High glucose does not significantly increase TGF-β1 in podocytes. Podocytes were exposed for 14 days to normal glucose (NG) or high glucose (HG). (*A*) TGF-β1 mRNA expression was analyzed by Northern blotting. The densitometric ratios of the TGF-β1 mRNA bands to 28S rRNA are shown as percentages of the control (NG). Results are the mean ± SEM of ten independent experiments. P = NS vs. NG. (*B*) Total TGF-β1 protein (latent plus active) in the cell culture supernatant was assayed by ELISA and performed according to the manufacturer's instructions (R&D Systems). The quantity of TGF-β1 protein (pg) was corrected for the total protein content (mg). Results are expressed as percentages of the control (NG) and are the mean ± SEM of six independent experiments. P = NS vs. NG.

in the differentiated podocyte. As shown in Figure 8A, high glucose increased the mRNA level of TGF-β1, but not by a statistically significant amount (HG, 120 ± 6% of normal glucose, P = NS, N = 10). To corroborate this finding, we measured the production of TGF-β1 by ELISA. The amounts of total TGF-β1 protein in both the cell lysate (data not shown) and the cell culture supernatant were not significantly different under high glucose vs. normal glucose conditions (HG, 110 ± 5% of normal glucose, P = NS, N = 6; Fig. 8B).

Although high glucose did not stimulate TGF-β1, it significantly increased the mRNA expression of TβRII by 70% compared with normal glucose ($P < 0.05$, N = 6; Fig. 9A). This high glucose effect translated into an increased level of TβRII protein on the cell surface (HG, 160 ± 26% of normal glucose, $P < 0.05$, N = 4, Fig. 9B), assessed by Western blotting of proteins isolated from the cell membrane.

The effects of exogenous TGF-β1 on the expression of TGF-β1 and TβRII were examined next. The addition of 2 ng/mL of TGF-β1 for 24 hours slightly increased the expression of TGF-β1 mRNA (TGF-β1, 118 ± 14% of control, P = NS, N = 4). Similarly, the mRNA level of TβRII was not significantly altered by exogenous TGF-β1 (TGF-β1, 97 ± 13% of control, P = NS, N = 4).

High glucose increases the effects of ambient TGF-β1

In this set of experiments, the dose of exogenous TGF-β1 was chosen according to the ambient concentration of endogenous TGF-β1. The mean concentration of active TGF-β1 in the conditioned media of podocytes after two weeks in normal glucose was 0.014 ± 0.004 ng/mL (N = 4). Therefore, we added 0.010 ng/mL of exogenous TGF-β1 to nearly double the level of active TGF-β1 in the next 24 hours. Both high glucose and TGF-β1 alone (that is, NG+TGF-β1) increased the

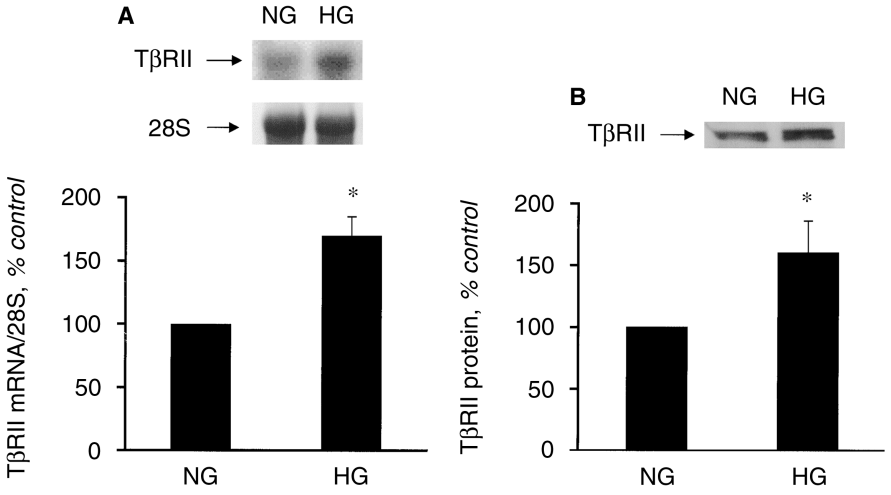

Fig. 9. High glucose stimulates expression and increases cell membrane quantity of TGF-β type II receptor (TβRII). Podocytes were exposed for 14 days to normal glucose (NG) or high glucose (HG). (*A*) TβRII mRNA expression was analyzed by Northern blotting. The densitometric ratios of the TβRII mRNA bands to 28S rRNA are shown as percentages of the control (NG). Results are expressed as the mean ± SEM of six independent experiments. *$P < 0.05$ vs. NG. (*B*) The amount of TβRII protein on the podocyte cell surface was assessed by Western blotting of proteins isolated from the plasma membranes as described. Results are expressed as percentages of the control (NG) and are the mean ± SEM of four independent experiments. *$P < 0.05$ vs. NG.

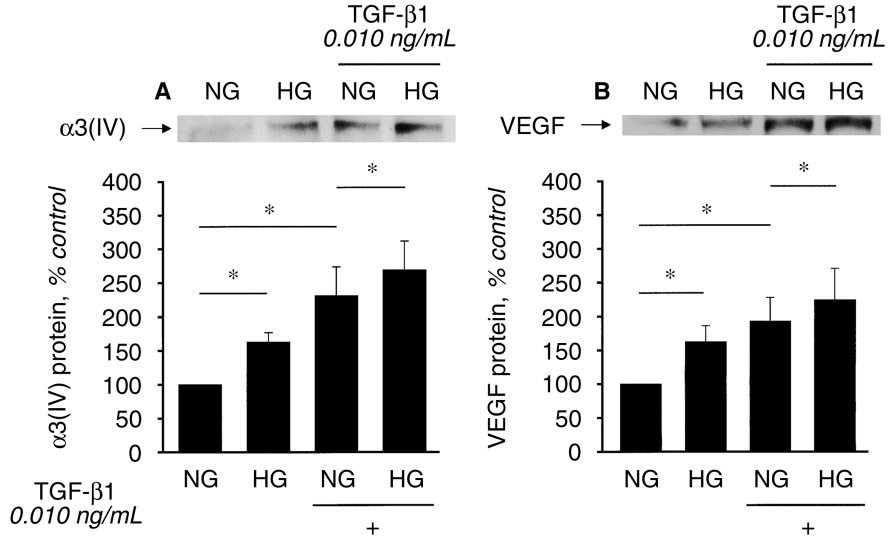

Fig. 10. Combined effects of high glucose and exogenous TGF-β1 on protein production of α3(IV) collagen and VEGF. Podocytes were exposed to normal glucose (NG) or high glucose (HG) for 14 days. In the last 24 hours, cells were treated with 0.010 ng/mL of exogenous TGF-β1, which approximates the physiological concentration of active TGF-β1 in podocyte cell culture. The protein production of α3(IV) collagen (*A*) and VEGF (*B*) was analyzed by Western blotting. Representative groups of α3(IV) collagen and VEGF bands, each from a single experiment, are shown. The densitometric values of the protein bands are reported as percentages of the control (NG) and are the mean ± SEM of four independent experiments. *$P < 0.05$ vs. NG or NG+TGF-β1 as indicated.

amounts of α3(IV) collagen (HG, 163 ± 14% of normal glucose; TGF-β1, 231 ± 43% of NG, both $P < 0.05$ vs. NG, $N = 4$; Fig. 10A) and VEGF protein (HG, 162 ± 24% of normal glucose; TGF-β1, 193 ± 35% of NG, both $P < 0.05$ vs. NG, $N = 4$; Fig. 10B). After two weeks in high glucose, the podocytes responded to 0.010 ng/mL exogenous TGF-β1 by producing more α3(IV) collagen (HG+TGF-β1, 269 ± 43% of normal glucose, $P < 0.05$ vs. NG+TGF-β1; Fig. 10A) and VEGF (225 ± 47% of normal glucose, $P < 0.05$ vs. NG+TGF-β1; Fig. 10B) than in response to either high glucose or TGF-β1 alone.

Localization of TGF-β1 and TβRII in podocytes in vivo

Our previous immunohistochemical study of the diabetic *db/db* mouse versus the non-diabetic *db/m* mouse showed that the increase in TGF-β1 protein was localized to the mesangium and the capillary loops of the glomerulus [18]. No appreciable increase in TGF-β1 protein was seen, however, in the podocytes (data not shown). On the other hand, immunohistochemistry did reveal an increased amount of the TβRII protein in the podocytes of diabetic *db/db* mice (Fig. 11). The in vivo observations thus agree with our in vitro data, demonstrating that high glucose stimulates the expression of TβRII but not TGF-β1 in the cultured differentiated podocyte.

DISCUSSION

Our conditionally immortalized cell model allowed the study of the podocyte in its fully differentiated state, yielding data that are more relevant to the investigation of many renal diseases including diabetic nephropathy. Previous studies investigating the effects of high glucose and TGF-β1 on the glomerular epithelial cell were ham-

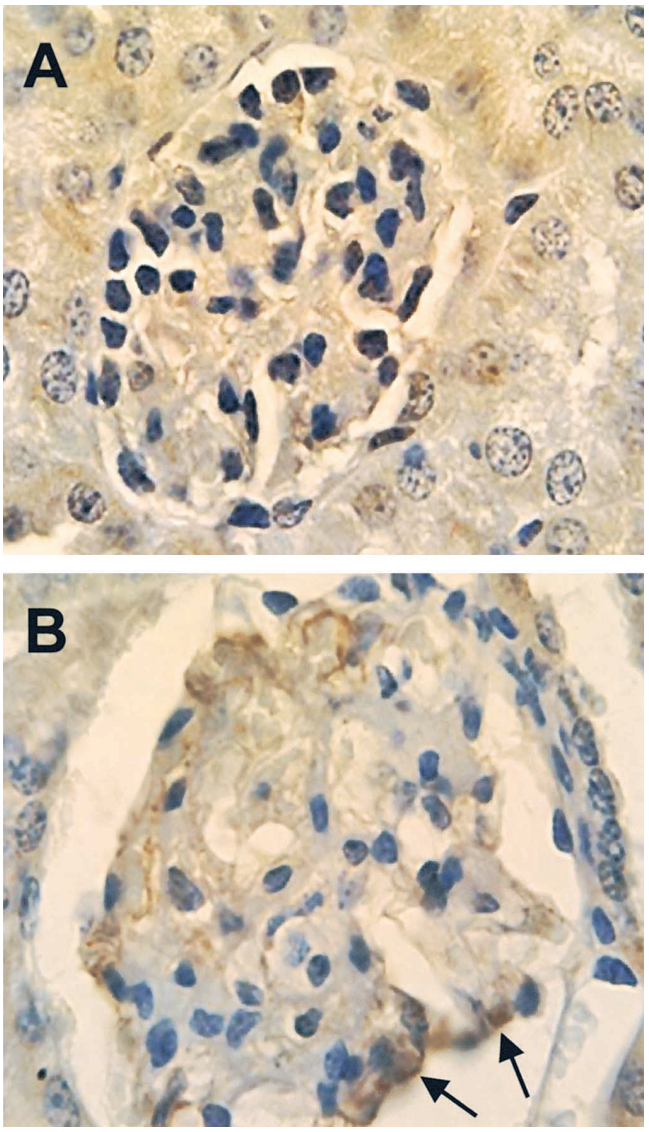

Fig. 11. Immunohistochemistry of kidney sections (magnification ×400) showing increased type II receptor protein in podocytes in diabetic *db/db* mice versus non-diabetic *db/m* mice. Kidney sections were incubated with a rabbit antibody against the TGF-β type II receptor (TβRII). A secondary biotinylated anti-rabbit antibody and then a streptavidin-horseradish peroxidase were subsequently added. The color reaction was developed with a diaminobenzidine detection kit (Vector Laboratories), and the sections were counterstained with hematoxylin. TβRII protein stained more abundantly in the glomeruli of the *db/db* vs. the *db/m* mouse (*A* and *B*). In particular, TβRII protein was increased in the podocytes (arrows) of the *db/db* mouse (panel B).

pered by the tendency of the podocyte to undergo dedifferentiation in culture [49]. Moreover, the glomerular epithelial cell may not have been a true podocyte [50]. Preparations of podocytes are often contaminated by glomerular parietal epithelial cells, which line Bowman's space and can overgrow the podocytes in culture [36].

To our knowledge, we show for the first time that exposure to high ambient glucose significantly increases the expression of collagen IV, VEGF, and TβRII in the differentiated mouse podocyte. The stimulatory effects of high glucose on collagen synthesis were seen in the increased proline incorporation, in the elevated levels of α1 and α5(IV) mRNA, and in the increased production of α1(IV), α3(IV), and α5(IV) proteins. These findings were not due to an osmotic effect of high glucose because an equivalent concentration of D-mannitol did not produce the above results. In many renal cell types, high glucose stimulates collagen IV production by activating the cellular TGF-β system [12]. Specifically, high glucose increases the secretion of endogenous TGF-β1 that then acts upon the cell in autocrine fashion to stimulate the expression of collagen IV and other extracellular matrix proteins [23]. In the case of the podocyte, however, high glucose does not increase TGF-β1 by either Northern analysis or ELISA, and high glucose and exogenous TGF-β1 have disparate effects on the expression of α1 and α5(IV) collagen. Therefore, despite its ability to promote matrix production in a wide variety of renal cells, the TGF-β system likely does not mediate all of the effects of high glucose to stimulate collagen IV expression in the podocyte. Of course, the α2, α4, and α6 chains will have to be studied to complete the profile of high glucose and TGF-β1 effects on collagen IV expression.

Besides an absolute increase in collagen IV production that may alter the quantity of GBM, a relative increase in one collagen IV alpha chain over another may affect the 'quality' of the GBM. The collagen IV alpha chains can self-assemble (via molecular recognition sequences in their NC1 domains) into a triple helical monomer in one of two ways: as an α1·α2·α1 network or as an α3·α4·α5 network [51]. The α3·α4·α5 network is mostly found in the subepithelial aspect of the GBM and is laid down by the overlying podocytes [2]. A relative deficiency of one of these chains may disrupt the incorporation of the other two chains into a collagen IV monomer [51]. On the other hand, an excess of one chain may competitively inhibit the NC1 interactions and prevent the normal assembly of a collagen IV network [51]. In either case, the high glucose- or TGF-β1–induced changes in the expression pattern of the collagen IV alpha chains may perturb the suprastructure of the GBM and affect its function as a filtration barrier.

In addition to structural and compositional changes in the GBM, the stimulated production of VEGF by the podocyte may play a role in the pathophysiology of diabetic proteinuria. While the mechanism of proteinuria is not entirely understood, VEGF may work by causing vasodilation through nitric oxide release [52], by inducing endothelial fenestrations [53], and by opening the junctions between endothelial cells [54]. All of these actions would allow more protein to leak across the glomerular endothelium. In health, the podocyte-derived VEGF, which is constitutively expressed, may travel across the

GBM and maintain the fenestrations of the glomerular endothelium [35]. How VEGF reaches the endothelial cell against the tide of glomerular filtration is not known, but we speculate that the heavier VEGF isoforms may be carried across the GBM. The $VEGF_{164, 188, 205}$ isoforms have heparin-binding domains that allow them to attach to the side chains of heparan sulfate proteoglycan, an abundant component of the GBM. The heparan sulfate side chains, varying in length from ~5 to ~70 kD, enjoy ample freedom to move laterally, and these linear polymers can swing around to reach distant targets, as far as several hundreds of nanometers away [55]. Thus, heparan sulfate could easily cross a full-thickness basement membrane [55] and perhaps deliver VEGF from the podocyte to the glomerular endothelial cell.

In diabetes, an inappropriate rise in VEGF production by the podocyte may increase the glomerular vascular permeability and exacerbate proteinuria. Indeed, histological evidence suggests that diabetes raises the VEGF gene expression and protein production in the podocyte [32, 56]. Consistent with this, we found that high glucose and TGF-β1 increase the mRNA and protein levels of VEGF in the mouse podocyte. In support of a role for VEGF in proteinuria, the expression of VEGF and its receptors is increased intrarenally in animal models of diabetes and in diabetic humans [32, 56]. The serum VEGF concentration and urinary VEGF excretion correlate with the risk for and degree of albuminuria [31, 56, 57]. Finally, treatment with a neutralizing anti-VEGF antibody completely corrected the glomerular hyperfiltration and partially corrected the albuminuria in streptozotocin (STZ)-diabetic rats, providing the strongest evidence to date that VEGF overexpression in the kidney contributes to diabetic proteinuria [33].

Because high glucose can modify the response of a renal cell to TGF-β [17], we examined the effect of high glucose on the expression of components of the TGF-β system. Even though high glucose does not raise the total amount of TGF-β1 protein in podocyte cell culture, it may still increase the bioactivity of TGF-β1. High glucose is known to stimulate the production of thrombospondin-1, a secreted matrix protein that binds and converts the latent form of TGF-β into the active form [58]. Thus, enough active TGF-β1 may accrue in the cell environment to mediate an effect of high glucose. In fact, our data show that high glucose acts mostly through the TGF-β system to induce production of α3(IV) collagen protein and partially through the TGF-β pathway to induce VEGF protein. On the other hand, other effects of high glucose are probably not mediated by TGF-β. For instance, the mRNA and protein levels of α1 and α5(IV) collagen are increased by high glucose, but decreased by exogenous TGF-β1. Given the opposing effects, high glucose is probably not stimulating the expression of these collagen IV chains via a TGF-β–dependent mechanism.

In another sense, high glucose does activate the TGF-β system in the podocyte, not at the ligand level but at the receptor level. The stimulation of TβRII expression, seen in this study, suggests that the readiness of the podocyte to respond to TGF-β1 also may underlie some of the high glucose effects. Given that high glucose increases the abundance of TβRII on the podocyte cell surface, we theorized that exogenous TGF-β1 would have a more pronounced effect in high glucose than in normal glucose. Consistent with this, the addition of a physiological concentration of exogenous TGF-β1 to podocytes that had been 'pre-treated' with high glucose resulted in larger absolute increases of α3(IV) collagen and VEGF proteins than the increases due to either high glucose or TGF-β1 in normal glucose. The interaction between high glucose and TGF-β1, however, cannot be construed as synergistic, but that might be expected, since the TGF-β system partly mediates the high glucose effects on α3(IV) collagen and VEGF.

Our data show that high ambient glucose and TGF-β1 coordinately induce the production of α3(IV) collagen and VEGF in the podocyte. However, high glucose and TGF-β1 exert disparate effects on the expression of α1 and α5(IV) collagen. This novel finding challenges the paradigm that high glucose acts through the TGF-β system to stimulate collagen IV production in renal cells, which include the mesangial [23], glomerular endothelial [59], and proximal tubular [60] cells. In this respect, the podocyte differs from the other renal cell types, but the intracellular pathway that mediates the high glucose effect on α1 and α5(IV) collagen remains unknown. As is the case with the mesangial cell [17], high glucose upregulates the expression of the TGF-β type II receptor in the podocyte, both in cell culture and in the *db/db* mouse. This phenomenon may allow the podocyte to react more vigorously to the effects of TGF-β1, and it sets up a glomerular paracrine system in which higher than normal levels of TGF-β1 coming from the mesangium or glomerular endothelium can affect the neighboring podocyte. Thus, high glucose may either accentuate or antagonize the effects of TGF-β1 depending on the collagen IV or VEGF parameter that is being studied. Such changes in the podocyte's expression of the collagen IV alpha chains and VEGF isoforms may trigger or worsen the GBM thickening and proteinuria of diabetic glomerulopathy.

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