Original Article

Mesangial Cell Hypertrophy by High Glucose Is Mediated by Downregulation of the Tumor Suppressor PTEN

Lenin Mahimainathan,[1] Falguni Das,[1] Balachandar Venkatesan,[1] and Goutam Ghosh Choudhury[1,2,3]

Diabetic nephropathy is characterized early in its course by glomerular hypertrophy and, importantly, mesangial hypertrophy, which correlate with eventual glomerulosclerosis. The mechanism of hypertrophy, however, is not known. Gene disruption of the tumor suppressor PTEN, a negative regulator of the phosphatidylinositol 3-kinase/Akt pathway, in fruit flies and mice demonstrated its role in size control in a cell-specific manner. Here, we investigated the mechanism of mesangial hypertrophy in response to high extracellular glucose. We link early renal hypertrophy with significant reduction in PTEN expression in the streptozotocin-induced diabetic kidney cortex and glomeruli, concomitant with activation of Akt. Similarly, exposure of mesangial cells to high concentrations of glucose also decreased PTEN expression and its phosphatase activity, resulting in increased Akt activity. Expression of PTEN inhibited high-glucose–induced mesangial cell hypertrophy, and expression of dominant-negative PTEN was sufficient to induce hypertrophy. In diabetic nephropathy, the hypertrophic effect of hyperglycemia is thought to be mediated by transforming growth factor-β (TGF-β). TGF-β significantly reduced PTEN expression in mesangial cells, with a reduction in its phosphatase activity and an increase in Akt activation. PTEN and dominant-negative Akt attenuated TGF-β–induced hypertrophy of mesangial cells. Finally, we show that inhibition of TGF-β signal transduction blocks the effect of high glucose on PTEN downregulation. These data identify a novel mechanism placing PTEN as a key regulator of diabetic mesangial hypertrophy involving TGF-β signaling. *Diabetes* 55:2115–2125, 2006

The early pathological manifestations of diabetic nephropathy consist of altered glomerular hemodynamics and whole-kidney and, in particular, glomerular hypertrophy (1–3). Later manifestations include microalbuminuria, frank proteinuria, and progressive fibrosis leading to end-stage renal disease. Increases in fractional volume of the mesangium correlate with mesangial cell hypertrophy and are characterized by an increase in overall protein synthesis in the absence of concomitant increases in DNA synthesis (4). Many hormones, cytokines, and growth factors, including transforming growth factor-β (TGF-β), are involved in the pathophysiology of diabetic mesangial cell hypertrophy and in subsequent steps that lead to glomerulosclerosis (5).

TGF-β elicits a myriad of pathological and physiological effects that include fibrosis, oncogenesis, immune suppression, and wound healing (6,7). The principal signaling pathway utilized by TGF-β involves the receptor-specific Smad2 and Smad3 (8,9), which on phosphorylation by the type I receptor translocate to the nucleus and regulate gene expression. In addition, TGF-β stimulates extracellular signal–regulated kinase 1/2 and p38 mitogen-activated protein kinases as well as c-Jun NH_2-terminal kinase, which in concert with Smad3 result in transcription of specific genes (10–13). In mesangial cells, we have recently shown that activation of phosphatidylinositol (PI) 3-kinase is necessary for fibronectin expression in response to TGF-β (14).

TGF-β receptor expression is increased in the diabetic kidney (15,16). Also, increased expression of TGF-β significantly correlates with mesangial hypertrophy (5,17–19). High glucose induces hypertrophy of mesangial cells concomitant with the production of TGF-β (2,5). Neutralizing TGF-β antibody in mesangial cells inhibits the hypertrophic effect of high glucose (20). Thus, TGF-β signaling represents a significant mechanism of mesangial cell hypertrophy in diabetic nephropathy.

Studies in *Drosophila* have demonstrated a role for type IA PI 3-kinase in cell size control. Overexpression of *Drosophila* catalytic subunit of PI 3-kinase in wing and eye imaginal discs induced enlarged wings and eye, respectively (21–23). In transgenic mice, heart-specific overexpression of constitutively active type IA PI 3-kinase catalytic subunit p110α resulted in increased heart size. In contrast, cardiac expression of dominant-negative p110α resulted in smaller heart size (23). In accordance with these results, expression of the downstream target of PI 3-kinase, Akt, in the imaginal discs increased cell size in the fruit fly (24). Similarly, overexpression of constitutively active Akt in mouse heart yielded a twofold increase in heart size as a result of cardiomyocyte hypertrophy (25). PI 3-kinase action is regulated by the tumor suppressor protein PTEN (26). The main biological activity of PTEN is to dephosphorylate PI 3,4,5-trisphosphate (PIP_3), resulting in inhibition of Akt (26,27). In *Drosophila*, PTEN has been shown to regulate cell size (28). Deletion of PTEN in cardiac and skeletal muscles of mice resulted in increased heart size caused by hypertrophy of the cardio-

From the [1]Department of Medicine, University of Texas Health Science Center at San Antonio, San Antonio, Texas; the [2]Geriatric Research, Education and Clinical Center, San Antonio, Texas; and the [3]South Texas Veterans Health Care System, San Antonio, Texas.

Address correspondence and reprint requests to Goutam Ghosh Choudhury, Department of Medicine, Mail Code 7882, 7703 Floyd Curl Dr., San Antonio, Texas 78229-3900. E-mail: choudhuryg@uthscsa.edu.

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HA, hemagglutinin; PDGF, platelet-derived growth factor; PI, phosphatidylinositol; PIP_3, PI 3,4,5-trisphosphate; TGF-β, transforming growth factor-β.

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myocytes, but no increase in cell size in the skeletal muscle was observed (29,30). These results demonstrate differential effects of PTEN in organ-specific cell hypertrophy. However, the role of PTEN in specific cellular hypertrophy has only been addressed in genetically manipulated animals.

In the current report, we examined the involvement of PTEN in glomerular mesangial cell hypertrophy in early diabetic kidney disease. We show in streptozotocin-induced type 1 diabetic rats that early kidney hypertrophy is associated with a significant decrease in the level of PTEN and that activation of Akt kinase is concomitantly increased in the glomeruli. Similarly, exposure of glomerular mesangial cells to high glucose significantly reduced expression of PTEN, resulting in activation of Akt. Introduction of PTEN or dominant-negative Akt significantly blocked high-glucose–induced mesangial cell hypertrophy. TGF-β, a major component in the hypertrophic response to high glucose, reduced PTEN expression and decreased its PIP_3 phosphatase activity before mesangial cells hypertrophied. We show that TGF-β–induced cellular hypertrophy is mediated by PTEN. Furthermore, we demonstrate that TGF-β mediates the effect of high glucose on PTEN downregulation. These results provide the first evidence that in a pathological setting, PTEN regulates mesangial cell hypertrophy caused by high glucose and that this effect of PTEN is mediated by TGF-β.

RESEARCH DESIGN AND METHODS

Streptozotocin, phenylmethylsulfonyl fluoride, Na_3VO_4, Nonidet-P40, PI, and anti-actin, -tubulin, and -FLAG antibodies were obtained from Sigma. Anti-PTEN and anti-hemagglutinin (HA) antibodies were purchased from Santa Cruz Biotechnology. Anti–phospho-473-Akt antibody, which recognizes the activated form of Akt, was obtained from Cell Signaling. Anti-PIP_3 antibody was purchased from Medical and Biological Laboratories. Tissue culture materials were obtained from Invitrogen. Recombinant TGF-β1 was obtained from R&D Systems. Adenovirus vectors expressing PTEN, a dominant-negative mutant of PTEN in which the catalytic site cysteine is mutated to serine (PTEN C/S), and dominant-negative Akt with HA tag have been described previously (31,32). Smad7 and Smad3 adenoviruses with FLAG tag were gifts from Dr. Anita Roberts, National Institutes of Health.

Animal protocol. Male Sprague-Dawley rats weighing 360–430 g were used. In one group, four to six rats were injected intravenously through the tail vein with 65 mg/kg body wt streptozotocin in sodium citrate buffer (pH 4.5). The control group received sodium citrate buffer alone. Blood glucose concentrations were monitored starting at 24 h postinjection, using a LifeScan One Touch glucometer (Johnson and Johnson). All rats were maintained in accordance with institutional animal care and use committee–approved procedures and had unrestricted access to food and water. No insulin was used in streptozotocin-induced diabetic animals. At the end of the experiment, 4 days after injection of streptozotocin, rats were euthanasized, and both kidneys were removed and weighed. Cortical sections of both kidneys from each rat were pooled. Glomeruli were isolated by differential sieving as previously described (33). Also, a slice of cortical tissue from each rat was frozen for biochemical analysis.

Cell culture and adenovirus infection. Sprague-Dawley rat glomerular mesangial cells were grown in RPMI 1640 medium with 5 mmol/l glucose and 17% fetal bovine serum (34). The cells were made quiescent by incubating in serum-free medium for 48 h. The cells were infected with 50 multiplicity of infection (moi) of indicated adenovirus vectors as previously described (35,36). The cells were maintained in serum-free medium with 5 mmol/l glucose for 24 h followed by incubation with fresh serum-free medium with 25 mmol/l glucose (high glucose) for 48 h. Control cells were incubated with 5 mmol/l glucose plus 20 mmol/l mannitol (low glucose) for 48 h.

Immunoblotting. Cortical and glomerular preparations were homogenized in radioimmunoprecipitation assay buffer (20 mmol/l Tris-HCl, pH 7.5, 150 mmol/l NaCl, 5 mmol/l EDTA, 1 mmol/l Na_3VO_4, 1 mmol/l phenylmethylsulfonylfluoride, 0.05% aprotinin, and 1% Nonidet P-40). Mesangial cells were also lysed in radioimmunoprecipitation assay buffer. Cleared lysates were prepared and protein content determined as previously described (31). Equal amounts of proteins present in the cell lysates were immunoblotted with the respective antibodies as previously described (32,35). For immunoblotting experiments with in vivo samples, the actin level was used for demonstrating loading (37). In some in vitro experiments, the level of tubulin was used for loading control. However, the level of both of these proteins did not change with the conditions of the experiments. For quantitation, the ratio of intensities of protein bands to the loading controls was determined.

PTEN lipid phosphatase assay. Radiolabeled [^{32}P]PI 3 was prepared for use in a PI 3-kinase assay with activated platelet-derived growth factor (PDGF) receptor immunoprecipitates from PDGF-stimulated mesangial cells. PTEN immunoprecipitates from equal amounts of proteins were incubated with equal amounts of radioactive [^{32}P]PI 3. The reaction products were extracted and separated by thin-layer chromatography as previously described (38). To quantitate the [^{32}P]PI 3, the spots were scraped and counted in a radioactive scintillation counter.

Hypertrophy assays. Mesangial cells in 12-well plates were starved in serum-free medium with 5 mmol/l glucose and incubated with medium containing 5 mmol/l glucose plus 20 mmol/l mannitol as control and with 25 mmol/l glucose for 48 h. In the last 2 h, the cells were labeled with 1 μCi/ml of [^{3}H]thymidine and [^{35}S]methionine. The washed cells were fixed in cold 10% trichloroacetic acid, and the precipitates were dissolved in 0.25 mol/l NaOH containing 0.1% SDS and then counted as previously described (34,39). Using this assay, there was no increase in DNA synthesis in response to 25 mmol/l glucose or TGF-β. Therefore, increase in protein synthesis was used as a surrogate for hypertrophy (39).

Statistical analysis. The significance of the data was determined by ANOVA followed by Student-Newman-Keuls analysis.

RESULTS

Downregulation of PTEN in diabetic nephropathy. In certain organs of genetically modified fruit flies and mice, PTEN has been implicated in cellular hypertrophy (28–30). However, its role in pathological hypertrophy is not known. Because early hypertrophy is a key pathological feature in diabetic nephropathy (3), we used the streptozotocin-induced type 1 model of diabetes in the rat. Administration of streptozotocin induced hyperglycemia in rats within 4 days (Fig. 1*A*). At this stage, kidneys showed significant hypertrophy as determined by the ratio of kidney weight to both post- and prestreptozotocin body weight of rats (Fig. 1*B* and online supplement Fig. S1 [available at http://diabetes.diabetesjournals.org]). To investigate the involvement of PTEN in this hypertrophy, we examined the expression of PTEN in the kidney cortex of diabetic animals. Hyperglycemia significantly reduced the expression of PTEN in the kidney cortex (Fig. 2*A*). Diabetic nephropathy is associated with characteristic glomerular hypertrophy, particularly of mesangial cells. Therefore, PTEN expression was examined in the glomerular preparation. Expression of PTEN in diabetic glomeruli was significantly decreased compared with that from the control animals (Fig. 2*B*). These data conclusively indicate that hyperglycemia-induced kidney hypertrophy is associated with a reduction in PTEN expression.

PTEN inactivates PI 3-kinase signaling by dephosphorylating PIP_3 at the D3 position (26,27). One of the downstream targets of PI 3-kinase is the serine threonine kinase Akt. We examined the activation of Akt in the glomerular lysates by immunoblotting, using Akt phospho-Ser 473 antibody, which specifically recognizes the activated form of this kinase. Akt was significantly activated in the diabetic glomeruli (Fig. 2*C*). These data demonstrate that downregulation of PTEN in diabetic glomeruli is associated with increased Akt activation.

High glucose inhibits PTEN expression in mesangial cells. To investigate the mechanism of glomerular downregulation of PTEN, we used cultured rat mesangial cells, which hypertrophy when exposed to high concentrations of glucose and thus mimic the effect of hyperglycemia in the animals. Incubation of mesangial cells with 25 mmol/l

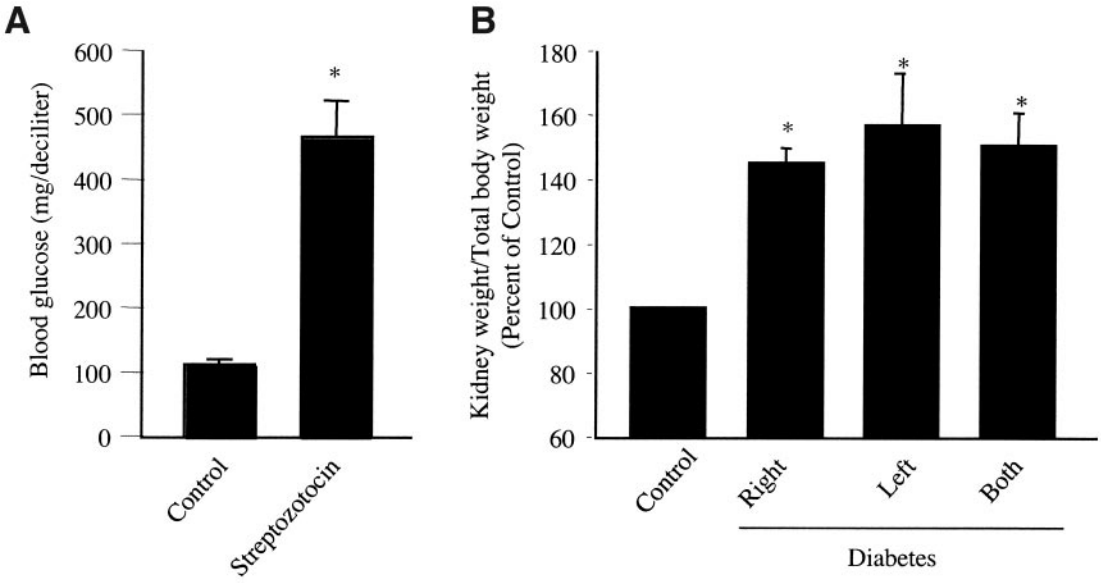

FIG. 1. Streptozotocin causes kidney hypertrophy in rats. Sprague-Dawley rats were injected with 65 mg/ml streptozotocin as described in RESEARCH DESIGN AND METHODS. The blood glucose concentration was determined 4 days later (*A*). *B*: The kidneys were harvested as described in RESEARCH DESIGN AND METHODS, and hypertrophy is expressed as a ratio of kidney weight to total body weight. Control: $n = 6$; streptozotocin: $n = 4$. *$P < 0.05$ vs. control by ANOVA.

glucose (high glucose) significantly reduced the amount of PTEN compared with cells incubated with 5 mmol/l glucose plus 20 mmol/l mannitol (low glucose, osmotic control) (Fig. 3*A*). RT-PCR of total RNA also showed a modest and significant decrease in PTEN mRNA in response to high glucose (online supplement Fig. S2). These data are consistent with our observation in rat that hyperglycemia induces downregulation of PTEN (Fig. 2*A* and *B*). To confirm the reduced expression of PTEN, we measured its phosphatase activity in the mesangial cells incubated with high glucose. PTEN was immunoprecipitated and assayed for its phosphatase activity in the presence of [^{32}P]PI 3. PTEN immunoprecipitates from cells exposed to high glucose (25 mmol/l) showed significantly reduced phosphatase activity compared with those from cells incubated in low glucose (Fig. 3*B*, compare *lane 3* with *lane 2*). To confirm the loss of function of PTEN in high-glucose–exposed cells, activation of Akt was determined, using phospho-Akt immunoblotting. High glucose significantly stimulated phosphorylation of Akt, suggesting its activation (Fig. 3*C*). These data indicate that reduction in PTEN in response to high glucose concentration results in functional activation of Akt kinase.

PTEN regulates high-glucose–induced hypertrophy of mesangial cells. Mesangial cell hypertrophy is defined by an increase in protein synthesis ([^{35}S]methionine uptake) in the absence of an increase in DNA synthesis ([^{3}H]thymidine incorporation). We routinely observed that 25 mmol/l glucose induces mesangial cell hypertrophy, manifested as an increase in protein synthesis and a slight nonsignificant decrease in DNA synthesis (Fig. 4*A*). This increase in protein synthesis also correlates well with hypertrophy measured as a ratio of total protein to cell number (online supplement Fig. S3).

Because PTEN downregulation is associated with hyperglycemia-induced kidney hypertrophy, and because PTEN was reduced in mesangial cells in response to high glucose, we examined the effect of introduction of PTEN, using an adenovirus vector (Fig. 4*B*), on mesangial cell hypertrophy. Expression of PTEN significantly inhibited high-glucose–induced protein synthesis without any significant effect on DNA synthesis (Fig. 4*C* and online supplement Fig. S4). Thus, downregulation of PTEN is necessary for glucose-induced hypertrophy of mesangial cells. Furthermore, these data indicate that basal PTEN activity may need to be inhibited for induction of hypertrophy. To confirm this notion, a dominant-negative mutant of PTEN in which the catalytic site cysteine is mutated to serine (PTEN C/S) was used (Fig. 4*D*). Expression of PTEN C/S was sufficient to cause hypertrophy of mesangial cells similar to that induced by high glucose (Fig. 4*E* and online supplement Fig. S5). These results conclusively demonstrate that downregulation of PTEN is necessary for mesangial cell hypertrophy.

The main biological function of PTEN is to block PI 3-kinase signaling, resulting in inhibition of Akt kinase (26,27). Because high glucose caused activation of Akt, we examined the effect of PTEN on activation of Akt in mesangial cells. Expression of PTEN inhibited high-glucose–induced phosphorylation of Akt, demonstrating its inhibition (Fig. 4*F*). The functional consequence of Akt inhibition on mesangial cell hypertrophy was then determined, using a dominant-negative Akt kinase. Expression of dominant-negative Akt (Fig. 4*G*) significantly inhibited high-glucose–induced hypertrophy of mesangial cells (Fig. 4*H*). These data indicate that along with PTEN downregulation, the resulting increase in Akt kinase regulates high-glucose–induced mesangial cell hypertrophy.

PTEN regulation of hypertrophy is mediated by TGF-β. TGF-β is a key regulator of diabetic nephropathy (3,5,15). Both TGF-β and its type II receptor are increased in the glomeruli of diabetic animals (18,40). Prolonged glucose exposure increases expression of TGF-β to mediate the hypertrophic effect. These results were obtained using TGF-β–neutralizing antibody (5,41). In our experimental condition, where glucose caused hypertrophy of mesangial cells within 48 h, we observed inhibition of protein synthesis by TGF-β–neutralizing antibody (Fig.

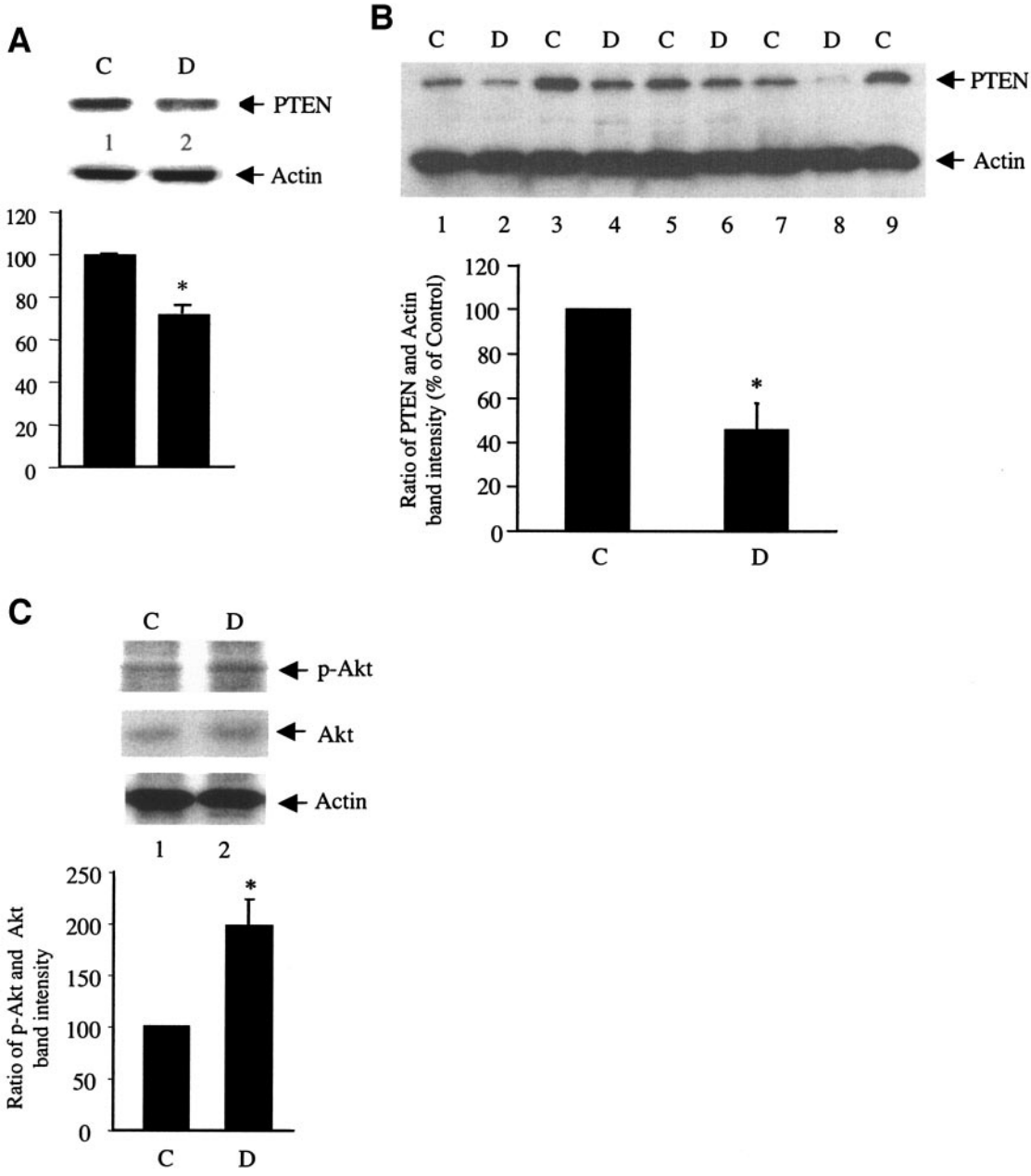

FIG. 2. Hyperglycemia reduces PTEN expression in kidney. We immunoblotted 75 μg of kidney cortical lysates (*A*) and glomerular lysates (*B*) from control (C) and diabetic (D) rats with anti-PTEN and anti-actin antibodies, respectively, and performed quantitation as described in RESEARCH DESIGN AND METHODS. Each lane represents a single animal. For cortex: $P = 0.001$ vs. control by ANOVA, $n = 9$. For glomeruli: $P = 0.001$ vs. control by ANOVA; control, $n = 5$; diabetes, $n = 4$. *C*: Glomerular lysates (75 μg) were immunoblotted with anti–phospho-Akt, anti-Akt, and anti-actin antibodies, respectively, and quantitation was performed as described in RESEARCH DESIGN AND METHODS. Control, $n = 4$; diabetes, $n = 4$. *$P = 0.001$ vs. control by ANOVA.

5*A*), confirming a role for TGF-β in high-glucose–induced mesangial cell hypertrophy. We further confirmed this observation, using the TGF-β receptor kinase inhibitor SB431542. Incubation of mesangial cells with SB431542 significantly blocked high-glucose–induced protein synthesis (Fig. 5*B*). Similarly, expression of Smad7 (Fig. 5*C*), which inhibits TGF-β signaling, also attenuated protein synthesis in response to high glucose (Fig. 5*D*). These data demonstrate that TGF-β signaling is necessary for the hypertrophic effect of glucose. Because we demonstrated that PTEN downregulation is necessary for high-glucose–induced hypertrophy, we examined the role of TGF-β in PTEN expression. TGF-β significantly reduced expression of PTEN (Fig. 6*A*), resulting in inhibition of its PIP_3 phosphatase activity (Fig. 6*B*, compare *lane 3* with *lane 2*). These data indicate that PTEN may regulate TGF-β–induced mesangial cell hypertrophy. To test this hypothesis, we examined the effect of PTEN on hypertrophy. Expression of PTEN significantly prevented protein synthesis in response to TGF-β without any significant effect on DNA synthesis (Fig. 6*C* and online supplement Fig. S6), indicating that PTEN plays an important role in TGF-β–induced mesangial cell hypertrophy.

We showed above that downregulation of PTEN by high glucose was associated with an increase in Akt activation in mesangial cells (Fig. 3*C*) and that Akt also regulates glucose-induced hypertrophy (Fig. 4*H*). Therefore, we examined the effect of TGF-β on activation of Akt. TGF-β

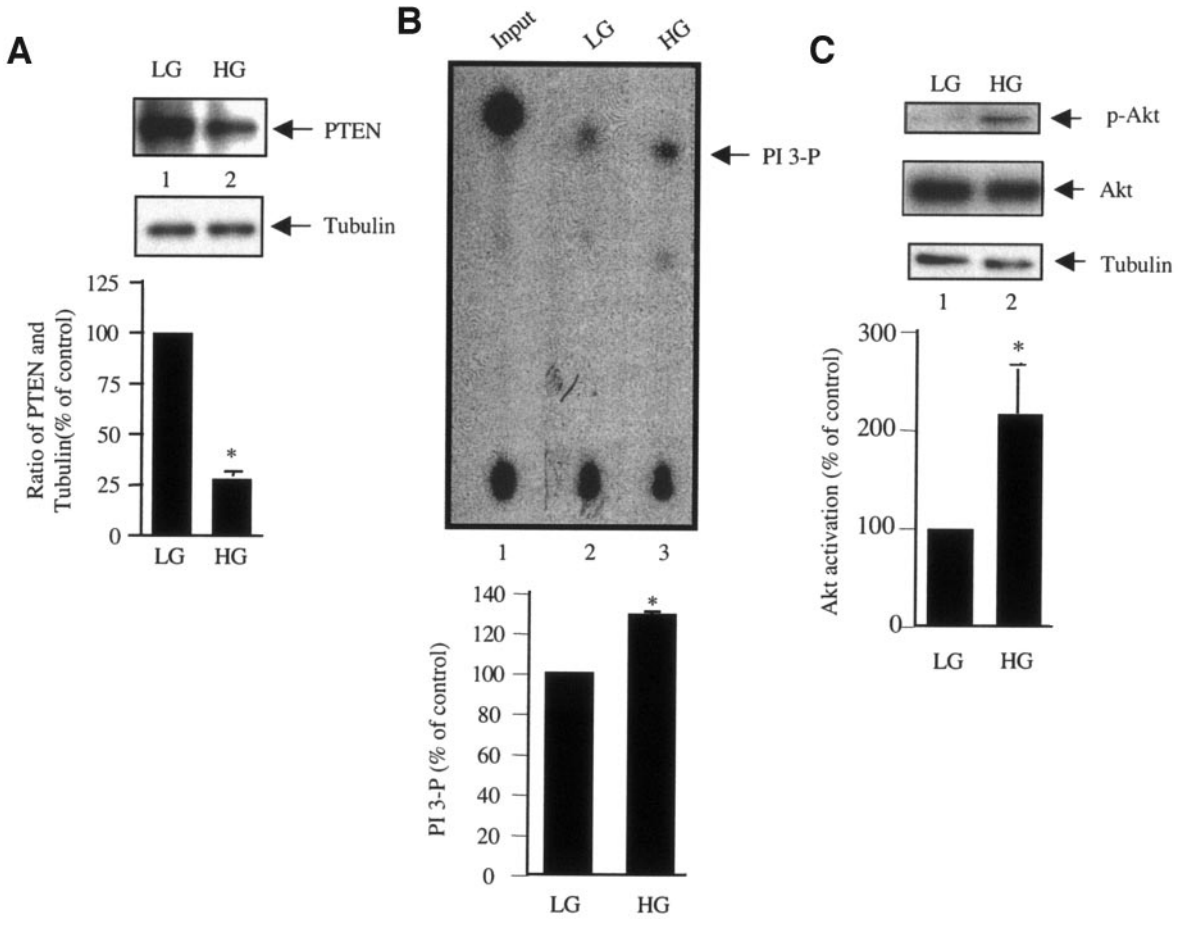

FIG. 3. High glucose downregulates PTEN in mesangial cells. *A*: Serum-deprived cells were incubated with 5 mmol/l glucose plus 20 mmol/l mannitol (low glucose [LG]) or 25 mmol/l glucose (high glucose [HG]) for 48 h. Then, 20 μg of cell lysates were immunoblotted with anti-PTEN and anti-tubulin antibodies. *Bottom panel* shows quantitation of protein bands. *$P < 0.05$ vs. control, $n = 17$. *B*: High glucose inhibits PTEN phosphatase activity. Mesangial cells were incubated with glucose as described in *A*. PTEN immunoprecipitates from 100 μg of lysates were assayed for phosphatase activity, using ^{32}P-labeled PI 3 (PI 3-P), as described in RESEARCH DESIGN AND METHODS. Arrow indicates the position of [^{32}P]PI 3. *Bottom panel* shows quantitation of [^{32}P]PI 3 spots. *$P < 0.05$ vs. control, $n = 3$. *C*: High glucose stimulates Akt. We immunoblotted 20 μg of cell lysates in *A* with phospho-Akt (pAkt), Akt, and tubulin antibodies, respectively. *Bottom panel* shows ratio of intensity of phospho-Akt and Akt as Akt activation. *$P < 0.05$ vs. control, $n = 8$.

increased phosphorylation of Akt with a time course similar to that of PTEN expression (Fig. 6*D*, compare with Fig. 6*A*). Expression of PTEN inhibited TGF-β–induced Akt activation (Fig. 6*E*). Because we also demonstrated that high-glucose–induced mesangial cell hypertrophy is mediated by PTEN-regulated Akt kinase (Fig. 4), we next examined the role of Akt in TGF-β–induced hypertrophy. Expression of dominant-negative Akt significantly blocked mesangial cell hypertrophy in response to TGF-β (Fig. 6*F*). These results provide the first evidence that a common signaling pathway, which involves downregulation of PTEN and resultant activation of Akt, is used by both high glucose and TGF-β in causing hypertrophy of mesangial cells.

Apart from hypertrophy, matrix expansion, including expression of fibronectin, is also a feature of diabetic nephropathy (2). Therefore, we examined the involvement of PTEN in expression of fibronectin in response to TGF-β. TGF-β increased expression of fibronectin in mesangial cells (Fig. 6*G*, compare *lane 2* with *lane 1*). Expression of PTEN inhibited TGF-β–induced fibronectin expression (Fig. 6*G*, compare *lane 4* with *lane 2*). These data indicate that along with hypertrophy, PTEN regulates expression of fibronectin in response to TGF-β.

We have established that TGF-β downregulates PTEN, resulting in hypertrophy of mesangial cells. TGF-β utilizes receptor-specific Smads for signal transduction. It is known that TGF-β receptor–specific Smad7, an inhibitory Smad, blocks TGF-β–induced Smad signaling (9). To elucidate the involvement of Smad signaling in TGF-β–induced downregulation of PTEN, we tested the effect of Smad7. As expected, TGF-β inhibited expression of PTEN (Fig. 7*A*). However, expression of Smad7 significantly attenuated downregulation of PTEN induced by TGF-β (Fig. 7*A*, compare *lane 4* with *lane 2*). To confirm this observation, we tested the effect of TGF-β receptor–specific Smad3 on PTEN downregulation. Expression of Smad3 alone inhibited the expression of PTEN similar to TGF-β (Fig. 7*B*, compare *lane 3* with *lane 1*). These data indicate that TGF-β utilizes Smad3 as the signaling molecule to downregulate PTEN in mesangial cells.

TGF-β regulates high-glucose–induced downregulation of PTEN. TGF-β acts downstream of high glucose to regulate the hypertrophy of mesangial cells (5). We have shown that both agonists independently downregulate PTEN, which is necessary for hypertrophy. Because TGF-β has been reported to regulate both negatively and positively the expression of PTEN in a cell-specific manner (42–44), we hypothesized that TGF-β may regulate high-glucose–induced PTEN downregulation. First, we as-

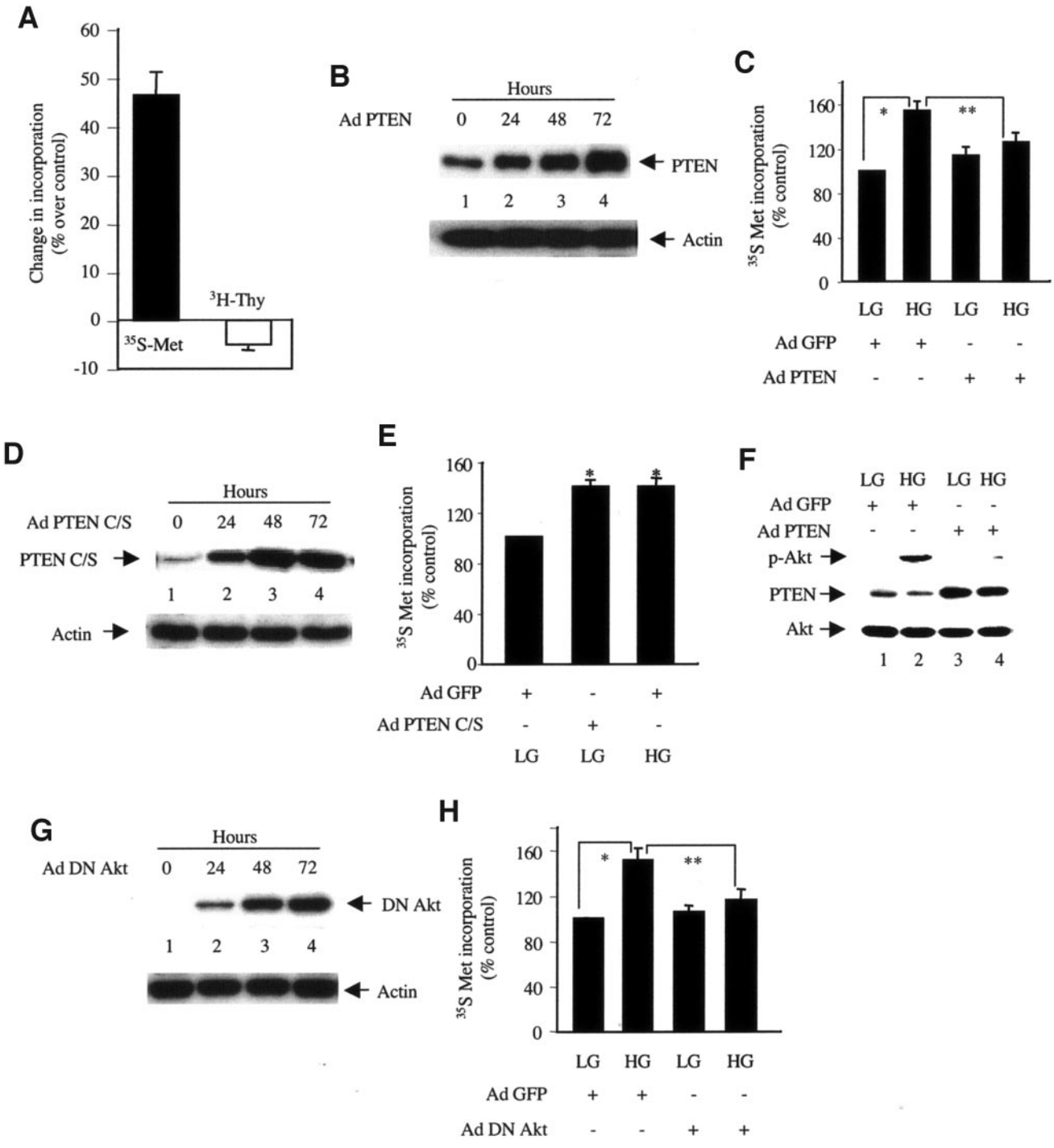

FIG. 4. PTEN regulates mesangial cell hypertrophy. *A*: Quiescent mesangial cells were incubated with 5 mmol/l glucose plus 20 mmol/l mannitol (low glucose [LG]) and 25 mmol/l glucose (high glucose [HG]) for 48 h. The cells were then incubated with [^{3}H]thymidine and [^{35}S]methionine, and the incorporation into DNA and protein was determined as described in RESEARCH DESIGN AND METHODS. *B*: Cells were infected with adenoviruses (Ad) (moi 50) and harvested at indicated times. Then, 20 μg of cell lysates were immunoblotted with anti-PTEN and anti-actin antibodies. *C*: Quiescent cells were infected with PTEN adenovirus or green fluorescent protein (GFP) adenovirus (moi 50) for 24 h followed by incubation with 5 mmol/l glucose plus 20 mmol/l mannitol and 25 mmol/l glucose for 48 h. [^{35}S]methionine (Met) incorporation was determined as described in RESEARCH DESIGN AND METHODS. Means of triplicate measurements are shown. *$P < 0.05$ and **$P < 0.05$ by ANOVA. *D*: Mesangial cells were infected with PTEN C/S adenovirus (moi 50), and 20 μg of lysates were immunoblotted as described in *B*. *E*: Quiescent mesangial cells were infected with green fluorescent protein adenovirus or PTEN C/S adenovirus (moi 50) for 72 h. For high glucose, the cells were infected with green fluorescent protein adenovirus for 24 h before incubation with 25 mmol/l glucose for 48 h. [^{35}S]methionine incorporation was determined as described in RESEARCH DESIGN AND METHODS. Means of triplicate measurements are shown. *$P < 0.05$ by ANOVA. *F*: Quiescent mesangial cells were infected with PTEN adenovirus (moi 50) and incubated with high and low glucose as described in *C*. Then, 20 μg of cell lysates were immunoblotted with phospho-Akt, PTEN, and Akt antibodies, respectively. *G*: Mesangial cells were infected with dominant-negative (DN) Akt adenovirus (moi 50), and the lysates were immunoblotted with anti-HA to detect dominant-negative Akt and actin antibody. *H*: Quiescent cells were infected with dominant-negative Akt adenovirus or green fluorescent protein adenovirus and incubated with high and low glucose as described in *C*. [^{35}S]methionine incorporation was determined as described in RESEARCH DESIGN AND METHODS. Means of triplicate measurements are shown. *$P < 0.01$, **$P < 0.01$ by ANOVA.

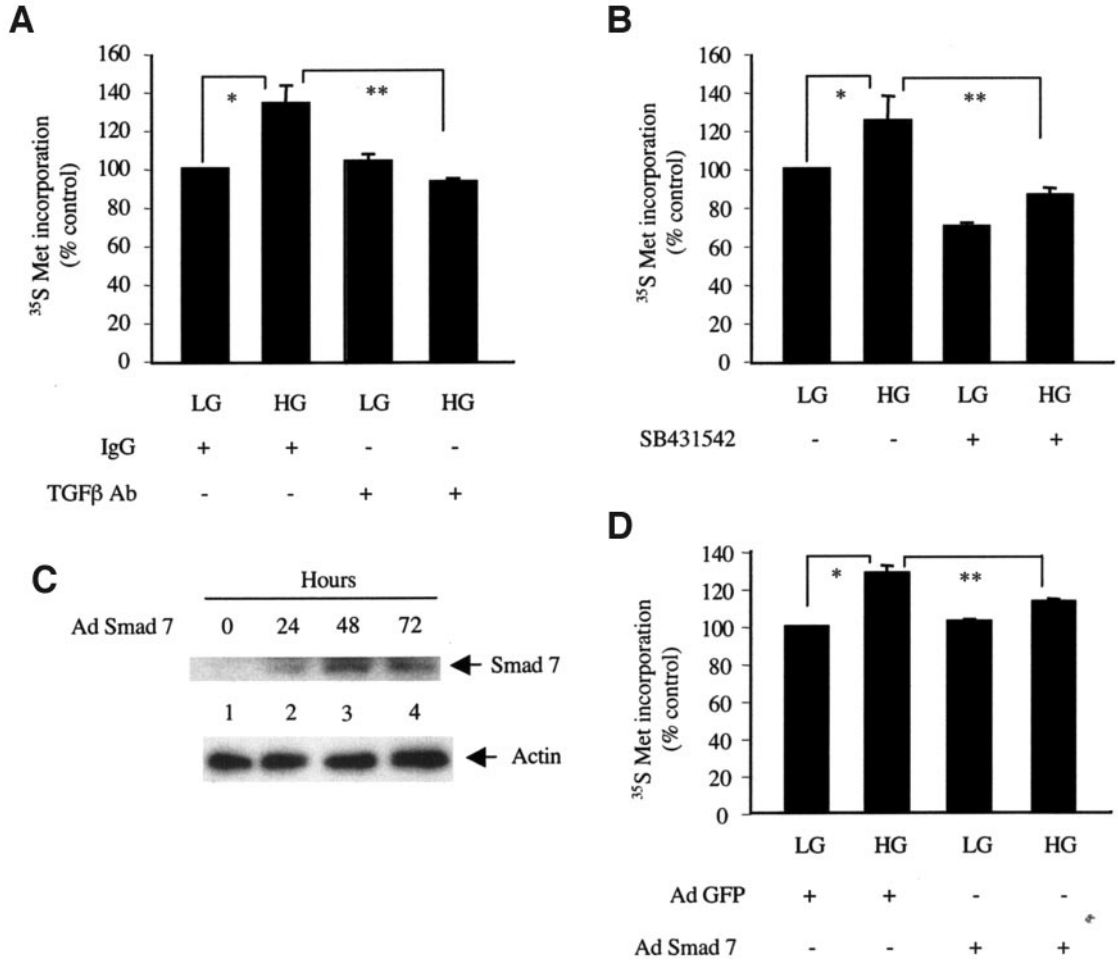

FIG. 5. TGF-β regulates high-glucose–induced mesangial cell hypertrophy. *A*: Serum-deprived mesangial cells were incubated with nonspecific IgG or TGF-β antibody (5 μg/ml) for 1 h before incubation with glucose as described in Fig. 4*A*, and [^{35}S]methionine (Met) incorporation was determined. The means of triplicate measurements are shown. **P* < 0.05 and ***P* < 0.05 by ANOVA. *B*: Quiescent mesangial cells were incubated with 2 μmol/l SB431542 for 1 h before incubation with glucose as described in Fig. 4*A*, and [^{35}S]methionine incorporation was determined. Means of triplicate measurements are shown. **P* < 0.05 and ***P* < 0.05 by ANOVA. *C*: Expression of Smad7. Mesangial cells were infected with Smad7 adenovirus (Ad) (moi 50). The cell lysates were immunoblotted with anti-FLAG to detect Smad7 and actin antibody, respectively. *D*: Mesangial cells were infected with Smad7 adenovirus or green fluorescent protein adenovirus (moi 50) for 24 h before incubation with glucose as described in Fig. 4*A*. [^{35}S]methionine incorporation was determined. Means of triplicate measurements are shown. **P* < 0.05 and ***P* < 0.05 by ANOVA. HG, high glucose; LG, low glucose.

sessed this notion, using a TGF-β–neutralizing antibody. As we had shown above, exposure of mesangial cells to high glucose reduced the expression of PTEN (Fig. 8*A*, compare *lane 3* with *lane 1*). Incubation of cells with TGF-β–neutralizing antibody significantly prevented the downregulation of PTEN induced by high glucose (Fig. 8*A*, compare *lane 4* with *lane 3*). Similarly, the TGF-β receptor kinase inhibitor SB431542 significantly reversed the inhibition of PTEN expression by high glucose (Fig. 8*B*, compare *lane 4* with *lane 3*). To further confirm the effects of TGF-β on PTEN, we used the TGF-β signaling inhibitor Smad7. Expression of Smad7 attenuated completely the downregulation of PTEN induced by high glucose (Fig. 8*C*, compare *lane 3* with *lane 1*). These data conclusively demonstrate that the effect of high glucose on PTEN downregulation is mediated by TGF-β.

DISCUSSION

We conclude that increased glucose concentration reduces the expression of PTEN, resulting in increased Akt activation, which induces hypertrophy of mesangial cells. This reduction in PTEN, and hence mesangial hypertrophy, is mimicked by TGF-β in these cells. Furthermore, we provide the first evidence that TGF-β mediates the reduction of PTEN in response to high glucose. These data place PTEN as the downstream target in the hypertrophic response of high glucose in the mesangial cells.

PTEN has been shown to regulate several cellular functions, including proliferation, apoptosis, and migration (45). Constitutive PTEN deletion in mouse results in embryonic lethality (46–48). PTEN null mouse embryo fibroblasts display increased PIP_3 expression (49). The biological response to PI 3-kinase activation is highly regulated and tissue specific (50,51). For example, PI 3-kinase/Akt signaling is important in mediating insulin actions in muscle, fat, and liver (52–55). Because PTEN is a negative regulator of PI 3-kinase signaling, its deletion in various tissues, such as liver, skin, endometrium, prostate, and breast, has permissive effects on tumor formation (56–59). On the other hand, reduction of PTEN in liver and fat by antisense oligonucleotides protected *db/db* mice from developing diabetes (60). Furthermore, muscle- and adipose tissue–specific deletion of PTEN protected mice against insulin resistance (30,61). In the case of muscle-

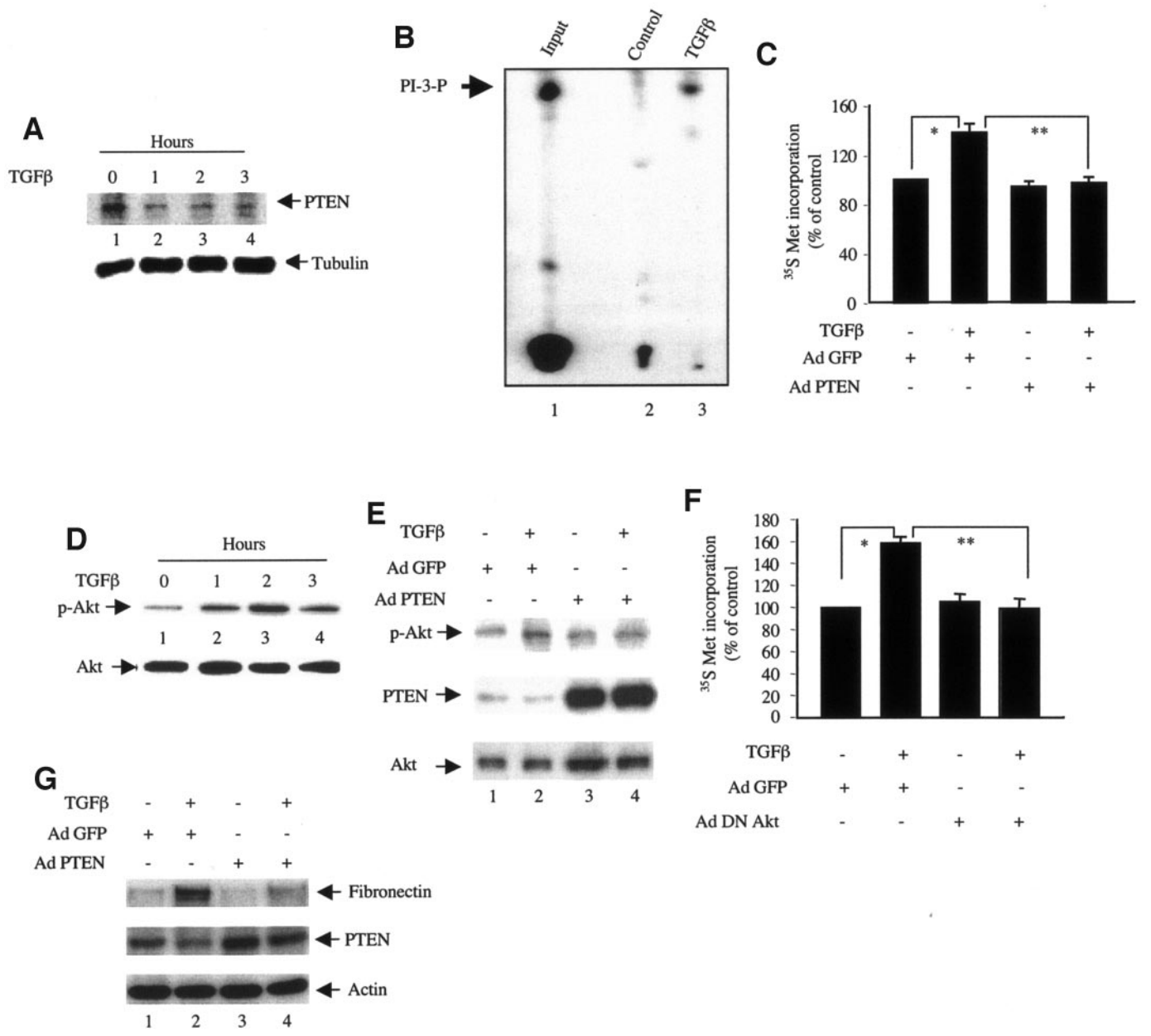

FIG. 6. TGF-β downregulates PTEN, resulting in mesangial cell hypertrophy. *A*: Quiescent anti-mesangial cells were incubated with 2 ng/ml TGF-β for indicated periods. Then, 20 μg cell lysates were immunoblotted with anti-PTEN and anti-tubulin antibodies. *B*: Mesangial cells were incubated with TGF-β for 2 h. PTEN immunoprecipitates from 100 μg lysates were assayed for phosphatase activity using ^{32}P-labeled PI 3 (PI 3-P) as described in RESEARCH DESIGN AND METHODS. Arrow indicates the position of [^{32}P]PI 3. *C*: Mesangial cells were incubated with green fluorescent protein (GFP) adenovirus (Ad) or PTEN adenovirus (moi 50) for 24 h before incubation with 2 ng/ml TGF-β for 48 h, and [^{35}S]methionine (Met) incorporation was determined. Means of triplicate measurements are shown. *$P < 0.05$ and **$P < 0.05$ by ANOVA. *D*: Quiescent mesangial cells were incubated with 2 ng/ml TGF-β for indicated times, and 20 μg lysates were immunoblotted with phospho-Akt and Akt antibodies, respectively. *E*: PTEN inhibits TGF-β–induced Akt activation. Quiescent mesangial cells were infected with PTEN adenovirus (moi 50) for 24 h before stimulating with 2 ng/ml TGF-β for 2 h. Then, 20 μg lysates were immunoblotted with phospho-Akt, PTEN, and Akt antibodies, respectively. *F*: Quiescent cells were infected with green fluorescent protein adenovirus or dominant-negative (DN) Akt adenovirus (moi 50) for 24 h before stimulation with TGF-β for 48 h. [^{35}S]methionine incorporation was determined. Means of triplicate measurements are shown. *$P < 0.05$ and **$P < 0.05$ by ANOVA. *G*: Mesangial cells were infected with green fluorescent protein adenovirus or PTEN adenovirus as described above. The infected cells were incubated with TGF-β for 24 h. Then, 5 μg cell lysates were immunoblotted with fibronectin, PTEN, and actin antibodies, respectively.

specific deletion of PTEN, insulin-stimulated glucose uptake was increased in soleus but not in extensor digitorum longus muscle (30). Adipose-specific PTEN deletion in mice elicits resistance to streptozotocin-induced diabetes (61). However, our data demonstrate that in rats, streptozotocin-induced diabetes is associated with a significant decrease in the cortical and glomerular PTEN level (Fig. 2). These results indicate that PTEN regulates cell function differently in different tissues (see below).

A significant role of PTEN in organ size was first identified in *Drosophila*. Deletion of PTEN in *Drosophila* eye and wing discs resulted in an increase in cell size and led to enlarged organ size (62,63). This cell growth effect of PTEN in *Drosophila* was not seen for its mammalian counterparts, i.e., in PTEN null embryonic stem cells, thymocytes, and fibroblasts, suggesting differential regulation of PTEN action in mammalian systems (50). However, more recent studies demonstrate that deletion of PTEN in

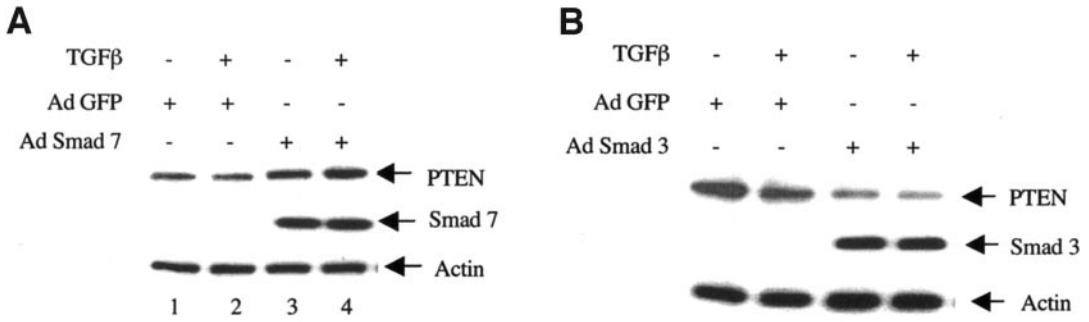

FIG. 7. TGF-β–specific Smads regulate PTEN downregulation. *A*: Mesangial cells were infected with Smad7 adenovirus (Ad) (*A*), Smad3 adenovirus (*B*), or green fluorescent protein (GFP) adenovirus (50 moi) followed by incubation with TGF-β for 2 h. Then, 20 μg lysates were immunoblotted with PTEN, actin, and FLAG (for detecting tagged Smad7 and Smad3) antibodies, respectively.

mouse brain increased the soma size of the neurons, which contributed to an increase in brain size (64,65). Furthermore, inactivation of PTEN in cardiomyocytes in mice resulted in hypertrophy (29). In contrast, deletion of PTEN in muscle did not induce any hypertrophy (30), thus demonstrating an organ-specific effect of PTEN in cell size control. These findings suggest that a lack of PTEN function because of its gene disruption results in differential effects in different tissues. Our data provide the first evidence that under a pathological form of renal hypertrophy, in the type 1 diabetic animal, PTEN expression is significantly reduced in the kidney cortex and glomeruli. Thus, PTEN plays a negative regulatory role in kidney hypertrophy (Figs. 1 and 2). Furthermore, we show that the hypertrophy of mesangial cells resulting from the exposure of these cells to high glucose is also associated with decreased PTEN expression and activity (Fig. 3). Introduction of PTEN blocked the hypertrophic response of glucose. These results conclusively demonstrate involvement of PTEN in the mesangial hypertrophy induced by high glucose. Furthermore, these data positively correlate with the role of Akt kinase in mesangial cell hypertrophy in response to high glucose (Fig. 4). To our knowledge, these results represent the first demonstration of involvement of PTEN in renal and mesangial hypertrophy during a pathological condition, namely diabetic kid-

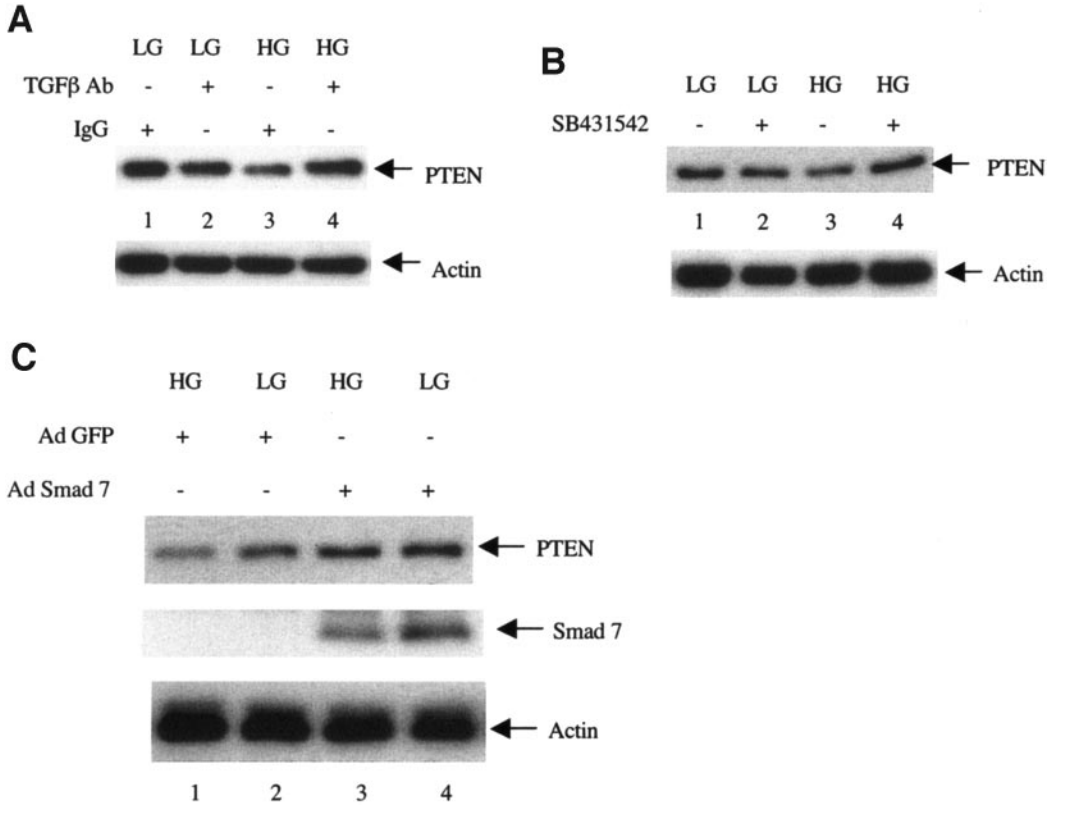

FIG. 8. TGF-β regulates PTEN expression in response to high glucose. *A*: Quiescent mesangial cells were incubated with nonspecific IgG or TGF-β neutralizing antibody (5 μg/ml) followed by incubation with glucose as described in Fig. 5*A*. Then, 20 μg lysates were immunoblotted with anti-PTEN and anti-actin antibodies. *B*: Quiescent mesangial cells were incubated with 2 μmol/l SB431542 for 1 h before incubation with high glucose, as described in Fig. 5*B*. Then, 20 μg lysates were immunoblotted with anti-PTEN and anti-actin antibodies. *C*: Quiescent mesangial cells were infected with green fluorescent protein (GFP) adenovirus (Ad) or Smad7 adenovirus (moi 50) for 24 h before incubation with high glucose as described in Fig. 5*D*. Then, 20 μg lysates were immunoblotted with anti-PTEN, anti-FLAG, and anti-actin antibodies, respectively. HG, high glucose; LG, low glucose.

ney disease. However, we have recently shown a role of PTEN in PDGF receptor–mediated signal transduction and DNA synthesis (32). However, expression of PTEN did not have any significant effect on basal DNA synthesis. We concluded that the inhibitory effect of PTEN was attributable to the protein phosphatase activity of PTEN on tyrosine-phosphorylated PDGF receptor. In the current study, we demonstrate that both high glucose and TGF-β inhibit expression of PTEN, resulting in activation of Akt kinase, which is associated with hypertrophy of mesangial cells. Whether tyrosine phosphorylation of growth factor receptor(s) is involved in this process is not known.

The pleiotropic cytokine TGF-β has been implicated in the pathogenesis of diabetic nephropathy (5,18,66,67). The hypertrophic effect of high glucose in mesangial cells is mediated by increased production of TGF-β (68–70). These results are also supported by recent results in which renal cells isolated from TGF-β1 null mice showed impairment in hypertrophy in response to high glucose (71). Additionally, TGF-β receptor II heterozygous mice with streptozotocin-induced type 1 diabetes showed significantly reduced glomerular hypertrophy, further providing evidence for the requirement of TGF-β signaling (40). However, the mechanism by which TGF-β regulates mesangial cell hypertrophy was not elucidated. In mesangial cells, we demonstrate here that TGF-β inhibits PTEN expression, resulting in activation of Akt (Fig. 6). These results link mesangial hypertrophy to PI 3-kinase/Akt signaling in response to TGF-β. Furthermore, we show that antagonizing TGF-β signaling prevents the inhibitory effect of high glucose on expression of PTEN (Fig. 8). Together, our data represent a potential mechanism to explain how PTEN downregulation by high glucose induces mesangial hypertrophy. Thus, modulation of PTEN function in diabetic nephropathy, where TGF-β signaling is a major cause of the disease, may prove beneficial.

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