RhoA/Rho-Kinase Contribute to the Pathogenesis of Diabetic Renal Disease

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OBJECTIVE—Accumulation of glomerular matrix proteins is central to the pathogenesis of diabetic nephropathy, with resident mesangial cells (MCs) known to upregulate matrix protein synthesis in response to high glucose. Because activation of the GTPase RhoA has been implicated in matrix upregulation, we studied its role in induction of the matrix protein fibronectin in diabetic MCs and in vivo in diabetic nephropathy.

RESEARCH DESIGN AND METHODS—Glucose (30 mmol/l)-induced RhoA/Rho-kinase, AP-1 activation, and fibronectin upregulation were assessed by immunoblotting, luciferase, electrophoretic mobility shift assay, enzyme-linked immunosorbent assay, real-time PCR, Northern blots, and immunofluorescence. Streptozotocin-induced diabetic rats were treated with the ρ-kinase inhibitor fasudil, which was compared with enalapril, and functional and pathologic parameters were assessed.

RESULTS—Glucose led to RhoA and downstream Rho-kinase activation. Mannitol was without effect. Activity of the transcription factor AP-1, increased in diabetic MCs and kidneys, is important in the profibrotic effects of glucose, and this was dependent on Rho-kinase signaling. Upregulation of fibronectin by glucose, shown to be mediated by activator protein-1 (AP-1), was prevented by Rho-kinase inhibition. RhoA siRNA and dominant-negative RhoA also markedly attenuated fibronectin upregulation by high glucose. Applicability of these findings were tested in vivo. Fasudil prevented glomerular fibronectin upregulation, glomerular sclerosis, and proteinuria in diabetic rats, with effectiveness similar to enalapril.

CONCLUSIONS—High glucose activates RhoA/Rho-kinase in MCs, leading to downstream AP-1 activation and fibronectin induction. Inhibition of this pathway in vivo prevents the pathologic changes of diabetic nephropathy, supporting a potential role for inhibitors of RhoA/Rho in the treatment of diabetic renal disease. ***Diabetes*** **57:1683–1692, 2008**

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AP-1, activator protein 1; ELISA, enzyme-linked immunosorbent assay; EMSA, electrophoretic mobility shift assay; MC, mesangial cell; MYPT, myosin phosphatase targeting subunit; STZ, streptozotocin; TGF, transforming growth factor; Thr, threonine; VEGF, vascular endothelial growth factor.

The kidney is a major site of diabetic microvascular complications, with diabetes being the leading cause of renal failure in the Western world. Glomerular matrix accumulation is the pathologic hallmark of diabetic nephropathy, and hyperglycemia is a primary pathogenetic factor in diabetic renal disease (1). Although maintenance of normoglycemia as well as interruption of angiotensin II signaling are effective in delaying onset of renal failure, these interventions at best only delay disease progression (1,2). Consequently, there is a need to identify new therapeutic strategies for diabetic nephropathy.

Elaboration of matrix proteins by glomerular mesangial cells (MCs) is an important factor in the development and progression of glomerular sclerosis (3). In elevated glucose concentrations, MCs synthesize extracellular matrix proteins including fibronectin (4). Increased fibronectin expression is also found in diabetic glomeruli in both humans and animal models (3). Recent studies have suggested a potential role for RhoA/Rho signaling in the elaboration of matrix proteins. In MCs, this pathway was required for the fibrotic response to transforming growth factor (TGF)-β and mechanical stress (5,6), but whether RhoA/Rho-kinase mediate the hyperglycemic effects on matrix elaboration by MCs and in diabetic nephropathy has not been addressed.

Rho GTPases are 20- to 24-kDa proteins that are essential in the regulation of diverse cellular functions. They cycle between an active GTP-bound and an inactive GDP-bound form, with their intrinsic hydrolytic activity affected by various Rho regulators. Membrane localization through posttranslational modification is a requirement for Rho activation (7). The best characterized Rho GTPases are Rac1, Cdc42, and RhoA. Recent studies have shown changes in RhoA activation in diabetes. In streptozotocin (STZ)-treated diabetic rats, an increase in membrane-bound RhoA was seen in the renal cortex (8,9). Similar observations, preceding overt pathologic changes, were made in the basilar artery and aorta (10,11). High glucose also led to RhoA membrane localization in MCs and to RhoA activation, as assessed by GTP binding in rat aortic smooth muscle cells (12,13).

RhoA is known to regulate the formation of F-actin stress fibers and focal adhesion complexes (7). A major role for RhoA in transcriptional gene regulation, however, is also increasingly recognized. Through activation of serum response factor, RhoA affects transcription of genes containing the serum response element (SRE) (14). The downstream RhoA effector Rho-kinase was shown to mediate this effect in some settings (15). The most well characterized genes upregulated through the serum response element are *c-fos* and *c-jun*, components of the

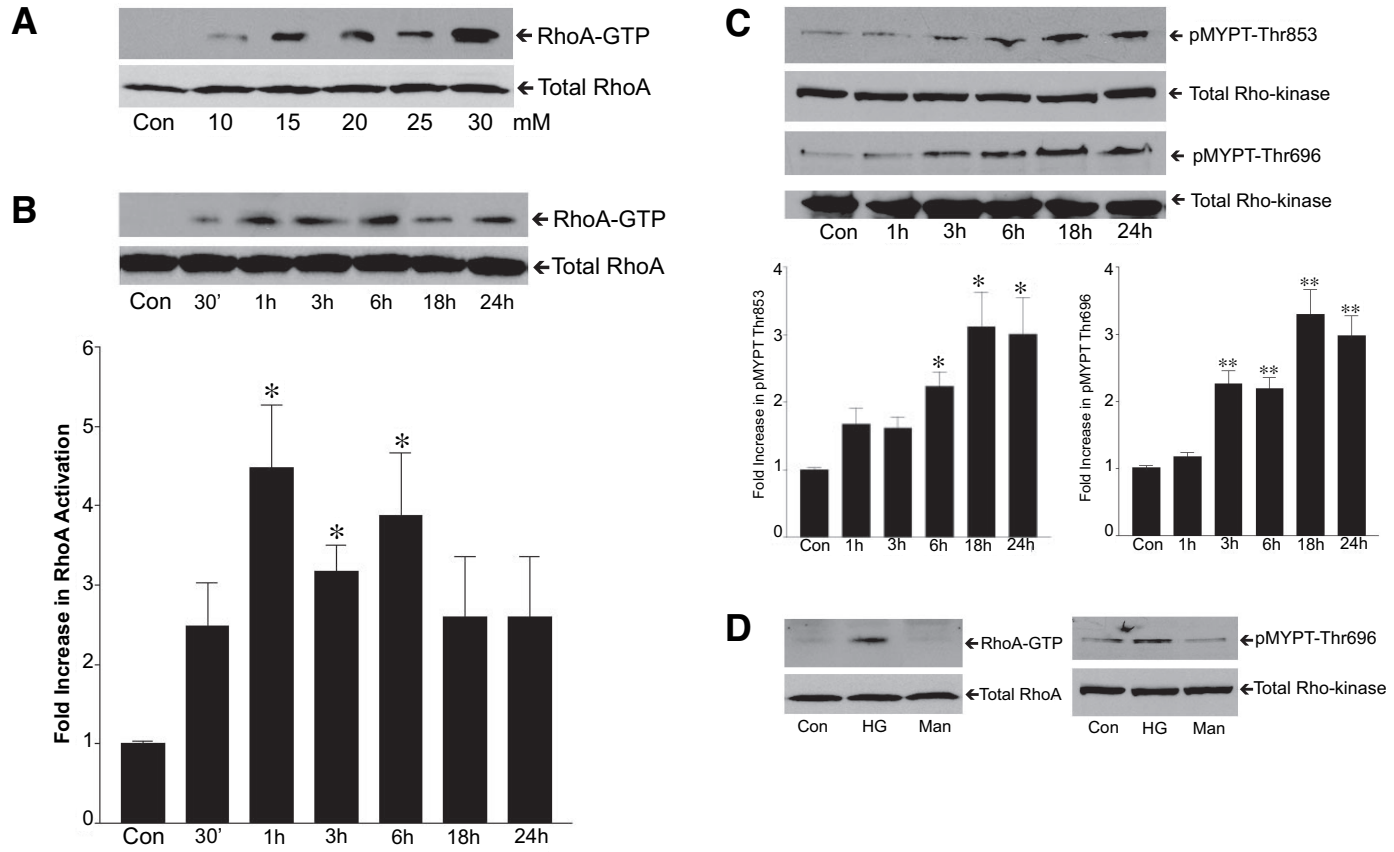

FIG. 1. High glucose–activated RhoA signaling in MCs. *A*: GTP-loaded RhoA was measured using a pull-down assay as described in RESEARCH DESIGN AND METHODS, after treatment of MCs for 6 h with increasing concentrations of glucose as indicated. *B*: RhoA activation was assessed after treatment for the indicated times with 30 mmol/l glucose, with densitometric data graphically displayed below (*$P < 0.05$ vs. control [Con], $n = 5$). *C*: Rho-Kinase activation was assessed by immunoblotting for phosphorylation of Thr853 or Thr696 on MYPT (pMYPT) after treatment for the indicated times with 30 mmol/l glucose (*$P < 0.05$ vs. Con, $n = 5$; **$P < 0.01$ vs. Con, $n = 5$). *D*: Treatment with equimolar mannitol for 6 h was without effect on both RhoA and Rho-kinase activation as compared with glucose. HG, high glucose.

heterodimeric transcription factor AP-1. Transcription of AP-1 component proteins is increased by high glucose in MCs, as is activator protein-1 (AP-1) activity (16,17). Numerous genes important in the fibrotic response in diabetic nephropathy are regulated by AP-1, including TGF-β, plasminogen activator inhibitor-1, and fibronectin (18–20). It is not known, however, whether RhoA/Rho-kinase signaling mediates high glucose–induced matrix upregulation by MCs, or the glomerular matrix accumulation observed in diabetic nephropathy.

Hence, we investigated whether high glucose activated RhoA/Rho-kinase in MCs and the potential involvement of this signaling pathway in matrix protein synthesis, with a focus on fibronectin. We sought to define a role for this signaling pathway in vivo in the STZ model of diabetic renal injury. Our studies, showing a requirement for RhoA/Rho-kinase in the fibrotic effects of high glucose both in vitro and in vivo, define a potential new target for therapeutic intervention in diabetic nephropathy.

RESEARCH DESIGN AND METHODS

Cell culture. Sprague-Dawley primary rat MCs were cultured in Dulbecco's modified Eagle's medium, 5.6 mmol/l glucose, supplemented with 20% fetal calf serum (Life Technologies), streptomycin (100 μg/ml), and penicillin (100 units/ml) at 37°C in 95% air/5% CO_2 and used between passages 6 and 15.

Confluent cells were rendered quiescent by incubation for 24 h in serum-free medium before treating with glucose (24.4 mmol/l for a final concentration of 30 mmol/l) or osmotic control (mannitol, 24.4 mmol/l) for various times as indicated. Rho-Kinase inhibitors were added before glucose: Y-27632 (Calbiochem), 10 μmol/l, 30 min, or HA-1077 (Calbiochem), 25 μmol/l, 30 min. The inhibitory activity and specificity of both inhibitors toward Rho-kinase has been demonstrated by others (21).

RhoA pull-down assay. This was performed as described previously (6). Briefly, cells were lysed in hypertonic buffer and GTP-bound RhoA was immunoprecipitated from cleared lysate with 30 μg glutathione S-transferase (GST)-tagged Rhotekin–Rho-binding domain protein bound to glutathione-agarose (Cytoskeleton). Beads were washed and the immunoprecipitate resolved on 15% SDS-PAGE. Membranes were probed with monoclonal anti-RhoA 1:500 (Santa Cruz). Lysate (40 μg) was also probed for RhoA to ensure equality across conditions.

Protein extraction and immunoblotting. Cells were lysed and protein was extracted as published (6). Supernatant (50 μg) was separated on 10% SDS-PAGE gels, and Western blotting was performed as described (6). Antibodies included polyclonal phospho–myosin phosphatase targeting subunit (MYPT) threonine (Thr)-696 (1 μg/ml; Upstate), polyclonal phospho-MYPT Thr853 (1:500, Santa Cruz), polyclonal ROCKα (2 μg/ml; Upstate), and monoclonal fibronectin (1:5,000, BD Biosciences). For assessment of secreted fibronectin, conditioned medium was collected after treatment with glucose for 48 h and centrifuged (4,000 rpm, 5 min) to pellet debris; 10 μg protein was separated on a 7.5% gel.

Luciferase assays. MCs plated to 85% subconfluence were transfected with 0.5 μg of AP-1-Luc (Stratagene) and 0.05 μg pCMV-β-galactosidase (β-gal) (Clontech) using LipofectAMINE (Qiagen). MCs were serum-deprived overnight 24 h after transfection and then exposed to glucose for the times and concentrations indicated. Lysis was achieved with Reporter Lysis Buffer (Promega) using one freeze-thaw cycle, and luciferase and β-galactosidase activities were measured on clarified lysate using specific kits (Promega) with a Berthold luminometer and a plate reader (420 nm), respectively. β-Galactosidase activity was used to adjust for transfection efficiency.

Electrophoretic mobility shift assay. After treatment, nuclear extracts were prepared as published (22). Cells were lysed in hypotonic buffer,

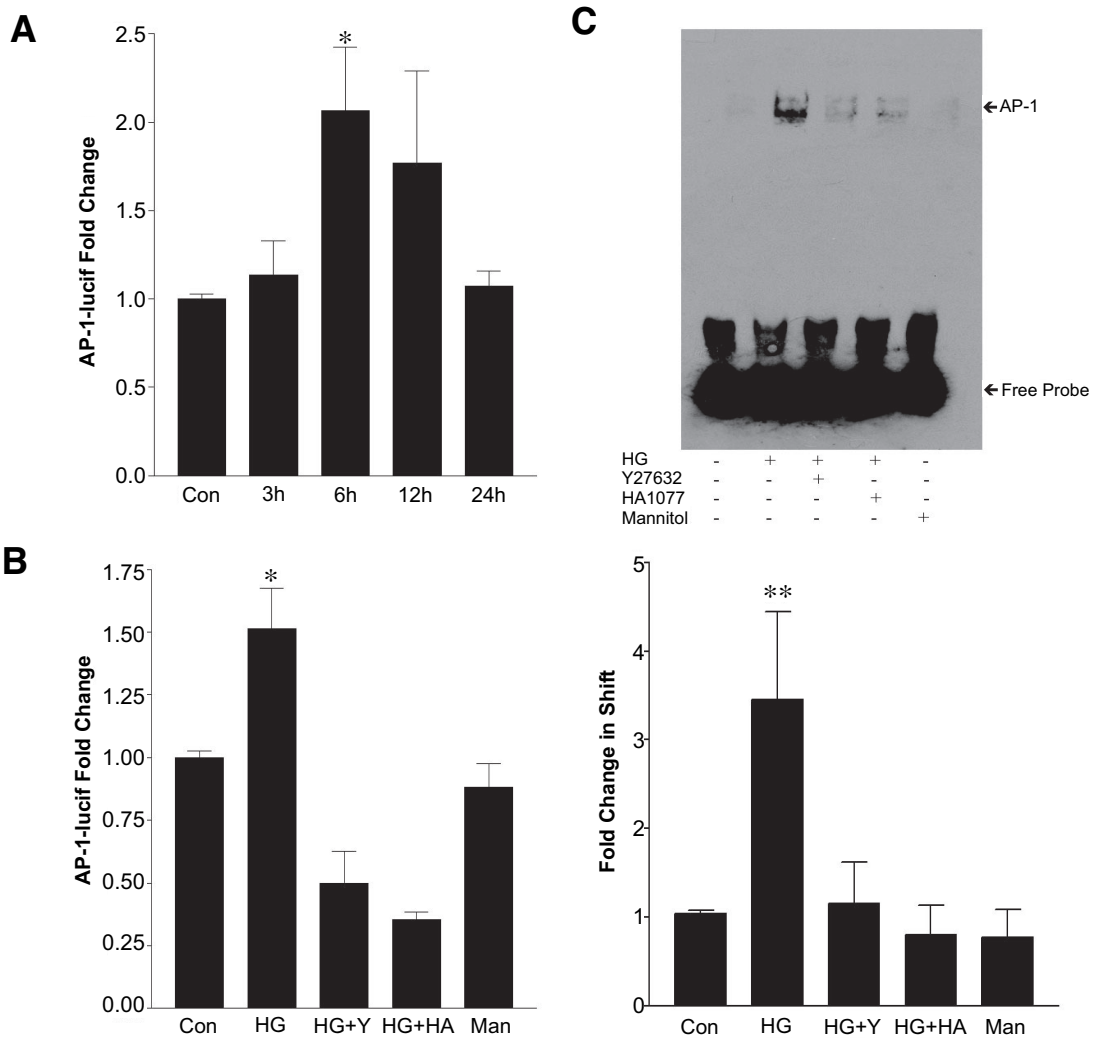

FIG. 2. Rho-Kinase mediates glucose-induced activation of the transcription factor AP-1 in MCs. *A*: MCs transiently transfected with AP-1-Luc were exposed to 30 mmol/l glucose for the times indicated, and luciferase activity was assayed (*$P < 0.05$ vs. control [Con], $n = 5$). *B*: MCs were incubated with the Rho-kinase inhibitors Y-27632 (10 μmol/l for 30 min) or HA-1077 (fasudil; 25 FM for 30 min) before treatment with 30 mmol/l glucose for 6 h and AP-1-luc activity was assayed (*$P < 0.05$ vs. all other conditions, $n = 4$). *C*: Nuclear proteins were isolated from MCs treated with glucose for 6 h with Rho-kinase inhibitors as above, and AP-1 activity was assessed by EMSA using an AP-1–specific sequence ($P < 0.01$ glucose versus all others, $n = 4$). In *B* and *C*, mannitol served as an osmotic control.**

homogenized, and sedimented at 16,000*g* for 20 min at 4°C. Pelleted nuclei were resuspended in hypotonic buffer containing 0.42 M NaCl and 20% glycerol, rotated for 30 min at 4°C, centrifuged as above, and supernatant containing nuclear proteins was collected. Nuclear proteins (3 μg) were incubated for 5 min at room temperature with a biotin-labeled AP-1 consensus oligonucleotide (Sigma) as per the manufacturer's instructions (Pierce). Reaction mixtures were electrophoresed in a 6% polyacrylamide gel, transferred, and DNA cross-linked to a nylon membrane (Amersham) and then probed with horseradish peroxidase–conjugated streptavidin antibodies (1:300) (Pierce).

Enzyme-linked immunosorbent assay. Debris was removed from conditioned media by low-speed centrifugation and fibronectin enzyme-linked immunosorbent assay (ELISA) performed as previously described (6). Briefly, 96-well microtiter plates were coated overnight at 4°C with media (1:6) in ELISA Coating Buffer (Sigma), blocked, and then incubated with monoclonal anti-fibronectin (1:5,000; BD Biosciences), which recognizes secreted fibronectin, followed by alkaline phosphatase-conjugated goat anti-mouse (1:30,000, Sigma). Finally, *p*-nitrophenyl phosphate was added, and reactions were read at 405 nm in a microplate autoreader.

Northern blot. Total RNA (10 μg), extracted using Trizol according to the manufacturer's instructions (Invitrogen), was separated on a formaldehyde-agarose gel and transferred to a nylon membrane (Hybond; Amersham Biosciences). Hybridization was performed with random primed digoxigenin-11-dUTP–labeled cDNA probes prepared from fibronectin cDNA amplified by PCR. Hybridized probes were detected using alkaline phosphatase-labeled anti-digoxigenin antibodies and CDP-star as substrate. Kits and reagents were from Roche Applied Science. Intensity of the 28S band was used as a loading control.

siRNA and real-time PCR. Rat RhoA on-target plus SMART pool siRNA and control nontargeting siRNA were purchased from Dharmacon. MCs were transfected with 100 nmol/l using GeneEraser siRNA reagent (Stratagene) at 60% confluence. After 24 h, cells were serum deprived in 0.5% fetal bovine serum for 24 h and then treated for 48 h with glucose. RNA was harvested as for Northern blots, RT was performed using standard methods (23), and cDNA was analyzed using real-time PCR for fibronectin (or vascular endothelial growth factor [VEGF] in other experiments) and 18S. Standard PCR was used to assess RhoA transcript downregulation by siRNA. This was normalized to β-actin.

In vivo studies. Animal studies were carried out in accordance with McMaster University and Canadian Council on Animal Care guidelines. Male Sprague-Dawley rats (Charles River, $n = 42$), 175–200 g, were housed under standard conditions with free access to regular food and water. Diabetes was induced by tail-vein injection of 55 mg/kg STZ (Sigma) and confirmed by tail-vein glucose measurement of >20 mmol/l (Precision XTra; Medisense) 48 h after injection. Control rats ($n = 9$) were injected with equal volume of citrate buffer. Diabetic rats were randomized (11/group) 48 h after induction to receive the Rho-kinase inhibitor fasudil (30 mg/kg daily; Asahi Kasei) or enalapril (10 mg/kg twice daily; Merck) by gavage feeding. Control rats were fed equal volumes of water. Diabetic rats also received low doses of insulin Lente (Eli Lilly) if required to prevent ketonuria as assessed by dipstick (Bayer Multistix), but to maintain hyperglycemia >20 mmol/l. Treatment was continued for 6.5 months, at which time rats were killed.

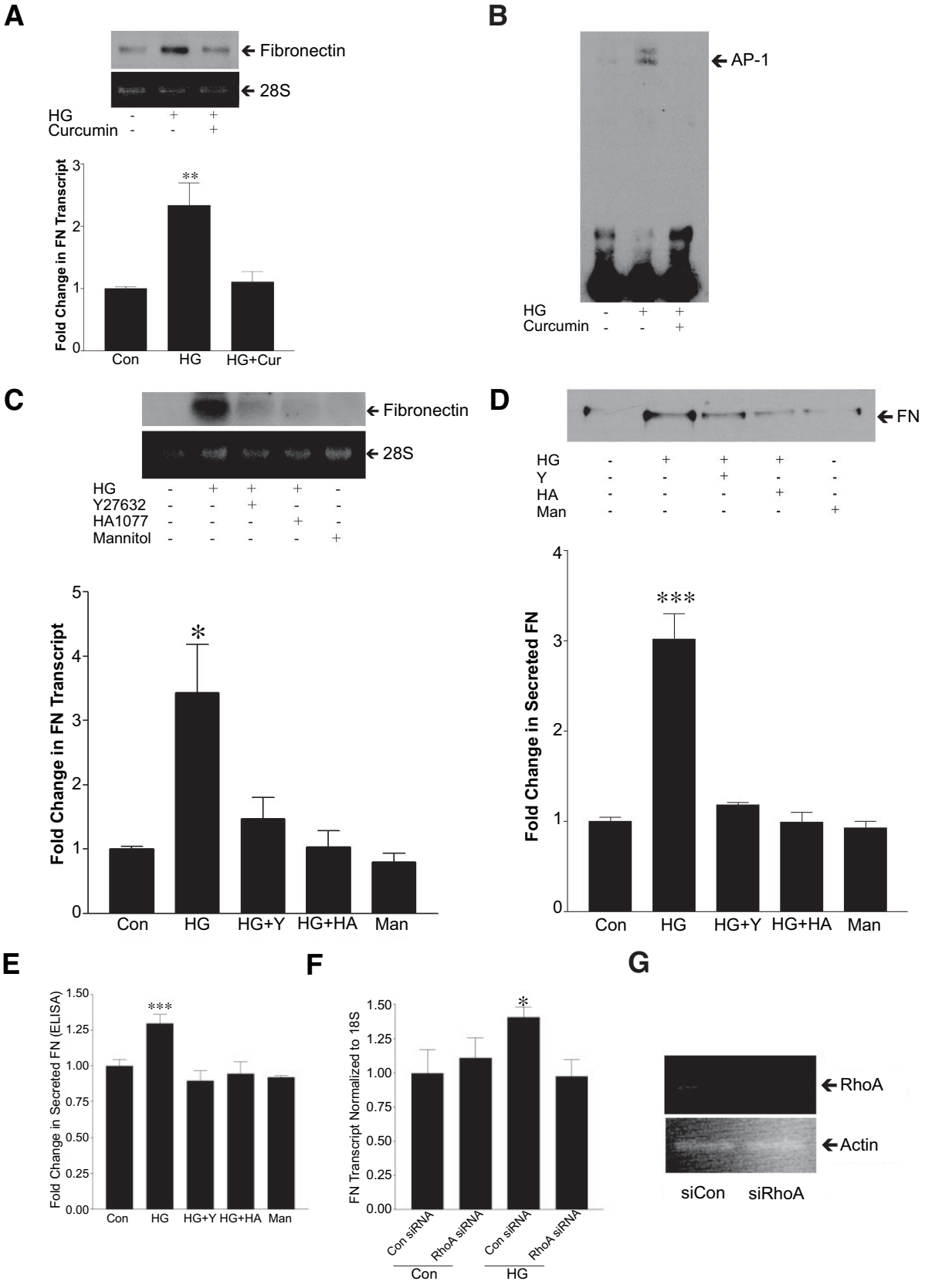

FIG. 3. Fibronectin production induced by high glucose in MCs is dependent on RhoA/Rho-kinase. *A*: MCs were treated with glucose for 48 h in the presence or absence of the AP-1 inhibitor curcumin (20 μmol/l, 30 min), after which fibronectin transcript levels were assessed by Northern analysis ($P < 0.01$ glucose vs. others, $n = 3$). 28S served as the loading control. *B*: Nuclear proteins were isolated from MCs treated with glucose for 6 h and curcumin as above, and AP-1 activity was assessed by EMSA using an AP-1–specific sequence. *C*: MCs were exposed to glucose for 48 h in the presence or absence of Rho-kinase inhibitors Y-27632 (10 μmol/l) or HA-1077 (25 μmol/l), or to mannitol as osmotic control. Fibronectin transcript levels were assessed by Northern analysis, with 28S serving as loading control (*$P < 0.05$ vs. all others, $n = 4$). *D*: Fibronectin protein secreted into the medium by MCs treated as in *C* was assessed by Western blot of 10 μg of medium; ***$P < 0.001$ vs all others. *E*: Fibronectin**

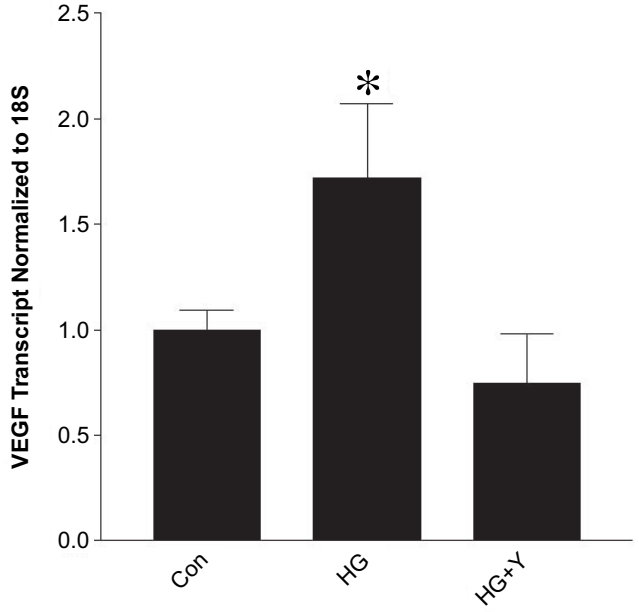

FIG. 4. Glucose-induced VEGF upregulation is dependent on Rho-kinase. MCs were treated with glucose for 24 h in the presence or absence of the Rho-kinase inhibitor Y-27632 (10 μmol/l). VEGF transcript, normalized to 18S, was determined by real-time PCR (*$P < 0.05$, $n = 4$). HG, high glucose; Y, Y-27632.

Rats were weighed weekly and urine ketones were assessed twice weekly. Blood pressure was determined by tail cuff plethysmography (Harvard Apparatus) monthly. At the end of the study, urine was collected for 6 h in a metabolic cage, and weight, blood pressure, blood glucose, and serum creatinine were obtained. Serum creatinine and urine protein were measured using Roche kits on a Modular P analyzer. Renal hypertrophy was assessed by kidney–to–body weight ratio (mg/g) at time of death. Formalin-fixed sections (3 μmol/l) were stained by periodic acid–Schiff reagent and glomerular matrix expansion scored for 20 glomeruli chosen at random from two to three slides as follows after blinding to treatment group: 0 = none, +1 = <25% glomerular area involved, +2 = 25–50%, +3 = >50% (24). Sections were scored by A.J.I. by visual inspection, a score was assigned for each glomerulus, and these values were averaged for a final score for each group. A small piece of cortex was also taken for electron microscopy, fixed in 0.2 M glutaraldehyde/0.1 M sodium cacodylate, pH 7.4, and samples were processed by the McMaster University electron microscopy facility. Basement membrane thickness was assessed on peripheral loops photographed randomly at 10,000× magnification, with calculation of the harmonic mean of measurements at 80–100 points, crossing a grid from one to two glomeruli. Glomeruli from the remainder of the kidney cortex were harvested by differential sieving. They were homogenized in lysis buffer or Trizol for immunoblotting and northern analysis, respectively.

Statistical analysis. Densitometry was obtained using Scion Image (National Institutes of Health), and analysis was performed using one-way ANOVA with Tukey's Honestly Significant Difference for post hoc studies (SPSS 14.0 for Windows). A P value <0.05 (two-tailed) was considered significant. Data are represented as the means ± SE. Experiments were repeated multiple times, with number of repetitions denoted by "n =".

RESULTS

RhoA and Rho-kinase are activated by high glucose in MCs. Increased membrane-bound RhoA has been observed in diabetic animals in major vessels and renal cortex (9–11) and in MCs after 24 h of high glucose exposure (12). This suggests increased RhoA activation in the diabetic milieu. We thus first sought to establish, using a direct assessment of RhoA activity, whether high glucose activates this GTPase. Primary rat MCs were exposed to increasing concentrations of glucose for 6 h, and activation of RhoA was measured using a pull-down assay. A dose-dependent increase in RhoA activity was observed (Fig. 1*A*). In subsequent experiments, 30 mmol/l glucose was used. A time course was next performed, in which MCs were treated with 30 mmol/l glucose for the times indicated (Fig. 1*B*). Significant RhoA activation was observed by 1 h. Downstream, Rho-kinase activation was measured by assessing phosphorylation on either Thr853 or Thr696 of MYPT, a specific Rho-kinase target (25,26). An increase of MYPT-Thr853/696 phosphorylation was seen after RhoA activation (Fig. 1*C*). Treatment with equimolar mannitol for 6 h did not induce RhoA or Rho-kinase activation (Fig. 1*D*).

High glucose activation of the transcription factor AP-1 in MCs requires RhoA signaling. The transcription factor AP-1 is activated by high glucose in MCs (17) and is an important mediator of TGF-β1 and fibronectin upregulation (18,20). Because RhoA signaling has been shown to regulate AP-1 transcriptional activation in other cell types in some settings (27,28), we first investigated whether glucose-induced AP-1 activation required RhoA/Rho-kinase.

Exposure of MCs transiently transfected with AP-1-Luc to glucose led to an increase in luciferase activity that was maximal at 6 h (Fig. 2*A*). This was abrogated by Rho-kinase inhibition with Y-27632 (10 μmol/l) or HA-1077 (25 μmol/l). Both inhibitors effectively blocked glucose-induced Rho-kinase activation, as assessed by MYPT-Thr853 phosphorylation (not shown). Mannitol was without effect (Fig. 2*B*). To confirm that Rho-kinase inhibition prevents AP-1 transcriptional activation, we next performed an electrophoretic mobility shift assay (EMSA) using an AP-1 consensus oligonucleotide. As seen in Fig. 2*C*, glucose-induced AP-1 nuclear protein binding was prevented by both Rho-kinase inhibitors and was not observed with mannitol. This binding and consequent shift were also prevented by 50× excess unlabeled oligonucleotide (not shown). Our data thus show that glucose-induced activation of AP-1 is dependent on RhoA signaling.

Fibronectin upregulation in response to high glucose requires RhoA signaling. Increased fibronectin expression is seen in human and animal diabetic glomeruli (3), and high glucose stimulates its upregulation in cultured MCs (29), perhaps through AP-1 (30). We first confirmed this, showing that the AP-1 inhibitor curcumin (20 μmol/l) prevented glucose-induced fibronectin upregulation (Fig. 3*A*). Curcumin effectively inhibited glucose-induced AP-1 activation, as assessed by EMSA (Fig. 3*B*). To determine whether fibronectin upregulation was also dependent on RhoA/Rho-kinase signaling, MCs were exposed to glucose for 48 h. High glucose induced a clear increase in fibronectin transcript by Northern analysis, prevented by Y-27632 and HA-1077 (Fig. 3*C*). No cell toxicity was observed with any treatment (not shown). Western blotting (Fig. 3*D*) and ELISA (Fig. 3*E*) of conditioned medium from MCs exposed to high glucose for 48 h showed an increase in fibronectin protein secretion, which was prevented by

secreted into the medium by MCs after treatment as above was also assessed by ELISA. Densitometry shows the results from five experiments performed in triplicate; ***$P < 0.001$ vs. all others. *F*: After MCs were transfected with control or RhoA siRNA and treated with glucose for 48 h, fibronectin transcript was assessed by real-time PCR. Values were normalized to 18S (*$P < 0.05$ vs. others, $n = 6$). *G*: Successful downregulation of RhoA transcript was shown by PCR, with actin serving as the loading control.

TABLE 1
Clinical characteristics at 7 months of control rats and STZ-induced diabetic rats untreated or treated with the Rho-kinase inhibitor fasudil (30 mg · kg^{-1} · day^{-1})

	Control	STZ	STZ + enalapril	STZ + fasudil	*P*
n	9	11	10	11	–
Glucose (mmol/l)	6.3 ± 0.20	19.5 ± 1.68*	20.3 ± 1.17*	19.8 ± 4.92*	*<0.001 vs. control
Weight (g)	740.0 ± 28.2	531.7 ± 21.4*	528.9 ± 13.1*	497.4 ± 16.3*	*<0.001 vs. control
Kidney/body weight (mg/g)	2.82 ± 0.09	5.10 ± 0.22*	5.26 ± 0.27*	5.11 ± 0.23*	*<0.001 vs. control
Systolic blood pressure (mmHg)	111.2 ± 1.7	116.0 ± 1.5	112.6 ± 1.7	112.0 ± 6.4	NS
Mean arterial pressure (mmHg)	85.6 ± 1.3	88.5 ± 1.1	86.1 ± 1.3	86.0 ± 1.5	NS
Creatinine (μmol/l)	29.4 ± 0.9	26.4 ± 1.1	28.3 ± 1.5	29.6 ± 1.9	NS

Data are means ± SE. *P = <0.001 vs. control.

both Rho-kinase inhibitors. In Fig. 3*C–E*, mannitol had no effect on fibronectin transcript or protein upregulation.

Subsequently, we assessed the effects of inhibiting upstream RhoA activation. MCs were transfected with either control or RhoA-specific siRNA. Figure 3*F* shows that glucose-induced fibronectin upregulation, as assessed by real-time PCR, was prevented by RhoA siRNA. Successful downregulation of RhoA was confirmed by RT-PCR, with actin serving as control (Fig. 3*G*). These results suggest an important role for RhoA/Rho-kinase in this characteristic fibrogenic response to high glucose.

The cytokine vascular endothelial growth factor (VEGF) has also been implicated in the pathogenesis of diabetic nephropathy and shown to be upregulated by high glucose in MCs (31,32). VEGF increases synthesis of the matrix protein collagen IV in MCs (33), and upregulation of VEGF itself was shown in other cells to be mediated by RhoA/Rho-kinase activation (34). Figure 4 shows that in MCs treated with glucose for 24 h, upregulation of VEGF transcription is prevented by Y-27632 and is thus also dependent on RhoA/Rho-kinase signaling.

Rho-kinase activation is important in diabetic nephropathy. Since RhoA/Rho-kinase signaling mediates key profibrotic responses to glucose in MCs, we sought to test the importance of this pathway in vivo. We used a model of type 1 diabetes, the STZ-treated rat, which is characterized by hyperglycemia and low circulating insulin levels. Treatment with the Rho-kinase inhibitor fasudil was compared with established therapy with the ACE inhibitor enalapril (2), initiated at the onset of hyperglycemia and continued for 6.5 months. The dose of fasudil used (30 mg · kg^{-1} · day^{-1}) was without effect on blood pressure when administered for 5 months in hypertensive rats (35).

Diabetic rats had significantly higher blood glucose levels and lower weights than their nondiabetic counterparts (Table 1), unaffected by either fasudil or enalapril. No difference in blood pressure was observed between any of the groups. Diabetic rats developed renal hypertrophy, as evidenced by a significantly higher kidney–to–body weight ratio. This was unaffected by either treatment, in keeping with observations using ACE inhibitor therapy (36). Serum creatinine at the end of study was also not different between groups.

Increased protein excretion, a hallmark of early diabetic nephropathy, was observed in the diabetic group and normalized by both fasudil and enalapril (5.68 ± 3.91 mg/6 h STZ vs. 2.55 ± 1.03 in control, 2.27 ± 0.80 in STZ + enalapril, and 2.29 ± 1.19 STZ + fasudil, $P < 0.03$, Fig. 5*A*). The degree of glomerular matrix accumulation was assessed by an observer blinded to treatment group (A.J.I.) according to the scale outlined in RESEARCH DESIGN AND METHODS. As shown graphically in Fig. 5*B*, increased glomerular sclerosis was observed in the diabetic group, and this was prevented in both treatment groups. Representative glomeruli stained with periodic acid–Schiff reagent are shown in Fig. 5*C*. Finally, ultrastructural examination of glomerular basement membrane thickness by electron microscopy showed this to be significantly increased in the diabetic animals, and this was reduced by treatment with fasudil (Fig. 5*D* and *E*). Focal areas of podocyte foot process effacement (arrows) were seen in the diabetic group (Fig. 5*E*).

We assessed glomerular Rho-kinase activation in each group by immunoblotting for phosphoMYPT-Thr696. Figure 6*A* shows significantly increased Rho-kinase activation in diabetic glomeruli, prevented by treatment with fasudil. Immunoblots from two separate rats are shown. Although there was a trend toward decreased Rho-kinase activation with enalapril, this was not statistically significant. Since elevated intraglomerular pressure is known to be a pathogenic factor in diabetic nephropathy (37), and corresponding in vitro hypertension models show Rho-kinase activation in MCs (6), it is possible that the trend toward decreased Rho-kinase activation is based on the favorable hemodynamic impact described for enalapril (37). It is clear, however, that other factors such as hyperglycemia also independently induce activation of this signaling pathway.

Finally, we assessed the degree of fibronectin upregulation in glomeruli. The graph in Fig. 6*B* summarizes results of Northern analysis for all animals, with two from each group shown in the blot above. Fibronectin transcript upregulation in diabetic glomeruli was prevented by treatment with either fasudil or enalapril. These data thus suggest that in several parameters, Rho-kinase inhibition is as effective as ACE inhibition in the prevention of early diabetic nephropathy.

DISCUSSION

Our studies clearly show a role for RhoA/Rho-kinase signaling in glucose-induced matrix upregulation in MCs, suggesting RhoA pathway inhibition merits consideration as a therapeutic target in the treatment of diabetic nephropathy.

RhoA activation in diabetic rodent models has been suggested in earlier studies (8–11). Similarly, RhoA membrane translocation was observed in MCs treated with high glucose for 24 h (12). We used a direct assay of RhoA activity, which isolates the active GTP-bound molecule, to show that glucose indeed activated RhoA in MCs. We also demonstrated activation of the downstream effector Rho-

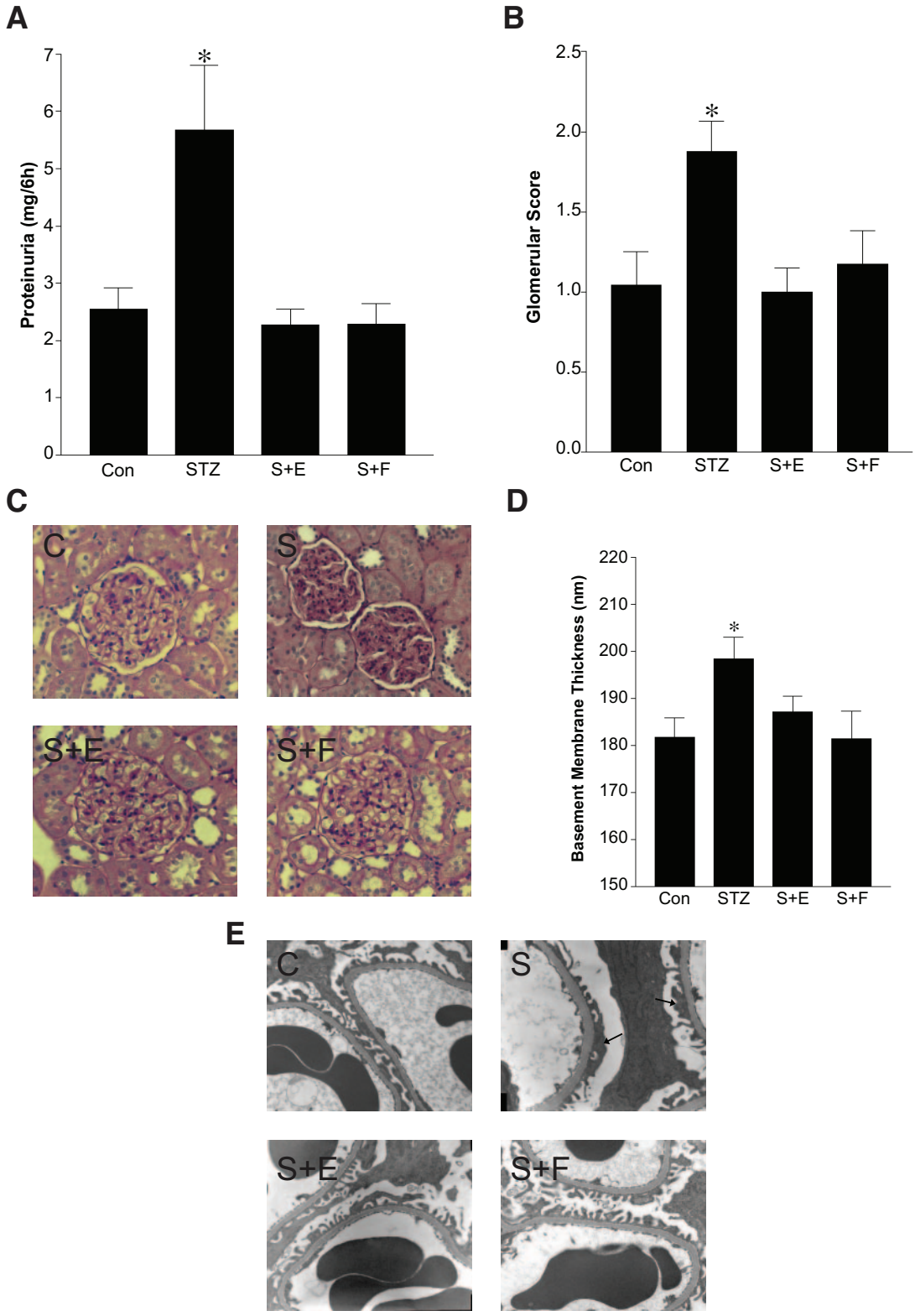

FIG. 5. Rho-Kinase inhibition reverses markers of diabetic nephropathy in vivo. *A*: Animals were placed in a metabolic cage and urine was collected for 6 h. The increased urinary protein excretion seen in diabetic rats was prevented by treatment with fasudil or enalapril (*$P < 0.05$ vs. all others). *B* and *C*: Glomerular matrix accumulation was assessed after staining of formalin-fixed sections with periodic acid–Schiff reagent. Scores were assigned in *B* after blinding to the treatment group according to the grading scheme outlined in RESEARCH DESIGN AND METHODS (*$P < 0.05$ vs. all others). Representative glomeruli from each group are shown in *C*. Glomerular basement membrane thickness was assessed by electron microscopy, with data summarized in the bar graph in *D*. *$P < 0.05$ vs. control (Con) and fasudil (F) groups. Representative photomicrographs are shown in *E*. Arrows denote focal areas of foot process effacement. *S*, STZ.

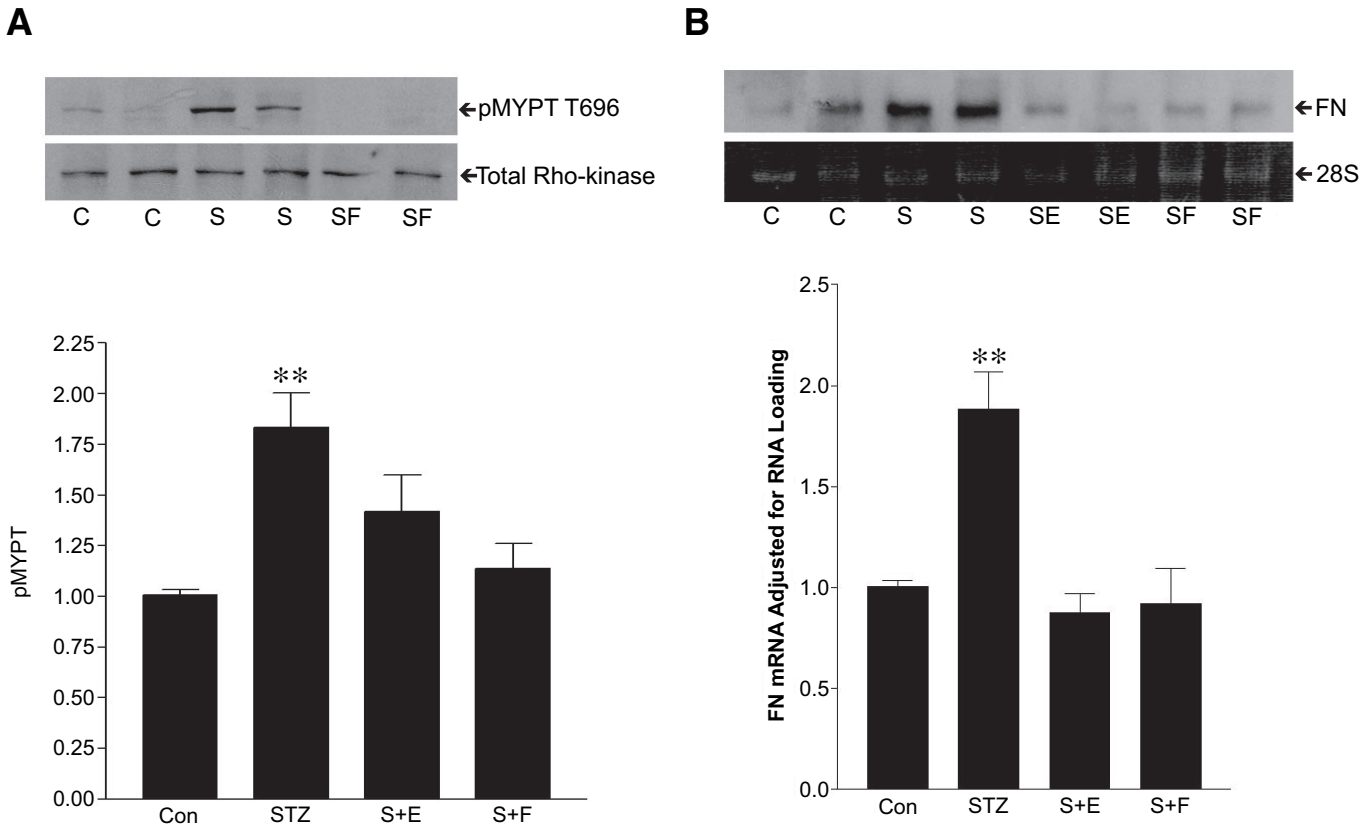

FIG. 6. Glomerular fibronectin upregulation in diabetes is prevented by Rho-kinase inhibition. *A*: Glomeruli were obtained by differential sieving at study termination, and activation of Rho-kinase was assessed by immunoblotting for its substrate phospho-MYPT ($P < 0.01$ for STZ vs. control [Con] and S + fasudil [F]). *B*: Glomerular fibronectin transcript levels were assessed by Northern analysis, with 28S used as a loading and normalization control (**$P < 0.01$ vs. all others). For both *A* and *B*, a representative blot is shown, with the graph below summarizing data from all animals.**

kinase by glucose in MCs, assessed by phosphorylation of its target MYPT (25,26).

RhoA regulates transcription of *c-fos* and *c-jun*, components of AP-1 (14). A requirement for RhoA in AP-1 transcriptional activity has also been demonstrated in T-cells and fibroblasts (27,28). AP-1, activated by high glucose in MCs and in diabetic kidneys (17,38), is a mediator of glucose responses in mesangial and other cells (18–20). Using a promoter with AP-1 binding sites driving luciferase production, as well as EMSA, we confirmed AP-1 activation by glucose in MCs and demonstrated a novel role for RhoA/Rho-kinase signaling in this activation. The effect on AP-1 is functionally relevant, since RhoA/Rho-kinase inhibition prevented glucose-induced upregulation of the AP-1 target gene fibronectin (20,30), as did AP-1 inhibition with curcumin.

Given these observations, we went on to assess the effects of fasudil, an orally bioavailable Rho-kinase inhibitor, in STZ-treated diabetic rats at doses that did not affect systemic blood pressure (35). Our data confirmed glomerular activation of Rho-kinase, abrogated by fasudil.

Treatment with either fasudil or enalapril decreased the matrix accumulation and fibronectin upregulation seen in diabetic glomeruli. Although our in vitro studies have shown a direct effect of Rho-kinase inhibition on limiting fibronectin upregulation, it is likely that the observed in vivo effects on suppression of glomerular sclerosis are the result of broader effects of this treatment. Indeed, we also show that Rho-kinase inhibition prevents glucose-induced VEGF upregulation, and VEGF has been shown to increase production of collagen IV by MCs (33). Glucose-induced TGF-β upregulation is also mediated by AP-1, and we have demonstrated the Rho-kinase dependence of AP-1 activation. Of interest, others have shown an early decrease in cortical TGF-β transcript levels in STZ diabetic rats treated with fasudil, although glomeruli were not specifically assessed (8). Furthermore, a role for RhoA signaling downstream of TGF-β, including collagen synthesis in MCs, has been shown (5). RhoA signaling in fibroblasts was also observed to promote matrix polymerization (39). Thus, the inhibitory effects of fasudil on diabetes-associated glomerular matrix expansion is likely multifactorial.

Importantly, both proteinuria and glomerular basement membrane thickening, well-established clinical and pathologic markers of early diabetic nephropathy, were normalized by both treatments. Early prevention of albuminuria by fasudil was also observed in type 1 diabetic rats at 1 month (8). These data thus support a potential role for Rho-kinase inhibition in preventing the progression of early diabetic nephropathy. Further studies would be needed to determine whether any further benefit might be gained from combination of fasudil with ACEI. Since Rho-kinase inhibition is not expected to result in hyperkalemia, an adverse effect often precluding ACEI use, Rho-kinase inhibitors may be of particular interest in this patient subset.

Therapeutic benefits of Rho-kinase inhibition may extend to insulin-resistant diabetes. Vascular smooth muscle cells from type 2 diabetic rats demonstrate increased RhoA/Rho-kinase activation, with Rho-kinase shown to

inhibit insulin signaling (40). In two different models of type 2 diabetes, fasudil improved metabolic parameters including glucose control and lipid profile, and insulin response in skeletal muscle was increased (41,42). Pathologic changes of diabetic nephropathy were also improved, although at significantly higher doses of fasudil than used in this study (100 mg/kg) (42). In a third model of type 2 diabetes (*db/db* mice), low-dose fasudil (10 mg/kg) reduced microalbuminuria, mesangial matrix expansion, and basement membrane thickening without any effect on metabolic parameters (43). These and our studies support a therapeutic role for Rho-kinase inhibition in both type 1 and type 2 diabetes.

The mechanism whereby RhoA is activated by high glucose remains to be defined. Protein kinase C is a possible candidate, since isoforms are implicated in glucose-induced matrix production in MCs (44), and protein kinase C was required for RhoA and Rho-kinase activation in response to various stimuli, including glucose-induced upregulation of osteopontin (13,45,46). Also possible is the inactivation by glucose of cyclic GMP-dependent protein kinase (cGK), an enzyme that phosphorylates and inactivates RhoA (47). In MCs, glucose decreased cyclic GMP-dependent protein kinase activity, and its overexpression blocked subsequent TGF-β1 and fibronectin upregulation (48). Finally, reactive oxygen species, thought to play a central role in the pathogenesis of diabetic nephropathy, have also been shown to activate RhoA (49,50). Further studies are required, however, to definitively delineate the upstream events leading to RhoA activation by glucose.

In conclusion, our studies demonstrate an important role for RhoA/Rho-kinase signaling in MCs in response to high glucose and in diabetic glomeruli. Because current treatments used to prevent the development or progression of diabetic nephropathy are not fully effective, the clinical use of Rho-kinase inhibitors represents a promising novel therapy.

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