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Original Article

Blocking VEGF/Caveolin-1 signaling contributes to renal protection of fasudil in streptozotocin-induced diabetic rats

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Aim: RhoA/ROCK signaling plays an important role in diabetic nephropathy, and ROCK inhibitor fasudil exerts nephroprotection in experimental diabetic nephropathy. In this study we investigated the molecular mechanisms underlying the protective actions of fasudil in a rat model of diabetic nephropathy.

Methods: Streptozotocin (STZ)-induced diabetic rats, to which fasudil or a positive control drug enalapril were orally administered for 8 months. Metabolic parameters and blood pressure were assessed during the treatments. After the rats were euthanized, kidney samples were collected for histological and molecular biological studies. VEGF, VEGFR1, VEGFR2 and fibronectin expression, and Src and caveolin-1 phosphorylation in the kidneys were assessed using RT-PCR, Western blot and immunohistochemistry assays. The association between VEGFR2 and caveolin-1 was analyzed with immunoprecipitation.

Results: Chronic administration of fasudil (30 and 100 mg·kg^{-1}·d^{-1}) or enalapril (10 mg/kg, bid) significantly attenuated the glomerular sclerosis and albuminuria in the diabetic rats. Furthermore, fasudil treatment prevented the upregulation of VEGF, VEGFR1, VEGFR2 and fibronectin, and the increased association between VEGFR2 and caveolin-1 in the renal cortices, and partially blocked Src activation and caveolin-1 phosphorylation on tyrosine 14 in the kidneys, whereas enalapril treatment had no effects on the VEGFR2/Src/caveolin-1 signaling pathway.

Conclusion: Fasudil exerts protective actions in STZ-induced diabetic nephropathy by blocking the VEGFR2/Src/caveolin-1 signaling pathway and fibronectin upregulation. Thus, VEGFR2 may be a potential therapeutic target for the treatment of diabetic nephropathy.

Keywords: diabetic nephropathy; glomerular sclerosis; RhoA/ROCK signaling; fasudil; enalapril; VEGF; Src; caveolin-1

Introduction

Diabetic nephropathy is one of the most serious microvascular complications of diabetes mellitus. The current treatments for diabetic nephropathy include optimization of glucose and blood pressure control by targeting the renin-angiotensin system (RAS) with angiotensin-converting enzyme (ACE) inhibitors and/or angiotensin II receptor blockers. Despite these measures, renal damage progresses in many patients. Novel strategies are therefore required, and some recent studies suggest that the inhibition of small G-protein RhoA and its downstream effector, Rho-associated kinase (ROCK), is a promising approach[1–5].

RhoA is the best studied member of the Rho GTPase family.

RhoA behaves as a "molecular switch" that cycles between an inactive GDP-bound state and an active GTP-bound state[6]. ROCK is a major RhoA effector. RhoA/ROCK signaling regulates numerous cell functions, including vascular contraction, actin cytoskeleton reorganization, cellular morphology, motility, adhesion and proliferation[7, 8]. Recent studies have indicated that RhoA/ROCK signaling is an important contributor to diabetic nephropathy[9, 10]. Fasudil, a relatively specific ROCK inhibitor, has nephroprotective actions during experimental diabetic nephropathy[11–13]. The possible mechanisms include the attenuation of diabetes-induced increases in renal TGF-β, CTGF and extracellular matrix (ECM) protein expression resulting in slower glomerulosclerosis and interstitial fibrosis development. Hemodynamic mechanisms and antiproteinuric effects might also be involved. Fasudil also inhibits other kinases, such as protein kinase C isoforms[14], which might contribute to the nephroprotective actions. However, the mechanisms underlying the activation of RhoA/ROCK

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signaling during diabetic nephropathy remain unknown, and the molecular mechanisms of fasudil should be characterized in more detail.

The cytokine vascular endothelial growth factor (VEGF) is an important regulator of normal kidney functions[15]. Recently, VEGF was demonstrated to be upregulated by high glucose in mesangial cells and was implicated in the pathogenesis of diabetic nephropathy. Increased VEGF involves glomerular hypertrophy, proteinuria and ECM production[16–18]. Peng *et al* demonstrated that the upregulation of VEGF transcription in mesangial cells induced by glucose was prevented by the Rho inhibitor Y-27632 indicating that VEGF upregulation is possibly dependent on RhoA/ROCK signaling[10]. There are three known VEGF receptors: VEGFR1/fms-related tyrosine kinase 1 (Flt1), VEGFR2/kinase insert domain receptor (KDR), and Neuropilin-1[19]. VEGF elicits its biological functions by activating specific transmembrane receptors (VEGFR1 and VEGFR2) with intrinsic tyrosine kinase activity[20]. Neuropilin-1 has no tyrosine kinase activity. There is only a small amount of data concerning the role of VEGF signaling pathways during diabetic nephropathy. The present study focused on the potential mechanisms of fasudil inhibition of VEGF upregulation and its receptors (VEGFR1 and VEGFR2) in the renal cortex of streptozotocin (STZ)-induced diabetic rats. The effects of fasudil were compared to the ACE inhibitor enalapril, which is an established therapeutic agent for both clinical and experimental diabetic nephropathy.

Materials and methods

Diabetic rat model

Experiments were performed with male Sprague-Dawley rats weighing 200–225 g (Animal Center, Wuhan University, China) in accordance with the official recommendations of the Chinese Community Guidelines. Diabetes was induced with a 55 mg/kg STZ (Sigma, St Louis, Missouri, USA) injection by tail vein. Control rats (*n*=10) were injected with equal volume of citrate buffer. Hyperglycemia (blood glucose >20 mmol/L) was confirmed (2 d after injection) with a reflectance meter (One Touch, Lifescan, Milpitas, California, USA). Diabetic rats were randomly divided into five groups: untreated, low-dose fasudil (10 mg·kg^{-1}·d^{-1}; Sigma), middle-dose fasudil (30 mg·kg^{-1}·d^{-1}), high-dose fasudil (100 mg·kg^{-1}·d^{-1}), and enalapril (10 mg/kg twice daily; Merck, Darmstadt, Germany) by intragastric gavage (ig). Treatment continued for 8 months. All rats had free access to regular lab chow and water. The diabetic rats also received low dose insulin if required to prevent ketonuria as assessed with a dipstick (Bayer Multistix) but to maintain hyperglycemia >20 mmol/L. The blood glucose levels were monitored weekly in all diabetic rats. Urine samples were collected for 24 h in metabolic cages at month 1, 3, and 8. Systolic blood pressure was determined by the tail-cuff method monthly. After anesthesia induction with an injection with sodium pentobarbital (50 mg/kg, ip), blood was collected from the aorta, and the kidneys were removed. The kidney samples were rapidly excised, weighed, frozen in liquid nitrogen and stored at -80 °C. Renal hypertrophy was assessed by the kidney to body weight ratio (mg/g) at the time of euthanasia. Some samples were fixed for immunohistochemistry or transmission electron microscopy.

Biochemical analysis and morphological studies

Blood glucose, serum or urinary creatinine, and urinary albumin levels were determined by the Department of Clinical Laboratory at Zhongnan Hospital, Wuhan University. Ccr (mL·min^{-1}·100 g body weight^{-1})=urine creatinine×urine volume (mL·min^{-1})/serum creatinine per 100 g body weight. For morphological studies, the excised kidneys were fixed in 10% formalin, dehydrated through a graded series of ethanol, embedded in paraffin, cut into 4 µm thick slices and mounted on glass slides. Some sections were stained with hematoxylin and eosin (H&E) to observe the tissue morphology. Some fixed sections were stained with periodic acid-Schiff (PAS) reagent. Briefly, the deparaffinized sections were oxidized in 0.5% periodic acid solution for 5 min and stained in Schiff reagent for 15 min. After washing in lukewarm tap water for 5 min, the sections were dehydrated through a graded series of ethanol. Finally, the cover slips were placed on the glass slides with a mounting medium (neutral balsam), and the slides were observed under a light microscope.

Glomerulosclerosis score

In total, 50 full-sized glomeruli were chosen randomly from 2–3 slides (PAS-stained sections) and were assessed at 400× magnification. The glomerular sclerosis was scored by an experimenter that was blind to the treatment groups) as follows: 0=no sclerosis, 1=25% glomerular area involved, 2=50%, 3=75%, 4=100%[21]. The sections were scored by visual inspection. A score was assigned for each glomerulus, and these values were averaged to obtain a final score for each group.

Immunohistochemistry

Formalin-fixed, paraffin-embedded renal cortical sections (4 µm) were routinely deparaffinized and rehydrated in distilled water. First, the deparaffinized sections were subjected to heat-induced antigen retrieval by incubation in citrate buffer solution (pH 6) for 20 min at 95 °C, cooled to room temperature, and washed with PBS. These sections were then treated with 3% H_2O_2 for 10 min to block endogenous peroxidase activity. After rinsing, the sections were blocked with 3% BSA in PBS and were incubated with monoclonal anti-rat VEGFR1 (1:100, Santa Cruz Biotechnology, Dallas, Texas, USA) or monoclonal anti-rat VEGFR2 (1:100, Santa Cruz Biotechnology) at 4 °C overnight. Thereafter, the sections were incubated within secondary antibody for 30 min. After washing with PBS, the sections were stained with a 3,3'-diaminobenzidine solution (DAB). Counterstaining for the nucleus was performed with hematoxylin. A negative control was performed with the absence of primary antibody. Positive staining (dark brown) was quantified by two investigators in a blinded manner under high-power magnification (×400) using the image analysis software Image-Pro Plus 7.0. The data are expressed as the average intensity of the threshold area.

Transmission electron microscopy

A small piece of kidney cortex was harvested for transmission electron microscopy (TEM). The tissue was fixed with 2.5% glutaraldehyde, post-fixed with 1% osmium tetroxide, and stained *en bloc* with 2% aqueous uranyl acetate. The samples were then processed by an EM facility at Wuhan University and viewed with an HT7700 transmission electron microscope (Hitachi, Tokyo, Japan) at 40–80 kV. The basement membrane thickness was assessed on the peripheral loops, which were photographed randomly at 10 000×magnification. The harmonic mean of measurements at 80–100 points crossing a grid from 1–2 glomeruli was calculated.

Protein extraction and Western blot

Kidney cortices or glomeruli were homogenized in lysis buffer, and the protein was extracted as published previously[22]. Briefly, the cells were lysed in buffer containing 50 mmol/L Tris pH 7.4, 150 mmol/L NaCl, 1% Triton X-100, 10% glycerol, 5 mmol/L EDTA, 100 µmol/L sodium vanadate, 1 mmol/L β-glycerophosphate, 1 mmol/L sodium fluoride, 2 µg/mL leupeptin, 10 µg/mL aprotinin, and 1 mmol/L PMSF. The lysates were collected and centrifuged at 10 000×g for 10 min at 4 °C to pellet the cell debris. The supernatant (50 µg) was separated on an SDS-PAGE, and Western blotting was performed as described previously[22]. The primary antibodies included monoclonal VEGFR1 (1:500, Santa Cruz Biotechnology), monoclonal VEGFR2 (1:500, Santa Cruz Biotechnology), monoclonal fibronectin (1:5000, BD Biosciences, San Jose, California, USA), monoclonal phospho-MYPT Thr853 (1:1000, Cell Signaling Technology, Danvers, MA, USA), monoclonal phospho-caveolin-1 Y14 (1:1000, BD Biosciences), polyclonal phospho-Src Y416 (1:1000, Cell Signaling Technology) and monoclonal β-actin (1:5000, Sigma).

Immunoprecipitation

The kidney cortices were lysed with lysis buffer with 60 mmol/L *N*-octyl-glucopyranoside as published previously[22]. After clarification, equal amounts of lysate were incubated overnight with 2 µg of primary antibody rotating at 4 °C followed by 25 µL of protein G-agarose slurry for 1.5 h at 4 °C. The immunoprecipitates were extensively washed, resuspended in 2×sample buffer, boiled, and analyzed by immunoblotting.

Quantitative real-time PCR

Quantitative polymerase chain reaction (qPCR) was performed with RNA extracted from the kidney cortices. Total RNA was isolated using TRIzol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, California, USA). cDNA was reversely transcribed using the Reverse Transcription kit (Promega, Madison, USA). Quantitative PCR was performed in duplicate using the MiniOpticon (Bio-Rad, Hercules, California, USA). Negative cDNA controls were included for each gene set in all PCR reactions to detect contamination. The thermo-cycle program was set as 10 min at 94 °C, followed by 30 cycles of at 94 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min.

The expression level for each gene was calculated using the ΔCt method relative to β-actin.

Statistical analysis

The data are presented as the mean±SEM. Statistical analysis was performed with a one-way ANOVA followed by Tukey's Honestly Significant Difference (HSD) tests for *post hoc* analysis (SPSS 17.0 for Windows). $P<0.05$ was considered significant. The experiments were repeated multiple times, and the number of repetitions is presented in the figure legends (*n*).

Results

Effects of fasudil on metabolic parameters and blood pressure

The metabolic parameters for all animal groups are summarized in Table 1. Diabetic rats had higher plasma glucose concentrations, increased renal hypertrophy (kidney/body weight ratios) and decreased body weights. Administration of fasudil and enalapril did not affect plasma glucose levels, body weights or renal hypertrophy in diabetic rats. Fasudil and enalapril did not affect blood pressure in diabetic rats. Serum creatinine (Table 2) and creatinine clearance (Table 3) were significantly elevated in diabetic rats at month 1 compared to controls. Administration of low-dose fasudil (10 mg/kg) and enalapril had no effect. Middle-dose (30 mg/kg) and high-dose fasudil (100 mg/kg) improved serum creatinine and creatinine clearance in diabetic rats. No difference in serum creatinine and creatinine clearance was observed at month 3 or 8 between groups. Increased albumin excretion, a hallmark of early diabetic nephropathy, was observed in the diabetic group and was prevented by treatment with middle-dose or high-dose fasudil (Table 4).

Effects of fasudil on renal morphology and ECM protein

The glomerular accumulation of PAS-positive matrix was prominent in STZ-induced diabetic rats compared to controls (Figure 1A). Increased glomerular sclerosis was observed in the diabetic group, and sclerosis was prevented in the middle-dose and high-dose fasudil groups but not in the low-dose fasudil group (Figure 1B). Glomerular basement membrane (GBM) thickness increased in diabetic rats (Figure 1C, 1D). In control animals, the glomerulus podocyte foot processes are arranged regularly and perpendicular to the basement membrane. In the diabetic group, the podocyte foot processes appeared fused together or effaced (arrows) along the surface of GBM (Figure 1D). Treatment with middle- or high-dose fasudil reduced GBM thickening and podocyte foot process effacement.

We assessed renal cortical ROCK activation in each group by Western blotting for phospho-MYPT Thr853. Increased ROCK activation in diabetic renal cortex is prevented by middle- and high-dose fasudil treatment. Although there was a trend towards decreased ROCK activation with enalapril, this was not significant (Figure 2A). In addition, fibronectin upregulation in diabetic renal cortex was prevented by middle- and high-dose fasudil treatment, and the effectiveness was similar to enalapril (Figure 2B). These results confirm that renal injury

Table 1. Clinical characteristics of control rats and STZ-induced diabetic rats untreated or treated with the ROCK inhibitor fasudil at month 8. Data are mean±SEM. [b]$P<0.05$ *vs* control.

	Control	STZ	STZ+F10	STZ+F30	STZ+F100	STZ+enalapril
n	10	11	10	10	8	9
Glucose (mmol/L)	3.66±0.42	29.11±2.81[b]	22.82±2.31[b]	31.11±3.19[b]	33.26±3.99[b]	27.57±2.32[b]
Weight (g)	416.5±17.7	227.9±22.4[b]	225.0±25.4[b]	244.1±28.1[b]	255.7±18.7[b]	236.2±22.8[b]
Kidney/body weight (mg/g)	2.50±0.05	5.74±0.28[b]	4.95±0.23[b]	5.66±0.25[b]	6.03±0.27[b]	4.63±0.22[b]
Systolic blood pressure (mmHg)	124.0±1.2	117.0±4.2	130.5±2.5	102±3.9	112.0±1.7	112.0±1.6

F10, F30, F100: SD rats treated with 10, 30, and 100 mg/kg fasudil dissolved in drinking water.

Table 2. Serum creatinine level (μmol/L) at month 1, 3, and 8. Data are mean±SEM. [b]$P<0.05$ *vs* control.

Time	Control	STZ	STZ+F10	STZ+F30	STZ+F100	STZ+enalapril
Month 1	50.8±6.84	120.9±42.53[b]	77.3±24.34[b]	63.2±5.29	61.2±6.67	79.4±13.03[b]
Month 3	53.3±5.32	57.0±11.07	52.7±3.22	50.1±7.25	48.5±7.72	46.1±4.66
Month 8	46.6±3.70	40.3±7.91	41.0±5.62	37.4±4.63	37.7±0.94	42.5±7.41

Table 3. The 24-h creatinine clearance (C_{cr}, mL·min^{-1}·100 g BW^{-1}) at month 1, 3, and 8. Data are mean±SEM. [b]$P<0.05$ *vs* control.

Time	Control	STZ	STZ+F10	STZ+F30	STZ+F100	STZ+enalapril
Month 1	4.43±0.52	8.29±1.40[b]	8.84±1.73[b]	4.61±0.55	4.22±0.47	7.55±0.81[b]
Month 3	3.56±0.67	4.83±0.43	4.54±0.66	3.96±0.58	4.35±0.53	4.47±0.62
Month 8	4.11±0.45	3.31±0.63	3.84±0.71	3.87±0.46	4.06±0.62	4.71±0.86

Table 4. Albuminuria (mg/24 h) at month 1, 3, and 8. Data are mean±SEM. [b]$P<0.05$ *vs* control.

Time	Control	STZ	STZ+F10	STZ+F30	STZ+F100	STZ+enalapril
Month 1	10.09±0.24	12.43±0.32	12.19±0.71	11.32±0.57	11.55±0.63	11.29±0.51
Month 3	10.35±0.41	26.31±0.73[b]	25.78±0.64[b]	11.38±0.44	11.40±0.72	11.65±0.47
Month 8	10.16±0.56	28.26±0.85[b]	27.06±0.93[b]	11.62±0.31	11.06±0.35	11.58±0.45

is characterized by glomerulosclerosis and ECM accumulation in STZ-induced diabetic rats (as expected). ROCK suppression with more than 30 mg/kg fasudil could ameliorate renal injury in diabetic rats.

Effects of fasudil on VEGF upregulation and its receptors in diabetic kidneys

VEGF and its receptors play a central role in the pathogenesis of diabetic nephropathy. However, whether Rho inhibitors prevent the upregulation of VEGF and VEGF receptors has not been addressed. We first determined the renal cortical expression of VEGF. The VEGF mRNA and protein levels were increased in diabetic rats compared to controls. These levels were restored by middle- and high-dose fasudil (Figure 3A, 3B). The VEGF receptor protein expression was determined in the renal cortex. Immunohistochemistry for VEGFR1 and VEGFR2 in cortical sections demonstrated strong VEGFR1 and VEGFR2 staining both in the glomeruli and tubular cells in diabetic rats compared to controls (Figure 4A). Middle- and high-dose fasudil prevented VEGFR1 and VEGFR2 upregulation, and enalapril did not affect VEGFR1 and VEGFR2 staining in the renal cortices of diabetic rats. We then determined the VEGFR1 and VEGFR2 protein levels by Western blotting (Figure 4B). Consistent with the immunohistochemistry results, increased VEGFR1 and VEGFR2 protein levels in diabetic renal cortices were inhibited by middle- and high-dose fasudil.

Effects of fasudil on the association of VEGF receptors and caveolin-1 in diabetic kidneys

Our previous data demonstrated that the association between caveolin-1 and VEGFR2 was involved in the VEGF-induced
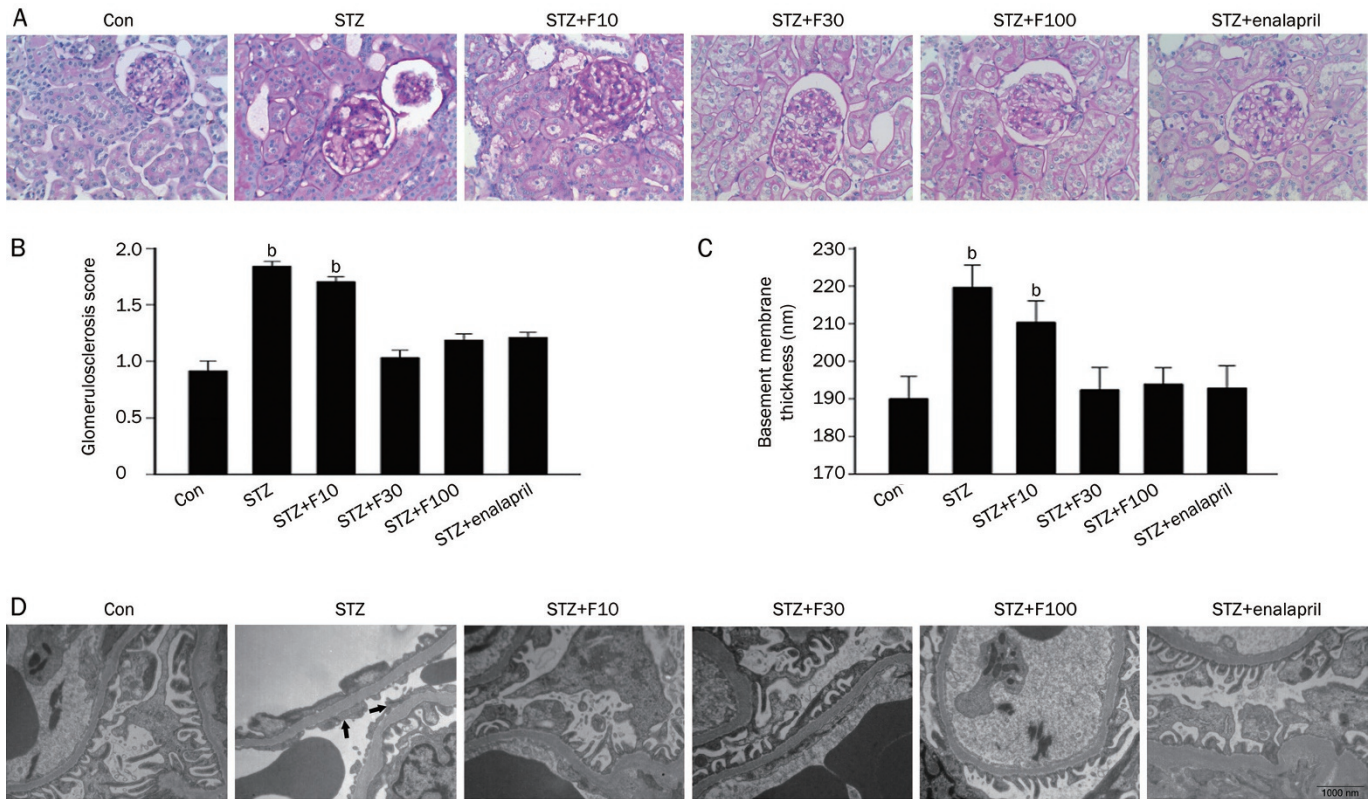
Figure 1. ROCK inhibitor fasudil improves glomerular injury of diabetic nephropathy *in vivo*. (A) Glomerular histopathology analysis by Periodic Acid-schiff (PAS) staining. Representative microphotographs of glomeruli from each group are shown at an original magnification of 400×. (B) Glomerular matrix accumulation was assessed and scores were assigned after blinding to treatment group according to the grading scheme outlined in Materials and methods ($^bP<0.05$ vs control). (C) Glomerular basement membrane (GBM) thickness was observed by transmission electron microscopy (TEM), with data summarized in the bar graph ($^bP<0.05$ vs control). (D) Representative microphotographs of GBM are shown at magnification 10 000×. Black arrows denote focal areas of foot process effacement. F10, F30, and F100: SD rats treated with 10, 30, and 100 mg/kg fasudil daily.

fibronectin upregulation within mesangial cells. This association was also increased in the renal cortices of diabetic rats, although both VEGFR1 and VEGFR2 receptor protein levels were increased in diabetic rats[23]. Here, we assessed the effects of fasudil on the association between VEGFR2 and caveolin-1 by immunoprecipitation of caveolin-1 from the renal cortices. The increased association between VEGFR2 and caveolin-1 in diabetic rats was prevented by middle- and high-dose fasudil but not by enalapril (Figure 5). These data indicate that the VEGFR2/caveolin-1 signaling pathway is likely blocked by ROCK inhibition during diabetic nephropathy.

Effects of fasudil on the phosphorylation of Src and caveolin-1 in diabetic glomeruli

Src-family kinases are the only known caveolin-1 Y14 kinases[24, 25], and our previous studies demonstrated that TGF-β- and VEGF-induced Src activation mediated caveolin-1 phosphorylation at Y14 followed by fibronectin synthesis in mesangial cells[23, 26]. Because fasudil prevented VEGF upregulation in diabetic kidneys, we investigated the effect of fasudil on Src and caveolin-1 phosphorylation levels. We examined the auto-phosphorylation of Y416 on Src, which is indicative

of increased Src activity, and caveolin-1 phosphorylation on Y14 in glomeruli by immunoblotting. Phosphorylated Src and caveolin-1 were significantly increased in glomerular isolates compared to controls. Treatment with middle- and high-dose fasudil partially blocked Src and caveolin-1 phosphorylation; enalapril had no effect (Figure 6A, 6B). These results suggest that the beneficial effect of fasudil is probably mediated through reversing the enhanced Src kinase activity and downstream caveolin-1 phosphorylation in diabetic rats.

Discussion

Diabetic nephropathy is the leading cause of end-stage renal disease, which is characterized by GBM thickening, mesangial expansion and glomerular hypertrophy in its early stages followed by ECM accumulation, glomerulosclerosis, and tubulointerstitial fibrosis. Recent studies have demonstrated that treatment with fasudil, a ROCK inhibitor, prevents the development of experimental diabetic nephropathy by inhibiting the signaling pathways involving angiotensin II, TGF-β, and ECM accumulation[9, 10, 27]. However, whether fasudil blocks VEGF signaling has not been addressed yet. We identified a previously unrecognized renoprotective mechanism for the
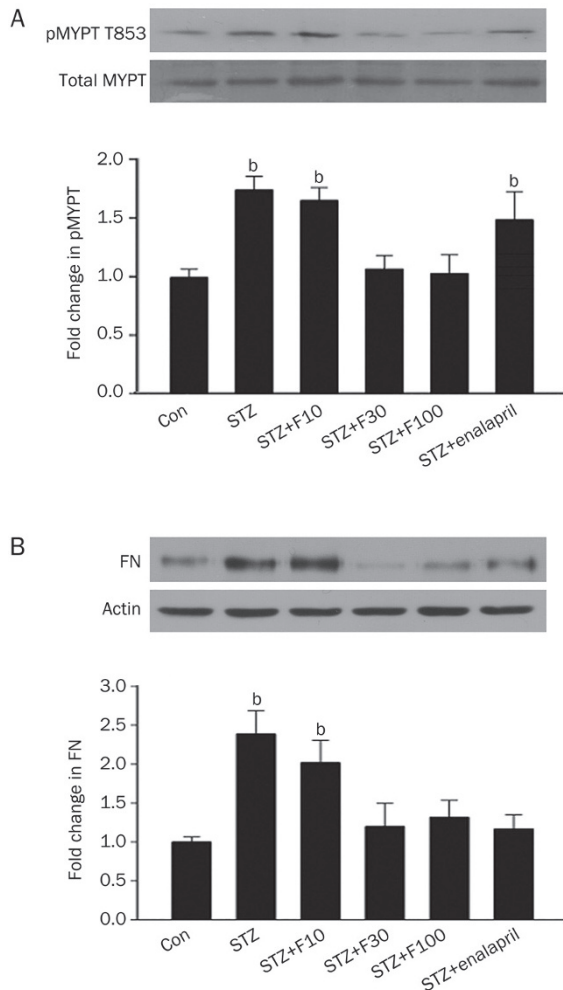
Figure 2. ROCK inhibitor fasudil prevents MYPT phosphorylation and fibronectin upregulation in diabetic kidneys. (A) MYPT phosphorylation (Thr 853) was determined by Western blotting as an indicator of renal cortical ROCK activity ([b]P<0.05 *vs* control). (B) The protein level of fibronectin (FN) was analyzed by Western blotting in renal cortical homogenates, with actin used as loading control ([b]P<0.05 *vs* control). Representative blots are shown in the upper panel for both A and B, with the graph below summarizing data from all animals.

Figure 3. Gene and protein expression of VEGF is reduced by ROCK inhibitor fasudil in diabetic kidneys. (A) VEGF gene expression was detected in renal cortical samples by real-time PCR. (B) VEGF protein level in renal cortex was assessed by Western blotting, with actin used as loading control. Data are shown as fold induction compared with control ([b]P<0.05 *vs* control).

effects of fasudil by preventing the upregulation of VEGF and its receptors, VEGFR2/caveolin-1 association and enhanced fibronectin synthesis during STZ-induced diabetic nephropathy.

The diabetic rats developed kidney hypertrophy, mild albuminuria, and progressive glomerulosclerosis. Chronic treatment with middle-dose (30 mg/kg) and high-dose (100 mg/kg) fasudil ameliorated the kidney injuries with no effect on blood pressure and glucose, which is consistent with previous studies[10, 12]. Although there was a trend towards decreased blood pressure in the middle-dose fasudil group, this trend was not significant. Komers *et al* reported that acute administration of fasudil decreased blood pressure in both control and diabetic rats[28]. However, hypertension is the most common adverse event associated with VEGFR-tyrosine
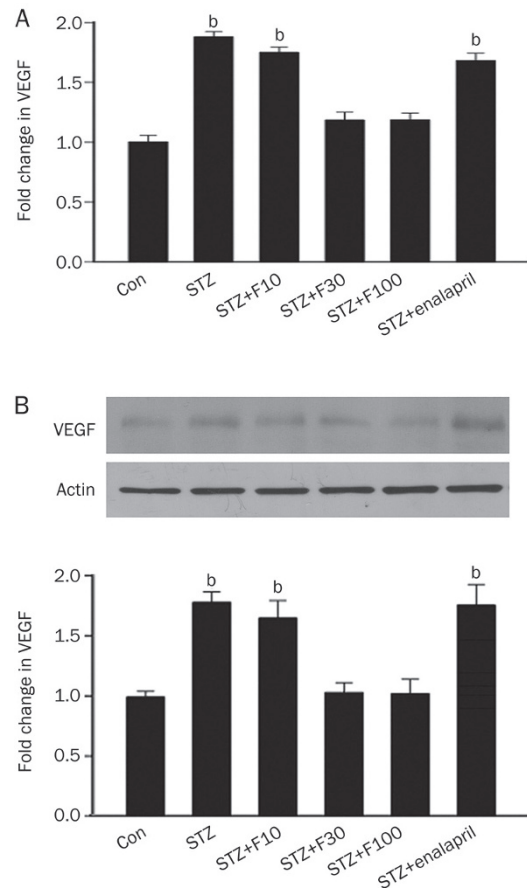
kinase inhibitors, and ROCK inhibition blunts sunitinib(VEGF antagonist) -induced hypertension[29]. Further studies are required to determine whether fasudil has specific role in the control of blood pressure. Low-dose (10 mg/kg) fasudil had no effect in this study, which is different from previous studies[4, 27], and may be related to the differences in animal models, treatment periods and protocols.

In the present study, we evaluated the effects of fasudil on serum creatinine and creatinine clearance throughout the course of the disease and extended previous results that have not been explored before. At month 1, diabetic rats demonstrated increased serum creatinine and creatinine clearance, which was improved by middle- and high-dose fasudil but not by enalapril. Our data indicated that fasudil may improve the enhanced GFR during the very early stage of nephropathy. No difference in serum creatinine or creatinine clearance was observed between groups at month 3 or 8, which agrees with previous studies[5, 27].

VEGF and its receptors are widely expressed in different cell types in the kidney[30, 31]. VEGF plays a central role in the
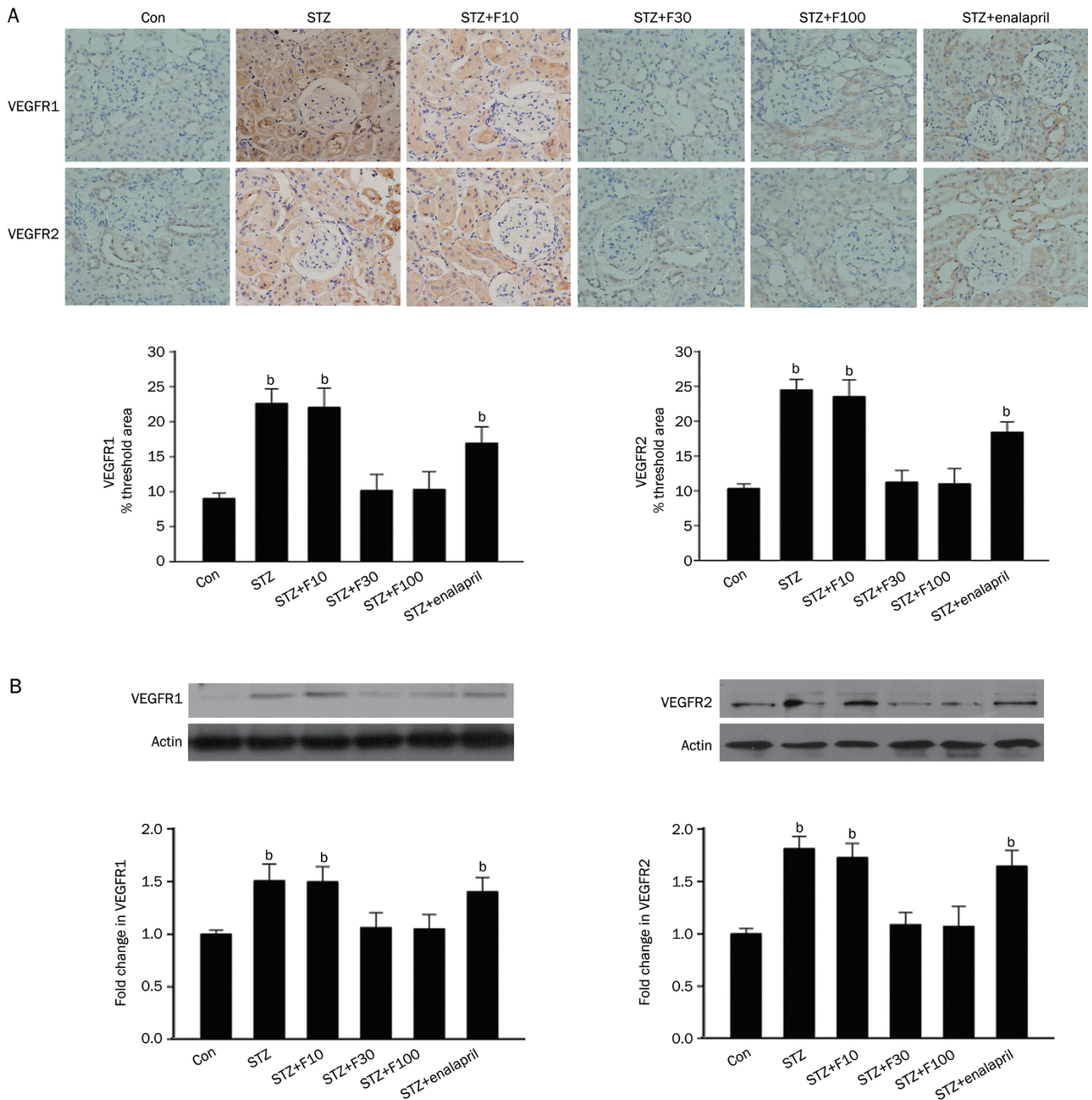
Figure 4. ROCK inhibitor fasudil prevents upregulation of VEGFR1 and VEGFR2 in diabetic kidneys. (A) Representative microphotographs of VEGFR1 and VEGFR2 immunostaining (400×) are shown. Computer-aided analysis of renal cortical VEGFR1 and VEGFR2 expressed as percent area ([b]$P<0.05$ *vs* control). (B) VEGFR1 and VEGFR2 protein levels in renal cortex were assessed by Western blotting, with actin used as loading control ([b]$P<0.05$ *vs* control).

pathogenesis of diabetic nephropathy through its modulatory effects on glomerular hypertrophy, proteinuria and ECM production[32–34]. However, little is known regarding the effects of fasudil on VEGF regulation. The present study demonstrated that fasudil inhibited VEGF upregulation in diabetic rat renal cortex. Similarly Peng *et al*[10] demonstrated that

the Rho inhibitor Y-27632 prevented high glucose-induced upregulation of VEGF transcription in mesangial cells. These results suggest that VEGF upregulation is probably dependent on RhoA/ROCK signaling. Given these observations, we also assessed the effects of fasudil on the protein levels of two VEGF receptors, VEGFR1 and VEGFR2, by immunohis-
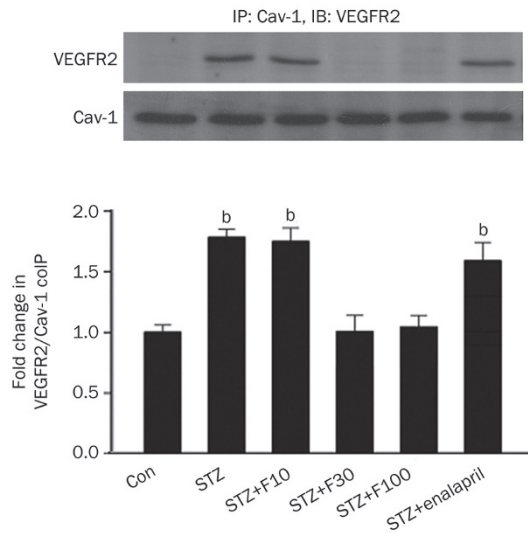
IP: Cav-1, IB: VEGFR2

VEGFR2

Cav-1

Figure 5. The association between VEGFR2 with caveolin-1 is inhibited by fasudil in diabetic kidneys. Caveolin-1 was immunopricipitated from renal cortical homogenates and its association with VEGFR2 was assessed by Western blotting. $^{b}P<0.05$ *vs* control..

tochemistry and Western blotting in diabetic rat renal cortex. Increased VEGFR1 and VEGFR2 protein levels in the diabetic group were inhibited by middle- and high-dose fasudil supporting a potential role of fasudil in preventing VEGF signaling.

Although VEGF signaling is activated during diabetic nephropathy, little is known regarding the function of different VEGF receptors. VEGF$_{165}$, the most biologically active VEGF isoform, predominantly signals through VEGFR2, although VEGF$_{165}$ can also be "retained" by VEGFR1, which has limited signaling potential[35]. VEGFR2 has been localized in caveolae in several cell lines, and caveolae have been implicated in VEGF-induced ERK2/1 signaling and permeability in vascular endothelial cells[36, 37]. Caveolae are specialized plasma membrane microdomains morphologically defined as 50- to 100-nm omega-shaped invaginations according to electron microscopy observations[38, 39]. Caveolin-1 functions as a scaffolding protein by directly interacting with various signaling molecules and integrates specific transmembrane signaling pathways in the caveolae, including VEGF signaling[40, 41]. Our previous studies demonstrated that the VEGFR2/caveolin-1 association plays an important role in ECM accumulation in mesangial cells[23]. Recent studies have demonstrated that selective stimulation of VEGFR2 accelerates progressive renal disease[42]. Thus, we investigated the effects of fasudil on the association between VEGFR2 and caveolin-1 within the diabetic renal cortex. Increased association between VEGFR2 and caveolin-1 in the diabetic groups was prevented by middle- and high-dose fasudil indicating that the VEGFR2/caveolin-1 signaling pathway is probably prevented by ROCK inhibition in diabetic nephropathy.

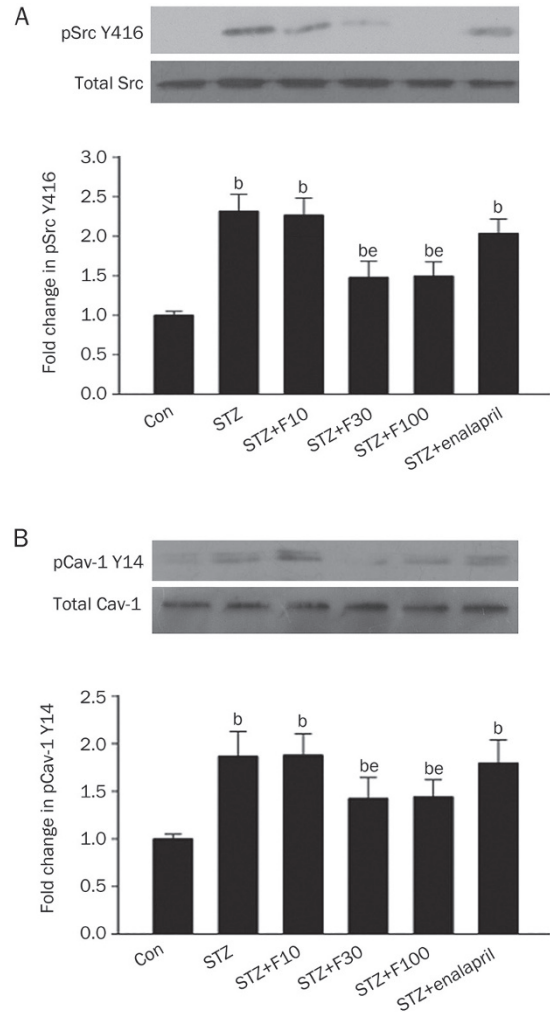We have previously demonstrated that the VEGFR2/caveo-

A

pSrc Y416

Total Src

B

pCav-1 Y14

Total Cav-1

Figure 6. Src activation and caveolin-1 phosphorylation on Y14 in diabetic glomeruli is partly blocked by ROCK inhibitor fasudil. (A) Y416-phosphorylated Src (pSrc Y416) in glomerular isolates was assessed by Western blotting. (B) Y14-phosphorylated caveolin-1 (pCav-1 Y14) in glomerular isolates was assessed by Western blotting. $^{b}P<0.05$ *vs* control. $^{e}P<0.05$ *vs* diabetic group.

lin-1 association is mediated by the caveolin-1/Src association, Src activation and further modification of caveolin-1 phosphorylation on tyrosine 14 via Src kinase during VEGF-induced matrix upregulation in mesangial cells[23]. Src activation also occurs in the kidneys of mice with STZ-induced type 1 diabetes. The Src inhibitor PP2 prevents albuminuria, glomerular matrix protein accumulation, GBM thickening, and podocyte depletion[43]. Therefore, this study investigated the effects of fasudil on Src/caveolin-1 signaling. Middle- and high-dose fasudil partially inhibited Src activation and caveolin-1 phosphorylation at Y14 suggesting that the renoprotective effect of fasudil is at least in partially mediated through blocking Src kinase activation and caveolin-1 phosphorylation in diabetic rats. Although the ACE inhibitor enalapril is effective in the prevention of early diabetic nephropathy[44], it cannot block VEGFR2/Src/caveolin-1 signaling.

In conclusion, the results of the present study provide evidence regarding the renoprotective mechanisms of the ROCK inhibitor fasudil concerning VEGF/Src/caveolin-1 signaling, which could be a potential therapeutic target for the treatment of diabetic nephropathy.

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Author contribution
Jing JIN and Chao PENG performed the experiments; Su-zhen WU and Hong-min CHEN collected and analyzed the data; Bai-fang ZHANG designed the experiments and prepared the manuscript.

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