Fasudil Inhibits Epithelial-Myofibroblast Transdifferentiation of Human Renal Tubular Epithelial HK-2 Cells Induced by High Glucose

Lingjia Gu, a Qian Gao, b Liasong Ni, * a Meirong Wang, a and Feixia Shen a

a Department of Endocrinology, The First Affiliated Hospital, Wenzhou Medical College, Wenzhou 325000, China; and b Department of Endocrinology, The Sixth of People’s Hospital; Shaxing 312000, China.

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Renal fibrosis is a crucial pathologic process underlying diabetic nephropathy (DN). Central to this process is the epithelial-mesenchymal transformation (EMT) of tubular epithelial cells. Fasudil, a Rho-associated coiled-coil forming protein serine/threonine kinase (ROCK) inhibitor, protects against renal fibrosis in a variety of renal injury models. However, fasudil’s effects on renal fibrosis in DN remain unknown. The aim of the present study was to investigate the effects of fasudil on high glucose-induced EMT in human renal tubular epithelial (HK-2) cells. HK-2 cells were exposed to 5.5 or 60 mmol/L glucose for 72h, or to mannitol (osmotic control). RhoA activity was assessed using a RhoA pull-down assay, and ROCK activity was determined by myosin phosphatase target subunit-1 (MYPT1) phosphorylation. Myofibroblast (vimentin and α-smooth muscle actin [α-SMA]) and epithelial (E-cadherin) markers expressions were detected by immunocytochemistry and Western blotting. Transforming growth factor (TGF)-β1 and fibroblastin secretion were determined by enzyme-linked immunosorbent assay (ELISA), and connective tissue growth factor (CTGF) was analyzed by Western blotting. Results showed that high glucose levels induced morphological changes, reduced E-cadherin expression (−73%), increased expression of vimentin (+148%) and α-SMA (+226%), increased TGF-β1 (from 116.0±5.2 µg/g to 351.0±3.2 µg/g) and CTGF (from 0.26±0.01 to 0.92±0.03) secretion, and increased RhoA and ROCK activation (p<0.05 for all). All these effects of high glucose stimulation were suppressed or abolished by fasudil. In conclusion, fasudil may attenuate EMT through reduced activation of RhoA/ROCK signaling, and decreased expression of TGF-β1 and CTGF. Thus, fasudil may be a renoprotective agent for the treatment of DN.

Key words diabetic nephropathy; fasudil; renal tubular epithelial cell; epithelial-myofibroblast transdifferentiation

The incidence of diabetes mellitus (DM) has been increasing worldwide.11 Kidneys are major targets of diabetic complications, and diabetic nephropathy (DN) is a leading cause of end-stage renal disease.2 Hyperglycemia is a primary factor causing DN. Drugs exist to control blood glucose levels and to help to protect renal function, thus delaying the progression of renal failure, but they are unable to fully prevent DN development.4 Consequently, there is a need to identify new therapeutic agents for the prevention and treatment of DN.

The pathogenesis of DN is complex and is not fully understood. One of the potential mechanisms involves hyperglycemia-induced epithelial-myofibroblast transdifferentiation (EMT) of renal tubular epithelial cells, which contributes to renal fibrosis.5 EMT has been identified in biopsies from patients with DN6 and in DM animal models.7,8 In DM, under specific stimulation, renal tubular epithelial cells may undergo a transformation into myofibroblasts, which are the main source of extracellular matrix (ECM) in the kidney.9 This transition is associated with a reduction in the expression of the epithelial marker, E-cadherin, and an increase in the expression of mesenchymal markers, such as vimentin and α-smooth muscle actin (α-SMA).10,11

Transforming growth factor (TGF)-β1 is an important fibrogenic growth factor known to play an important role in EMT development.12,13 Although TGF-β1 is thought to act via a number of cellular signaling pathways, connective tissue growth factor (CTGF) is a downstream target responsible for many of the pro-fibrotic effects of TGF-β1.14 TGF-β1 and CTGF have been found to be up-regulated in both experimental and human DN, and to be associated with EMT;10,11,15–21 supporting a key role for these signaling molecules in DN.

Whether RhoA/Rho-associated coiled-coil forming protein serine/threonine kinase (ROCK) mediates high glucose-induced EMT has not been fully addressed. The small Rho GTPase family comprises 20- to 40-kDa monomeric G proteins that cycle between an active guanosine 5′-triphosphate (GTP)-bound and an inactive guanosine 5′-diphosphate (GDP)-bound state, and play important roles in the regulation of numerous cell functions.22–24 The most recognized members of the Rho GTPase family are Rho A, Rac1 and Cdc42. ROCK was the first identified RhoA effector, and is the best characterized. A recent study suggested that RhoA is activated in the renal cortex of diabetic rats,25 and that high glucose levels activate RhoA in renal mesangial cells.26 Most in vitro studies of high glucose-induced RhoA/ROCK activation in the kidney focused on glomerular mesangial cells.27–29 In recent years, evidences suggested that proteinuria and deterioration of renal function in DM are more closely related to the extent and severity of tubulointerstitial lesions than to glomerulospathy.30

Fasudil is the most commonly used pharmacological ROCK inhibitor, both for in vitro and in vivo studies.27,28 Interestingly, fasudil has been demonstrated to protect against renal fibrosis in a variety of renal injury models, including rats with unilateral ureteral ligation, nephrectomized spontaneously hypertensive rats, and Dahl salt-sensitive rats.31–34 These studies suggest the possibility that RhoA and ROCK may also contribute to the development of EMT that occurs in response to hyperglycemia in DM.

In the present study, we investigated high glucose-activated...
RhoA/ROCK in human tubule epithelial (HK-2) cells. We also studied the influence of fasudil on high glucose-induced EMT in HK-2 cells, and explored the potential mechanisms by which fasudil exerts its effects.

**Experimental**

**Reagents and Antibodies** The following reagents were used: fasudil hydrochloride for injection (lot number: 091114; Hongri Medicine Co., Ltd., Tianjing, China); Dulbecco’s modified Eagle’s medium (DMEM) containing 5.5 mmol/L d-glucose and fetal bovine serum (FBS) (Gibco Invitrogen, Grand Island, NY, U.S.A.); d-glucose (analytical grade; Guanghua Chemical Factory Co., Ltd., Guangdong, China); mannitol (Amresco, Solon, OH, U.S.A.); mouse monoclonal anti-α-SMA antibody, mouse monoclonal anti-E-cadherin antibody, mouse monoclonal anti-vimentin antibody, mouse monoclonal anti-RhoA antibody, anti-CTGF antibody and goat polyclonal anti-phosphorylated-mysosin phosphatase target subunit-1 (MYPT1) (Thr⁶⁹⁶) antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.); anti-β-actin antibody, anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody, lysis buffer, horseradish peroxidase (HRP)-conjugated anti-mouse immunoglobulin G (IgG) and enhanced chemiluminescence (ECL) detection kit (Beyotime Institute of Biotechnology, Jiangsu, China); fluorescein isothiocyanate (FITC)-labeled secondary antibodies (Multi Sciences Biotech Co., Ltd., Zhejiang, China); Rho pull down assay kit (Upstate Biotechnology, Lake Placid, NY, U.S.A.); rabbit monoclonal anti-phosphorylated-MYPT1 (Thr⁸⁵³) antibody (Cell Signaling Technology, Danvers, MA, U.S.A.); TGF-β₁ and fibronectin ELISA (Xitang, Shanghai, China). All chemicals and reagents used were of analytical grade.

**Cell Culture** HK-2 cells were purchased from the China Center for Type Culture Collection (CCTCC, Wuhan, China). Cells were cultured in DMEM containing 5.5 mmol/L d-glucose (normal glucose, NG), supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin and 10% FBS, at 37°C in humidified air containing 5% CO₂; cells were passaged when 80% confluent. In order to induce EMT, cells were cultured for 72 h in high glucose (HG) medium containing 60 mmol/L d-glucose; the culture duration and glucose concentration were chosen based on preliminary experiments and on previously published studies. Cells were synchronized with FBS-free medium (5.5 mmol/L d-glucose) for 24 h once they reached 80% confluence, and then cultured with HG for 0, 30 min, 1, 3, 7 h, 12 h or 24 h; these durations were chosen to observe the early events in RhoA/ROCK and because it has been shown that these effects occur early. HK-2 cells were then lysed in ice-cold lysis buffer. Samples were centrifuged and incubated overnight at 4°C with Rhotekin Rho binding domain-agarose beads to precipitate GTP-RhoA. The precipitates were washed and re-suspended in 1× loading buffer. Total lysates and precipitates were analyzed by Western blot, using a mouse monoclonal anti-RhoA antibody (1:200 dilution).

**ROCK Activity** ROCK activity was determined with a co-immunoprecipitation assay. The antibody against ROCK recognized both isoforms. Briefly, HK-2 cells were plated in 10-cm culture plates and cultured in DMEM containing 5.5 mmol/L d-glucose. Cells were synchronized with FBS-free medium for 24 h once they reached 80% confluence, and were then cultured with HG for 0, 30 min, 1, 3, 7 h, 12 h or 24 h; these durations were chosen to observe the early events in RhoA/ROCK and because it has been shown that these effects occur early. HK-2 cells were then lysed in ice-cold lysis buffer. Samples were centrifuged and incubated overnight at 4°C with anti-phospho-MYPT1 (Thr⁸⁵³) antibody or anti-phospho-MYPT1 (Thr⁶⁹⁶) antibody. Then, protein A+G was added to precipitate the immunocomplex. Precipitates were washed, re-suspended in 1× loading buffer, and analyzed by Western blot using anti-phospho-MYPT1 (Thr⁸⁵³) or anti-phospho-MYPT1 (Thr⁶⁹⁶) antibody. The lystate was also analyzed to ensure equality across conditions. ROCK activity was expressed as the ratio of phospho-MYPT1 to ROCK.

**Measurement of TGF-β₁ and Fibronectin Secretion** HK-2 cells were plated in 6-well culture plates at a density of 50,000 cells/well and cultured in DMEM containing 5.5 mmol/L d-glucose. Cells were synchronized with FBS-free medium (5.5 mmol/L d-glucose) for 24 h once they reached 80% confluence, and were then cultured in NG, HG or HM medium for 72 h. The concentrations of TGF-β₁ and fibronectin secreted by cultured cells into the culture medium were determined by ELISA. The final absorbance was measured at 450 nm using a TECAN Infinite M200 microplate reader (Thermo Fisher Scientific, Waltham, MA, U.S.A.). Concentrations of TGF-β₁ and fibronectin were extrapolated from a standard curve constructed in the same plate, and expressed relative to the cell protein concentration.

**Western Blotting** HK-2 cells were plated in 10-cm culture plates and cultured in DMEM containing 5.5 mmol/L d-glucose. Cells were synchronized with FBS-free medium (5.5 mmol/L d-glucose) for 24 h once they reached 80% confluence, and were then cultured with NG, HG or HM medium for 72 h. HK-2 cells were subsequently lysed in ice-cold lysis buffer.
buffer. Equal amounts of protein (30 µg) were separated on a 12% polyacrylamide sodium dodecyl sulfate gel, and transferred on a polyvinylidene difluoride membrane (Amersham, Piscataway, NJ, U.S.A.). Non-specific binding was blocked using 5% non-fat dry milk in Tris-buffered saline and 0.1% Tween 20 (TBST), and samples were then incubated overnight at 4°C with one of the following primary antibodies: mouse anti-α-SMA (1 : 200), anti-E-cadherin (1 : 200), anti-vimentin (1 : 200), anti-CTGF (1 : 200), anti-β-actin (1 : 1000) or anti-GAPDH (1 : 1000). After four washes in TBST, membranes were incubated with a secondary antibody (HRP-conjugated anti-mouse IgG; 1 : 2000 dilution) in TBST (pH 7.4). Protein bands were visualized using an ECL detection kit, and semi-quantitated by densitometry using analysis software (ImagLab). β-Actin or GAPDH were used to normalize the data.

Statistical Analysis
Results are expressed as mean ± S.E.M. Statistical analyses were performed using SPSS 17.0 for Windows (SPSS Inc., Chicago, IL, U.S.A.). Statistical significance was determined using one-way ANOVA followed by the Least Significant Difference (LSD) test. A p-value <0.05 (two tailed) was considered statistically significant.

Results
Fasudil Reverses the Morphological Changes Caused by HG-Induced HK-2 Cell Injury
HK-2 cells were serum-deprived for 24 h and then exposed to HG for 9 d, to make the morphological changes more obvious. The culture medium was changed every two days and HM served as the osmotic control. Cell morphology was observed with phase-contrast microscopy. HK-2 cells in NG exhibited a cobblestone-like appearance (Fig. 1A), and a similar morphology was observed for cells exposed to HM (Fig. 1B). In contrast, cells exposed to HG exhibited an elongated, fibroblast-like phenotype (Fig. 1C). Fasudil exposition (20 µmol/L) with HG prevented these morphological changes in most cells (Fig. 1D).

Rho A and ROCK Are Activated by HG in HK-2 Cells
Since increased RhoA activation has been observed in the renal cortex and mesangial cells of diabetic animals exposed to high glucose, we investigated whether high glucose would activate RhoA in HK-2 cells, using a pull-down assay.
Exposure of cells to HG resulted in RhoA activation: after 3 h of exposure to HG, the GTP-RhoA : total RhoA ratio was significantly increased from 1.58±0.03 to 7.57±1.20 (p<0.05), and remained elevated throughout the remainder of the 24 h exposure (Fig. 2A). Downstream ROCK activation was assessed by the amount of phosphorylation at either Thr696 of MYPT1, a specific target of ROCK. Phosphorylation at Thr696 was enhanced after 7 h exposure to high glucose (an increase in the GTP-RhoA : total RhoA ratio from 0.57±0.01 to 1.45±0.14; p<0.05), and Thr696 phosphorylation was enhanced after 3 h (from 1.08±0.09 to 2.40±0.09; p<0.05). Furthermore, these increases were maintained for the remainder of the 24 h experimental period (Fig. 2B). As shown in Fig. 2, after 12 h of exposure to high glucose, Thr853 MYPT1 phosphorylation was significantly increased from 0.48±0.03 to 1.31±0.02 (p<0.01). Treatment with fasudil (20 μmol/L) significantly reduced the increase of phosphorylated MYPT1 by 62%.

Fasudil Reverses HG-Induced EMT in HK-2 Cells

To confirm the transformation of cells into a fibroblast-like phenotype, the expressions of the epithelial marker, E-cadherin, and of the mesenchymal markers, vimentin and α-SMA, were determined by fluorescence immunocytochemistry and Western blotting. In addition, fibronectin, the most important component of the ECM, was evaluated using ELISA. Fluorescence immunocytochemistry (Fig. 4A) demonstrated that cells exposed to HG for 72 h had decreased E-cadherin expression and increased vimentin and α-SMA expressions, which were not observed in NG and HM cells. Fasudil reversed these changes induced by HG. Western blotting demonstrated that compared with the NG control, HG down-regulated the expression of E-cadherin protein by 73%, and increased the expressions of vimentin and α-SMA proteins by 148% and 226%, respectively (Figs. 4B–D). Furthermore, fasudil was able to attenuate these changes in a dose-dependent manner. ELISA showed that expression of fibronectin was enhanced significantly in HK-2 cells exposed to HG (from 449.0±23.0 to 681.0±13.7 μg/mL, p<0.05), and that co-exposure to HG and fasudil (20 μmol/L) abolished this increase in fibronectin expression (419.0±14.9 μg/mL, Fig. 4E).

Fasudil Suppresses the Secretion of TGF-β1 and CTGF

Expression of TGF-β1 and CTGF were measured using ELISA and Western blotting, respectively. Levels of TGF-β1 and CTGF secreted into the culture medium by HK-2 cells were significantly increased by exposure to HG (Fig. 4A, B), compared with NG (TGF-β1: 351.0±3.2 μg/g vs. 116.0±5.2 μg/g; CTGF: 0.92±0.03 vs. 0.26±0.01; p<0.05). In contrast, HM was without effect on TGF-β1 and CTGF expression. Fasudil suppressed the increase in TGF-β1 and CTGF expression by HG, in a dose-dependent manner (Figs. 5A, B).

Discussion

EMT is believed to play an important role in the pathogenesis of DN. Over-accumulation of ECM in the tubulointerstitium is known to lead to tubulointerstitial fibrosis, and this is thought to be an important contributor to DN pathogenesis. There are emerging evidences that a large proportion of the myofibroblasts that secrete ECM are derived from EMT of tubular epithelial cells. Consistent with this, we observed that HK-2 cells that had undergone EMT lost epithelial features and gained fibroblastic features.

Four key steps are involved in EMT: loss of epithelial cell adhesion, de novo α-SMA expression and actin reorganization, disruption of the tubular basement membrane, and enhanced cell migration. In our study, we found that exposure to HG resulted in a change in HK-2 cell morphology to a fibroblast-like phenotype, with reduced expression of the epithelial marker, E-cadherin, and enhanced expression of mesenchymal markers, vimentin and α-SMA. We also observed that fibronectin, the most important component of ECM, was increased in HK-2 cells exposed to HG. Taken together, these data suggest that high glucose was able to induce EMT in HK-2 cells, consistent with previous studies.

The molecular mechanisms underlying EMT remain incompletely understood. Multiple intracellular signaling pathways are thought to be involved in EMT regulation, including TGF-β1/Smad, Wnt and p38 MAPK. More recently, the roles of the Rho/ROCK signal transduction pathway in EMT and...
renal interstitial fibrosis received increasing attention.\textsuperscript{23,39,40} Masszi \textit{et al.} reported that Rho plays a central role in TGF-β\textsubscript{1}-induced α-SMA expression during EMT,\textsuperscript{39} and Patel \textit{et al.}\textsuperscript{41} determined that RhoGTPase activation is a key step in renal EMT. The Rho/ROCK pathway generated significant interest as a key regulator in a variety of renal injury models, and may also play a critical role in many of the chronic complications of DM.\textsuperscript{42} A previous study showed that the RhoA/ROCK pathway is involved in the up-regulation of TGF-β, CTGF and reduced nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidase in diabetic kidney, and that fasudil could inhibit these factors to prevent DN.\textsuperscript{20,28,29,42–44} RhoA/ROCK pathway is involved in NAD(P)H expression, as well as Angiotensin-2 and the platelet-activated growth factor.\textsuperscript{45} However, it is still unknown how these factors interact to affect TGF-β and CTGF. Also, in STZ-induced diabetic rats, statins were shown to decrease TGF-β, CTGF and NOX4 expressions through Rhoa/ROCK inhibition, and these effects were similar to fasudil.\textsuperscript{20}

A major finding of our study is that RhoA/ROCK activation is increased in HK-2 cells exposed to HG, supporting the hypothesis that the RhoA/ROCK pathway is involved in

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**Fig. 4. Effects of Fasudil on High Glucose-Induced EMT in HK-2 Cells**

(A) E-Cadherin, vimentin and α-SMA expression in HK-2 cells, exposed for 72 h to NG, HM, HG, or HG plus 20 μmol/L fasudil, detected using fluorescence immuno-histochemistry. Propidium iodide staining is shown in red; FITC-labeling proteins are shown in green. Original magnification, ×400. (Color images were converted into gray scale.) (B–D) Western blot analysis of E-cadherin (B), vimentin (C) and α-SMA (D) protein levels in HK-2 cells. FA(5): 60 mmol/L D-glucose+5 μmol/L fasudil; FA(10): 60 mmol/L D-glucose+10 μmol/L fasudil; FA(20): 60 mmol/L D-glucose+20 μmol/L fasudil. \*\*\* \(p<0.01\) compared with NG; \#\#\# \(p<0.01\) compared with HG. (E) ELISA of fibronectin in HK-2 cells exposed to NG, HM, HG or HG plus FA(20). \* \(p<0.05\), \*\* \(p<0.01\) compared with NG; \#\# \(p<0.01\) compared with HG.
diabetic rats, and diabetic rats with unilateral nephrectomy have been observed, 29) due to the fact that ROCK is the downstream effector of RhoA. Based on these evidences, inhibition of ROCK, suppressed the up-regulation of TGF-β1 and CTGF and the activation of the Rho/ROCK pathway. Therefore, fasudil’s renal protective actions may be due, in part, to a reduced expression of TGF-β1 and CTGF.

CTGF seems to be induced by high mannitol, but this increase was not significant. Previous studies examined the effect of high glucose levels in human vascular smooth muscle cells and in human mesangial cells. They used mannitol 25 mmol/L to confirm that the effect they observed on CTGF was not due to the osmotic pressure. 48,49) In our study, we used a mannitol concentration of 54 mmol/L and we cannot exclude that this non-statistically significant increase is due to the osmotic pressure. However, high mannitol had comparable effects to NG on the other studied parameters. Fasudil only partially inhibited the effects of high glucose. We cannot exclude the fact that other unknown or ill known downstream effectors may exist and be responsible for this partial effect of fasudil. However, we showed that fasudil had a dose-dependent effect, and it is thus possible that the dose at which fasudil completely inhibits the effects of high glucose is higher than the highest dose used in the present study.

Fig. 5. Effects of Fasudil on High Glucose-Induced TGF-β1 and CTGF Expression in HK-2 Cells
(A) Expression of TGF-β1 in the culture media (ELISA assay) detected in HK-2 cells exposed to NG, HM, HG or HG plus FA(20). **p<0.01 compared with NG; ††p<0.01 compared with HG. (B) Expression of CTGF (Western blotting) in HK-2 cells exposed to NG, HM, HG, or HG plus FA(5), FA(10) or FA(20). *p<0.05, **p<0.01 compared with NG; ††p<0.01 compared with HG.

hyperglycemia-induced EMT. We also observed a time lag between RhoA activation and MYPT-Thr853/696, which has been previously observed, 29) due to the fact that ROCK is the downstream effector of RhoA. Based on these evidences, inhibition of the RhoA/ROCK pathway may be a promising approach for preventing the development and progression of DN.

Five recent studies examined the renal protective effects of fasudil in experimental models of DM, including rats with streptozotocin-induced DM, db/db diabetic mice, OLETF diabetic rats, and diabetic rats with unilateral nephrectomy. 20,28,29,43,46) Our study results provide strong evidences that fasudil is able to prevent high glucose-induced EMT. Compared with the HG group, fasudil was found to increase the expression of E-cadherin and to decrease the expressions of vimentin, α-SMA and fibronectin, suggesting that the protective effect of fasudil against DN may, at least in part, be due to a reduction in ECM over-accumulation. In DN, fibronectin accumulation in the tubulointerstitial space leads to impaired kidney function and CTGF and TGF-β1 are involved in fibrotic processes observed in DN. 14) TGF-β1 and CTGF have been found to be up-regulated in both experimental and human DN, and to be associated with EMT, 7,10,11,15,21) supporting a key role for these signaling molecules. A previous study in diabetic rats showed that using fasudil as a treatment also decreased EMT. 47) In the present study, fasudil was used in a prevention setting, but we might expect similar results if we use it in a treatment setting in HK-2 cells.

To study the mechanisms underlying the protective effects of fasudil in more details, we examined the expression of TGF-β1, and CTGF. TGF-β1 is a key fibrogenic growth factor, and the most important inducer of EMT, 12,13) while CTGF is a downstream target of TGF-β1 that is responsible for many of its pro-fibrotic effects. 10) TGF-β1 and CTGF have been shown to be up-regulated in both experimental and human DN. 20,21) In our study, the expressions of TGF-β1 and CTGF were increased in HK-2 cells exposed to HG, while fasudil partially reduced this enhanced expression. These data suggest a correlation between the hyperglycemia-induced increases in TGF-β1 and CTGF and the activation of the Rho/ROCK pathway. Therefore, fasudil’s renal protective actions may be due, in part, to a reduced expression of TGF-β1 and CTGF.

Conclusion
Our study showed that high glucose can induce EMT in HK-2 cells and activate the RhoA/ROCK pathway, demonstrating that the RhoA/ROCK pathway is involved in high glucose-induced EMT. Fasudil, a pharmacological inhibitor of ROCK, suppressed the up-regulation of TGF-β1 and CTGF and attenuated EMT. Pharmacological inhibition of these pathways may thus be an important future therapeutic strategy for the management of DN.

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