Int J Clin Exp Med 2017;10(9):13224-13234
www.ijcem.com /ISSN:1940-5901/IJCEM0053591

Original Article

Effect of the Rho kinase inhibitor Y-27632 and fasudil on inflammation and fibrosis in human mesangial cells (HMCs) under high glucose via the Rho/ROCK signaling pathway

Fenqin Chen[1], Ning Zhang[3], Dongwei Ma[2], Xiaoyu Ma[1], Ting Huang[1], Qiuyue Wang[2]

Departments of [1]Geriatrics, [2]Endocrinology, The First Hospital, China Medical University, Shenyang 110001, China; [3]Department of Pathophysiology, College of Basic Medicine, China Medical University, Shenyang 110001, China

Received May 3, 2016; Accepted August 5, 2017; Epub September 15, 2017; Published September 30, 2017

Abstract: This study investigated the effect of the Rho kinase inhibitor Y-27632 and fasudil on the development of human mesangial cell (HMC) inflammation and fibrosis induced by high glucose and to clarify the contribution of the Rho/ROCK signaling pathway in the pathogenesis of diabetic kidney disease (DKD). High glucose (30 mmol/l) induced the Rho/ROCK signaling pathway. Western blotting was used to detect active and total RhoA, ROCK-I, tumor necrosis factor-α (TNF-α), connective tissue growth factor (CTGF) activation, and fibronectin (FN) up-regulation as assessed by enzyme-linked immunosorbent assay (ELISA) and real-time polymerase chain reaction (PCR). Lipofectamine™ 2000 was applied to transfect RhoA-small interfering RNA (siRNA), which could inhibit RhoA expression. RhoA, ROCK, FN, CTGF, and TNF-α expression was detected by real-time reverse transcription-PCR (RT-PCR) and ELISA. High glucose up-regulated RhoA and downstream ROCK-I expression ($P<0.05$). RhoA, CTGF, and TNF-α of HMCs cultured under high glucose were expressed significantly more than those of HMCs in the normal glucose group ($P<0.05$); this was dependent on ROCK signaling. FN up-regulation by high glucose, shown to be mediated by CTGF and TNF-α, was prevented by Y-27632 or fasudil ($P<0.05$). RhoA siRNA was also markedly attenuated by ROCK-I, FN, CTGF, and TNF-α up-regulation by high glucose ($P<0.05$). The present study demonstrates that the Rho/ROCK signaling pathway is involved in the up-regulation of TNF-α, CTGF, and FN in DKD. The ROCK inhibitors Y-27632 and fasudil, in addition to RhoA siRNA, could effectively reverse the high glucose-induced expression of ROCK-I, FN, CTGF, and TNF-α and reduce glomerular fibrosis and inflammation. We conclude that suppression of the Rho/ROCK signaling pathway could provide a new intervention target for preventing and treating DKD.

Keywords: Rho/ROCK signaling pathway, inflammatory response, fibrosis, diabetic kidney disease

Introduction

Diabetic kidney disease (DKD) is one of the important microvascular complications of diabetes mellitus (DM), the main cause of end-stage renal disease (ESRD), and one of the leading causes of death for patients with DM. Its main pathological features include glomerular basement membrane thickening and mesangial matrix expansion. Early lesions may be compensated for by hyperplasia of the glomeruli. However, with disease progression, lesions increase, leading to decompensated hemodynamics and a decreased glomerular filtration rate. Renal function will ultimately be affected and subsequent hyperglycemia is the major factor that causes DKD [1]. The pathogenesis of DKD is complex and currently considered to be the result of many factors. In recent years, the signaling pathway in the pathogenesis of diabetic microvascular complications has aroused extensive attention [2]. Research has found that the Rho/ROCK signaling pathway is related to vascular and myocardial reorganization and renal fibrosis [3]. Hyperglycemia can stimulate the activation of the Rho/ROCK signaling pathway in renal cells. The Rho/ROCK signaling pathway could raise gene expression through the regulation of DNA transcription factor activity, including extracellular matrix (ECM),

types I and III collagen, fibronectin (FN), and laminin (LN). This may cause release of inflammatory factors, such as tumor necrosis factor-α (TNF-α) and increased concentrations of connective tissue growth factor (CTGF) [4].

In the present study, the Rho/ROCK signaling pathway was induced in cultured human mesangial cells (HMCs) by culture in a high glucose concentration. A variety of inflammatory factors and changes in cytokine secretion induced fibrosis, which was inhibited in the treatment group by adding an exogenous agent that blocked the signaling pathway. Changes in relevant indicators were measured to study the possible molecular mechanisms aimed at the cellular and gene levels. The relationship between Rho/ROCK signaling pathway activation and the pathogenesis of DKD further clarifies that suppression of the Rho/ROCK signaling pathway is able to delay the occurrence and development of DKD.

Materials and methods

Materials

HMC 4200, MCM 4201 medium, and fetal bovine serum (Sciencell, USA), improved type RPMI 1640 medium, and 0.25% trypsin were purchased from Hyclone (USA). Trizol was purchased from Invitrogen (USA). Fasudil (Rho kinase inhibitor) was obtained from Asahi Kasei Pharmaceutical Co. (Tokyo, Japan). RhoA antibodies were purchased from Cell Signaling (USA). Y-27632 was purchased from Calbiochem (Germany). The Western blot detection kit was purchased from Amersham (England). The Plasma Membrane Protein Extraction Kit was from Bio Vision (USA). The SYBR® PrimeScript™ reverse transcription-polymerase chain reaction (RT-PCR) Kit II (Perfect Real-time) was purchased from Takara (Japan). Enzyme-linked immunosorbent assay (ELISA) kits were purchased from R&D (USA).

Cell culture and grouping

HMCs were cultured in MCM 4201 medium, 5.5 or 30 mmol/l glucose, or 5.5 mmol/l glucose and 24.5 mmol/l mannitol supplemented with 20% fetal bovine serum at 37°C, 1% mesangial cell growth supplement and streptomycin (100 μg/ml), and penicillin (100 units/ml) in 95% air/5% CO_2 incubator. Confluent cells were rendered quiescent by incubation for 24 h in serum-free medium before treatment with glucose (24.5 mmol/l for a final concentration of 30 mmol/l) or mannitol osmotic control (mannitol, 24.5 mmol/l) for various times (12, 24, 36, 48, and 72 h) as indicated. Rho kinase inhibitors were added before glucose: Y-27632 (10 μmol/l, 30 min) or fasudil (25, 50, and 100 μmmol/l, 30 min). The inhibitory activity and specificity of both inhibitors toward Rho kinase have been demonstrated in other experiments [5-8].

Expression of active RhoA

This was performed as described previously [9]. In elevated glucose concentrations, cells were collected at 0.5, 1, 6, 12, 24, 36, and 48 h and membrane protein was extracted. The procedures were performed in accordance with the instructions in the Membrane Protein Extraction Kit. Protein concentration was measured using the BCA method, and RhoA expression activity was detected by Western blot.

RhoA-stealth RNA synthesis and transfection

Human RhoA on-target plus SMARTpool stealth-RNA (RhoA siRNA), control nontargeting small interfering RNA (siRNA), and Lipofectamine™ 2000 reagent were purchased from Invitrogen (USA). HMCs were transfected with 100 nmol/L siRNA using Lipofectamine™ 2000 reagent at 60% confluence. After 6 h, cells were serum deprived in Opti-MEM (Invitrogen, USA) for 24 h and then treated for 48 h with high glucose. RNA was harvested as in reverse transcription, which was performed using standard methods, and cDNA was analyzed using real-time PCR. Standard PCR was used to assess RhoA transcript down-regulation by siRNA. The MTT method was used to detect the proliferation of cells and describe the cell growth curve. The supernatants were collected for ELISA.

Detection of RhoA, ROCK-I, CTGF, and TNF-α mRNA by real-time RT-PCR

Total RNA was extracted from cells using Trizol agent (Invitrogen, USA) according to the manufacturer's guidelines. The purity and concentration of the RNA samples were detected by spectrophotometry. Simultaneously, the integrity of total RNA was also detected in a denaturing agarose gel. Real-time RT-PCR was performed with total RNA using an SYBR PrimeScript RT-PCR kit. Briefly, reverse transcription was

Table 1. Real-time RT-PCR test primer sequence

Genes	Primer sequence	Amplification length	Annealing temperature
RhoA	For: 5'-GACTCGGATTCGTTGCCTGA-3' Rev: 5'-TGGGAACTGGTCCTTGCTGA-3'	116 bp	60°C
ROCK-I	For: 5'-CTGCAACTGGAACTCAACCAAGAA -3' Rev: 5'-GCTGGCCAACTGCATCTGAA-3'	138 bp	55°C
CTGF	For: 5'-CTGCAACTGGAACTCAACCAAGAA -3' Rev: 5'-GCTGGCCAACTGCATCTGAA-3'	90 bp	58°C
TNF-α	For: 5'-GTGACAAGCCTGTAGCCCATGTT-3' Rev: 5'-TTATCTCTCAGCTCCACGCCATT-3'	112 bp	58°C
β-actin	For: 5'-TGGCACCCAGCACAA TGAA-3' Rev: 5'-CTAAGTCATAGTCCGCCTAGAAGCA-3'	186 bp	55~60°C
GAPDH	For: 5'-GCACCGTCAAGGCTGAGAAC-3' Rev: 5'-TGGTGAAGACGCCAGTGGA-3'	138 bp	55~60°C

RhoA: GTPase; ROCK-I: Rho kinase-I; CTGF: Connective tissue growth factor; TNF-α: Tumor necrosis factor alpha; Forward: upstream; Reverse: downstream.

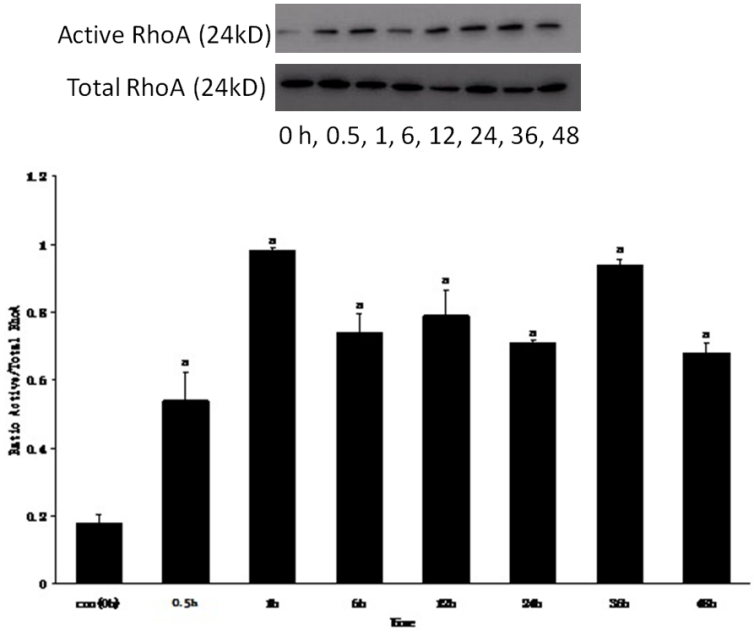

Figure 1. Expression of GTP-RhoA at different time point (Western blot, n=3) vs. con (0 h) [a]P<0.01.

performed in a total volume of 10 μl containing 500 ng total RNA. Then, 25 μl PCR included 10 pmol/μl of each primer, 1 μl cDNA template, 12.5 μl SYBR Green Supermix, and 8.5 μl dd H_2O and run for 40 cycles at 95°C for 5 s, 60°C for 20 s, and 72°C for 30 s. PCR efficiency was detected by the serial dilution cDNA template. Melting curve data were used to check the PCR specificity. β-actin and GAPDH primers were used as internal controls. Each sample was run in triplicate. For primer sequences of human RhoA, ROCK, CTGF, TNF-α, β-actin, and GAPDH see **Table 1**. The relative mRNA level was presented as unit values of $2^{-\Delta\Delta CT}$.

ELISA

Debris was removed from the conditioned media by low-speed centrifugation (20 min at the speed of 2000-3000 rpm). The supernatant was taken and stored at -80°C. FN, TNF-α, and CTGF levels were measured using a quantitative sandwich ELISA. Finally, reactions were read at 450 nm in a microplate autoreader.

Statistical analysis

Quantitative results are shown as means ± standard deviations. Statistical analysis was performed using one-way analysis of variance (ANOVA) with Tukey's honestly significant difference for post hoc studies (SPSS 17.0 for Windows). A *p*-value of <0.05 was considered statistically significant. Experiments were repeated multiple times, with number of repetitions denoted by "n=3" or "n=6".

Results

Activation of RhoA by high glucose in HMCs

HMCs stimulated by high glucose at different times showed a time-dependent increase in

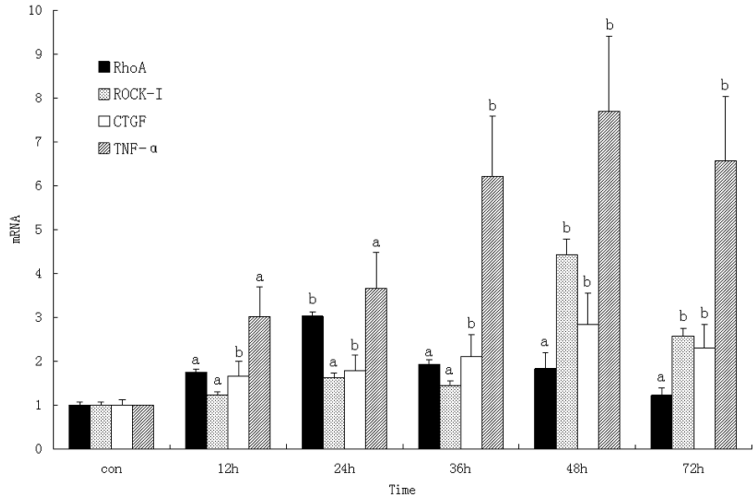

Figure 2. Expression of each mRNA at different time point (n=6). con: Normal glucose group, vs. con [a]P<0.05, vs. con [b]P<0.01.

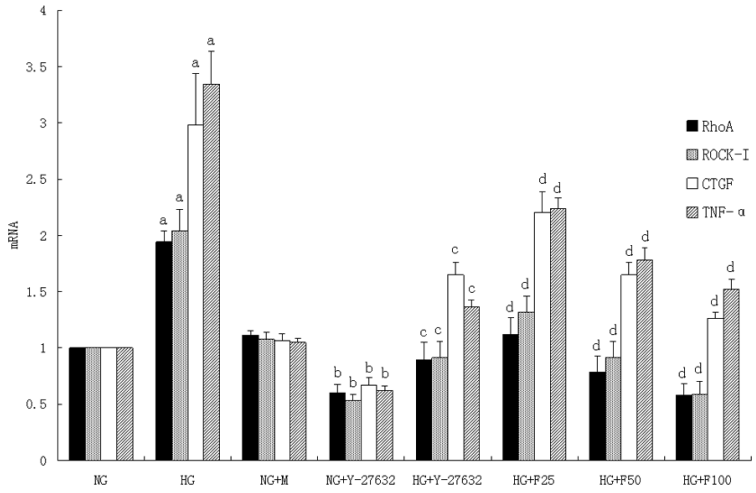

Figure 3. Expression of each mRNA at 24 h or 48 h in the supenatant of each group (n=6). NG: Normal glucose group, HG: High glucose group, NG+M: Mannitol group; NG+Y-27632: Normal glucose+Y-27632 group (10 μMol), HG+Y-27632: High glucose+Y-27632 group (10 μMol), HG+F25: High glucose+fasudil-25 group, Fasudil (25 μMol), HG+F50: High glucose+fasudil-50 group, Fasudil (50 μMol), HG+F100: High glucose+fasudil-100 group, Fasudil (100 μMol). vs. NG group, [a]P<0.05, [b]P<0.01, vs. HG group, [c]P<0.01, [d]P<0.001 (RhoA was assessed after 24 h, others were assessed after 48 h).

RhoA activity compared to the control group cultured in complete medium. RhoA could be activated within 30 min of high glucose induction and increased by 2.95±0.47 times (P< 0.05). Significant RhoA activation was observed at 1 h after exposure to high glucose (5.44±0.42 times vs. control group; P<0.05). Thereafter, RhoA expression was decreased. However, total RhoA protein expression in each group showed no significant changes (P>0.05; **Figure 1**).

Expression of each mRNA under the stimulus of high glucose by real-time RT-PCR

Real-time RT-PCR was used to detect the expression of each mRNA (RhoA, ROCK-I, CTGF, and TNF-α) in HMCs at different time points. A time-dependent increase in mRNA expression was observed. The results showed that RhoA mRNA expression was highest at 24 h in HMCs exposed to 30 mmol/l glucose. Otherwise, ROCK-I, CTGF, and TNF-α mRNA expression was significantly enhanced at 48 h (**Figure 2**). Treatment with equimolar mannitol for 6 h did not induce RhoA or Rho kinase activation.

Expression of each mRNA in different groups as indicated in 24 or 48 h

According to the above groups, after HMCs were exposed to glucose for 24 h, RhoA mRNA expression was up-regulated significantly in the high glucose-induced group compared to the normal glucose-induced group (5.5 mmol/l glucose). ROCK-I, CTGF, and TNF-α mRNA expression was significantly enhanced in the high glucose-induced group compared to the normal glucose-induced group. However, treatment with equimolar mannitol for 24 or 48 h did not induce each mRNA. Administration of Y-27632 or fasudil abolished the enhancement of RhoA, ROCK, TNF-α, and CTGF mRNA expression in HMCs exposed to normal or high glucose (**Figure 3**). In addition, different concentrations of fasudil in the treatment groups compared to the high glucose group were concentration dependent, but there were no differences in the normal glucose group (P>0.05).

Table 2. The concentration of FN in the supernatant of each group ($\bar{x}$±s μg/ml, n=3)

Group	Culture time (h)				
	12	24	36	48	72
NG	1.277±0.021	1.358±0.088	2.099±0.173	6.983±0.142	3.316±0.060
HG	1.430±0.031[a]	1.645±0.124[a]	2.919±0.031[a]	8.059±0.041[a]	4.314±0.125[a]
Man	1.329±0.011[b]	1.309±0.050[b]	1.995±0.102[b]	6.983±0.193[b]	3.113±0.176[b]
NY	0.820±0.03[c]	1.067±0.06[c]	1.125±0.09[c]	6.424±0.28[c]	2.532±0.08[c]
HY	0.921±0.04[d]	1.290±0.08[d]	1.338±0.05[d]	5.152±0.38[d]	2.747±0.20[d]
H-F25	1.018±0.046[e]	1.329±0.064[e]	1.530±0.124[e]	6.727±0.298[e]	3.404±0.171[e]
H-F50	0.912±0.047[e]	1.290±0.087[e]	1.338±0.054[e]	5.186±0.200[e]	2.610±0.278[e]
H-F100	0.691±0.118[e]	1.045±0.09[e]	1.277±0.082[e]	4.739±0.320[e]	2.592±0.185[e]

NG: Normal glucose group, 5.5 mmol/L glucose; HG: High glucose group, 30 mmol/L glucose; Man: 5.5 mmol/L glucose+24.5 mmol/L mannitol; NY: 5.5 mmol/L glucose+10 μmol/L Y-27632; HY: High glucose+Y-27632 group, 30 mmol/L glucose+10 μmol/L Y-27632; H-F25: High glucose+fasudil-25 group, Fasudil (25 μMol); H-F50: High glucose+fasudil-50 group, Fasudil (50 μMol); H-F100: High glucose+fasudil-100 group, Fasudil (100 μMol). vs. NG group, [a]P<0.01, [b]P>0.05, [c]*P*<0.01; vs. HG group, [d]P<0.01, [e]P<0.01.

Table 3. The concentration of CTGF protein in the supematant of each group ($\bar{x}$±s μg/ml, n=3)

Group	Culture time (h)				
	12	24	36	48	72
NG	4.544±0.051	5.004±0.208	5.432±0.114	9.305±0.519	6.032±0.117
HG	5.606±0.098[a]	6.056±0.087[a]	5.947±0.125[a]	12.836±0.433[a]	7.820±0.136[a]
Man	4.802±0.085[b]	4.907±0.076[b]	5.322±0.186[b]	9.126±0.548[b]	6.137±0.045[b]
NY	3.845±0.03[c]	4.080±0.15[c]	4.806±0.09[c]	6.432±0.392[c]	4.952±0.19[c]
HY	4.888±0.13[d]	4.821±0.12[d]	4.982±0.04[d]	8.677±0.391[d]	5.722±0.15[d]
H-F25	5.131±0.076[e]	5.049±0.070[e]	5.202±0.142[e]	9.278±0.266[e]	6.575±0.283[e]
H-F50	4.888±0.136[e]	4.821±0.126[e]	4.982±0.049[e]	8.677±0.392[e]	5.109±0.034[e]
H-F100	4.039±0.070[e]	4.158±0.095[e]	4.013±0.097[e]	6.029±0.202[e]	4.791±0.085[e]

NG: Normal glucose group, 5.5 mmol/L glucose; HG: High glucose group, 30 mmol/L glucose; Man: 5.5 mmol/L glucose+24.5 mmol/L mannitol; NY: 5.5 mmol/L glucose+10 μmol/L Y-27632; HY: High glucose+Y-27632 group, 30 mmol/L glucose+10 μmol/L Y-27632; H-F25: High glucose+fasudil-25 group, Fasudil (25 μMol); H-F50: High glucose+fasudil-50 group, Fasudil (50 μMol); H-F100: High glucose+fasudil-100 group, Fasudil (100 μMol).vs. NG group, [a]P<0. 01, [b]P>0.05, [c]P<0.01; vs. HG group, [d]P<0. 01, [e]P<0.01.

Changes of each protein (FN, CTGF, and TNF-α) in the supernatant of each group at various time points

HMCs cultured in high glucose with protein in the supernatant (including FN, CTGF, and TNF-α) were increased from 12 h and significantly enhanced at 48 h, after which, protein expression started to decrease (P<0.01). Mannitol had no effect on FN transcript or protein up-regulation at different time points (P>0.05). In the normal glucose+Y-27632 group compared to the normal glucose and the high glucose+Y-27632 group compared to the high glucose group, protein expression was significantly decreased (P<0.01; **Tables 2**-**4**). After treatment by different concentrations of fasudil, compared to the high glucose group, each protein level was significantly decreased (P<0.01) and was concentration dependent (**Tables 2**-**4**).

RhoA siRNA and the expression of various factors

Subsequently, the study assessed the effects of inhibition of upstream RhoA activation. HMCs were transfected with either control or RhoA-specific siRNA (**Figure 4**). **Figure 5** shows that glucose induced the up-regulation of each mRNA, as assessed by real-time PCR. The up-regulation of FN and each protein, as detected by ELISA (**Tables 5**-**7**), was prevented by RhoA siRNA. The successful down-regulation of RhoA was confirmed by real-time PCR, with β-actin

Table 4. The concentration of TNF-α protein in the supernatant of each group ($\bar{x}$±s ng/ml, n=3)

Group	Culture time (h)				
	12	24	36	48	72
NG	8.118±0.865	16.614±5.730	35.306±3.685	59.997±2.144	34.173±4.817
HG	34.550±2.265[a]	38.515±2.042[a]	59.850±2.907[a]	98.176±5.588[a]	80.618±9.634[a]
Man	12.650±7.648[b]	17.558±5.191[b]	39.459±4.949[b]	55.696±7.690[b]	42.102±6.366[b]
NY	2.077±0.865[c]	3.021±1.989[c]	9.251±2.554[c]	35.494±1.42[c]	16.427±4.49[c]
HY	13.782±1.17[d]	27.565±2.61[d]	34.928±2.90[d]	53.997±3.31[d]	55.885±3.46[d]
H-F25	19.635±0.865[e]	30.774±1.179[e]	45.690±4.175[e]	67.968±3.537[e]	59.661±1.730[e]
H-F50	14.349±3.319[e]	26.432±2.677[e]	34.928±2.907[e]	53.997±3.319[e]	51.920±3.119[e]
H-F100	3.398±2.266[e]	7.930±2.997[e]	22.090±4.276[e]	33.040±3.856[e]	32.662±4.326[e]

NG: Normal glucose group, 5.5 mmol/L glucose; HG: High glucose group, 30 mmol/L glucose; Man: 5.5 mmol/L glucose+24.5 mmol/L mannitol; NY: 5.5 mmol/L glucose+10 μmol/L Y-27632; HY: High glucose+Y-27632 group, 30 mmol/L glucose+10 μmol/L Y-27632; H-F25: High glucose+fasudil-25 group, Fasudil (25 μMol); H-F50: High glucose+fasudil-50 group, Fasudil (50 μMol); H-F100: High glucose+fasudil-100 group, Fasudil (100 μMol). vs. NG group, [a]P<0. 01, [b]P>0.05, [c]P<0.01; vs. HG group, [d]P<0. 01, [e]P<0.01.

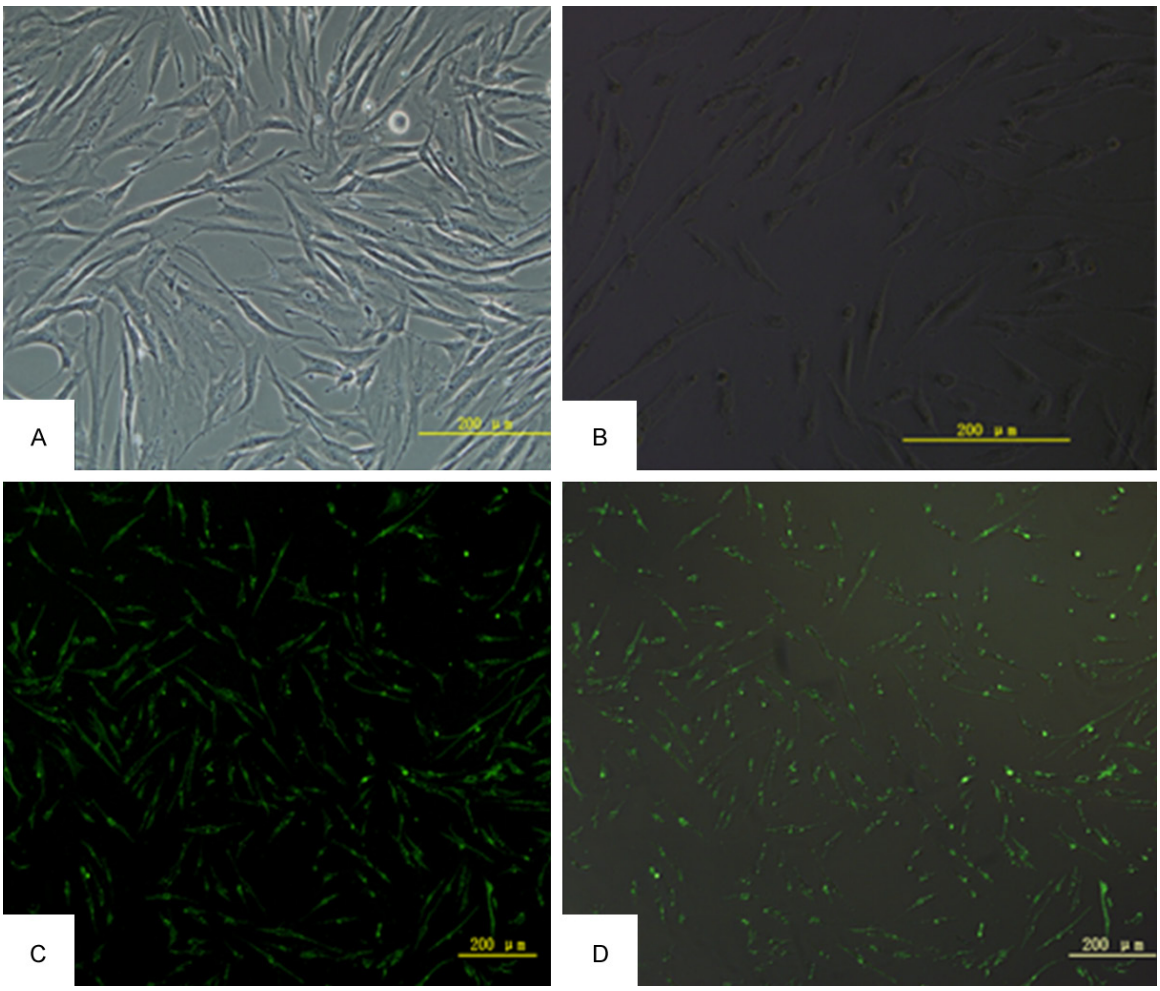

Figure 4. HMCs transfection screening and transfection efficiency. (A) HMCs were cultured in normal medium (under normal light microscope, 100×). (B) HMCs were transfected by RhoA Stealth-siRNA (under normal light microscope, 100×). (C) Inverted fluorescence microscope siRNA transfection rate of 90% (GFP marker 100×) siRNA (μl): Lipofectamine™2000=1:2. (D) Synthesized by (B and C).

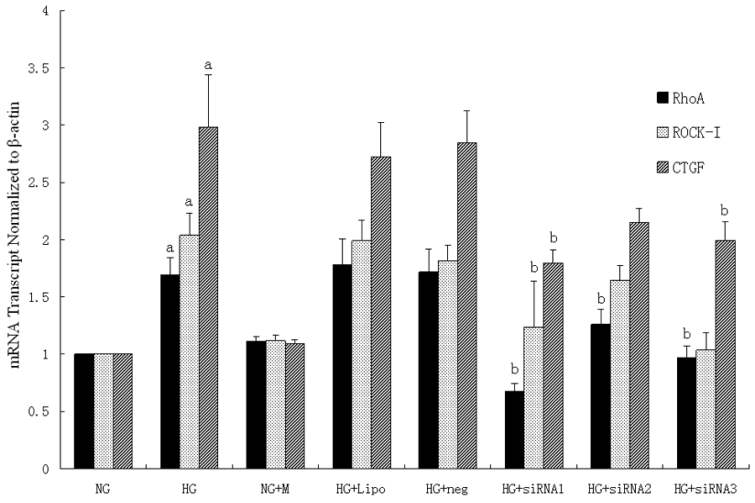

Figure 5. Expression of mRNA at 48 h in each group. NG: Normal glucose group; HG: High glucose group; NG+M: 5.5 mmol/L glucose+24.5 mmol/L mannitol; HG+Lipo: High glucose+Lipofectamine group; HG+neg: High glucose+negative siRNA group; HG+siRNA1: High glucose+RhoA-1siRNA group; HG+siRNA2: High glucose+RhoA-2siRNA group; HG+siRNA3: High glucose+RhoA-3siRNA group. vs. NG group, $^{a}P<0.01$; vs. HG group, $^{b}P<0.01$.

and GAPDH serving as controls (**Table 1**). These results suggest an important role for RhoA/ROCK in this characteristic fibrogenic response and inflammatory reaction to high glucose.

Discussion

This study demonstrated that the Rho/ROCK signaling pathway can be activated in HMCs in vitro by high glucose. The level of RhoA and the proliferation and division rate were significantly increased in HMCs induced by high glucose under an inverted microscope, which is consistent with previous in vivo and in vitro studies [10-15]. By activating the Rho/ROCK pathway, the expression of downstream inflammatory and fibrotic factors was increased. This led to a series of inflammatory reactions and glomerular fibrosis, accelerating the occurrence and development of DKD. The ROCK inhibitors Y-27632 and fasudil, in addition to RhoA siRNA, effectively reversed the high glucose-induced expression of ROCK-I, FN, CTGF, and TNF-α and then reduced glomerular fibrosis and inflammation, delaying the progress of glomerular sclerosis and glomerular hypertrophy.

DKD, as the major microvascular complication of DM, is an important cause of disability and death in patients with DM. DM could cause kidney damage through various mechanisms, and the occurrence of DKD is the result of many factors. In recent years, the role of the Rho/ROCK signaling pathway, which contributes to the microvascular complications of DM, has attracted widespread attention.

RhoA is a GTPase that has been shown to play a key role in both actin stress fiber formation and focal adhesion complex assembly in fibroblasts [16, 17]. Recent studies demonstrate that RhoA is an important target in the cell signaling pathway and that RhoA plays a pivotal role in cell function. Rho kinase, that is, ROCK, which is the most well studied downstream factor of Rho, consists of an N-terminal kinase domain followed by a central coiled coil-forming region containing an Rho-binding domain (RBD) and C-terminal cysteine-rich domain (CRD) located within the pleckstrin homology (PH) motif. The Rho/ROCK signaling pathway not only has a relationship and regulation with many vasoactive factors [18] but also plays a key role in the regulation of actin cytoskeleton formation, cell motility, proliferation, and apoptosis [19], and is also involved in smooth muscle cell contraction, gene expression, and regulation of cytokinesis [20].

The Rho/ROCK signaling pathway can be activated by high glucose, high concentrations of amino acids, and mechanical damage. These downstream cytokines and vasoactive substances are important contributors to inflammatory injury and tissue fibrosis. Accordingly, the Rho/ROCK signaling pathway may be integrated in the after effects of inflammatory receptors, which are involved in the pathophysiology of these diseases [16, 21].

In our study, the activation of RhoA was induced by 30 min exposure of HMCs to high glucose in vitro. GTP-RhoA expression was high with 1 h treatment. Both a dose-dependent decrease with the extension of duration of high glucose and a time-dependent decrease on the level of activation of protein expression were observed. RhoA expression reached the highest level in

Table 5. The concentration of TNF-α in the supernatant of each group ($\bar{x}$±s, ng/mL, n=3)

Group	Culture time (h)			
	12	24	48	72
NG	50.78±4.40	58.15±1.73	68.34±4.02	74.19±5.09
HG	90.62±4.28[a]	100.44±5.59[a]	91.76±2.27[a]	121.97±3.98[a]
NG+M	57.63±3.09[c]	60.10±2.09[c]	69.89±3.01[c]	79.77±2.11[c]
HG+Lipo	91.57±1.98[c]	101.01±5.67[c]	80.05±7.71[c]	116.11±4.53[c]
HG+neg	88.17±2.68[c]	104.78±2.04[c]	72.12±2.85[c]	126.31±6.31[c]
HG+siRNA1	83.07±2.29[b]	80.81±3.98[b]	61.17±2.59[b]	93.83±2.29[b]
HG+siRNA2	64.76±0.87[b]	68.72±1.73[b]	76.46±1.69[b]	107.24±3.98[b]
HG+siRNA3	58.71±1.99[b]	58.91±1.13[b]	71.37±1.69[b]	99.69±2.27[b]

NG: Normal glucose group; HG: High glucose group; NG+M: 5.5 mmol/L glucose+24.5 mmol/L mannitol; HG+Lipo: High glucose+Lipofectamine group; HG+neg: High glucose+negative siRNA group; HG+siRNA1: High glucose+RhoA-1siRNA group; HG+siRNA2: High glucose+RhoA-2siRNA group; HG+siRNA3: High glucose+RhoA-3siRNA group. [a]P<0.01, vs. NG group; [b]P<0.01, [c]P>0.05. vs. HG group.

Table 6. The concentration of FN in the supernatant of each group ($\bar{x}$±s, μg/mL, n=3)

Group	Culture time (h)			
	12	24	48	72
NG	1.35±0.09	1.86±0.06	3.31±0.06	2.10±0.17
HG	1.65±1.23[a]	2.68±0.09[a]	4.31±0.13[a]	2.92±0.03[a]
NG+M	1.40±0.09[c]	1.90±0.10[c]	3.45±0.88[c]	2.20±0.09[c]
HG+Lipo	1.69±0.09[c]	2.61±0.05[c]	4.16±0.21[c]	2.85±0.03[c]
HG+neg	1.52±0.05[c]	2.69±0.04[c]	4.13±0.27[c]	2.80±0.04[c]
HG+siRNA1	0.56±0.05[b]	1.59±0.05[b]	2.56±0.17[b]	1.38±0.07[b]
HG+siRNA2	0.84±0.09[b]	2.08±0.04[b]	3.14±0.22[b]	1.48±0.04[b]
HG+siRNA3	0.54±1.03[b]	1.43±0.04[b]	1.99±0.04[b]	1.06±0.06[b]

NG: Normal glucose group; HG: High glucose group; NG+M: 5.5 mmol/L glucose+24.5 mmol/L mannitol; HG+Lipo: High glucose+Lipofectamine group; HG+neg: High glucose+negative siRNA group; HG+siRNA1: High glucose+RhoA-1siRNA group; HG+siRNA2: High glucose+RhoA-2siRNA group; HG+siRNA3: High glucose+RhoA-3siRNA group. vs. NG group, [a]P<0.01; vs. HG group, [b]P<0.01, [c]P>0.05.

HMCs stimulated by 24 h exposure to high glucose in RNA levels. However, high glucose was depleted and RhoA expression was decreased gradually over time. Its downstream factor, ROCK-I, also showed high expression along with the increase of RhoA, which had the highest expression level at 48 h. RhoA and ROCK-I activation was not induced by equimolar mannitol for 24 h. It can be inferred that high glucose may induce the activation of the Rho/ROCK signaling pathway in HMCs.

In addition, high glucose could decrease the activity of the GMP cyclin-dependent protein kinase and then increase the transforming growth factor-β (TGF-β) and FN [22]. It has been reported that some ECM components, especially FN, could enhance cell adhesion and motion, and FN produces its marked effect via the integrin family. The increase of ECM proteins is the significant factor in the development of glomerulosclerosis. Therefore, the expression level of FN and LN in the kidney glomerulus increases greatly in DM patients or in DM models [23].

The results of this study revealed that the expression of the inflammatory factor TNF-α, fibrosis factor CTGF, and ECM component FN was significantly increased under high glucose-induced HMC and this further accelerated HMC proliferation, inflammatory reactions, and fibrosis, which might result in glomerulosclerosis development. Besides, we found that TNF-α, CTGF, and FN expression in the supernatant fluid of HMC cultivated under normal glucose increased with incubation time. This might be attributed to cell division growth during the prolonged incubation time of HMC.

Y-27632 is a kind of synthetic pyridine derivative and is usually used as a selective inhibitor of Rho kinase. It is absorbed by cells via carrier-mediated facilitated diffusion and combines with the effective apparatus kinase catalysis sites of downstream Rho. Fasudil is a Rho kinase inhibitor, and its derivative, fasudil hydrochloride, has been used clinically as a drug aimed to heal post-subarachnoid hemorrhage cerebral arterial spasm.

This study used Y-27632 to interfere with HMC incubated with normal and high levels of glucose, and the high glucose groups were given different concentrations of fasudil. We found

Table 7. The concentration of CTGF in the supernatant of each group ($\bar{x}$±s, μg/mL, n=3)

Group	Culture time (h)			
	12	24	48	72
NG	4.59±0.09	4.91±0.11	5.42±0.11	6.03±0.12
HG	5.71±0.09[a]	6.06±0.09[a]	6.06±0.09[a]	7.82±0.14[a]
NG+M	4.62±0.10[c]	5.01±0.07[c]	5.33±0.20[c]	6.13±0.20[c]
HG+Lipo	5.52±0.10[c]	5.78±0.09[c]	5.92±0.14[c]	7.70±0.11[c]
HG+neg	5.80±0.11[c]	6.14±0.10[c]	5.86±0.14[c]	7.71±0.16[c]
HG+siRNA1	4.14±0.11[b]	5.05±0.10[b]	5.05±0.08[b]	5.50±0.20[b]
HG+siRNA2	5.18±0.10[b]	5.29±0.10[b]	5.26±0.08[b]	6.16±0.12[b]
HG+siRNA3	4.53±0.14[b]	4.66±0.16[b]	4.98±0.17[b]	5.34±0.15[b]

NG: Normal glucose group; HG: High glucose group; NG+M: 5.5 mmol/L glucose+24.5 mmol/L mannitol; HG+Lipo: High glucose+Lipofectamine group; HG+neg: High glucose+negative siRNA group; HG+siRNA1: High glucose+RhoA-1siRNA group; HG+siRNA2: High glucose+RhoA-2siRNA group; HG+siRNA3: High glucose+RhoA-3siRNA group. vs. NG group, [a]P<0.01; vs. HG group, [b]P<0.01, [c]P>0.05.

that RhoA and ROCK-I expression was significantly decreased compared to the level before treatment. The results indicated that Y-27632 and fasudil could block the signaling pathway of the activated high glucose by inhibiting ROCK-I expression. We also determined that the multiplicative division speed of HMC was slower as observed with an inverted microscope. The decrease of TNF-α, FN, and CTGF expression was also detected. Hence, the results of our research revealed that one could decrease the expression of some inflammatory factor and cytokine that lead to glomerulosclerosis by inhibiting activation of the Rho/ROCK signaling pathway. The decrease of fibrosis and inflammatory reactions in mesangial cells might prevent the development of glomerulosclerosis and delay the process of DKD.

In addition, some improvements on the metabolic index were determined under large doses of fasudil in type 2 DM, such as control of blood glucose, blood lipids increase, decrease of insulin resistance, and improvement of DKD pathologic modification [24, 25]. Although a low dose of fasudil could decrease the thickness of the glomerular basement membrane and the expansion of mesenteric substrate, it had no great effect on the metabolic index [26] but could prevent the increase of FN in the kidney glomerulus and kidney glomerular cirrhosis. It had a therapeutic effect similar to that of enalapril [27].

RNA interference (RNAi) is a new molecular biology technique for gene silencing developed in recent years. RNAi effect has high sequence specificity, and any misalliance in base would result in the loss of RNAi effect [28, 29]. RNAi is more effective and thorough than the ribozyme and antisense oligonucleotide, and it has been the most important strategy for gene analysis and gene therapy.

Previous studies of the inhibitor of signaling molecule ROCK in downstream small G protein have reported that the inhibitor had an inhibitory action for the ROCK downstream signal. Meanwhile, human glomerular mesangial cells transiently transfected by RhoA siRNA were established by chemical synthesis. As observed under an inverted microscope, some post-transfection cells became round and floated. The cell-cell contact was no longer close, and proliferation was slow. Besides, the particle number in cells increased and vacuolization was observed. It was demonstrated that after 12 h, siRNA transiently transfected mammalian cells displayed a gene silencing effect. The transfection efficiency increased gradually with transfection time and reached a maximum after 48 to 72 h. The RhoA mRNA level of the HG+RhoA siRNA group after 12 h transfection as determined by real-time RT-PCR was lower than that of the untransfected HG group. This result implied that RhoA expression was inhibited at the gene level. After 48 h transfection, the silencing efficiency of the RhoA of the HG+RhoA siRNA group was higher than 80% and greater than that of the HG group. Meanwhile, no silencing effect was observed for the control of the blank liposomes and the missense negative control. Furthermore, we found that the expression level of ROCK-I, CTGF, FN, and TNF-α of the transfection group was lower than that of the untransfected high glucose group in the gene and protein levels, indicating that the inflammatory reaction and fibrotic effect of the HMCs under high glucose can be reduced by high glucose transfection and induce the protective effect on the kidney.

It has been reported that the Rho/ROCK signaling pathway could contact with Smads, NF-κB,

JAK/STAT, MAPK, mTOR, and the integrin receptor to form complex networks that influence signal transduction [30]. The effect of Y-27632 can markedly reduce the inflammatory reaction and fibrotic effect of intercapillary cells, which might be the result of the interaction of the Rho/ROCK and ERK/MAPK signaling pathways. Fasudil could reduce the phosphoric acid level of Smad2/3 [31], indicating that Smads might be one substrate of ROCK.

In conclusion, this study demonstrates the important role of the Rho/ROCK signaling pathway in the development of DKD and suggests that inhibition of this pathway is a potential target point to retard the development of DKD. Future studies should focus on the therapeutic effect of the RhoA kinase inhibitor on DKD. The influence of the signal networks formed by the Rho/ROCK signaling pathway and other signaling pathways on the molecular mechanism of DKD development should be evaluated as well in future research.

Acknowledgements

This work was supported by grants from Liaoning's Department of Education (L20-15564).

Disclosure of conflict of interest

None.

Address correspondence to: Qiuyue Wang, Department of Endocrinology, The First Hospital, China Medical University, Shenyang 110001, China. Tel: +0086-24-83282301; Fax: +0086-24-83282908; E-mail: wqycmu123@163.com

References

[1] Chen FQ, Wang J, Liu XB, Ma XY, Zhang XB, Huang T, Ma DW and Wang QY. Levels of inflammatory cytokines in type 2 diabetes patients with different urinary albumin excretion rates and their correlation with clinical variables. J Diabetes Res 2013; 2013: 138969.

[2] Jo M, Thomas KS, Somlyo AV, Somlyo AP and Gonias SL. Cooperativity between the Ras-ERK and Rho-Rho kinase pathways in urokinase-type plasminogen activator-stimulated cell migration. J Biol Chem 2002; 277: 12479-12485.

[3] Hayashi K, Wakino S, Kanda T, Homma K, Sugano N and Saruta T. Molecular mechanisms and therapeutic strategies of chronic renal injury: role of Rho-kinase in the development of renal injury. J Pharmacol Sci 2006; 100: 29-33.

[4] Zeng L, Xu H, Chew TL, Eng E, Sadeghi MM, Adler S, Kanwar YS and Danesh FR. HMG CoA reductase inhibition modulates VEGF-induced endothelial cell hyperpermeability by preventing RhoA activation and myosin regulatory light chain phosphorylation. FASEB J 2005; 19: 1845-1847.

[5] Zhou L, Liu F, Huang XR, Liu F, Chen H, Chung AC, Shi J, Wei L, Lan HY and Fu P. Amelioration of albuminoidal in ROCK1 knockout mice with streptozotocin-induced diabetic kidney disease. Am J Nephrol 2011; 34: 468-475.

[6] Zhang Y, Duan X, Xiong B, Cui XS, Kim NH, Rui R and Sun SC. ROCk inhibitor Y-27632 prevents porcine oocyte maturation. Theriogenology 2014; 82: 49-56.

[7] Burger D, Thibodeau JF, Holterman CE, Burns KD, Touyz RM and Kennedy CR. Urinary podocyte microparticles identify prealbuminuric diabetic glomerular injury. J Am Soc Nephrol 2014; 25: 1401-1407.

[8] Wu G, Xu M, Xu K and Hu Y. Benidipine protects kidney through inhibiting ROCK1 activity and reducing the epithelium-mesenchymal transdifferentiation in type 1 diabetic rats. J Diabetes Res 2013; 2013: 174526.

[9] Krepinsky JC, Ingram AJ, Tang D, Wu D, Liu L and Scholey JW. Nitric oxide inhibits stretch-induced MAPK activation in mesangial cells through RhoA inactivation. J Am Soc Nephrol 2003; 14: 2790-2800.

[10] Massey AR, Miao L, Smith BN, Liu J, Kusaka I, Zhang JH and Tang J. Increased RhoA translocation in renal cortex of diabetic rats. Life Sci 2003; 72: 2943-2952.

[11] Danesh FR, Sadeghi MM, Amro N, Philips C, Zeng L, Lin S, Sahai A and Kanwar YS. 3-Hydroxy-3-methylglutaryl CoA reductase inhibitors prevent high glucose-induced proliferation of mesangial cells via modulation of Rho GTPase/p21 signaling pathway: implications for diabetic nephropathy. Proc Natl Acad Sci U S A 2002; 99: 8301- 8305.

[12] Yang R, Chang L, Liu S, Jin X and Li Y. High glucose induces Rho/ROCK-dependent visfatin and type I procollagen expression in rat primary cardiac fibroblasts. Mol Med Rep 2014; 10: 1992-1998.

[13] Su J, Zou W, Cai W, Chen X, Wang F, Li S, Ma W and Cao Y. Atorvastatin ameliorates contrast medium-induced renal tubular cell apoptosis in diabetic rats via suppression of Rho-kinase pathway. Eur J Pharmacol 2014; 723: 15-22.

[14] Danesh FR, Sadeghi MM, Anlro N, Philips C, Zeng L, Lin S and Kanwar YS. 3-Hydroxy-3-methylglutaryl CoA reductase inhibitors pre-

vent high glucose-induced proliferation of mesangial cells via modulation of Rho GTPase/p21 signaling pathway: implications for diabetic nephropathy. Proc Natl Acad Sci U S A 2002; 99: 8301-8305.

[15] Kawamura H, Yokote K, Asaumi S, Kobayashi K, Fujimoto M, Maezawa Y, Saito Y and Mori S. High glucose-induced upregulation of osteopontin is mediated via Rho/Rho kinase pathway in cultured rat aortic smooth muscle cells. Arterioscler Thromb Vasc Biol 2004; 24: 276-281.

[16] Wang C, Song S, Zhang Y, Ge Y, Fang X, Huang T, Du J and Gao J. Inhibition of the Rho/Rho kinase pathway prevents lipopolysaccharide-induced hyperalgesia and the release of TNF-α and IL-1β in the mouse spinal cord. Sci Rep 2015; 5: 14553.

[17] Hu H, Chen W, Ding J, Jia M, Yin J and Guo Z. Fasudil prevents calcium oxalate crystal deposit and renal fibrogenesis in glyoxylate-induced nephrolithic mice. Exp Mol Pathol 2015; 98: 277-285.

[18] Funakoshi Y, Ichiki T, Shimokawa H, Egashira K, Takeda K, Kaibuchi K, Takeya M, Yoshimura T and Takeshita A. Rho-kinase mediated angiotensin II-induced monocyte chemoattractant orotein-1 expression in rat vascular smooth muscle cells. Hypertension 2001; 38: 100-104.

[19] Jafe AB and Hall A. Rho GTPase: biochemistry and biology. Annu Rev Cell Dev Biol 2005; 21: 247-269.

[20] Somlyo AP and Somlyo AV. Signal transduction by G-protein, Rho-kinase and protein phosphatase to smooth muscle and non-musclemyosin II. J Physiol 2000; 522: 177-185.

[21] Guo J, Wang SB and Yuan TY. Coptisine protects rat heart against myocardial ischemia/reperfusion injury by suppressing myocardial apoptosis and inflammation. Atherosclerosis 2013; 231: 384-91.

[22] Peng FF, Wu DC, Gao B, Ingram AJ, Zhang B, Chorneyko K, McKenzie R and Krepinsky JC. RhoA/Rho-Kinase contribute to the pathogenesis of diabetic renal disease. Diabetes 2008; 57: 1683-1692.

[23] Wang S, Wu X, Lincoln TM and Murphy-Ullrich JE. Expression of constitutively active cGMP-dependent protein kinase prevents glucose stimulation of thrombospondin 1 expression and TGF-beta activity. Diabetes 2003; 52: 2144-2150.

[24] Kanda T, Wakino S, Homma K, Yoshioka K, Tatematsu S, Hasegawa K, Takamatsu I, Sugano N, Hayashi K and Saruta T. Rho-kinase as a molecular target for insulin resistance and hypertension. FASEB J 2006; 20: 169-171.

[25] Kikuchi Y, Yamada M, Imakiire T, Kushiyama T, Higashi K, Hyodo N, Yamamoto K, Oda T, Suzuki S and Miura S. A Rho-kinase inhibitor, fasudil, prevents development of diabetes and nephropathy in insulin-resistant diabetic rats. J Endocrinol 2007; 192: 595-603.

[26] Kolavennu V, Zeng L, Peng H, Wang Y and Danesh FR. Targeting of RhoA/ROCK signaling ameliorates progression of diabetic nephropathy independent of glucose control. Diabetes 2008; 57: 714-723.

[27] Tijsterman M and Plasterk RH. Dicers at RISC: the mechanism of RNAi. Cell 2004; 117: 1-3.

[28] Caplen NJ. Gene therapy progress and prospects downregulating gene expression: the impact of RNA interference. Gene Ther 2004; 11: 1241-1248.

[29] Fukushima M, Nakamuta M, Kohjima M, Kotoh K, Enjoji M, Kobayashi N and Nawata H. Fasudil hydrochloride hydrate, a Rho-kinase (ROCK) inhibitor, suppresses collagen production and enhances collagenase activity in hepatic stellate cells. Liver Int 2005; 25: 829-838.

[30] Sánchez-López E, Rodriguez-Vita J, Cartier C, Rupérez M, Esteban V, Carvajal G, Rodrígues-Díez R, Plaza JJ, Egido J and Ruiz-Ortega M. Inhibitory effect of interleukin-1β on angiotensin II-induced connective tissue growth factor and type IV collagen production in cultured mesangial cells. Am J Physiol Renal Physiol 2008; 294: F149-F160.

[31] Katsuma S, Ruike Y, Yano T, Kimura M, Hirasawa A and Tsujimoto G. Transcriptional regulation of connective tissue growth factor by sphingosine 1-phosphate in rat cultured mesangial cells. FEBS Lett 2005; 579: 2576-2582.