Fasudil alleviates hepatic fibrosis in type 1 diabetic rats: involvement of the inflammation and RhoA/ROCK pathway

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Abstract. – **OBJECTIVE:** Rho-associated kinases (ROCKs) are recognized to be involved in many pathophysiological processes caused by hyperglycemia. We performed experiments to evaluate the effects of fasudil, the Rho/ROCK inhibitor, on preventing hepatic fibrosis in type 1 diabetic rats and to elucidate the underlying mechanisms.

MATERIALS AND METHODS: Sprague-Dawley (SD) rats were randomly divided into five groups: normal control (NC), untreated diabetic (DM), low-dose fasudil-treated (L-Fas), high-dose fasudil-treated (H-Fas) and captopril-treated (Cap) groups. Streptozotocin was injected to establish the diabetes model. Alanine aminotransferase (ALT), aspartate aminotransferase (AST) and inflammatory factors such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), were analyzed. Hematoxylin and eosin (HE) and Masson's trichrome staining were used for histological observations. The expression of transforming growth factor-β (TGF-β1), metalloproteinase-9 (MMP-9)/tissue inhibitor of metalloproteinase-1 (TIMP-1), collagen type Iα (Coll α1), nuclear factor-kappa B (NF-κB) and ROCK-1 were measured to investigate the mechanisms involved in fibrosis.

RESULTS: The DM group exhibited hepatic fibrosis with remarkable liver damage and inflammation reaction by the activation of the NF-κB pathway. Treatment with fasudil or captopril suppressed not only the inflammation reaction but also the accumulation of the extracellular matrix due to the downregulation of TGF-β1 and MMP-9/TIMP-1, which induces the amelioration of the liver fibrosis with diabetes. Furthermore, fasudil significantly attenuated the activation of ROCK-1 and NF-κB in the livers of diabetic rats.

CONCLUSIONS: These results suggest that fasudil exert anti-inflammation actions and markedly decrease the accumulation of extracellular matrix. Fasudil is a good candidate agent for treating hepatic fibrosis in diabetes.

Key Words:
   Hepatic fibrosis, Type 1 diabetes, Fasudil, Inflammation, Extracellular matrix.

Introduction

Diabetes is a serious threat to human health and has become the most important non-communicable disease. Aside from micro- and macro-vascular diabetic complications, non-alcoholic fatty liver diseases (NAFLD) are strongly associated with hyperglycemia[1,2]. Targher et al[3] found that NAFLD is very common in type 1 diabetic subjects. The prevalence of NAFLD was 44.4%, and NAFLD was the most common cause (69.8%) of liver steatosis on ultrasound examination in patients with confirmed type 1 diabetes (T1DM). The David group investigated patients with T1DM who underwent liver biopsy, and found that 73.5% of the patients had hepatic fibrosis[4]. A minority of diabetic patients has more severe forms of NAFLD including progressive

fibrosis that can sometimes lead to cirrhosis[5]. Many experimental and clinical researches[6,7] have confirmed that diabetes can promote and increase the occurrence of hepatic fibrosis, but the precise interaction mechanism is unclear.

Hepatic fibrosis is caused by inflammation and the accumulation of extracellular matrix (ECM), which are the major characteristics of chronic liver disease[8,9]. The most likely possible mechanisms involve inflammatory reaction, the activation of hepatic stellate cells (HSC), insulin resistance and the effects of various cytokines that promote hepatic fibrosis. Previous studies have shown that the high glucose levels trigger the activation of inflammatory factors such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), which contribute to the activation of hepatic fibrosis[10]. Nuclear factor-kappaB (NF-κB) pathway plays an important role in regulating functions in liver physiology and pathology. It leads to different gene transcription in the liver, which is involved in the regulation of inflammation, cell survival and immune response[11].

RhoA is a member of the Ras superfamily of guanosine triphosphate (GTP)-binding proteins, which acts as molecular switches and cycle between an inactive guanosine diphosphate (GDP)-bound and an active GTP-bound conformation. Rho-associated coiled coil-forming protein kinase (ROCK), the Rho downstream effector molecule, belongs to the serine/threonine kinase family. Recent studies[12] have confirmed RhoA/ROCK exerts a positive role in numerous cell regulation behaviors and functions, involving contraction, adhesion, transmigration, maintenance, growth, division, endotheliocyte transformation, and fibroblastic hyperplasia. Some cytokines and inflammatory mediators can active the Rho/ROCK pathway[13]. Furthermore, cytokines and vascular active materials, such as platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β) and angiotensin II, play an important role in the inflammatory lesions and fiber hyperplasia diseases. Therefore, the Rho/ROCK pathway may participate in the pathophysiological processes in diseases induced by the inflammatory mediators.

Fasudil has been used in the clinic as the first-generation selective Rho/ROCK inhibitor[14]. The safety and efficacy of fasudil has been identified by some clinical trials in treating pulmonary arterial hypertension, cerebrovascular and cardiovascular diseases[15-17]. Our previous investigations[18,19] have also shown the anti-inflammatory and antioxidant effects of fasudil in hypercholesterolemic rats. Previous studies have suggested that fasudil can postpone and prevent fibrosis in heart and kidney diseases[20]. It has been reported[21,22] that the RhoA/ROCK pathway has important roles in renal fibrosis in models of both type 1 and type 2 diabetes, suggesting that fasudil is also a beneficial adjuvant drug for diabetes and its complications. Moreover, it has been demonstrated that captopril, the angiotensin converting enzyme inhibitor, has anti-hepatic fibrosis effects[23-25]. Kitamura et al [26] showed that the Rho/ROCK is a key enzyme system involved in the angiotensin II signaling pathway of liver fibrosis. In the present work we chose captopril as the positive control agent and investigated the effects of fasudil on preventing the hepatic fibrosis in type 1 diabetic rats, elucidating the possible mechanisms involved.

Materials and Methods

Animals

All animal handling procedures in this study were carried out in strict accordance with the Guidelines of Animal Experiments from the Committee of Medical Ethics at the National Health Department of China. The experiments were approved by the Ethics Committee for Animal Experiments of Hebei Medical University (approval number: HEBMU-2012-02; approval date: Feb. 16, 2012). All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

Male Sprague Dawley (SD) rats aged 5-6 weeks were obtained from the Experimental Animal Center, Hebei Medical University and reared at room temperature with a 12-hour light/dark cycle. The rats were fed with normal rat chow and drank water ad libitum.

Induction of Diabetes and Drug Treatment

Sprague Dawley (SD) rats were randomly divided into normal control and diabetes groups. We used streptozotocin (STZ), a particularly toxic chemical to the insulin-producing beta cells of the pancreas, to induce diabetes. 10 rats with normal rat chow were included into the normal control (NC) group. Other rats received an intraperitoneal injection of 60 mg/kg STZ (Sigma-Aldrich, St. Louis, MO, USA) dissolved in 0.01 M sodium citrate buffer (pH 4.5). Rats should fast for 6 hours prior to STZ induction. STZ-injected rats

were included in the study after confirmation of hyperglycemia (>15 mM glucose, 5 days later) by a portable glucometer (Accu-Chek Active, Roche Diagnostics Limited, Mannheim, Germany).

Subsequently, the diabetic rats were weighted and randomly divided into four groups: untreated diabetic (DM), low-dose fasudil-treated (L-Fas), high-dose fasudil-treated (H-Fas) and captopril-treated (Cap) groups. The rats in the two fasudil (Chase Sun Pharmaceutical Co., Ltd. Tianjin, China) groups received fasudil (2 mg/kg b.i.d., L-Fas group and 10 mg/kg b.i.d., H-Fas group) separately by intraperitoneal injection as described previously[27]. The rats of Cap group were gavaged with captopril (30 mg/kg, Changzhou Pharmaceutical Factory Co., Ltd. Changzhou, China) once a day, whereas the rats of DM and NC rats were treated with intraperitoneal injection of equivalent volumes of normal saline for 8 weeks.

Food iIntake and Activities of the Rats were Observed Carefully Every Day, and the Experiment Lasted for 8 Weeks.

At the end of the experiment, the body weight of each animal was recorded. The rats were anesthetized by intraperitoneal injection of 3% pentobarbital (30 mg/kg) and then sacrificed. Blood (8-10 mL) from each rat's femoral artery was immediately collected and processed into serum for further analysis. The livers of the animals were washed by ice-cold saline solution, weighed and frozen in liquid nitrogen, then stored at -80°C until further analyze.

Assessment of Biochemical Indices in Serum

Alanine aminotransferase (ALT), aspartate aminotransferase (AST) and glycosylated hemoglobin (HbA_1c) levels were measured with commercially available kits (Jian Cheng Biological Engineering Institute, Nanjing, China).

Enzyme-Linked Immunosorbent Assay of Inflammatory Markers

Each hepatic tissue sample was weighed to prepare a 10% (w/v) buffered homogenate (100 mg tissue/mL buffer 50 mM phosphate buffer, pH 7.2) as described before. The supernatant centrifuged from the homogenate was used for biochemical assays[28]. The commercially available kits (Jian Cheng Biological Engineering Institute, Nanjing, China) were used to estimate the concentrations of tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) in the liver.

Histopathology Analysis of Liver Tissue

Liver specimens were fixed in 4% buffered formalin and embedded in paraffin for histological analysis. All samples were cut to 4 μm-thick sections and stained with hematoxylin and eosin (HE). Masson's trichrome staining was to present collagen deposition of the hepatic fibrosis. The area of fibrosis or Prussian blue staining was quantified in a digital microscope system (Olympus Japan Co., Tokyo, Japan) and a true color multi-functional image analysis system (Image-ProPlus6.0, Media Cybernetics, Rockville, MD, USA). 50 randomly selected fields at 400× magnification were taken to analyze with the following formula: % positive area = stained area/ (parenchymal area-luminal area) ×100.

Western Blot Analysis

The bicinchoninic acid (BCA0 protein assay kit (Pierce Chemical Company, Rockford, USA) was used to quantitate the protein concentrations. Tissue homogenates (40 μg of protein) were separated by sodium dodecyl sulphate (SDS)-polyacrylamide gel (5% spacer gel and 10% separation gel) electrophoresis at the constant voltage of 50 V and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). Next, the membranes were washed and blocked with 5% defatted milk powder for 1 hour. As for phosphorylated myosin phosphatase targe (p-MYPT-1) staining, 5% bovine serum albumin (BSA) in Tris-buffered saline and Tween-20 (TBST-20) was performed for blocking instead of with defatted milk powder. After the membranes were incubated with the appropriate primary antibody at 4°C overnight, they were washed and hatched with secondary antibody conjugated to horseradish peroxidase (HRP) for 1 h at room temperature. The blots were then developed with ECL system (Amersham, Piscataway, NJ, USA). The anti-MMP-9, anti-TIMP-1, anti-TGF-β1, anti-NF-κBp65, anti-IκBα, anti-p-MYPT-1 and anti-β-actin antibodies (1:500 dilution) used in this study were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The signals were quantified by densitometry. β-actin was used for as an internal protein control for normalization[29].

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from liver tissues using the Maxwell 16 Tissue LEV Total RNA Purification Kit (Promega, Cat. No. AS1220, Madison, WI, USA). Primers for collagen type Iα (Coll α1), ROCK-

Table I. Upstream and downstream primer sequences for Coll α1, ROCK-I, and GAPDH.

Primer	Sequence
Coll α1-F	CGAAGGCAACAGTCGATTCA
Coll α1-R	GGTCTTGGTGGTTTTGTATTCGAT
ROCK-1-F	GAGCAACTATGATGTGCCTGAAAAT
ROCK-1-R	GATGTCGTTTGATTTCTTCTAC
GAPDH-F	ACCACAGTCCATGCCATCAC
GAPDH-R	TCCACCACCCTGTTGCTGTA

1, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were synthesized by Shanghai Sangon Biological Engineering Technology and Services Co., Ltd., (Shanghai, China) (Table I). Total RNA (0.5 μg) was amplified using the Qiagen One Step RT-PCR kit (Qiagen GmbH, Hilden, Germany). The amplification cycle number was 35. The products were checked by agarose-gel electrophoresis and ethidium bromide staining. Hybridized bands were visualized using UVP gel-imaging system (Thermo Fisher, Waltham, MA, USA). PCR products were standardized against GAPDH.

Statistical Analysis

Results were presented as mean ± standard error of the mean (S.E.M.) based on experiments repeated in triplicate. Multiple comparisons were analyzed using one-way analysis of variance (ANOVA), followed by the least significance difference (LSD) test with Statistical Package for the Social Sciences (SPSS) 19.0 software (IBM, Armonk, NY, USA). p-values < 0.05 were set as statistically significant.

Results

Effects of Fasudil on General State and Liver Function

The general state of the rats in the NC group was normal. Their fur was smooth and had a shiny gloss. They ate and urinated normally. On the other hand, diabetic rats were tired with decreased activity, slow response, and dark/rough fur. The BW was significantly lower than that of the NC group. Similarly, the LW and LW/BW in untreated diabetic rats were significantly increased. The high dose of fasudil increased the BW and decreased the LW/BW compared with diabetic rats (Table II). We used the measure known as hemoglobin A_1C (HbA_1C) to detect the blood sugar according to the American Diabetes Association (ADA). All of the diabetic rats revealed a dramatically upregulation of HbA_1C. In contrast, neither of the fasudil treated groups and captopril treated group showed a significantly altered HbA_1C level, suggesting that both fasudil and captopril had no direct effect on glucose metabolism and insulin resistance (Table III). ALT and AST are representative enzymes that reflect the degree of liver damage. Activities of serum ALT and AST in the DM group were higher than the NC group, suggesting that the high level of glucose resulted in a reduction in liver function. Similar to the protective effects exerted by captopril, fasudil treatment resulted in markedly decreased levels of ALT and AST in serum, and the high dose (10 mg/kg) group appeared more effective than the low dose group (2 mg/kg) (Table III).

Effects of Fasudil on Inflammatory Reaction in the Liver

The results of HE staining showed that the hepatocytes in the DM rats were generally abnormal compared with the NC group. The structure of the liver lobule was destroyed, vacuoles can be found in hepatocytes, and inflammatory cell infiltration was present. Fiber hyperplasia was observed. These alterations were significantly ameliorated in fasudil and captopril treated diabetic rats (Figure 1).

TNF-α and IL-6 in the liver were tested as the inflammatory markers. The concentrations of TNF-α and IL-6 of the DM group had signifi-

Table II. Effects of fasudil on body weights, liver weights, and liver coefficients.

Group	BW (g)	LW (g)	LW/BW (%)
NC	234.89±13.61	9.51±1.09	4.04±0.29
DM	171.13±16.42##	17.94±2.00##	10.48±0.52##
L-Fas	180.63±9.89##	15.66±1.05##**	8.69±0.76##**
H-Fas	194.79±11.55##**	13.56±1.80##**	6.94±0.62##**
Cap	205.16±16.77##**	10.90±1.12#**	5.32±0.48##**

Results are expressed as the mean ± SEM. #p<0.05 vs. NC group; ##p<0.01 vs. NC group; *p<0.05 vs. DM group; **p<0.01 vs. DM group; n=10.
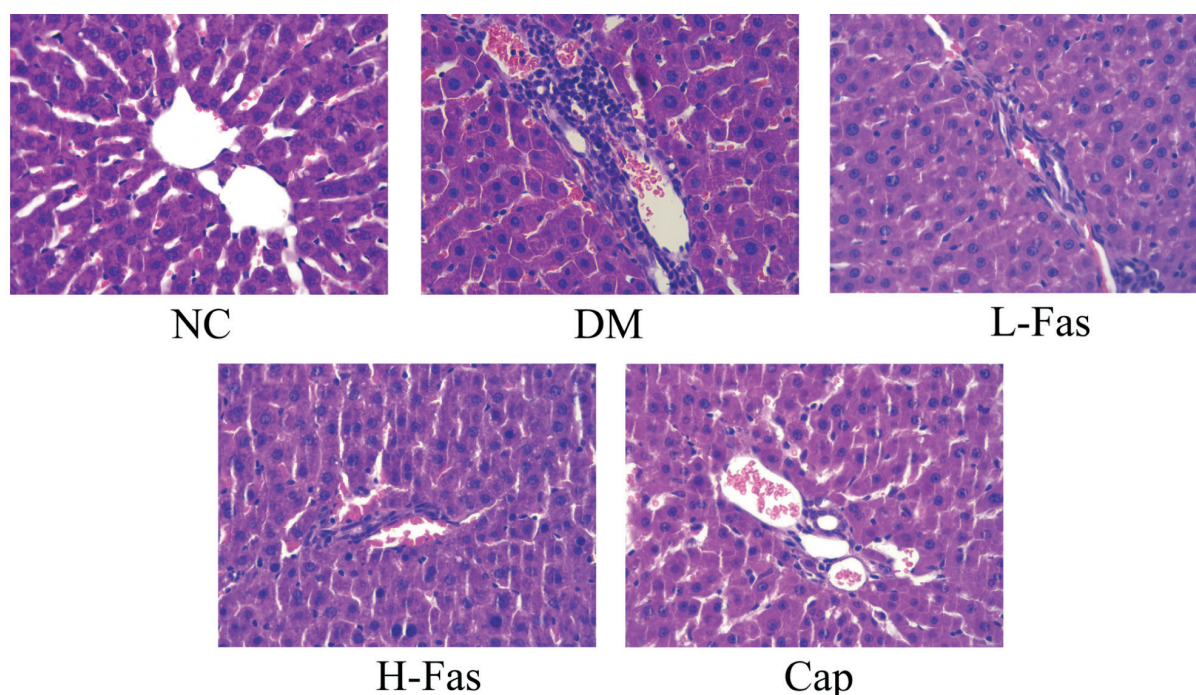
Figure 1. Fasudil-induced histopathological changes in rat livers stained with HE. Representative microscopic photographs of liver stained with HE (400×). Livers were obtained from the normal control (NC), untreated diabetic (DM), low-dose fasudil-treated (L-Fas), high-dose fasudil-treated (H-Fas), and captopril-treated (Cap) groups. Scale bar, 50 μm.

cantly elevated than those of the NC group. Both dosages of fasudil led to remarkable suppression of TNF-α and IL-6 levels, with the high dose (10 mg/kg) more effective (Table IV).

The NF-κB pathway plays a crucial role in inflammatory responses. Under normal circumstances, NF-κB combined with IκB to maintain in the inactive state. In response to specific stimuli, NF-κB needs to first be separated from its inhibitory IκB partner. In our research, the expression of NF-κBp65 in the liver tissues of DM group notably enhanced than that in the NC group, but this upregulation was inhibited by treatment with fasudil (low-dose and high-dose) and captopril

(Figure 4A). The expression of IκB followed a pattern opposite to that of NF-κBp65 (Figure 4B).

Table IV. Effects of fasudil on TNF-α and IL-6 in liver tissue supernatant.

Group	TNF-α (pg/mL)	IL-6 (pg/mL)
NC	15.65±3.15	51.77±7.67
DM	78.14±8.02##	196.16±16.44##
L-Fas	60.35±7.67##**	158.84±15.69##**
H-Fas	31.65±7.13##**	83.70±10.97##**
Cap	40.07±9.47##**	100.66±18.38##**

Results are expressed as the mean ± SEM. ##$p<0.01$ vs. NC group; **$p<0.01$ vs. DM group; n=10.

Table III. Effects of fasudil on serum parameters.

Group	ALT (U/L)	AST (U/L)	HbA1c (%)
NC	15.78±3.34	68.09±8.80	5.55±1.10
DM	47.80±5.02##	121.85±11.15##	18.39±1.41##
L-Fas	30.61±5.58##**	110.36±10.54##*	17.32±0.94##
H-Fas	20.67±4.21#**	84.49±12.52##**	17.41±1.33##
Cap	39.95±6.39##**	109.66±13.28##*	18.00±1.28##

Results are expressed as the mean ± SEM. #$p<0.05$ vs. NC group; ##$p<0.01$ vs. NC group; *$p<0.05$ vs. DM group; **$p<0.01$ vs. DM group; n=10.
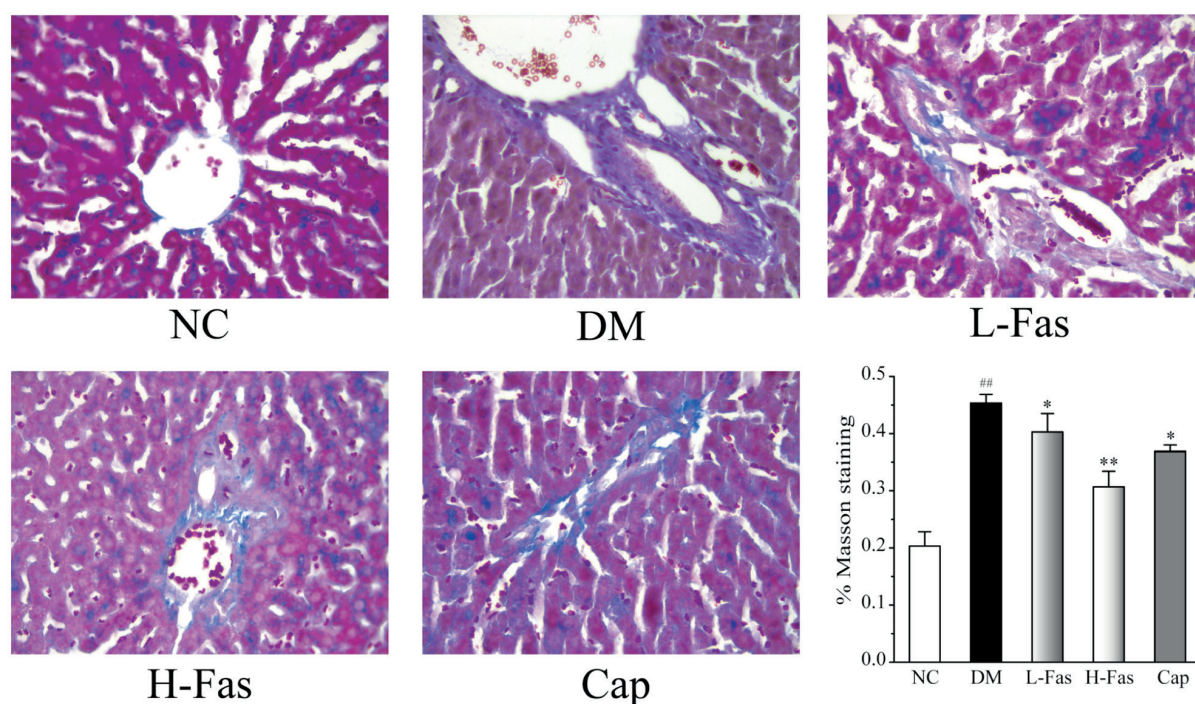
Figure 2. Effects of fasudil onhepatic fibrosis as assessed by staining with Masson's trichrome. Representative microscopic photographs of liver stained with Masson's trichrome (400×). Livers were obtained from the normal control (NC), untreated diabetic (DM), low-dose fasudil-treated (L-Fas), high-dose fasudil-treated (H-Fas), and captopril-treated (Cap) groups. Scale bar, 50 μm. The average optical density (IOD) values were observed using the Image-Pro Plus Image analysis software. #$p<0.05$ *vs.* NC group; ##$p<0.01$ *vs.* NC group; *$p<0.05$ *vs.* DM group; **$p<0.01$ *vs.* DM group; n=10.

Effects of Fasudil on the Formation of Liver Fibrosis

Liver biopsy is the gold diagnosed method to detect the changes in collagens indicative of fibrosis development. Histologically, we employed Masson's trichrome staining to present the distribution of collagen fibers (bright blue) in the background of the liver (dark purple and pink). Compared with the NC group, the livers of DM rats developed typical hepatic fibrosis, which revealed rapid increases of fibrotic tissue area (Figure 2). Cord-like collagen fibers could be found in the disrupted lobular areas in the liver sections of DM group. Treatment with fasudil at both doses significantly ameliorated these alterations. Meanwhile, fibrotic tissue areas in the Cap group were significantly lower than that in the DM group.

To further assess the level of collagens mediated by high glucose, we detected the expression of Coll α1. Diabetes markedly increased the expression levels of Coll α1 mRNA. Furthermore, fasudil treatment at either the high dose or low dose markedly reduced the gene expression of Coll α1 as compared to the DM group, suggesting a compensatory mechanism to prevent fibrosis (Figure 3D).

TGF-β1 and MMP-9, contributing to the pathogenesis of fibrotic disorders, associated with fibrosis extent closely. Western blot analysis showed that diabetes dramatically increased the expression levels of TGF-β1 (Figure 3A) and TIMP-1 (Figure 3C), and decreased the expression of MMP-9 (Figure 3B) in a statistically significant manner, indicating an increase of fibrosis of the liver. However, treatment with fasudil prominently reduced TGF-β1 and TIMP-1 levels in the livers of rats, and the expression of MMP-9 was enhanced correspondingly, with the effects of the high dose being greater than those of the low dose.

Effects of Fasudil on RhoA/ROCK in the Liver

We detected the expression quantity of phosphorylated myosin phosphatase target (p-MYPT1) at Ser-695 of myosin light chain (MLC) to assess ROCK activity in liver tissue. The expression of p-MYPT-1 (Figure 4C) and ROCK-1 (Figure 4D) mRNA were both prominently elevated in the DM group compare to the NC group, suggesting that the RhoA/ROCK

pathway was activated in the diabetic livers. However, the treatment with fasudil resulted in a remarkably reduction in p-MYPT-1 and ROCK-1 expression in a dose-dependent manner.

Discussion

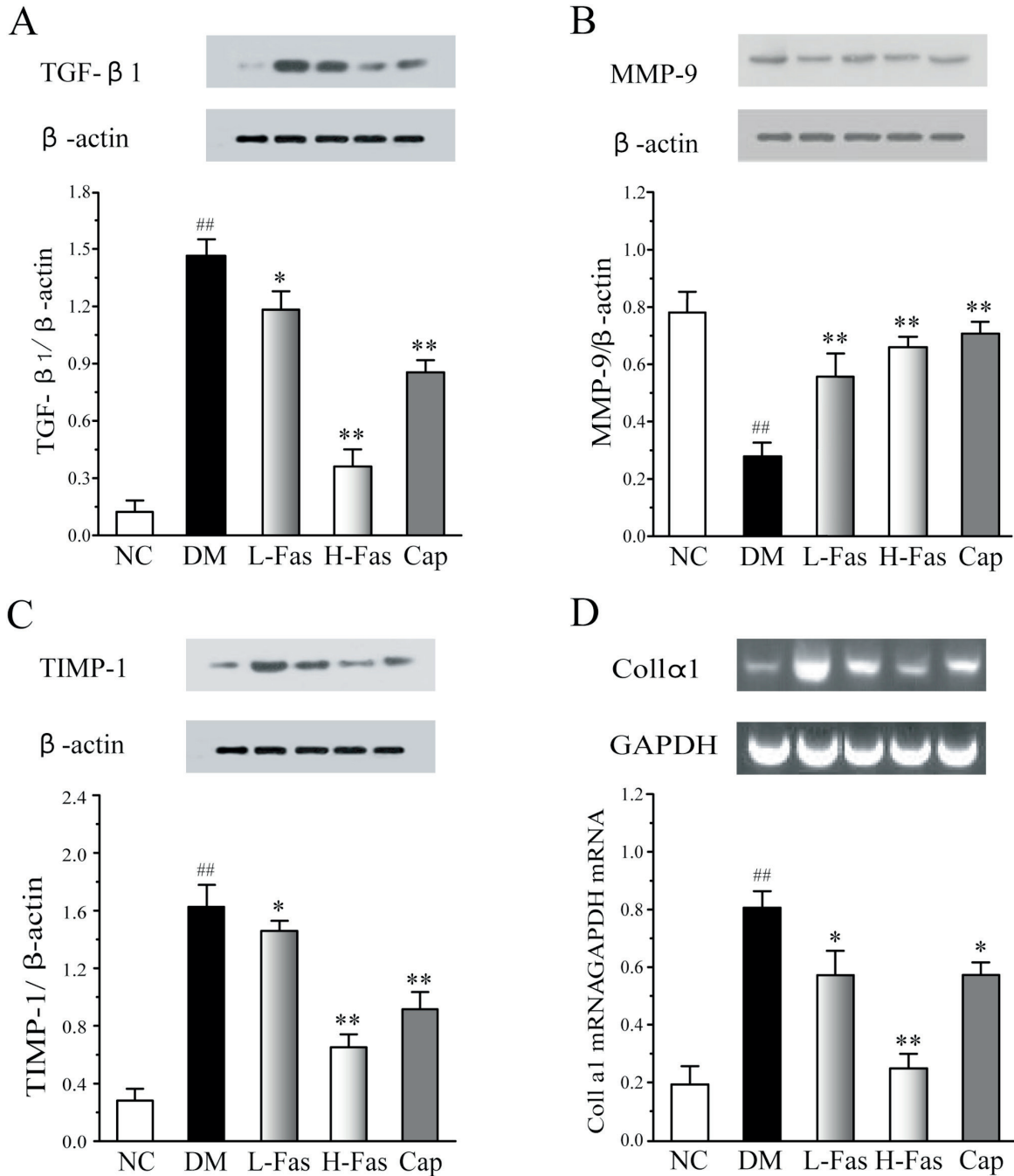In this investigation, the LW and LW/BW values in untreated diabetic rats were significantly

Figure 3. Effects of fasudil on TGF-β1, MMP-9, TIMP-1 protein and Coll α1 mRNA levels in rat livers. Expression of TGF-β1 (**A**), MMP-9 (**B**), TIMP-1 (**C**) protein, and Coll α1 (**D**) in livers obtained from the normal control (NC), untreated diabetic (DM), low-dose fasudil-treated (L-Fas), high-dose fasudil-treated (H-Fas), and captopril-treated (Cap) groups. Intensities of target proteins were standardized to that of β-actin / GAPDH. The proportion showed in the histogram below the target bands. Results are expressed as the mean ± SEM. ##$p<0.01$ *vs.* NC group; *$p<0.05$ *vs.* DM group; **$p<0.01$ *vs.* DM group; n=10.
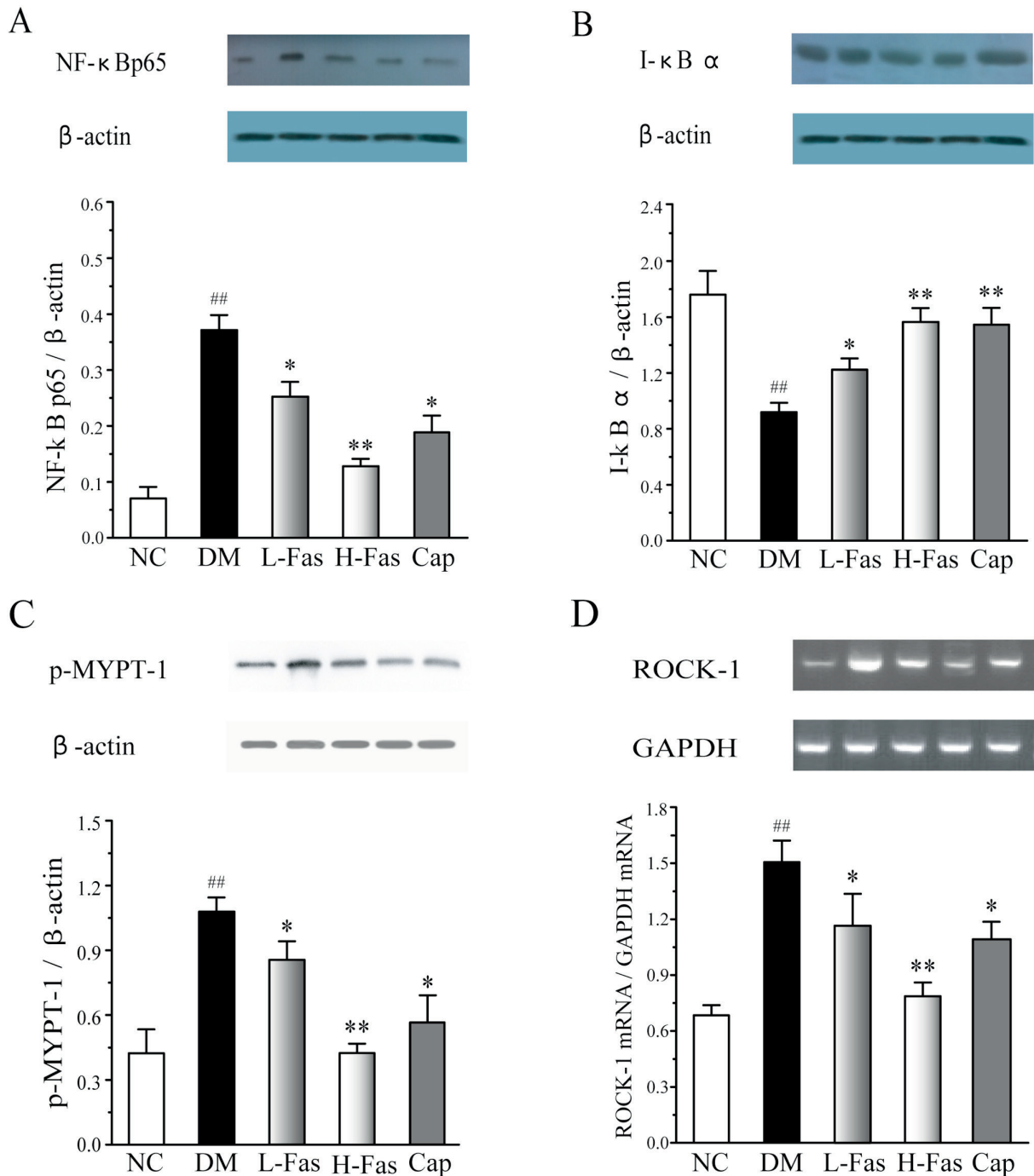
A

B

C

D

Figure 4. Effects of fasudil on NF-κB p65, IκB, p-MYPT-1 protein and ROCK-1 mRNA expression in rat livers treated with fasudil. Expression of NF-κB p65 (*A*), IκB (*B*), p-MYPT-1 (*C*) and ROCK-1 mRNA (*D*) in livers obtained from the normal control (NC), untreated diabetic (DM), low-dose fasudil-treated (L-Fas), high-dose fasudil-treated (H-Fas), and captopril-treated (Cap) groups. Intensities of target mRNAs were standardized to that of β-actin / GAPDH. Results are expressed as the mean ± S.E.M. #*p*<0.05 *vs.* NC group; ##*p*<0.01 *vs.* NC group; *p*<0.05 *vs.* DM group; **p*<0.01 *vs.* DM group; n=10.

increased. The inflammatory cell infiltration was observed, and hepatic collagen accumulation was confirmed by Masson staining. These histopathological characteristics suggested that experimen-

tally induced hepatic fibrosis developed in the diabetic rats, concomitant with the development of abnormal liver function. Treatment with fasudil led to the reversion on both structure and fun-

ction of diabetic liver. The results demonstrated that RhoA/ROCK signaling pathway activation played an important role in the development of diabetic hepatic fibrosis. Administration of fasudil, a specific ROCK inhibitor, exerted anti-inflammation actions, markedly decreased collagen synthesis and postponed hepatic fibrosis.

A growing number of studies have implicated that diabetes is associated with an exacerbated inflammatory process. In clinical studies, patients with high glucose also have liver inflammation and hepatic fibrosis. In the three steps of activation model put forward by Gressner et al[30], the activation of former inflammatory factors of TNF-α and IL-6 can further prompt kupffer cells to release TGF-β1 and TNF-α to necrosis area, thus promoting the transformation of HSC into myofibroblast, and for the development of liver fibrosis. Many studies[31-33] suggest that ROCK play a key role in the TNF-α induced inflammation in diabetes. Our findings indicated that the levels of

inflammatory makers (TNF-α and IL-6) markedly elevated in the DM group with the development of liver fibrosis. Treatment with fasudil led to significant reduction of TNF-α and IL-6. These data indicated that fasudil result in the alleviation of the liver inflammation in diabetic rats through inhibiting ROCK activity.

It is recognized that NF-κB is a vital mediator in the inflammatory processes. The activation of NF-κB can results in the up-regulation of inflammatory (TNF-α, IL-1 and IL-6) and TGF-β1. Otherwise, those cytokines, as stimulus signals, activate NF-κB in return, thus aggravate inflammation[20,21]. Previous researches[34-37] have shown NF-κB pathway can be activated by hyperglycemia. Our study also showed that the expression of NF-κBp65 was up-regulated in the diabetic rats, while the expression of I-κB was reduced. Several researches[38,39] reported RhoA can activate NF-κB in murine NIH-3T3 fibroblasts and simian COS-7 fibroblast-like cells. Our previous[18,19] studies
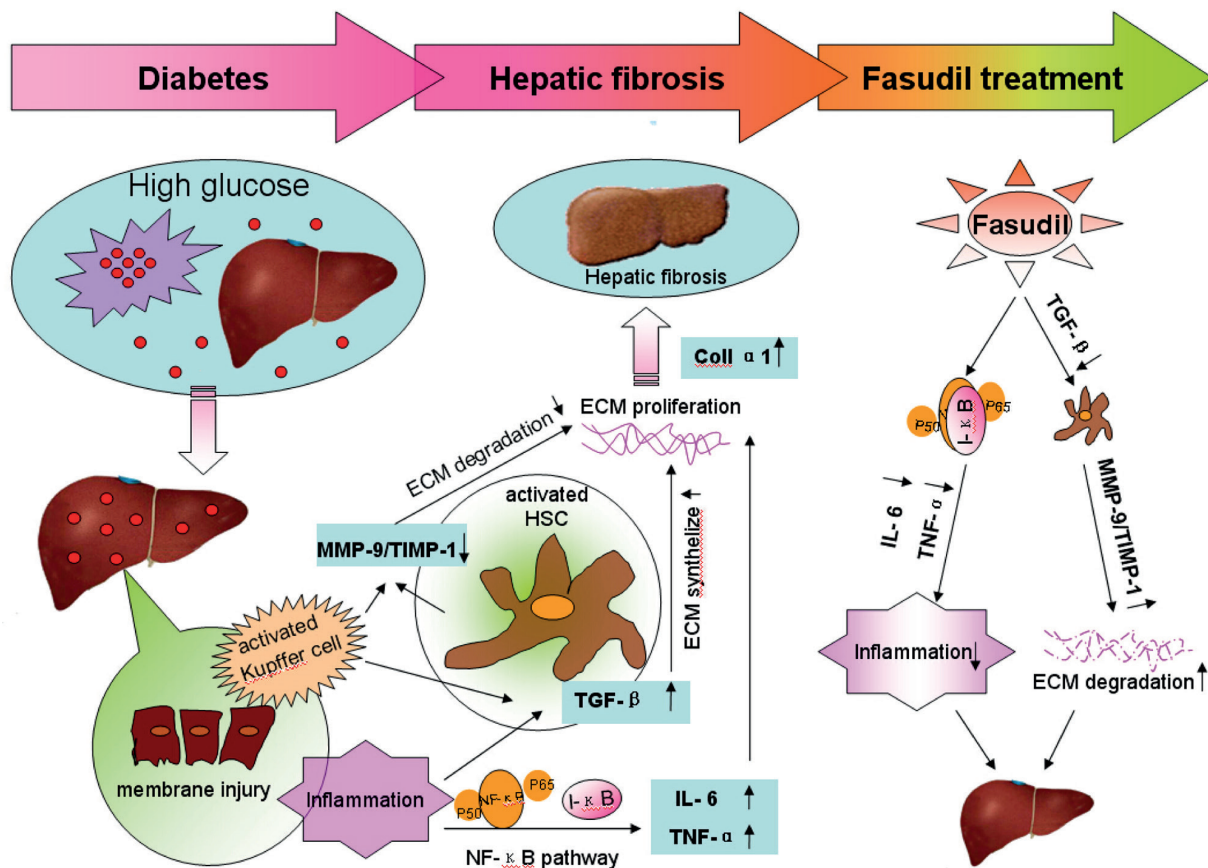

Figure 5. Multitargeted effects of fasudil treatment on hepatic fibrosis in diabetes. Proposed signaling pathways involved in the protective effects of fasudil on hepatic fibrosis in type 1 diabetes. RhoA/ROCK is activated by high glucose and its activation is essential for hepatic fibrosis in diabetes.

demonstrated that fasudil, the highly selective ROCK inhibitor, has anti-inflammatory and anti-oxidant effects through its modulatory activity on NF-κB activation in high cholesterol diet-induced hypercholesterolemic rats. In this work, we also observed that fasudil acted antagonistically through the NF-κB signaling pathway to ameliorate the inflammatory reaction in diabtic rats.

TGF-β1 is the most important activation factor that can promote hepatic fibrosis. In experimental and human hepatic fibrosis, expression of TGF-β1 is observably increased and stimulates pro-collagen I mRNA expression and collagen synthesis in cultured HSCs[40,41]. TGF-β1 can also inhibit the generation of collagen enzyme and MMPs[42], promote the secretion of TIMP-1[43,44] and PAI-1, and reduce ECM degradation, which enable a large sedimentary of ECM in the liver and cause of liver fibrosis, eventually lead to cirrhosis of the liver[45]. MMPs are a family of ECM degradation enzymes, of which MMP-9 is an important member. MMP-9 is also known as gelatinase B and degrades type IV collagen. TIMP-1, which acts as the key endogenous inhibitor of most MMPs, plays a crucial role in the pathogenesis of hepatic fibrosis. It is reported that mutant matrix metalloproteinase-9 (mMMP-9) enhances ECM degradation by neutralizing the elevated TIMP-1 in hepatic fibrosis. Our previous investigations[46,47] have confirmed that liver fibrosis reduced the expression of MMP-9, MMP-13 and increased the expression of TIMP-1. In the current study, we found that the expression levels of TGF-β1 and MMP-9/TIMP-1 were significantly higher in rat livers with diabetes. The histopathology changes and the high expression of Coll α1 in diabetic rats revealed these animals developed hepatic fibrosis. Notably, our data indicated that fasudil significantly ameliorated the elevation of these indexes above. This suggested that ROCK inhibition could reduce the production of collagen and promote degradation of ECM to exert the protective effect of hepatic fibrosis.

Previous studies[21,22,48-50] have suggested that Rho-ROCK proteins can be stimulated by high glucose and other components of the diabetic milieu. Moreover, some hormones, cytokines, and physical forces implicated, such as angiotensin II[51], aldosterone, vascular endothelial growth factor[52,53], TGF-β[54,55], and mechanical stress[56] can be activated by the Rho-ROCK pathway in the pathophysiology of diabetic complications. In this study, up-regulated ROCK-1 and elevated p-MYPT1 were observed in diabetic rat livers in

the process of increased inflammation, collagen synthesis, and hepatic fibrosis. Meanwhile, Fasudil inhibited not only the activation of ROCK but also the inflammation and the accumulation of ECM in a dose-dependent manner in hepatic fibrosis with diabetes. We also found that fasudil has little effect on blood glucose levels, which agrees with other reports[57,58]. We deduce that the effects of fasudil in inhibiting hepatic fibrosis may be mediated at least in part by blocking the RhoA/ROCK pathway, as indicated by the suppression of the inflammation and the accumulation of ECM.

Conclusions

We showed that RhoA/ROCK pathway participate in the process of hepatic fibrosis with type 1 diabetes. Fasudil, the ROCK inhibitor, could prevent hepatic fibrosis in diabetic rats both through reducing the production of inflammatory factors to alleviate the inflammation and inhibiting the accumulation of ECM (Figure 5). Activation of RhoA/ROCK pathway, TNF-α/IL-6-NF-κB pathway and TGF-β1-MMP-9/TIMP-1 pathway might be involved in the potential mechanisms. Therefore, blockade of the ROCK pathway may be a therapeutic option for treating hepatic fibrosis in diabetes

Conflict of Interest
The Authors declare that they have no conflict of interest.

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