Abstract. Myocardial fibrosis and excessive proliferation of cardiac fibroblasts (CFs) contribute to diabetic cardiomyopathy (DCM). However, the underlying mechanism is still not completely clear. The aim of this study was to investigate the relationship between high-glucose treatment and the expression of visfatin and type I procollagen in rat CFs, and examine the regulatory effects of high-glucose treatment on the Rho/ROCK signaling pathway. CFs from newborn Sprague Dawley rats were treated with high concentrations of glucose (10, 30 and 50 mmol/l D-glucose), a baseline concentration of glucose (5.5 mmol/l D-glucose + 44.5 mmol/l mannitol) as an osmotic control. CFs were also treated with 30 mmol/l D-glucose for 6, 12, 24 and 48 h. The proliferation of CFs was determined by the MTT assay. The mRNA and protein expression of visfatin and type I procollagen were quantified by RT-qPCR and western blot analysis, respectively. Cardiac fibroblast proliferation reached a peak at 30 mmol/l D-glucose, and visfatin and type I procollagen expression were significantly increased upon treatment with high concentrations of glucose (10 and 30 mmol/l) compared to baseline glucose treatment. Treatment with 30 mmol/l D-glucose time-dependently promoted cardiac fibroblast proliferation. The mRNA and protein expression of visfatin and type I procollagen were significantly increased compared to the control at 24 h after 30 mmol/l D-glucose treatment. Y27632, a Rho-associated protein kinase (ROCK) inhibitor, significantly decreased the mRNA and protein levels of visfatin and type I procollagen, induced by 30 mmol/l D-glucose (all P<0.05). In conclusion, a high level of glucose promotes cardiac fibroblast proliferation, and induces visfatin and type I procollagen expression in CFs, at least partially via the Rho/ROCK signaling pathway. These results may be helpful in developing an appropriate therapeutic strategy for DCM.

Introduction

Diabetic cardiomyopathy (DCM) is a heart failure disorder arising as a complication of diabetes mellitus; it is described as a number of functional and structural changes in the heart due to diabetes in the absence of other cardiac pathologies (1). Since the prevalence of diabetes increases worldwide, estimated to affect approximately 5% of the global population by 2025 (2), the mortality due to DCM is also likely to increase. In patients with diabetes, myocardial damage involves both myocardial and cardiac interstitial cell damage. Most research has concentrated on the damage to cardiac cells (3,4). Myocardial fibrosis, excessive proliferation of cardiac fibroblasts (CFs), and oversecretion and overexpression of collagen, are considered the major pathological changes resulting from DCM. These changes are associated with the risks of deterioration of the cardiac function and cardiac decompensation (5,6).

The expression of numerous growth factors is higher in patients with diabetes compared to healthy subjects. A newly discovered adipocyte factor, visfatin, regulates blood sugar levels, as well as insulin secretion (7). The plasma levels of visfatin are associated with a wide range of diseases, including cardiovascular diseases, endothelial dysfunction, insulin resistance, and the occurrence and progression of diabetes. Visfatin also plays a role in vulnerable plaque formation and vascular proliferation and inflammation, by exerting an insulin-like effect (8), facilitating adipocyte differentiation, inhibiting cell apoptosis, and promoting cell maturation and proliferation as a pro-inflammatory factor (9,10). It has been suggested that visfatin is overexpressed in obese patients and in patients with diabetes, causing myocardial fibrosis, causing an increase in extracellular matrix synthesis, or even leading to heart failure (11-13). However, there are few studies on visfatin expression in cardiac cells. To our knowledge, the high glucose-induced expression of visfatin in CFs and visfatin synthesis in vitro have not been reported to date.

Interstitial fibrosis is an important factor in the occurrence and progression of DCM. Myocardial fibrosis in DCM usually leads to ventricular diastole and contractile function disorders, and ultimately to congestive heart failure (14). Myocardial fibrosis is tightly linked to the high incidence and mortality.
associated with DCM. Sufficient knowledge of the pathogenesis of DCM is the basis for appropriate clinical treatment. Collagen is the major component of the extracellular matrix, and the ratio of type I to type III collagen indicates the severity of myocardial fibrosis. Increased blood sugar levels induce the synthesis and secretion of myocardial interstitial collagen. Type I collagen, which accounts for the biggest proportion of total collagen, is more closely related to myocardial stiffness than type III collagen. Patients with diabetes are commonly at higher risk of myocardial fibrosis and greater ventricular stiffness (15). A study of the signal transduction pathways involved in myocardial fibrosis is the basis for the identification of therapeutic targets for DCM.

The Rho/Rho-associated protein kinase (ROCK) signaling pathway has been shown to be an important pathway in hypertension, cerebral apoplexy, and chronic heart failure (16-18). The Rho/ROCK signaling pathway also regulates contraction, adhesion, proliferation, and apoptosis of cells. Dysfunctions of this pathway are predicted to lead to cell function disorders and pathological changes. The Rho/ROCK signaling pathway is implicated in chronic fibrosis of the liver and kidneys (19,20). Previous studies showed that the ROCK pathway is also activated in the cardiac cells of rats with diabetes (21,22). The expression of the Ras homolog gene family, member A (RhoA) protein was upregulated and its activity was enhanced, resulting in increased polymerization of the actin cytoskeleton. Rho kinase exists in the form of two isomers, ROCK1 (or ROCKα) and ROCK2 (or ROCKβ), which are expressed in vascular smooth muscle cells and cardiac cells. The ROCK2 mRNA is predominately detected in the brain and skeletal muscle. It has been suggested that ROCK1 plays a key role in pathological fibrosis of the organs, whereas ROCK2 is related to organ hypertrophy (23,24). Other studies found that ROCK not only increases the polymerization of the actin cytoskeleton, but also induces the expression of fibrosis-related genes and proteins (19,23,25,26). Phrommintikul et al (23) confirmed in the animal model of stress-induced cardiac hypertrophy that the ROCK inhibitor reduces the synthesis of cardiac collagen, and improves the diastolic function.

This study investigated visfatin and type I procollagen expression, following high-glucose treatment at different concentrations and for different durations, in CFs from newborn rats, and examined the effects of these treatments on the Rho/ROCK signaling pathway. Our results highlight a mechanism involved in DCM that may be targeted in the context of new therapeutic interventions.

Materials and methods

Animals. Clean-grade, male and female Sprague Dawley (SD) rats, aged 2 to 3 days, were provided by the Experimental Animal Center of the Hebei Medical University. The experiments were carried out in accordance with the Hebei Animal Management Regulations and all procedures and animal experiments were approved by the Animal Ethics Committee of the Hebei Medical University. The rats were housed before the experiment in room temperature and humidity controlled cages with a 12-h day-night cycle. No narcotics were used to avoid potential influences on the experimental data.

Primary culture of CFs. The newborn SD rats were soaked with 70% alcohol for 10 sec, and then, sterile eye surgery scissors were used to cut the anterior third of the ventricular portion of the heart, which was placed on a Petri dish filled with 5 ml D-Hanks solution precooled at 4°C. The ventricular muscles were cut into pieces of ~1-3 mm, and then washed twice to remove blood cells. The non-adherent myocardial cells were removed by enzymatic digestion and the differential adhesion method as in (27). The tissue samples were digested into single cell suspensions with 0.125% trypsin (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and 0.04% collagenase II (Gibco Life Technologies, Carlsbad, CA, USA) at 37°C. The supernatant was collected from each digestion, and an equal volume of low-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) was added. After two centrifugations at 1,200 x g for 5 min, the cells were resuspended, filtered through a 200 mesh sieve, and seeded in 100 mm² Petri dishes at 37°C, in a 5% CO₂ incubator. Non-adherent cardiomyocytes were removed using differential adhesion after 90 min of culture. The cells were cultured in low-glucose DMEM with 10% FBS at 37°C, in a 5% CO₂ incubator. The cells from passages 2 and 3 were used in the following experiments.

Immunocytochemical staining of CFs. The primary CFs were identified by immunocytochemical staining (28) for vimentin and α-smooth muscle actin (α-SMA). α-actin protein levels were evaluated by immunocytochemical examination. Slides were placed into 6-well culture plates and CFs at passages 2-3 (2x10⁵ cells/well) were inoculated into each well. Following attachment of CFs, slides were fixed in 4% paraformaldehyde. Streptavidin-biotin complex immunocytochemical staining for α-actin was performed. The α-actin content of the cells was expressed as positive staining areas/the number of positive cells from 10 random fields for each slide using the Motic Med 6.0 digital medical image analysis system (Motic Incorporation, Ltd., Causeway Bay, Hong Kong).

Study design. The extracted CFs were treated with high levels of glucose (HG groups, 10, 30 and 50 mmol/l D-glucose; Sigma-Aldrich, St. Louis, MO, USA), 5.5 mmol/l of D-glucose (normal glucose or NG group), or 5.5 mmol/l D-glucose and 44.5 mmol/l mannitol (high osmotic pressure or HOP group), and were further cultured for 48 h. The concentration of 5.5 mmol/l D-glucose was used to simulate the baseline sugar level of healthy human blood cells. The concentration of 45.5 mmol/l mannitol (Sigma-Aldrich), combined with 5.5 mmol/l D-glucose was used to establish equivalent osmosis to that caused by 50 mmol/l D-glucose and avoid confusion with the effects of osmosis on the expression of visfatin and type I procollagen in the CFs. The CFs were also treated with 30 mmol/l D-glucose for 6, 12, 24 and 48 h. The CFs were cultured with 10 µmol/l of the ROCK inhibitor Y27632, dissolved in 100% dimethyl sulfoxide (DMSO; both from Santa Cruz Biotechnology, Inc.), or 0.1% DMSO for 30 min, and then induced with 5.5 and 30 mmol/l D-glucose, respectively. They were further cultured in DMEM containing 0.1% FBS for 24 h.
Determination of cardiac fibroblast proliferation by the MTT assay. CFs at the logarithmic phase of growth were seeded at a density of 5x10^5 cells/well (200 µl) in a 96-well plate for 48 h. Blank wells were also prepared with culture medium without cells. After culturing in serum-free medium for 24 h, the cells were treated with different concentrations of D-glucose for 48 h, or 30 mmol/l D-glucose for 6, 12, 24 and 48 h, according to the planned experiments. Next, the CFs were treated with 20 µl of 5 mg/ml HyClone™ 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Thermo Fisher Scientific, Logan, UT, USA) for 4 h. DMSO (150 ml) was added to each well, and the plate was agitated gently for 15 min. The absorbance was measured at 490 nm using the Biochrom Anthos Zenyth 340rt microplate reader (Biochrom Ltd., Cambridge, UK).

RNA extraction and determination of visfatin and type I procollagen mRNA expression by reverse transcription-quantitative PCR (RT-qPCR). The primers for the qPCR amplification of visfatin, type I procollagen and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were designed by GeneCopoeia (catalog nos. RQP053337, RQP054226 and RQP049537, respectively; Guangzhou, China). Total RNA was extracted from CFs using the TRIzol reagent (SBS Genetech Co, Ltd., Beijing, China), and the concentration and purity of the extracted RNA were measured using a spectrophotometer (Beckman Coulter, Miami, FL, USA). The integrity of RNA was assessed by electrophoresis on 1% agarose gel. Total RNA was reversetranscribed into cDNA using oligo(dT) primers (Invitrogen Life Technologies, Carlsbad, CA, USA). mRNA quantification was performed by qPCR using the All-in-One™ qPCR mix (GeneCopoeia) in an IQ5 Real-Time PCR system (Bio-Rad, Hercules, CA, USA), according to the guidelines provided by the manufacturer. Forty cycles were run with the following parameters: 95°C for 10 sec, 60°C for 20 sec, and 72°C for 15 sec. The dissociation curve was analyzed immediately after qPCR. The mRNA expression level of the treated group was calculated relative to that of the control group using the ΔΔCt method (29), with the 2^(-ΔΔCt) calculated based on the formula: ΔΔCt = (Ct target gene-Ct GAPDH)treated group - (Ct target gene-Ct GAPDH)control group. Where Ct denotes the threshold cycle.

Determination of visfatin, type I procollagen, and ROCK1 protein expression using western blot analysis. The CFs were lysed with RIPA buffer (Promega, Madison, WI, USA), and total protein was extracted by centrifugation (4°C, 12,000 x g, 10 min). Proteins were quantified using the Coomassie blue method according to the manufacturer's instructions (Life Technologies). Equal amounts of protein (80 µg) were loaded and separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis. The proteins were electroblotted onto a polyvinylidene difluoride membrane (EMD Millipore, Bedford, MA, USA). Next, the non-specific sites on each blot were blocked with 5% non-fat milk diluted in TBS with 0.05% Tween-20 (TBST) for 1 h. The membrane was then incubated at 4°C overnight with the primary antibodies goat anti-rat anti-visfatin and -procollagen type I (both from Santa Cruz Biotechnology, Inc.); rabbit anti-rat anti-ROCK1 (Abbiotec, San Diego, CA, USA); and rabbit anti-rat anti-GAPDH (Santa Cruz Biotechnology, Inc.). The membranes were washed for 30 min with TBST at room temperature, and then incubated with the secondary antibody (horseradish peroxidase-labeled IgG) for 1 h. The chemiluminescence detection of the antibody complex was carried out with the chemiluminescence kit (Jei Daniel Biotech Corp., Shandong, China) and the Odyssey 9120 imaging system (LI-COR, Lincoln, NE, USA). The Gel-Pro Analyzer 3.1 (Media Cybernetics, Rockville, MD, USA) was used to measure the absorbance. Protein expressions were normalized to that of GAPDH.

Statistical analysis. All data were statistically analyzed using the SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). The data were expressed as means ± standard deviation (SD). Statistical significance was evaluated by one-way analysis of variance (ANOVA) with Student-Newman-Keuls (SNK) test for post-hoc analysis. The significance level was set at P<0.05.

Results

Identification of CFs. Immunocytochemical staining was positive for vimentin (Fig. 1A), and negative for α-SMA (Fig. 1B). The final purity of CFs was ~95%, as indicated by the vimentin-positive status of the cells.

Figure 1. Identification of cardiac fibroblasts (CFs). (A) Immunocytochemical staining for vimentin and (B) α-smooth muscle actin (α-SMA) in the CFs (magnification, x200). Arrow, vimentin-positive expression.
Effect of glucose concentration on the proliferation of CFs. Cardiac fibroblast proliferation was reflected by the absorbance value measured in the MTT assay. When the cells were treated with different concentrations of D-glucose for 48 h, the absorbance values of the 30 and 50 mmol/l D-glucose treatment groups were significantly higher compared to the 5.5 mmol/l D-glucose treatment group, serving as the control (both P<0.01). The highest absorbance value was observed in the 30 mmol/l D-glucose-treated group, while the absorbance value of the 50 mmol/l D-glucose-treated group was slightly lower; no significant difference was identified between these two groups (P>0.05). Compared to the control group, the absorbance value of the high osmotic group was not significantly different (P>0.05) (Fig. 2A).

Effect of glucose concentration on mRNA and protein expression of type I procollagen and visfatin in CFs. When the cells were treated with different concentrations of D-glucose, the mRNA and protein expression of type I procollagen was increased in a dose-dependent manner (Fig. 2B and C). The protein expression of the 10, 30 and 50 mmol/l groups were significantly different (0.398±0.008, 0.792±0.014, 0.875±0.023 and 0.261±0.007, respectively, all P<0.01) from that of the control group (NG, 5.5 mmol/l). The expression of type I procollagen in the high osmotic pressure group was not significantly different from that of the control group (0.267±0.005, P>0.05) (Fig. 2C).

All high-glucose treatments (10, 30 and 50 mmol/l) were accompanied by significantly increased (all P<0.01) mRNA and protein expression of visfatin compared to the control group (Fig. 2D and E). The 30 mmol/l group reached the highest level of protein expression (0.339±0.008), the 50 mmol/l group had a slightly lower level of protein expression (0.271±0.0055), whereas the difference between these two concentrations was not significant (P>0.05). The protein expression of visfatin in the high osmotic pressure group (0.205±0.0049) was not significantly different from that of the control group (P>0.05) (Fig. 2E).

Effect of the duration of high-glucose treatment on the proliferation of CFs. When the cells were treated with 30 mmol/l D-glucose for different durations (6, 12, 24 and 48 h), the absorbance values increased with treatment time (Fig. 3A).

Effect of high-glucose treatment duration on mRNA and protein expression of type I procollagen and visfatin in CFs. For all treatment durations (6, 12, 24 and 48 h) with 30 mmol/l D-glucose, the mRNA and protein expression of type I procollagen were significantly decreased (all P<0.01) compared to the control group (Fig. 3B and C). The protein expression of
type I procollagen decreased in a time-dependent manner until 24 h (3.70±0.39), while its expression was even more decreased 48 h later (3.69±0.42); the difference between 24 and 48 h was however not significant (P>0.05) (Fig. 3C).

For all durations (6, 12, 24 and 48 h) of treatment with 30 mmol/l D-glucose, the mRNA and protein expression of visfatin were significantly decreased compared to the control group, except for 6 h (Fig. 3D and E). As shown in Fig. 3E, the protein expression levels of the 12-, 24- and 48 h-treated groups were significantly different from that of the control group (0.282±0.008, 0.340±0.009, 0.335±0.001 and 0.097±0.003, respectively, all P<0.01). The visfatin protein expression level steadily decreased until 24 h and was further reduced after 48 h of treatment, but the difference between these time-points was not significant (P>0.05).

**mRNA and protein expression levels of type I procollagen and visfatin in the presence of a ROCK inhibitor.** Compared to the control group (5.5 mmol/l D-glucose), the high-glucose (30 mmol/l)-treated group had a significantly increased protein expression of ROCK1 (0.323±0.010 vs. 0.160±0.011, P<0.01). Pretreatment with 10 µmol/l Y27632 significantly reduced the ROCK1 protein expression compared to high-glucose treatment (0.153±0.015 vs. 0.323±0.010, P<0.01), but did not cause a significant change (P>0.05) compared to the control group (Fig. 4E).

Pretreatment with Y27632 also significantly reduced type I procollagen mRNA and protein expression induced by high glucose (0.286±0.031 vs. 0.367±0.033 for the protein, P<0.05), and the level of the protein in the HG+Y27632 group was significantly higher compared to the NG group (0.145±0.035, P<0.05) (Fig. 4A and B).

Pretreatment with Y27632 significantly reduced the visfatin mRNA expression level induced by high-glucose treatment (0.907±0.098 vs. 1.675±0.179, P<0.01), but the level of the HG + Y27632 group did not significantly differ (P>0.05) from that of the control group NG + Y27632 (Fig. 4C). Pretreatment with Y27632 also significantly reduced the visfatin protein expression induced by high glucose (0.284±0.005 vs. 0.330±0.007, P<0.01), and the level of the HG + Y27632 group was significantly different (P<0.05) from that of the NG + Y27632 group (0.227±0.005) (Fig. 4D).

**Discussion**

The aim of this study was to enhance our understanding on the pathological mechanism underlying DCM by investigating whether high glucose affects the proliferation of CFs and the expression levels of visfatin and type I procollagen in CFs, and by further investigating whether these changes can be inhibited by Y27632, a ROCK inhibitor. This study showed that high glucose promotes cardiac fibroblast proliferation and
induces visfatin and type I procollagen expression in CFs, and Y27632 inhibited these effects.

Visfatin may be upregulated as a compensatory mechanism to reduce high blood sugar levels. Previous studies showed that visfatin promotes the proliferation of vascular smooth muscle cells and endothelium (9,10) and facilitates the synthesis of type I collagen (28,30). There are relatively few studies on visfatin expression in cardiac tissues and the pathological and physiological involvement of visfatin in myocardial fibrosis in DCM remains a disputed issue.

The CFs used in this study were extracted from newborn rats. This was to avoid the interference of the in vivo environment. qRT-PCR and western blot analysis showed that the CFs of rats express visfatin. The high glucose concentration affected the synthesis of visfatin and type I procollagen, which may further cause insulin resistance and myocardial fibrosis in patients with diabetes and DCM. Three different concentrations of glucose (10, 30 and 50 mmol/l) were prepared for the high-glucose treatment and administered to the rats. The glucose concentration in the control group was set at 5.5 mmol/l, which is the baseline blood sugar level, and then induced with 5.5 (normal glucose; NG) and 30 mmol/l D-glucose (high glucose; HG) for 24 h, respectively. A, Type I procollagen mRNA and (B) protein expression were determined by reverse transcription-quantitative PCR and western blot analysis, respectively. Visfatin (C) mRNA and (D) protein expression, and (E) ROCK1 protein expression. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. The shown data represent means ± standard deviation. *P<0.05 and **P<0.01, vs. NG groups; *P<0.05 and **P<0.01, HG + Y27632 group vs. HG group.

In order to develop a potential therapeutic target for DCM, it is important to identify the relevant signaling pathways. We hypothesized that the Rho/ROCK signaling pathway may be involved in the increase in visfatin and type I procollagen levels, since previous studies have shown a role for this pathway in chronic fibrosis of the liver and kidney (19,25), and that it is activated in the heart of diabetic rats (21,22). The present study showed that high-glucose treatment increased the expression...
leakage. Overall, these results will contribute to the future development of therapeutic agents for the treatment of DCM.

References