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Repression of autophagy in diabetic cardiomyopathy via RhoA/ROCK2 signaling pathways

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Activated RhoA and ROCK is associated with many cardiovascular diseases (CVD) such as congestive heart failure (CHF), atherosclerosis and hypertension. However, the role of RhoA/ROCK2 signaling pathway in initiating diabetic cardiomyopathy (DCM) has not been fully elucidated. Here, we studied the role of RhoA/ROCK2 signaling pathway in induction of DCM through autophagy suppression in diabetic rat animal models. Broadly, we investigated the potential role and mechanism of diabetes induced myocardial dysfunction in rats. DCM was induced by injections of streptozocin (STZ) in experimental Wistar rats. The experimental rats were randomized to be treated with fasudil and lentivirus carrying the RhoA cDNA. Haemodynamic changes, assessment of cardiac weight index, histopathological examinations, cardiomyocyte autophagy and expression of RhoA and ROCK2 mRNAs were compared between groups. The expression of RhoA and ROCK mRNAs was found significantly increased in cardiac tissues compared with control group. The RhoA overexpression significantly decreased the values of left ventricular ejection fraction (LVEF), $\pm dp/dt_{max}$ and repressed autophagy. RhoA/ROCK2 signaling pathway repressed autophagy in diabetic cardiomyopathy indicating that it may serve as a potential therapeutic target for DCM treatment.

Keywords: CVD, Diabetes, LVEF, RhoA/ROCK2 signaling, Streptozocin

Diabetic cardiomyopathy (DCM) has been defined as the cardiac dysfunction in diabetic patients in the absence of coronary artery disease, hypertension or changes in blood pressure[1]. DCM, as a chronic complication of diabetes mellitus, is highly characterized by abnormalities in both myocardial structure as well as function[2]. The main clinical features of DCM are decreased values of left ventricular ejection fraction (LVEF), cardiomyocyte hypertrophy and myocardial fibrosis[3]. Other hallmarks of DCM include hyperglycemia, insulin resistance, excessive free radical production, mitochondrial and endothelial dysfunction and cell death, and cell death is considered to be the terminal pathway of cardiomyocytes during DCM[2,4,5]. Dysregulation in cardiovascular and reproductive systems along with nephropathy, retinopathy, neuropathy and diabetic foot ulcer may arise in the advanced stages of diabetes. Furthermore, high glucose level also encourages proliferation of cancer cells, development of osteoarthritis and facilitates multiple infections[6]. Fan *et al*.[7] found that glucagon-like peptide 1 (GLP-1), a glucagon incretin hormone released from the gut endocrine L-cells, might reverse myocardial hypertrophy through the PKA/RhoA/ROCK2 signaling pathway.

While investigating the role of cardiomyocyte RhoA and of ROCK2 in DCM development, the effects of heterozygous deletion of ROCK2 (ROCK2+/-) on cardiac function in a CD1 mouse model of type 1 diabetes induced by streptozotocin (STZ) showed that deletion of ROCK2 and cardiomyocyte RhoA protects the diabetic heart[8]. Further, Shimokawa *et al*.[9] suggest that Rho-kinase is substantially involved in the pathogenesis of a wide range of cardiovascular diseases, and Rho-kinase inhibitors may be useful for treating cardiovascular diseases (CVD)[9].

Morphologically, cells death may occur due to apoptosis, autophagy, necrosis or entosis. However, the specific mechanism of DCM has not been fully elucidated, and there is no effective treatment available for treating DCM in diabetic patients. Autophagic cell death is a degradation process to remove damaged proteins and dysfunctional organelles initiated by the formation of auto-phagosomes[2]. Small guanosine triphosphate (GTP)

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binding protein Ras homolog gene family member A (RhoA) and its effector Rho-associated kinase (ROCK) play a significant role in cardiovascular systems. RhoA has been reported to regulate cell functions such as cell movement, autophagy and apoptosis[10], while as ROCK is one of the most important effectors of RhoA, accumulating evidence has demonstrated that RhoA/ROCK2 signaling pathway is implicated in hypertension, cerebral vasospasm, heart failure, pulmonary hypertension, atherosclerosis, hemangioma, ischemia reperfusion injury and so on[11,12]. There is growing evidence that the ROCK pathway plays an important role in the development of cardiovascular diseases and that inhibition of ROCK activity by selective ROCK inhibitors would be beneficial in treating cardio-vascular diseases[13].

ROCK1 is preferentially expressed in the liver, lung, kidney, spleen and testes, whereas ROCK2 is most highly expressed in the heart and brain, and ROCK2 acts as a prominent regulator of autophagic flux and caspase-3 activities in neurodegenerative diseases[14]. Moreover, the RhoA/ROCK2 pathway is implicated in DCM and acute inhibition of ROCK2 improves contractile function of hearts in type 1 diabetic rats both *in vitro* and *in vivo*[15]. Activation of RhoA/ROCK2 signaling pathway has been found to play a critical role in the development of DCM, and the ROCK2 might be a significant therapeutic target for treating DCM condition[16].

Lin28, a highly conserved RNA-binding protein, protects against DCM through PKA/ROCK2 dependent pathway. Its overexpression significantly improves left ventricular ejection fraction (LVEF), and promotes autophagy in diabetic mice, and might serve as a potential therapeutic target[17]. Though the RhoA/ROCK2 signaling pathway is known to play an important role in DCM, its mechanism still remains unelucidated. Hence, in the present study, we tried to understand the role of the RhoA/ROCK2 signaling pathway in induction of DCM through autophagy suppression in diabetic rat animal models.

Materials and Methods

Animal model preparation for *in vivo* experiments

Forty male Wistar rats (200-220 g) were purchased from Beijing Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China). Diabetes was induced by intraperitoneal injection of streptozotocin (STZ, Sigma, St. Louis, MO) dissolved in 0.1 mL citrate buffer (pH 4.5) at 50 mg/kg for 5 days, while the control group was injected with citrate buffer[17]. Blood sample was obtained from all rats through the caudal vein after one week of STZ injections and the values of blood glucose were measured using a reflectance meter (Accu-Chek, Roche Diagnostic, Mannheim, Germany)[17]. The values above 16.6 mmol/L were considered as diabetes. After the induction of diabetes for 8 weeks, the rats were randomly divided into four groups (n=10): (i) Control group; (ii) STZ group; (iii) Fasudil group (fasudil is an inhibitor of RhoA/ROCK2 signaling pathway); and (iv) Over-expression of RhoA group. Lentivirus containing RhoA cDNA (30 µL, 1×10^9 TU/mL) was delivered via three separate intramyocardial injections (designed and purchased from Genechem, Shanghai, China). After 4 weeks, the Wistar rats were sacrificed.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total-RNA from the left ventricular myocardial tissue was extracted using Trizol reagent (1596-026) (Invitrogen Life Technologies, Carlsbad, CA, USA), and reverse transcription was conducted using RevertAid™ Reverse Transcriptase (EP0441) (Thermo Fisher Scientific Inc., Rockford, IL, USA). Quantitative PCR was conducted using FastStart Universal SYBR Green PCR Master mix (4913914001) (Rox; 11929100) (Roche, Indianapolis, IN, USA), according to manufacturer's procedure. Forward (F) and reverse (R) primers were used as follows: RhoA, F: 5'-ACCAGTTCCCAGAGGTGTA TGT-3' and R: 5'-TTGGGACAGAAGTGCTTGAC TTC-3'; ROCK, F: 5'-GAG CAACTATGATGTGCCTG AAAAAT-3' and R: 5'-GATGTCGTTTGATTTCTT CTAC-3'; GAPDH, F, 5'-ATGGGGAAGGTGAAGG TCG-3' and R, 5'-GGGGTCATTGATGGCAACA ATA-3' (Invitrogen Life Technologies, Carlsbad, CA, USA). All measurements were performed in triplicate, and measured by ABI Prism 7300 sequence detection system (Applied Biosystems). The fold-change of RhoA and ROCK was calculated using the 2-ΔΔCT method.

Measurement of cardiac function

Wistar rats were anesthetized with 25% urethane (1.0 g/kg). Cardiac dimensions and function were detected by M-mode echocardiography. The values of left ventricular ejection fraction (LVEF), maximal rate of ventricular pressure rise ($+dp/dt_{max}$) and decline ($-dp/dt_{max}$) were obtained according to the manufacturer's procedure, which represents three continuous cardiac cycles[17].

Evaluation of autophagosomes by TEM

The rats' hearts were retrograde perfused using phosphate buffer saline (PBS; pH 7.4) and 2% glutaraldehyde diluted in 0.1 M cacodylate buffer. Using 2% osmium tetroxide in 0.1M cacodylate buffer and 1% aqueous uranyl acetate was used to fix the tissue[16]. Then, the tissue was prepared for transmission electron microscopy (TEM). A JEOL 1200 EX TEM (operating at 30-120 KV with a digital camera) was used to image the sections. Ultrastructural studies were adopted to explore the double membrane-bound autophagic vacuoles. The alternations of myofibril and mitochondrial ultrastructure were detected by electron micrographs (original magnification X43000).

Western blot analysis

Whole-tissue protein lysates were prepared and subjected to western blotting[18]. Primary antibody LC3, Beclin-1, and RhoA were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Antibodies GAPDH and β-actin were purchased from Bioworld Technology, Inc. (Louis Park, MN, USA).

Lentivirus containing RhoA cDNA

Lentivirus carrying RhoA cDNA was designed and purchased from Genechem Company (Shanghai, China).

Statistical analysis

The statistical significance of differences between different treatments was analyzed with two-sided unpaired Student's t tests. Results were considered to be statistically significant at $P < 0.05$.

Results

Effect of RhoA/ROCK2 signaling pathway on blood glucose levels

After four weeks of intraperitoneal injection of STZ, the Wistar rats had high values of blood glucose, compared to the control group injected with citrate buffer. Student's t tests showed a statistical significance, suggesting successful model of DCM rats (Table 1). The STZ also significantly induces the decrease of body weight. However, compared with the STZ group, fasudil group and overexpression of RhoA group could not alter the values of blood glucose and the body weight. Results indicated that both activated or inactivated RhoA/ROCK2 signaling pathway did not affect blood glucose and body weight in DCM rats.

RhoA/ROCK2 signaling pathway is activated in DCM

The expression of RhoA was detected by western blot analysis and qRT-PCR analysis (Fig. 1 A and B). The RNA and protein levels of RhoA were elevated in STZ group, which indicates that RhoA/ROCK2 signaling pathway was activated in DCM rats. Fasudil partly abrogated STZ induced elevated expression of RhoA (compare lanes 3 and 2 in Fig. 1A), which indicates that although fasudil is a universally recognized inhibitor of RhoA/ROCK2 signaling pathway, it could not fully inhibit RhoA/ROCK2 pathways in the current study. Fasudil could not treat diabetic patients, and lentivirus carrying the RhoA cDNA greatly promoted the expression of RhoA, suggesting that the lentivirus worked in the current experiment (compare lanes 4 and 2 in Fig. 1A).

A significant effector of RhoA called ROCK2 was elevated in three groups (STZ, fasudil, and over-expression of RhoA group) is represented in Fig. 1C. It confirmed that in diabetic rats, RhoA/ROCK2

Table 1—Effects of over expression of RhoA on the body weight and blood glucose (X±SD) in all four groups (Control, STZ, fasudil and RhoA expression group)

Group	No.	Body weight (g)			Blood glucose (mmol/L)		
Cont.	10	4w	8w	12w	4w	8w	12w
STZ	10	239±13	251±18	286±14	5.7±0.7	5.5±0.3	5.6±0.5
fasudil	10	212±16*	190±14*	182±11*	19.5±0.4*	18.7±0.3*	18.1±0.5*
RhoA	10	205±10	200±13	191±14	18.0±0.3	18.2±0.5	18.3±0.2

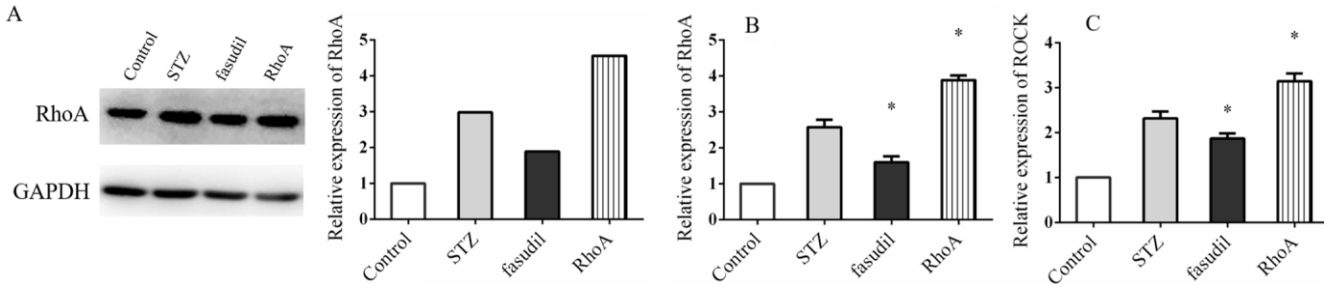

Fig. 1 — RhoA/ROCK signaling pathway is activated in DCM rats. Wistar rat's left ventricular myocardial tissue was extracted for detect of expression of RhoA and ROCK by western blot analysis and qRT-PCR analysis. (A) Expression of RhoA was detected by Western blot analysis, and the semi-quantified expression of RhoA is presented on the right; Expression of (B) RhoA; and (C) ROCK were detected by qRT-PCR analysis. [Columns, means three independent tests; bars, S.D. *P <0.05 *vs.* STZ]

pathway was activated, and fasudil alone could not inactive RhoA/ROCK2 pathway.

Effect of RhoA/ROCK2 signaling pathway on DCM rat's cardiac functions

Hemodynamic measurements were performed in DCM rats to measures the blood pressure. Compared to the control group, the DCM rats have lower percentage of LVEF (Fig. 2A, column 2), which is consistent with the clinical features in DCM patients. Treatment with fasudil partly improved the percentage of LVEF, suggesting that the activity of RhoA/ROCK2 signaling pathway is related to the cardiac function of DCM rats (compare columns 3 and 2 in Fig. 2A). On the other hand, overexpression of RhoA aggravated the heart failure in DCM rats. The percentage of LVEF was lower than the STZ group.

The $\pm dp/dt_{max}$ was decreased in DCM rats (compare columns 2 and 1 in Fig. 2 B and C). Fasudil significantly enhanced $\pm dp/dt_{max}$ compared with the STZ group. The $\pm dP/dt_{max}$ was further decreased in the overexpression of RhoA group compared with the STZ group (columns 3 and 4 in Fig. 2 B and C). All results indicated that RhoA/ROCK2 signaling pathway is activated in DCM, and manipulating the RhoA/ROCK2 signaling pathway activity can reverse cardiac functions in DCM condition.

RhoA/ROCK2 signaling pathway repressed autophagy in DCM

The RhoA/ROCK2 signaling pathway has been reported to regulate cell functions such as cell movement, autophagy and apoptosis[10]. However, in DCM, it is not clear whether the RhoA/ROCK2 signaling pathway regulates autophagy or not. Therefore, expression of LC3 and Beclin 1 was studied. Compared with the control group, LC3 II was decreased together with Beclin 1, indicating that autophagy was repressed (compare lanes 2 and 1 in Fig. 3). Fasudil partly abrogated STZ induced autophagy suppression, the

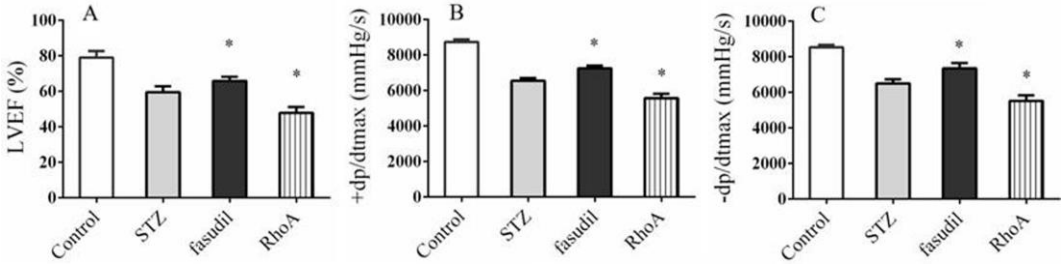

Fig. 2 — RhoA/ROCK signaling pathway affects the cardiac functions. (A) Left ventricular ejection fraction (LVEF) of each group; (B and C) First derivative of the left ventricular pressure ($\pm dp/dt_{max}$). [Columns and error bars represent means and SD. n = 10 rats per group. *P <0.05 *vs*. STZ]

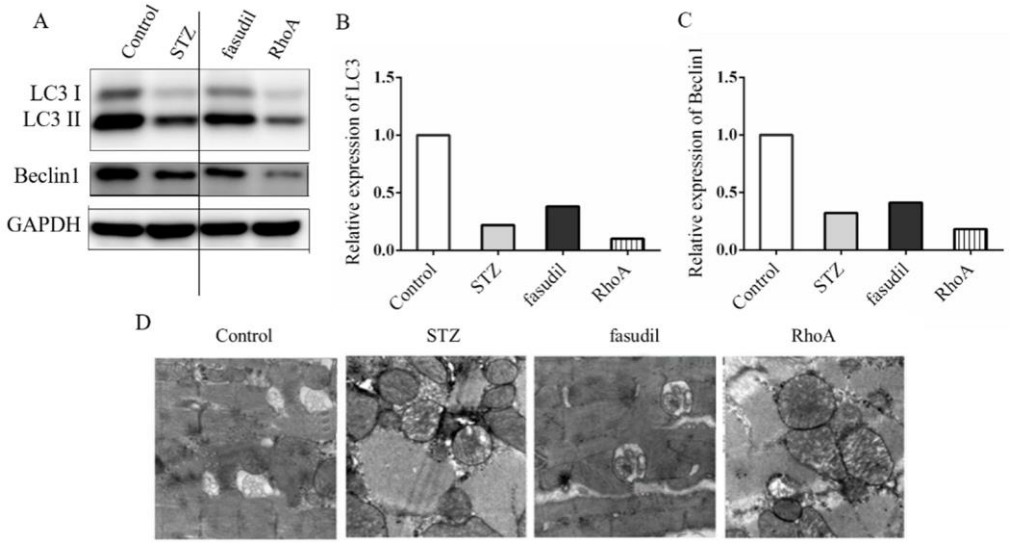

Fig. 3 — RhoA/ROCK signaling pathway repressed autophagy in DCM. (A) Wistar rat's left ventricular myocardial tissue was extracted to detect the expression of LC3 and Beclin1 by western blot analysis; Semi-quantified expression of (B) LC3 II; and (C) Beclin1; and (D) Effect of RhoA/ROCK signaling pathway on the number of intracellular autophagosomes in the DCM rat. [Activated RhoA/ROCK signaling pathway suppressed the formation of intracellular autophagosomes. Representative TEM images from different groups]

expression of LC3 II and Beclin 1 were partly reversed (compare lanes 2 and 3 in Fig. 3). Results suggested that manipulation of RhoA/ROCK2 signaling pathway may reverse the autophagy suppression. Collectively, results indicated that activated RhoA/ROCK2 signaling pathway aggravated the heart failure in DCM.

Discussion

Among the Ras and Ras-like proteins such as ras homolog gene family (Rho), Rab, Ran, Arf, ras homolog gene family member A (RhoA) and the effector Rho-associated kinase (ROCK) have been investigated in detail[19-22]. Accumulating evidence has demonstrated that RhoA/ROCK2 signaling pathway play vital roles in regulating cells adhesion, migration, motility, contraction, apoptosis, and proliferation through regulating the organization of actin cytoskeleton[23]. RhoA/ROCK2 signaling pathway also regulates autophagy[19]. On other hand, RhoA/ROCK2 signaling pathway is responsible for modulating the cardiovascular system by control gene and protein expression. So far, RhoA/ROCK2 signaling pathway has been reported to participate in the development of hypertension[24], metabolic syndrome[25], coronary artery spasm[26], acute ischemic stroke[27], pulmonary hypertension[28] and congestive heart failure[29].

The current study investigated the relation between RhoA/ROCK2 signaling pathway and DCM. The Framingham Heart study[1] showed that the incidence of heart failure (HF) is 2-5 times higher in diabetic men and women. The DCM mainly impaired left ventricular and passive relaxion, cardiomyocyte hypertrophy and myocardial fibrosis[30]. The DCM has been reported to be associated with inflammation, oxidative stress, myocardial fibrosis, mitochondrial damage, myocardial apoptosis and autophagy. The present study found that RhoA/ROCK2 signaling pathway plays a role in regulating autophagy in DCM.

Body weight (g) and blood glucose level (mmol/L) of DCM Wistar rats were measured on a daily basis. Both body weight and blood glucose level recorded for 4, 8 and 12 weeks are given in Table 1. Results indicated that STZ group rats have lower body weight and higher blood glucose (above 16.6 mmol/L). The DCM rats were used as successful animal models.

The study investigated role of RhoA/ROCK2 signaling pathway and overexpression of RhoA on the body weight and the blood glucose levels in DCM rats as compared to the STZ group. The results indicated that the RhoA/ROCK2 signaling pathway did not regulate the glycometabolism. Furthermore, hemodynamic measurements were performed in DCM rats. It was found that when compared to the STZ group, fasudil could partly increase the percentage of LVEF and maximum rate of ventricular pressure ($\pm dp/dt_{max}$) (Fig. 2). However, fasudil could not completely improve the cardiac functions, whereas overexpression of RhoA aggravated the heat failure in DCM rats.

The left ventricular myocardial tissue specimen was collected after sacrifice of rats and the western blotting and qRT-PCR analysis was carried out (Fig. 1). It was found that the RhoA expression was elevated in STZ group. Fasudil partly abrogated the high expression of RhoA in DCM, and the ROCK2 was regulated by RhoA.

Role of the RhoA/ROCK2 signaling pathway in autophagy was studied using expression of LC3 and Beclin 1 and the results are presented in Fig. 3. Results showed that the autophagy was suppressed in DCM, and the overexpression aggravated the autophagy repression (compare lanes 4 and 2 in Fig 3A, the expression of LC3 II and Beclin 1 was decreased) as reported earlier by Sun *et al*.[17], and inhibition of the RhoA/ROCK2 signaling pathway could partly reverse the autophagy repression (compare lanes 3 and 2 in Fig. 3A, the expression of LC3 II and Beclin 1 were increased). The TEM results showed that activated RhoA/ROCK2 signaling pathway suppressed the formation of intracellular autophagosomes (Fig. 3D). Gao *et al*.[31] also found that fasudil suppressed ROCK levels, promoted autophagy *via* increasing the LC3-II/LC3-I ratio, Beclin-1 expression, and the number of autophagosomes in H9c2 cells treated with high glucose, and such effects could be abrogated by inhibitors of autophagy.

Conclusion

In this study, results indicated that both activated or inactivated RhoA/ROCK2 signaling pathway did not affect blood glucose and body weight in DCM rats. Significant effector of RhoA called ROCK2 was elevated in three experimental groups STZ, fasudil, and RhoA overexpression group. Additionally, RhoA/ROCK2 pathway was activated in diabetic rats,

and fasudil alone could not inactive RhoA/ROCK2 pathway. Levels of microtubule-associated protein light chain 3 II (LC3 II) and Beclin 1 were decreased, indicating repression of autophagy. Manipulating the activity of RhoA/ROCK2 signaling pathway may release the autophagy suppression. Conclusively, when RhoA/ROCK2 signaling pathway was activated in DCM, it aggravated the heart failure through repression of autophagy, which provided a new target for clinical treatment in DCM patients.

Conflict of interest

Authors declare no conflict of interests.

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