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Rosuvastatin inhibits TGF-β1 expression and alleviates myocardial fibrosis in diabetic rats

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This study aimed to investigate the effects of rosuvastatin on TGF-β1 expression, cardiac fibrosis, ventricular remodeling and cardiac function in diabetic cardiomyopathy rats. Twenty-seven diabetic rats induced by streptozotocin intraperitoneal injection were randomly divided into three groups, viz. diabetic, rosuvastatin low-dose (Ros-L) and high dose group (Ros-H). Intervention group were given rosuvastatin 2 mg/kg/d and 5 mg/kg/d orally, respectively. After 10 weeks, the levels of glycosylated hemoglobin (HbA1c), creatine phosphokinase isoenzyme (CK-MB), plasma brain natriuretic peptide (BNP), myocardial collagen volume fraction (CVF) and left ventricular mass index (LVWI) were measured. CK-MB levels in Ros-H and Ros-L rats were lower than in the diabetic group. Rosuvastatin alleviated myofibrosis cordis and fibroplastic proliferation. LVWI, BNP, CVF and TGF-β1 mRNA and protein levels in the diabetic group were higher than in the control, but were reduced after rosuvastatin treatment. These results demonstrate that rosuvastatin dose-dependently reduces TGF-β1 expression and inhibits the development of myocardial fibrosis in diabetic cardiomyopathy.

1. Introduction

Cardiovascular disease is a major complication of diabetes and the leading cause of death among diabetic people—about 65% of people with diabetes die from heart disease and stroke. Adults with diabetes are 2–4 times more likely to have heart disease or suffer a stroke than normal people. High blood glucose in diabetes increases the risk for heart attack, stroke, angina, and coronary artery disease (Nathan et al. 2005). This risk is independent of other cardiovascular risk factors such as age, sex, hypertension, obesity and hyperlipidemia, suggesting that DM causes damage to the heart in a unique way.

To date, it is well known that diabetic patients suffer from an additional cardiac insult termed 'diabetic cardiomyopathy' (DC) (Rubler et al. 1972). In DM, myocardial tissue structure change and dysfunction induced by factors other than coronary artery disease and cardiac neuropathy are defined as DC. The first clinical manifestation of DC is diastolic dysfunction, which may be accompanied by systolic dysfunction later. Myocardial cell degeneration and necrosis are the major factors causing pathophysiological changes in DC (Fang et al. 2004).

Transforming growth factor beta 1 (TGF-β1) is a member of the transforming growth factor beta superfamily of cytokines. TGF-β1 is highly expressed in the heart in experimental diabetes in association with cell proliferation, recognition, apoptosis, special differentiation and extracellular matrix accumulation. At present, a large number of studies have shown that TGF-β1 is increased in myocardial tissues of diabetic patients and TGF-β1 is expected to become a new target for the treatment of diabetic cardiomyopathy (van Bilsen et al. 2004; Bujak et al. 2007).

Recently, statins have shown protective effects on cardiovascular system such as anti-inflammation, anti-platelet aggregation, inhibition of vascular smooth muscle cell proliferation and improvement of vascular endothelial function. Especially, statins can alleviate ventricular remodeling caused by a variety of aberrant processes. However, the mechanism by which statins modulate ventricular remodeling remains elusive. The present study aimed to characterize the effect of rosuvastatin on myocardial fibrosis in DC rat and investigate the underlying mechanism with the focus on the role of TGF-β1 in this process.

2. Investigations and results

2.1. Characteristics of experimental animals

The rats injected with STZ gradually presented a dull response, hidrosis, sparse hair, polydipsia, polyuria and weight loss. These symptoms were gradually improved after 5–6 weeks of rosuvastatin treatment. The glucose levels of both fasting and fed rats were significantly higher ($P < 0.05$) in diabetic rats (DM) than in normal control rats (control). The levels of glucose in fasting and fed diabetic rats were reduced after treatment with different dosage of rosuvastatin, but the differences were not statistically significant.

2.2. Glycated hemoglobin (HbA1c), CK-MB and BNP in rats

HbA1c, CK-MB and BNP levels in the diabetic, Ros-H and Ros-L rats were significantly increased ($P < 0.01$) compared with controls. There was no significant difference in HbA1c

Table 1: HbA_1C, CK-MB and BNP levels in the experimental animals

Groups	n	HbA_1C (%)	CK-MB(μ/L)	BNP(ng/L)
Normal	10	6.30 ± 0.57	16.00 ± 4.10	34.2 ± 13.1
Diabetes	9	$11.83 \pm 0.26^{\Delta}$	$72.36 \pm 5.47^{\Delta}$	$276.3 \pm 91.4^{\Delta}$
Ros-L	9	$12.32 \pm 0.43^{\Delta\ *}$	$50.62 \pm 3.92^{\Delta\ \circ}$	$205.7 \pm 67.3^{\Delta\ \circ}$
Ros-H	9	$12.03 \pm 0.37^{\Delta\ *}$	$48.88 \pm 3.87^{\Delta\ \circ\ \Diamond}$	$166.3 \pm 58.6^{\Delta\ \circ\ \star}$

$^{\Delta}$ $P < 0.01$ vs normal group; * $P > 0.05$ vs diabetic group; $^{\circ}$ $P < 0.05$ vs diabetic group; $^{\Diamond}$ $P > 0.05$ vs Ros-L group; * $P < 0.05$ vs Ros-L group.

levels between the diabetic, Ros-H and Ros-L rats. CK-MB level in the Ros-H and Ros-L rats was significantly lower than in the diabetic group ($P < 0.05$). However, there was no significant difference between Ros-H and Ros-L rats. Both high- and low-dose of rosuvastatin could decrease BNP level in diabetic rats ($P < 0.05$). Moreover, high dose of rosuvastatin showed a stronger inhibitory effect on BNP than low dose ($P < 0.05$) (Table 1).

2.3. Body weight (BW), left ventricular weight (LVW) and left ventricular weight index (LVWI) in rats

Compared with control group, a significant increase ($P < 0.01$) of LVWI was noted in diabetic, Ros-H and Ros-L group. Importantly, both high and low dosage of rosuvastatin decreased LVWI in diabetic rats ($P < 0.05$). However, there was no significant difference between Ros-H and Ros-L group (Table 2).

2.4. Myocardial pathology and myocardial collagen volume fraction (CVF) in rats

The cardiomyocytes in normal rats presented a regular arrangement, clear stripes without myofilament fracture, and uniform intercellular space. In contrast, cardiomyocytes in the diabetic group showed myofibrillar disarray and myocardial fibrosis, scattered muscle fiber degeneration, coarse granules in cytoplasm, nucleus swelling and deformation, loss of cardiac muscle fiber stripes, interstitial edema and hyperplasia, and lymphocytic infiltration. Myocardial cell arrangement in diabetic rats treated with high- and low-dose of rosuvastatin was more regular than that in diabetic group. In addition, rosuvastatin alleviated myofibrosis cordis and fibroplastic proliferation in a dose dependent manner (Fig. 1). Compared with control group, CVF levels in diabetic, Ros-H and Ros-L group were significantly increased ($p < 0.01$). However, Rosuvastatin could suppress CVF levels in diabetic rats in a dose dependent manner (Table 3).

2.5. Rosuvastatin inhibits TGF-β1 expression in diabetic rats

To investigate the mechanism by which rosuvastatin has beneficial effects on myocardial fibrosis in DC rats, we focused on TGF-β1 since it has been shown to be involved in myocardial fibrosis. We examined the effect of rosuvastatin on TGF-β1 expression in cardiac muscular tissues obtained from the normal, diabetic, Ros-H and Ros-L rats. RT-PCR analysis showed that TGF-β1 mRNA level in diabetic, Ros-H and Ros-L rats were greater than that in control animals ($P < 0.01$). However, rosuvastatin decreased TGF-β1 mRNA level in diabetic rats in a dose dependent manner ($P < 0.05$) (Fig. 2A). Furthermore, Western blot analysis showed that rosuvastatin decreased TGF-β1 protein level in diabetic rats in a dose dependent manner ($P < 0.05$) (Fig. 2B). Collectively, these results suggest that rosuvastatin inhibits TGF-β1 expression in diabetic rats.

3. Discussion

Diabetic cardiomyopathy (DC) is one of the important cardiovascular complications causing cardiac dysfunction in DM patients. The main pathological changes include myocardial cell focal hypertrophy, degeneration, necrosis, apoptosis, and myocardial remodeling. A series of pathophysiological changes caused by myocardial interstitial remodeling play an important role in the pathogenesis of DC.

TGF-β1 is an important fibrogenic factor that promotes the synthesis and secretion of collagen I and III by myocardial CFbs and induces myocardial fibrosis. In this study, LVWI, BNP, CVF and TGF-β1 mRNA and protein levels in the diabetic group are significantly higher than those in control group, suggesting that the increase in TGF-β1 in DM rats is closely associated with myocardial remodeling.

Statins are a large class of medications known as HMG-CoA reductase inhibitors, which essentially block the metabolic pathway that produces cholesterol in the body and are clearly the most effective drugs for lowering LDL cholesterol. In addition to inhibiting cholesterol synthesis, statins may exert other cardiovascular protective effects, e.g. in myocardial infarction (Vincze and Brugos 2012). Statins inhibit cardiac hypertrophy and apoptosis by inhibiting Ang II-, inflammatory factor- and reactive oxygen species (ROS)-mediated ventricular remodeling, blocking synthesis of isoprenoid intermediates, and regulating eNOS-NO and endothelin-1 (ET-1) system (Ichiki et al. 2001; Planavila et al. 2005; Zhao et al. 2008).

Taking into account the role of myocardial interstitial remodeling in DC development, this study focused on exploring the beneficial effects of rosuvastatin on DC myocardial remodeling. We found that 10 weeks of intervention with high-dose or low-dose of rosuvastatin significantly reduced LVWI, BNP,

Table 2: BW, LVW and LVWI in the experimental animals

Groups	n	BW (g)	LVW (mg)	LVWI (mg/g)
Normal	10	382.20 ± 26.34	718.20 ± 40.72	1.88 ± 0.18
Diabetes	9	233.10 ± 23.31	692.31 ± 50.01	$2.97 \pm 0.21^{\Delta}$
Ros-L	9	286.32 ± 24.65	635.60 ± 49.84	$2.22 \pm 0.31^{\Delta\ \circ}$
Ros-H	9	290.22 ± 27.36	670.40 ± 62.37	$2.31 \pm 0.17^{\Delta\ \circ\ \Diamond}$

$^{\Delta}$ $P < 0.01$ vs normal group; $^{\circ}$ $P < 0.05$ vs diabetic group; $^{\Diamond}$ $P > 0.05$ vs Ros-L group.

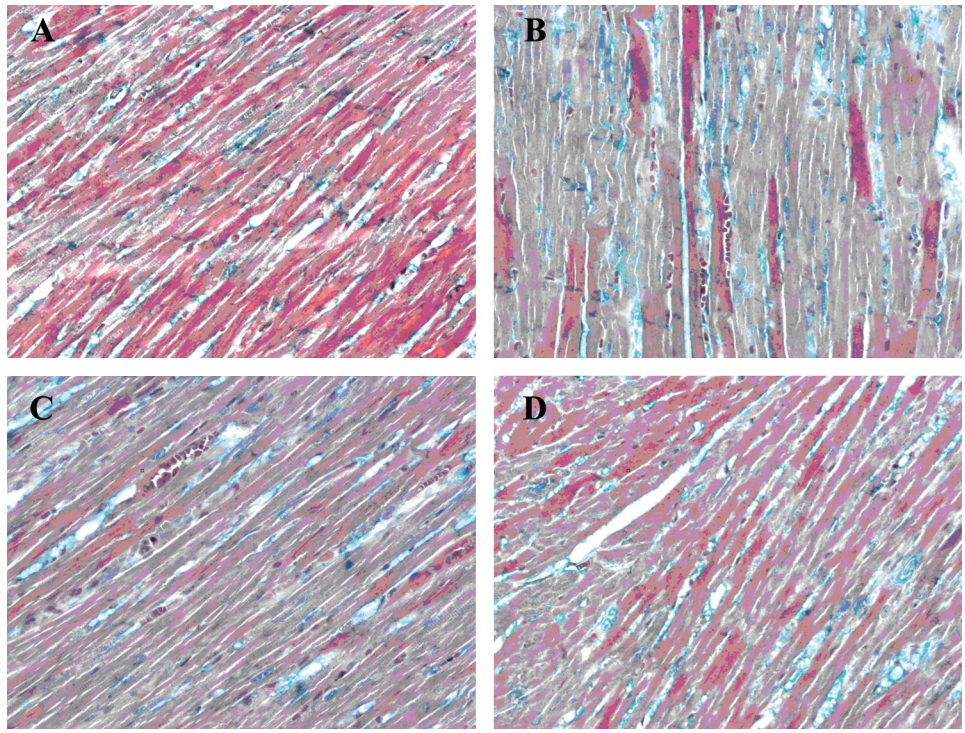

Fig. 1: Rosuvastatin alleviated myofibrosis cordis and fibroplastic proliferation (Masson stain, x200) A, normal group; B, diabetic group; C, Ros-L group; D, Ros-H group

myocardial collagen volume fraction, and TGF-β1 mRNA and protein levels in diabetic rats. In addition, compared with low dose group, high dose of rosuvastatin had stronger inhibitory effects on BNP, myocardial collagen volume fraction, TGF-β1 mRNA and protein levels. These results are consistent with recent reports that pharmaceutics suppressed myocardial fibrosis by inhibiting TGF-β1 transcription (Gu et al. 2010; Guo et al. 2011).

In conclusion, we demonstrated that rosuvastatin inhibits the development of myocardial fibrosis in DC and this is associated with the inhibition of TGF-β1 expression. These findings provide new guidance for the clinical treatment of DC.

4. Experimental

4.1. Materials and chemicals

Streptozotocin (STZ), Tris, chloral hydrate, and nitrocellulose (NC) membranes were obtained from Sigma Chemical Co (St Louis, MO, USA). Rosuvastatin (H-8661) was provided by AstraZeneca (USA). Prime Script RT reagent Kit and SYBR Premix Ex Taq II were from TaKaRa (Dalian, China). β-Actin and TGFβ1 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Enhanced chemiluminescence (ECL) detection reagents were from Kirkegaard & Perry Laboratories (Gaithersburg, MA, USA). All other chemicals were of the highest analytical grade.

4.2. Diabetic rat model and treatment

Healthy male Wistar rats (8 weeks old and 251.31 ± 16.22 g weight) were provided by Shanghai SLAC Laboratory animal Co. Ltd. All procedures were in accordance with the Institute Ethical Committee for the Experimental Use of Animals of the First Affiliated Hospital of Xiamen University. Rats were housed five per cage in a room with a 12:12 hour light:dark cycle at 22–25 °C. After one week on adaptive feeding diet, the animals were

Table 3: CVF levels in the experimental animals

Group	n	CVF (%)
Normal	10	3.92 ± 0.68
Diabetes	9	$12.31 \pm 0.55^{\Delta}$
Ros-L	9	$10.02 \pm 0.47^{\Delta\,\circ}$
Ros-H	9	$7.29 \pm 0.83^{\Delta\,\circ\,\star}$

$^{\Delta}$ P < 0.01 vs normal group; ○ P < 0.05 vs diabetic group; ☆ P < 0.05 vs Ros-L group.

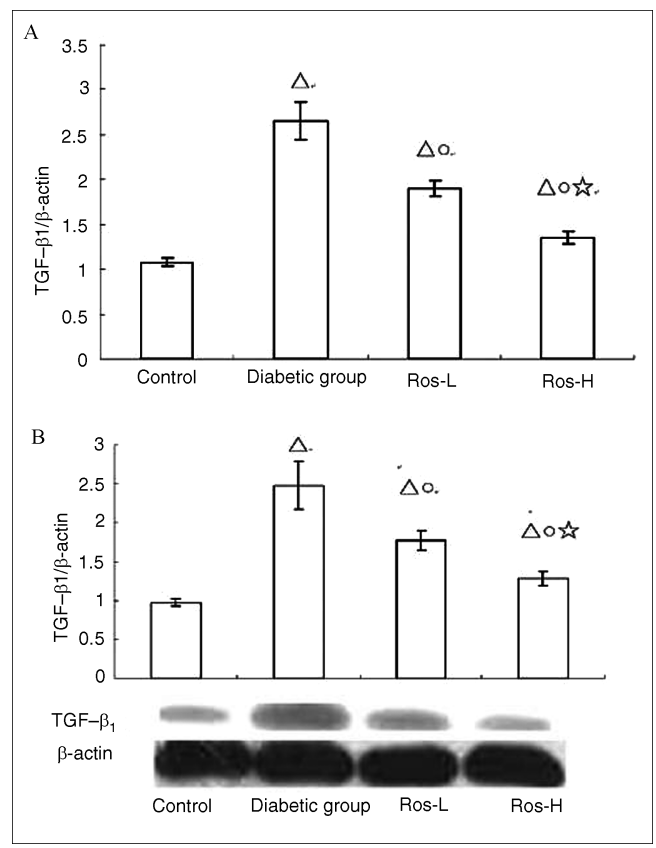

Fig. 2: Rosuvastatin inhibited TGF-β1 expression in cardiac muscular tissues of diabetic rats. A, mRNA level of TGF-β1 in different groups detected by RT-PCR. △P < 0.01 vs. normal group; ◯ P < 0.05 vs. diabetic group; ☆P < 0.05 vs. Ros-L. B, protein level of TGF-β1 in different groups detected by Western blot analysis. △P < 0.01 vs. normal group; ◯ P < 0.05 vs. diabetic group; ☆P < 0.05 vs. Ros-L

injected with STZ (50 mg/kg in 0.1 mol/L citrate-buffered saline, pH 4.5) or the same volume of citrate-buffered saline into the lower left abdominal. Animals had free access to food and water after the STZ injection, and both STZ-injected and saline-injected animals continued on their original diets for the duration of the study. After a 12-h fast, animals showing fasting glucose level > 11.1 mmol/L and non-fasting blood glucose > 16.7 mmol/L for three times at 72 h after STZ injection were considered diabetic.

The diabetic groups were then further subdivided into treated and untreated groups: Model (n = 9); diabetic group treated with low dosage of rosuvastatin by oral gavage (Ros-L, n = 9); diabetic group treated with high dosage of rosuvastatin (Ros-H, n = 9). All rats were fed with normal diet and drinking water. Treatment was given daily for 10 weeks. The control group received an equal volume of vehicle (saline). At the end of each week, individual body weights were recorded, and glucose and insulin levels were determined under fasting and non-fasting conditions. Glycemia was assessed on blood collected from the tail vein using an OneTouch Ultra blood glucose meter (LifeScan, Milpitas, CA, USA). After the full course of treatment, the animals were weighed and anesthetized with 3.5% chloral hydrate (10 ml/kg) intraperitoneally. Blood samples were withdrawn from the hearts and serum was isolated. Creatine phosphokinase isoenzyme (CK-MB) and brain natriuretic peptide (BNP) were assayed. The heart was quickly removed under strictly aseptic conditions and perfused with ice-cold DEPC distilled water. The great vessels, atrial and right ventricular tissues were removed. The left ventricular tissues were sucked up with a sterile filter paper and weighed (LVW; mg). The left ventricular weight/body weight, i.e. left ventricular mass index (LVWI; mg/g) was calculated. The bottom of heart was fixed in 4% paraformaldehyde and embedded in paraffin. The vertex cordis was stored in liquid nitrogen for further molecular biology experiments.

4.3. Myocardial collagen analysis

The heart tissues were paraffin-embedded and cut into sections followed by haemotoxylin staining. In addition, the collagen in the heart was specifically stained by ponceau red and the myocardial collagen volume fraction (CVF) was determined. In brief, the sections were captured and analyzed with the Image-Pro plus 6.0 image analysis system. Five fields were randomly selected and the CVF was calculated as collagen area/total area followed by averaging. But the area of collagens surrounding the vessels was not included in the CVF. Four arterioles in the ventricular wall were selected and the cross-section area was measured.

4.4. RT-PCR

Total RNA was isolated using Trizol reagent and purified with QIAGEN RNeasy (Qiagen, Valencia, CA) according to the manufacturer's instructions. Reverse transcription of 500 ng total RNA was performed in a total volume of 20 μL using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). One microliter of cDNA was amplified by PCR in 20 μL reactions containing specific primers and iQ SYBR Green Supermix (Bio-Rad). PCR was performed for 40 cycles consisting of 95 °C for 15 s, 94 °C for 5 s, 58 °C for 15 s and 72 °C for 15 s using iCycle iQ Real Time Detection System (Bio-Rad). The primers used for PCR were purchased from Invitrogen (Grand Island, NY) with the following sequences: TGF-β1 forward primer 5'-GCTCGCTTTGTACAACAGCA-3' and reverse primer 5'-GAGTTCTACGTGTTGCTCCA-3'; β-actin forward primer 5'-CCTCTATGCCAACACAGTGC-3' and reverse primer 5'-GTACTCCTGCTTGCTGATCC-3'. Relative mRNA levels were determined using the comparative CT method with data normalized to 36B4 riboprotein mRNA and calibrated to the average ΔCT of untreated controls. Data were expressed as percentage of control that was set to 100%.

4.5. Western Blot analysis

Proteins were extracted from heart homogenates and aliquots (50 μg) were subjected to SDS-PAGE (7.5% gel) and transferred onto nitrocellulose membranes. The membranes were blocked for 2 h at room temperature with block solution provided in the ECL kits, then incubated with primary antibody overnight at 4 °C. The membranes were then washed for 30 min in wash solution (ECL kit), and incubated with IgG conjugated with horseradish-peroxidase in block solution. The membranes were washed for 30 min in wash solution, and the immunoreactive bands were detected with ECL kit.

4.6. Statistical analysis

Statistical analysis was performed with SPSS version 14.0 statistics software package and data were expressed as means ± standard deviation (SD). Comparisons of means between multiple groups were performed with one-way ANOVA analysis of variance. A value of $P < 0.05$ was considered statistically significant.

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