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Cardiac Fibrosis in Diabetic Rats: Regulation and Mechanism of Activation of the PPARγ Signal Pathway

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Abstract

Peroxisome proliferator-activated receptor-gamma (PPARγ) is a member of the nuclear hormone receptor superfamily which affects organic fibrosis. The aims of the study were to approach the effects of activation of the PPARγ signal pathway on cardiac fibrosis in diabetic rats, and also the effects on cardiac remodeling and function. Type 1 diabetic models were used in the study. All the animals were divided into 3 groups: I : control group; II : diabetic group; III: diabetes+Pioglitazone (Piog, a PPARγ ligand) administration group. After 14 weeks of feeding, general condition, fibrosis indices, echocardiography and interventricular pressures parameters were detected. At the 14th week, compared with group I, the hydroxyproline concentration in group II significantly increased, and CO I and III distribution was more obvious by sirius red staining. Reduction of LVSP (left ventricular systolic pressure) and increase of LVEDP (left ventricular end-diastolic pressure) were also significant in group II. But these situations were changed by the administration of Piog in group III. Furthermore, results of RT-PCR and immunohistochemistry showed that Piog administration reduced angiotensin II type 1 receptor (AT1-R) expression in diabetic models. Hence, activation of the PPARγ signal pathway could repress cardiac fibrosis in diabetic rats, and partly improve cardiac remodeling and function by down-regulating activity of RAS at the receptor level.

Key Words: PPARγ signal pathway, cardiac fibrosis, angiotensin II type 1 receptor (AT1-R), diabetes

Introduction

Peroxisome proliferator-activated receptors (PPARs) are transcription factors belonging to the nuclear receptors superfamily. PPARγ regulates gene transcription and expression by binding to peroxisome proliferator response elements (PPRE) in target gene promoters or upstream enhancer regions as heterodimeric complexes with the retinoid X receptor (RXR). PPARγ ligands can improve insulin sensitivity and protect the function of pancreatic islets β cells. The synthetic ligands-Thiazolidinediones (TZDs) were widely used in the treatment of diabetes, and it has been shown recently that TZDs can repress organic fibrosis (7, 11) and protect normal functions. TZDs were also proved to have many positive effects on cardiovascular systems such as decrease of blood pressure, suppression of atherosclerosis and reduction of myocardial infarction size (6, 12). The aims of the study were to investigate whether cardiac fibrosis induced by diabetes was regulated by activation of the PPARγ signal pathway and possible mechanism of regulation.

Ang II plays an important role in the development of cardiac fibrosis (2, 4). In diabetic individuals, tissue concentration of Ang II increases, and the activity of the renin-angiotensin system (RAS) elevates remarkably, which may be the major reason

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for diabetes-induced cardiac fibrosis. As shown in recent studies, there are complicated interactions between Ang II and PPARγ(5, 8). Hence, we hypothesized that PPARγ agonists might exert effects on diabetes-induced cardiac fibrosis by intervention of RAS activity.

Materials and Methods

Animals

The study was conducted according to recommendations from the Animal Care Committee of the BeiJing Friendship Hospital. Male Sprague-Dawley rats (SD, 200 ± 20 g) were used. Diabetes (type 1) was induced with a single intraperitoneal injection of streptozotocin (STZ, 60 mg/kg) (Sigma, St. Louis, MO, USA) (10). Rats with blood glucose concentrations above 16.7 mM after 72 h were defined as diabetic rats. All the rats were divided into 3 groups: I. Control group (n = 16); II. Diabetic group (n = 16); III. Diabetic + Piog group (TaiYang Pharmaceuticals Company, BeiJing, PRC, n = 16). These animals were used to perform the studies after 14 weeks of intragastric administration [physiologic saline for groups I and II, Piog (10 mg/kg) for group III] and under standard feeding conditions.

Cardiac Fibrosis Assay

Hydroxyproline is a specific amino acid of cardiac collagen protein. So degrees of fibrosis in fresh myocardial tissues were determined by the hydroxyproline content assay. It was measured with a commercial hydroxyproline detection kit (JianCheng Institute of Biotechnology, Nanjing, Jiansu PRC) following the manufacturer's instructions.

Myocardial tissue samples fixed in 4% paraformaldehyde were embedded in paraffin and cut into 5 μm-thick sections. Using a standard procedure as previously described (1), the sections were stained with Sirius red for 1 h and Mayer haematoxylin for 1 min. Micropolarimete were used to observe collagen distribution, and the severity of cardiac fibrosis was evaluated with an image analysis system (Northern Eclipse 5.0, EMPIX Imaging Inc, Mississauga, Canada).

Echocardiographic and Hemodynamic Study

Echocardiographic (echo) and hemodynamic studies were performed to evaluated cardiac remodeling and function. The rats were anesthetized with 6% hydral. Two-dimensional and M-mode echo measurements were carried out with a VEVO 770 (VisualSonics, Toronto, Canada) machine. The tested parameters included left ventricular (LV) end-diastolic diameter (EDD), intraventricular septum thickness (IVS) and ejection fraction (EF). To correct for body weight (BW) disparities, parameter/BW as adjusted indices (EDD-I, IVS-I) were used to compare among groups.

A ultraminiature catheter connecting to a polygraph (BL-420,TaiMeng, Chengdu, PRC) was inserted into the carotid artery and then to the left ventricle of the anesthetic animals. After a 5-min stabilization period, LV pressure and ±dP/dTmax were recorded, respectively.

Expression of the Angiotensin II Type 1 Receptor (AT1-R)

AT1-R cDNA was amplified in each group to assess the impacts of diabetes and Piog treatment on RAS at the mRNA level. The forward and reverse primers of AT1-R were 5'-CACCCAATGAAGTC TCGC-3' and 5'- AAGGAAAGGGAACACGAA-3' to generate a 234-bp fragment. The forward and reverse primers of β-actin were 5'-GAAATCGTGCGT GACATTA-3' and 5'-TAGGAGCCAGGGCAGTAA-3' to produce a 349-bp fragment. The RT-PCR reaction was carried out in a standard buffer with 30 ng of each primers, dNTPs (10 mM) 0.5 µl, and 1 µl Taq DNA polymerase for 30 cycles. PCR products were analyzed on a 2% agarose gel.

AT1-R immunohistochemical staining was performed with 5 µm paraffin sections of myocardial tissues with standard methods. Rabbit anti-mouse AT1-R monoclonal antibody was purchased from Abcam Company (Cambridge, UK). The results were evaluated by a image analysis system (Northern Eclipse 5.0, EMPIX Imaging Inc, Mississauga, Canada).

Statistical Analysis

All values were analyzed using the SPSS12.0 software. Data were expressed as means ± SD. Statistical analysis was performed by one-way analysis of variance and the SNK test. A value of $P < 0.05$ was considered significant.

Results

General Characteristics of Each Group

After 14 weeks of feeding, the survival rate in group II was much lower than that in group I. But Piog treatment significantly elevated the survival rate of diabetic rats in group III ($\chi^2 = 4.80$, $P < 0.05$) (Table 1). At the 14th week, compared with group I , the blood glucose concentrations in group II and III were higher, and those of serum insulin were

Table 1. General characteristics of each group

	Survival rate	Body weight (g)	Heart weight (g)
Group I	100%	589.83 ± 37.4	7.8 ± 0.35
Group II	44% (7/16)*	238.50 ± 20.8*	3.9 ± 0.45*
Group III	81% (13/16)#	285.17 ± 58.2*	4.6 ± 0.64*#

*The differences were significant comparing with group I ($P < 0.05$).
#The differences were significant comparing with group II ($P < 0.05$).

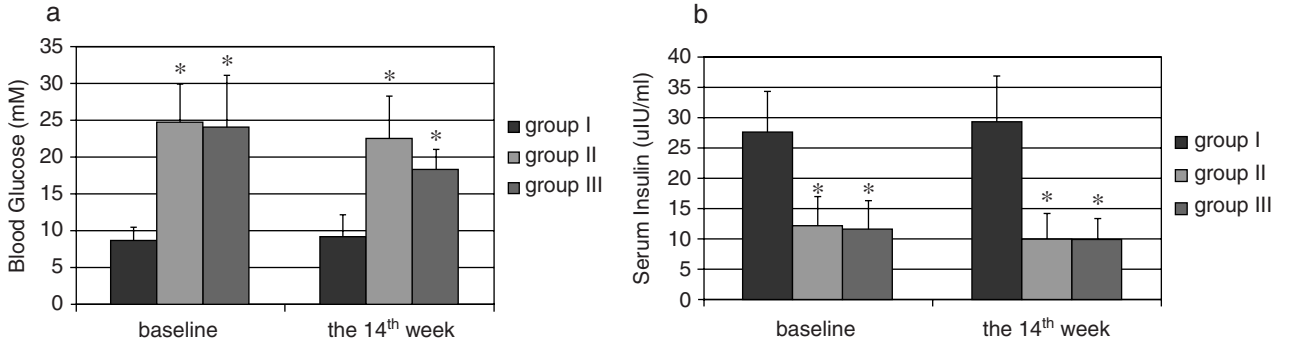

Fig. 1. a. The results of blood glucose level. At the baseline and the 14th week, compared with group I, blood glucose levels in groups II and III increased significantly ($P < 0.05$). There was no significant difference in the blood glucose levels between groups II and III ($P > 0.05$). b. The results of serum insulin level. At the baseline and the 14th week, compared with group I, serum insulin in groups II and III decreased significantly ($P < 0.05$). There was no significant difference in the serum insulin levels between groups II and III ($P > 0.05$).

lower, but the results in group II and III were similar ($P = 0.845$, $P = 0.971$, Fig. 1, a and b).

Assessment of Tissue Fibrosis

Sirius red staining revealed an increase of collagen accumulation in the diabetic rats (Fig. 2b, Collagen I- reddish yellow; Collagen III- green). Administration of Piog attenuated the collagen content in group III (Fig. 2c). Hydroxyproline contents (HC) of each group (from I to III) were (in mg/g) 0.035 ± 0.005, 0.046 ± 0.005, 0.039 ± 0.008, respectively. HC in group II was significantly higher than that in group I ($P < 0.01$) and group III ($P < 0.05$). And there was no longer obvious differences in HC between groups I and III ($P = 0.197$).

Echo and Hemodynamic Studies

Compared with group I, LVSP and –dP/dtmax of group II decreased significantly ($P = 0.00$, $P = 0.005$), and LVEDP increased significantly ($P < 0.01$). After Piog treatment in group III, LVSP increased (II *vs.* III: $P < 0.05$), and LVEDP decreased (II *vs.* III: $P = 0.029$), and the differences were significant (Table 2).

There were no obvious differences in EDD-I and EF in the 3 groups ($P > 0.05$); but IVS-I in groups II and III were higher than that in group I ($P < 0.01$, $P < 0.05$) (Table 2).

Expression of AT1-R

AT1-R mRNA expression in group II was higher than that in group I ($P = 0.01$). Compared with group II, expression of AT1-R mRNA in group III attenuated significantly ($P < 0.05$) (Fig. 3, a and b). Immunohistochemistry staining revealed AT1-R expression as brown stains. More positive staining result could be detected in group II (Fig. 4b). After Piog administration, AT1-R expression was found to be inhibited in group III (Fig. 4c).

Discussion

Cardiac fibrosis is characterized by the proliferation of cardiac fibroblasts and excessive accumulation of matrix proteins collagen I and III in the extracellular space (9). It is a result of an imbalance in extracellular matrix (ECM) metabolism involving increase of collagen synthesis and decrease of collagen degradation. Cardiac fibrosis contributes to stiffness of the ventricular wall and to diastolic dysfunction. It is also associated with systolic heart failure, arrhythmia and cardiac sudden death. The development and mechanism of cardiac fibrosis have been inves-

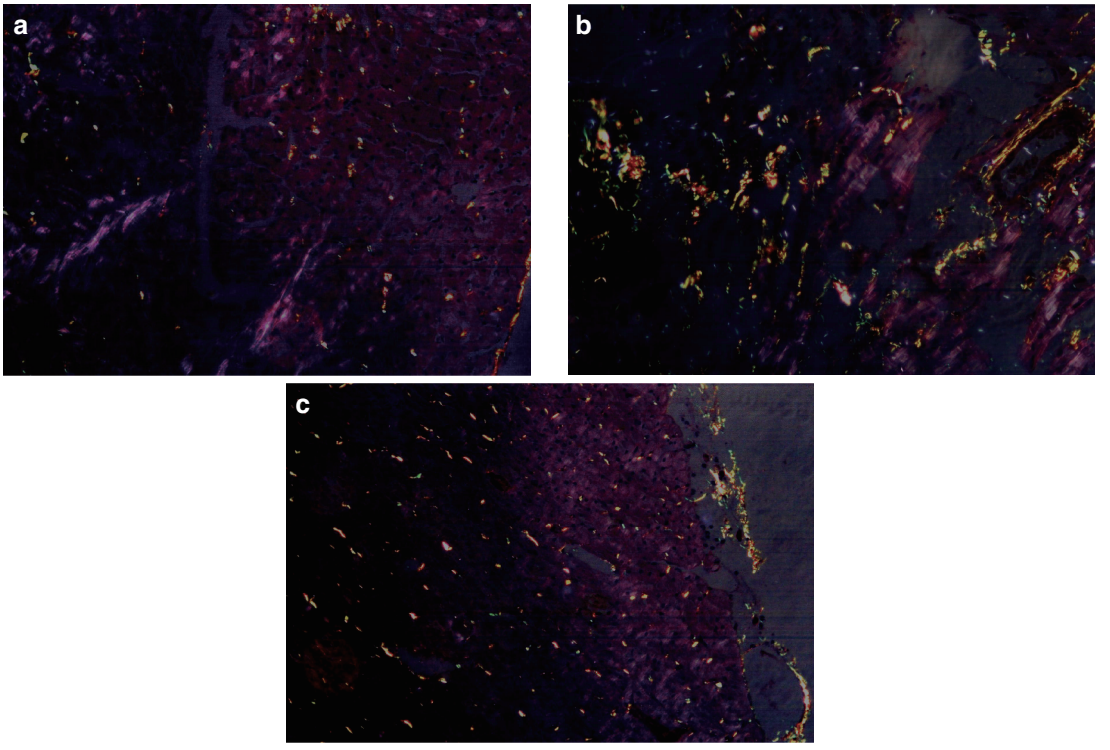

Fig. 2. a. Sirius red staining of Myocardial tissue sample in group I. b. Sirius red staining of Myocardial tissue sample in group II. c. Sirius red staining of Myocardial tissue sample in group III. a-c. COI & III expression appeared as reddish yellow and green stains, respectively. Sirius red staining revealed an increase of collagen accumulation in group II while administration of Piog attenuated collagen content in group III.

Table 2. The results of Echo and hemodynamic studies

	Group I	Group II	Group III
LVSP (mmHg)	52.8 ± 7.5	27.1 ± 7.5*	41.4 ± 9.2*#
LVEDP	1.07 ± 1.56	5.88 ± 2.3*	2.48 ± 2.1#
+dp/dtmax	1.49 ± 0.79	0.87 ± 0.43	0.93 ± 0.35
–dp/dtmax	1.13 ± 0.29	0.58 ± 0.20*	0.69 ± 0.22*
EDD-I	13.67 ± 3.06	19.41 ± 4.00	20.54 ± 3.96
IVS-I	2.75 ± 0.46	5.38 ± 0.61*	4.46 ± 1.08*
EF (%)	70.6 ± 8.1	58.1 ± 19.6	73.9 ± 17.4

*The differences were significant comparing with group I ($P < 0.05$).
#The differences were significant comparing with group II ($P < 0.05$).

tigated for many years, but diagnostic standards and effective therapy strategies are still unsatisfactory.

It has been proved that diabetes mellitus is one of the main reasons to induce cardiac fibrosis. TZDs as synthetic ligands of PPARγ was widely used as antidiabetic agent, and it has been reported to repress organic fibrosis in liver, kidney and lung. Hence, we wondered if TZDs had effects on cardiac fibrosis as a treatment of diabetes.

In the present study, myocardial tissues of diabetic rats were used to investigate the genesis and progression of cardiac fibrosis, and also the impacts of PPAR ligands. After 14 weeks of feeding, body weight and heart weight in group II decreased significantly and the mortality was markedly higher than that in groups I and III. The survival rate was only 44% in group II while it was 81% in group III ($P < 0.05$). Piog as an insulin sensitizer did not have an impact on blood glucose level in type 1 diabetic model in the study. Compared with group I, random blood glucose concentrations in groups II and III were higher, but the difference between the two groups

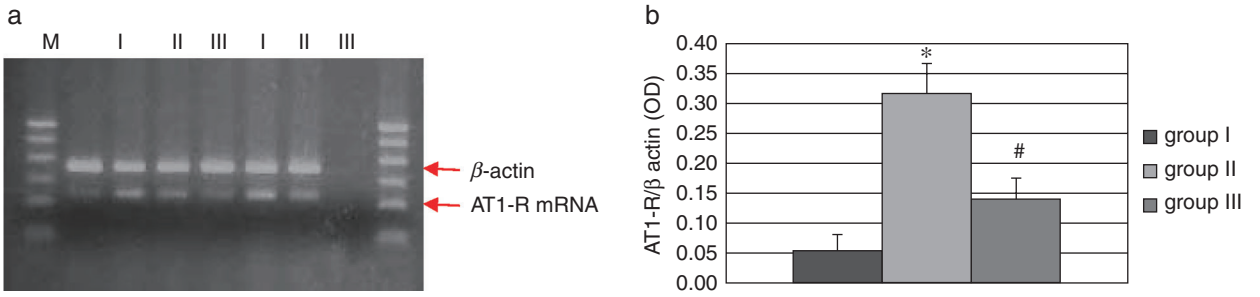

Fig. 3. a. Expression of AT1-R mRNA (in RT-PCR) in each group. b. AT1-R mRNA expression in each group. Compared with group I, AT1-R mRNA expression in group II elevated significantly ($P = 0.01$). The expression was obviously attenuated in group III ($P = 0.011$).

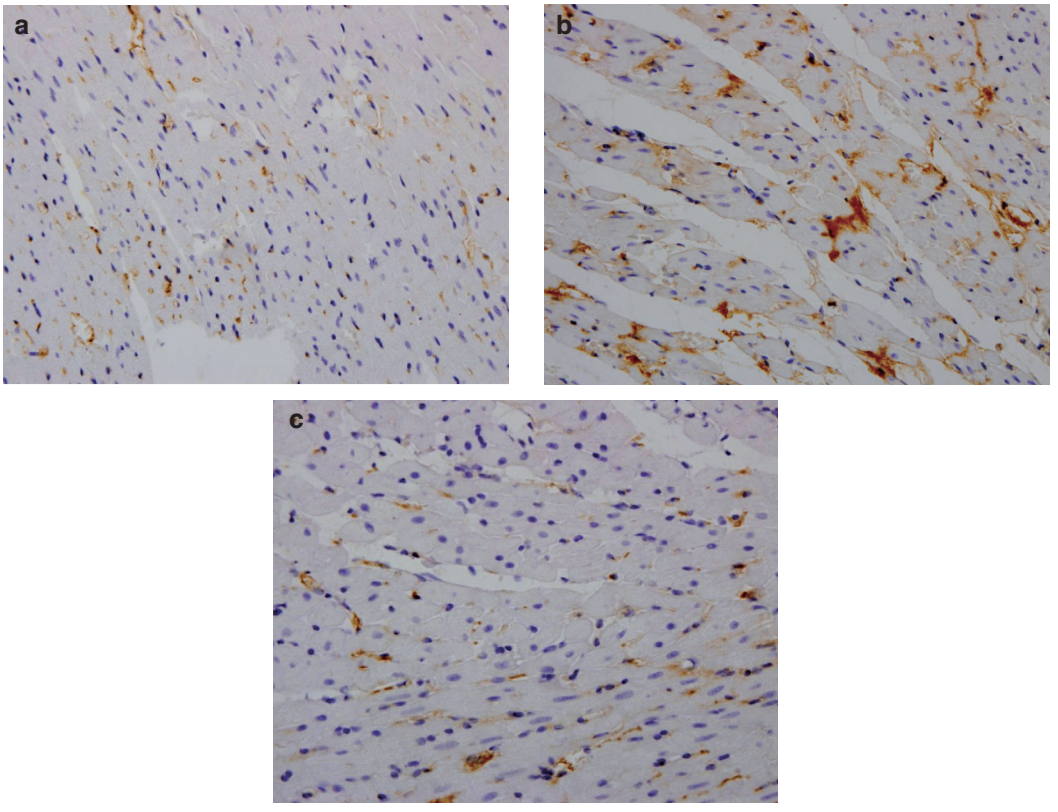

Fig. 4. a-c. AT1-R immunohistochemistry stain (× 40) of myocardial tissue sample in groups I-3, respectively. Brown stain was AT1-R expression. More positive staining result could be detected in group II. After Piog treatment, AT1-R expression was inhibited in group III. *, the differences were significant comparing with group I ($P < 0.05$); #, the differences were significant comparing with group II ($P < 0.05$).

was not significant ($P = 0.845$) at the end. The results indicated that the difference of cardiac fibrosis and mortality in groups II and III were not due to the difference of blood glucose and insulin levels between the two groups.

Hydroxyproline is a specific amino acid of collagen proteins and constitutes about 13% in collagen amino acid content. Measurement of hydroxyproline reflects tissue collagen content and the degree of tissue fibrosis. In the present study, the hydroxyproline content in group II was obviously higher than that in group I, but Piog intervention decreased it significantly in group III. The phenomenon indicated that the PPARγ ligand depressed myocardial fibrosis in the diabetic model, which was also proved by Sirius red staining. We also found that fibrosis mainly occurred under epicardium and surrounding small coronary arteries under micropo-

larimete.

At the 14th week, myocardial fibrosis induced cardiac remodeling and alteration of cardiac functions. Although EDD-Is were similar in groups II and I, IVS-I elevated significantly in group II as detected by echocardiography. Although EFs were similar in the 3 groups, LVEDP increased and LVSP decreased markedly in group II. The results highlighted diabetes impaired mechanical function of myocardium. At the same time, −dp/dtmax decreased significantly in group II which indicated that diabetes depressed myocardium diastolic function in the early stage, but Piog treatment did not improve the condition.

It has established that Ang II concentration in cardiac tissues and in circulation increased in diabetic individuals. Increase of RAS activity is closely associated with myocardial fibrosis. Excessive activation of RAS in diabetic model plays an important role in myocardial fibrosis. The present study revealed that AT1-R expression increased in diabetic myocardial tissue which may upregulate RAS activity at the receptor level while Piog administration could attenuate AT1-R expression, depressed activity of RAS at the receptor level, and inhibited cardiac fibrosis induced by diabetes.

Development and progression of cardiac fibrosis were associated with complicated mechanisms involving expression of nuclear transcription factors, inflammatory factors and pro-fibrosis factors. As shown in our previous study (13), activation of the PPARγsignal pathway could attenuate AngII-induced collagen synthesis in cardiac fibroblasts through down-regulation of AT1-R expression, which repressed the activity of RAS at the receptor level and the degree of fibrosis. Diabetic myocardium fibrosis is just related to excessive activation of tissue RAS. In the present study, activation of the PPARγ signal pathway could inhibit the process of myocardium fibrosis and could partly improve cardiac functions in the diabetic model. One of the mechanisms was the decrease of AT1-R expression. Additionally, the formation of advanced glycosylation end products (AGEs) played an important role in diabetes-induced myocardial fibrosis. There was evidence that PPARγ ligands also inhibited the formation of AGEs receptor in blood vessel endothelium of diabetic rats, and in ameliorated vessel function (3). The relationship between PPARγ ligands and diabetic myocardium fibrosis need to be further investigated.

Since the duration of the study was relative short, the effects of diabetes and Piog intervention on myocardium might not have been completely displayed. Prolonging of the observation time might lead to obtaining more truthful results.

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