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Effects of PPARγ agonist pioglitazone on cardiac fibrosis in diabetic mice by regulating PTEN/AKT/FAK pathway

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Abstract. – OBJECTIVE: The aim of this study was to explore the role of pioglitazone (PIO), a peroxisome proliferator-activated receptor-gamma (PPARγ) agonist, in cardiac fibrosis of diabetic mice.

MATERIALS AND METHODS: A total of 60 adult male C57/B6 mice were divided into 3 groups using a random number table, namely, control group (Sham group, n=20), diabetic cardiomyopathy group (DCM group, n=20), DCM + PIO group (n=20). Streptozocin (STZ) was injected into mice at a dose of 125 mg/Kg to induce the model of diabetes *in vivo*. After successful induction, mice in DCM + PIO group were intragastrically given PIO at 10 mg/kg/d once a day for 6 weeks. Meanwhile, those in Sham group and DCM group were given the same volume of normal saline. After 6 weeks, ejection fraction % (EF%), fraction shortening % (FS%) and heart rate of mice in each group were examined via echocardiography. Picrosirius red (PSR) staining assay was conducted to detect collagen deposition in myocardial tissues of mice in each group. The protein expression level of PPARγ in mouse myocardial tissues in each group was measured through Western blotting and immunohistochemical staining assays. Hematoxylin-eosin (H&E) staining assay was carried out to evaluate the myocardial hypertrophy of mice in each group. The protein expression level of transforming growth factor-β (TGF-β) in mouse myocardial tissues in each group was measured through immunohistochemical staining assay. In addition, Western blotting was employed to detect the expression of proteins related to the phosphate and tension homology deleted on chromsome ten (PTEN)/protein kinase B (AKT)/focal adhesion kinase (FAK) signaling pathway in myocardial tissues of mice in each group.

RESULTS: The messenger ribonucleic acid (mRNA) and protein expression levels of PPARγ in mouse myocardial tissues were significantly lower in DCM group than those in Sham group ($p<0.05$). PPARγ agonist PIO could significantly increase the protein expression of PPARγ in myocardial tissues of DCM mice. The results of cardiac Doppler ultrasound revealed that PIO significantly upregulated EF% and FS% in DCM mice ($p<0.05$). Besides, PIO remarkably reduced collagen deposition and TGF-β protein expression in myocardial tissues in DCM mice ($p<0.05$). H&E staining results showed that PIO notably attenuated myocardial hypertrophy in DCM mice ($p<0.05$). Furthermore, it was discovered that PIO markedly elevated PTEN protein in myocardial tissues of DCM mice and inhibited the phosphorylation of AKT and FAK proteins ($p<0.05$).

CONCLUSIONS: The protective effect of PIO against cardiac fibrosis in diabetic mice may be related to its regulation on the PTEN/AKT/FAK signaling pathway. Our findings suggest that PIO is expected to become a targeted drug for the treatment of DCM in clinical practice.

Key Words:
Pioglitazone, PPARĐ, Diabetic cardiomyopathy, Cardiac fibrosis, PTEN.

Introduction

Diabetes mellitus has an increasing incidence rate in recent years. Its complications, especially cardiac dysfunction and chronic heart failure caused by cardiac fibrosis, seriously endanger the health of millions of people worldwide[1]. Cardiac fibrosis is a vital player in the development and progression of diabetic heart failure. In the case of diabetic cardiomyopathy (DCM), extracellular matrix (ECM) protein deposition and matrix cross-linking may increase myocardial bluntness, thereby mediating diastolic dysfunction[2-4]. Therefore, further exploration of the pathogenesis of cardiac fibrosis in DCM is of great significance for the prevention and treatment of diabetes-induced cardiac dysfunction.

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Peroxisome proliferator-activated receptor-gamma (PPARγ) is an important member of the nuclear hormone receptor superfamily. Some scholars have shown that it is able to affect the fibrosis of many organs[5]. Meanwhile, its expression is prominently inhibited in hypertension-induced or stress-induced cardiac fibrosis[6]. Severe cardiac fibrosis may occur under the stimulation of angiotensin II if the mouse PPARγ gene is defective[7]. In contrast, drug activation or genetically overexpression of PPARγ is capable of signally repressing the production of collagen fibers in cardiac fibroblasts[8]. However, no reports have elucidated the role of PPARγ in DCM.

In this study, the expression of PPARγ in myocardial tissues of healthy and DCM mice was first detected. The role of pioglitazone (PIO), a PPARγ agonist, in fibrosis in DCM mice was observed. Moreover, the potential mechanism of PIO in resisting cardiac fibrosis was further investigated. All our findings might help to provide a certain reference for the clinical treatment of DCM in the future.

Materials and Methods

Animal Grouping and Modeling

A total of 60 male C57/B6 mice weighing (21.11±1.83) g and aged 6-8 weeks old were divided into 3 groups using a random number table, including: control group (Sham group, n=20), diabetic cardiomyopathy group (DCM group, n=20), DCM + PIO group (n=20). All mice were fasted for 12 h, and streptozocin (STZ) was intraperitoneally injected into the mice at a dose of 125 mg/Kg, according to a previous study[9]. After that, the model of diabetes was successfully established in mice. Meanwhile, mice in Sham group were injected with the same volume of normal saline. 3 days later, fasting blood glucose level of mice in each group was measured and recorded for 1 week. Afterwards, mice with a stable blood glucose level greater than 11.1 mmol/L were selected as experimental subjects. This study was approved by the Animal Ethics Committee of Qingdao University Animal Center.

Examination of Cardiac Function of Mice in Each Group by Doppler Ultrasound

To examine the cardiac function of mice in each group, echocardiogram was obtained using a Mylab 30CV ultrasound system (Esaote S.P.A, Genoa, Italy) and a 10-MHz linear ultrasound transducer. To be specific, the mice were anesthetized after shaving the anterior chest hair. Next, they were placed on a hot plate at 37°C with the left side facing up. Finally, parameters such as ejection fraction % (EF%), fractional shortening (FS%), and heart rate were examined.

Measurement of PPARγ Expression Via Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total ribonucleic acid (RNA) was firstly extracted from freshly frozen mouse ventricular tissues using a TRIzol™ kit (Invitrogen, Carlsbad, CA, USA). The concentration and purity of extracted RNA were detected using a spectrophotometer. Next, RNAs with qualified concentration and purity (2 μg from each sample) were reversely transcribed into complementary deoxyribonucleic acid (cDNA). RT-PCR was conducted using the following system, including: 2.5 μL of 10 × Buffer, 1 μL of cDNA, 0.5 μL of forward primers (20 μmol/L), 0.5 μL of reverse primers (20 μmol/L), 10 μL of LightCycler® 480 SYBR Green I Master (2X), and 5.5 μL of ddH_2O. The RT-PCR amplification system was the same for all samples. Primers used in this study were shown in Table I.

Western Blotting

Ventricular tissues of each group of mice freshly frozen in a refrigerator at -80°C were

Table I. Primer sequence of each index in RT-PCR.

Target gene		Primer sequence
GAPDH	Forward	5'-GACATGCCGCCTGGAGAAACCC-3'
	Reverse	5'-AGCCCAGGATGCCCTTTAGTCCA-3'
PPARγ	Forward	5'-CAGCTGATAGTCGTGATGTCGTAG-3'
	Reverse	5'-ACGTAGTCGGCTGATATATGCCCA-3'

Note: For each independent sample, two replicates were set.

first taken out. After cut into pieces with scissors, the tissues were fully ground using a grinder, followed by ultrasonic lysis. Next, the lysate was centrifuged, and the supernatant was collected into Eppendorf (EP; Hamburg, Germany) tubes in aliquots. Protein concentration was detected by the bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA) and UV spectrometry. Then, the proteins were dissolved to the constant concentration, sub-packaged and stored in a refrigerator at -80°C. Total proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto cellulose acetate/polyvinylidene difluoride (PVDF) membranes (Roche, Basel, Switzerland). After that, the membranes were incubated with primary antibodies at 4°C overnight. On the next day, the membranes were incubated with goat anti-rabbit secondary antibody in dark for 1 h. Immuno-reactive bands were finally scanned and quantified using an Odyssey (Seattle, WA, USA) membrane scanner.

Evaluation of Myocardial Hypertrophy

Heart samples obtained from each group were first placed in 10% formalin overnight, dehydrated, and embedded in paraffin blocks. Next, myocardial tissues were sliced into sections (5 μm in thickness), fixed on glass slides, and dried for staining. According to the instructions, the sections were soaked in xylene, a gradient ethanol series, and hematoxylin, respectively. Then, the sections were mounted with resin. After drying, the sections were observed and photographed under an optical microscope. Finally, the morphology of myocardial cells, cardiac interstitial and myofilaments was observed.

Determination of Cardiac Fibrosis

Myocardial tissues cut were baked in an oven at 60°C for 30 min, deparaffinized with xylene (5 min ×3 times), and dehydrated with 100%, 95% and 70% ethanol (3 times for each concentration), respectively. Next, the tissues were added with picrosirius red (PSR) stain, followed by incubation for 1 h. Thereafter, the tissues were rinsed slowly with running water for 10 min to remove the staining solution on the surface. Finally, the tissues were added with hematoxylin to stain the nucleus and mounted, followed by observation under an optical microscope (Olympus, Tokyo, Japan).

Detection of Pparγ Protein Expression Through Immunohistochemistry

Mouse myocardial sections were first deparaffinized in an incubator at 60°C for 30 min, and subjected to antigen retrieval *via* citrate high pressure method. Subsequently, the sections were covered and incubated with 3% hydrogen peroxide for 20 min, followed by blocking with 8% goat serum for 30 min. After that, PPARγ [diluted at 1:200 in phosphate-buffered saline (PBS)] and transforming growth factor-β (TGF-β) (diluted at 1:100 in PBS) primary antibodies were added dropwise to completely cover the sections, followed by incubation in a refrigerator at 4°C overnight. On the next day, the sections were rewarmed and incubated with secondary antibodies added in drops at room temperature for 30 min. After washing, the sections were added drop-wise with the developer diaminobenzidine (DAB) working solution (Solarbio, Beijing, China). The time of color development was strictly controlled under an optical microscope. Lastly, the sections were counter-stained with hematoxylin, dehydrated with a gradient ethanol series and mounted. Cardiac tissues in ten randomly selected fields of view were photographed under an optical microscope (200×), and the protein positive rate in each group was analyzed by Image J software.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 software (IBM Corp., Armonk, NY, USA) was utilized for all statistical analysis. Measurement data were expressed as mean ± standard deviation. *t*-test was employed to compare the difference between two groups. $p<0.05$ was considered statistically significant.

Results

Expression of PPARγ in Myocardial Tissues of DCM Mice

The protein and messenger RNA (mRNA) expression levels of PPARγ in myocardial tissues were dramatically lower in DCM group than those in Sham group ($p<0.05$) (Figure 1A-1B). The results of immunohistochemical staining assay further uncovered that in myocardial cells, PPARγ was mainly expressed in the nucleus.

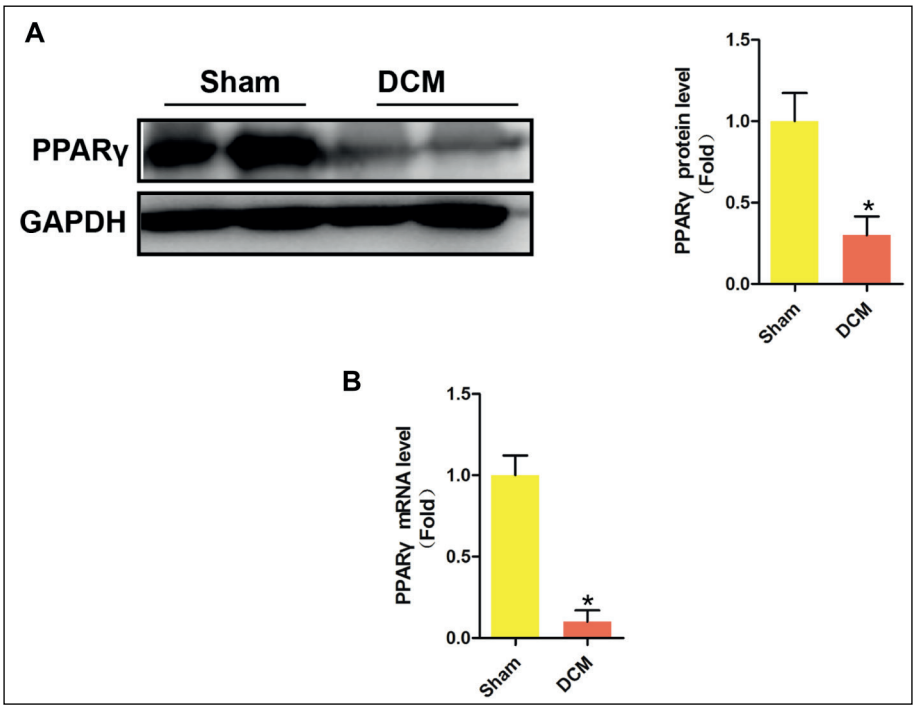

Figure 1. Expression of PPARγ in myocardial tissues of DCM mice. **A,** protein expression of PPARγ detected by Western blot, **B,** mRNA expression of PPARγ detected by PCR, (Sham: Sham group, and DCM: DCM group. $^{*}p<0.05$ *vs.* Sham group).

Effect of PIO on PPARγ in Myocardial Tissues of DCM Mice

PIO is a typical hypoglycemic drug and also a potent PPARγ agonist. Western blotting results (Figure 2) demonstrated that PIO significantly increased the protein expression level of PPARγ in myocardial tissues of DCM mice ($p<0.05$)

Effect of PIO on Cardiac Function of DCM Mice

According to echocardiograms (Figure 3), there was no statistically significant difference in the heart rate among the three groups of mice. Therefore, it could be ruled out that the differences in EF% and FS% of mice among groups were caused by different heart rates. In comparison with Sham group, DCM group displayed remarkably enlarged ventricular cavity and thinned wall of the heart. PIO notably improved the abnormal changes in the cardiac structure of DCM mice. Meanwhile, the levels of FS% and EF% in each group of mice were detected. It was found that PIO distinctly increased FS% and EF% in DCM mice ($p<0.05$). All these results suggest that PIO can ameliorate the deterioration of cardiac function in DCM mice.

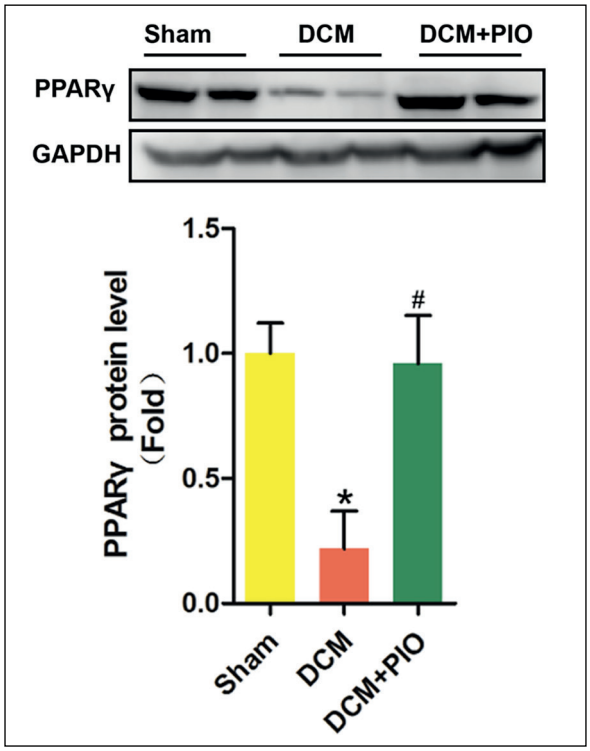

Figure 2. Effect of PIO on PPARγ in myocardial tissues of DCM mice. Sham: Sham group, DCM: DCM group, and DCM + PIO: DCM + PIO group. $^{*}p<0.05$ *vs.* Sham group, $^{\#}p<0.05$ *vs.* DCM group.

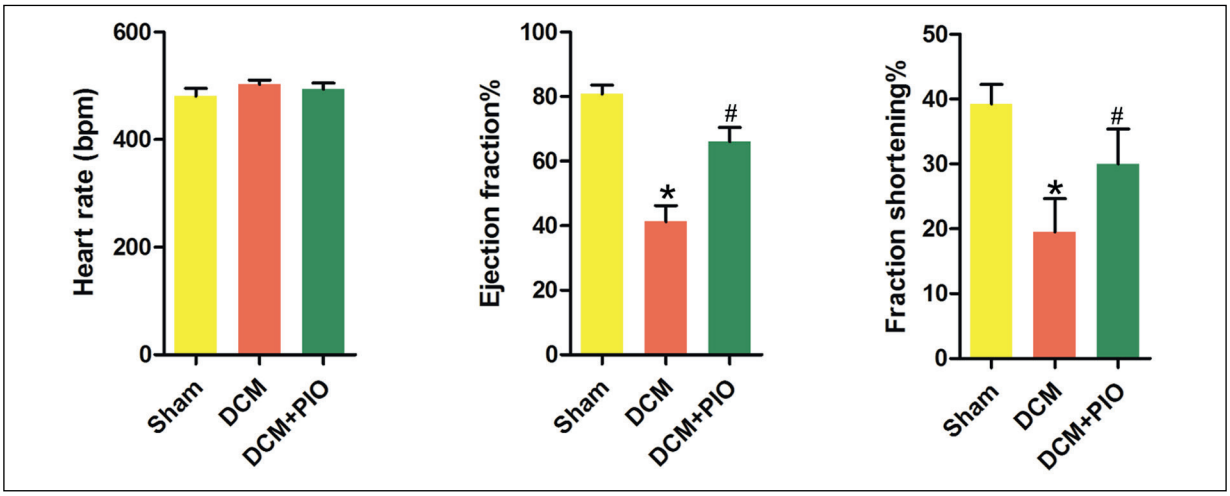

Figure 3. Effect of PIO on cardiac function of DCM mice. Sham: Sham group, DCM: DCM group, and DCM + PIO: DCM + PIO group. $^{*}p<0.05$ *vs.* Sham group, $^{\#}p<0.05$ *vs.* DCM group.

Effect of PIO on Collagen Deposition in the Myocardium of DCM Mice

PSR staining assay indicated that collagen content in myocardial tissues was significantly higher in DCM group than that in Sham group ($p<0.05$). However, PIO visibly inhibited collagen deposition in the myocardium of DCM mice ($p<0.05$) (Figure 4).

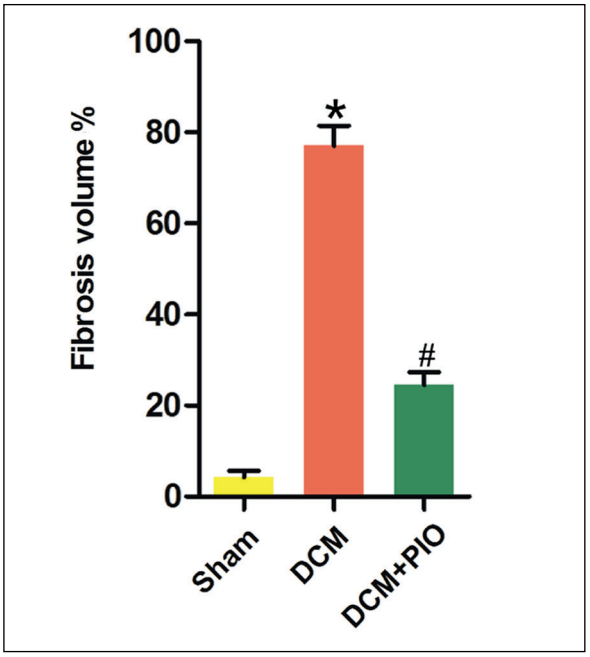

Figure 4. Effect of PIO on collagen deposition in the myocardium of DCM mice. (magnification: 200×). Sham: Sham group, DCM: DCM group, and DCM + PIO: DCM + PIO group. $^{*}p<0.05$ *vs.* Sham group, $^{\#}p<0.05$ *vs.* DCM group.

Effect of PIO on TGF-β Expression in the Myocardium of DCM Mice

TGF-β is a key factor leading to the phenotypic transformation of fibroblasts to myofibroblasts in the heart. Meanwhile, it is also a stimulating factor for collagen synthesis in myocardial tissues. It was discovered in the present study that PIO considerably reduced the protein level of TGF-β in myocardial tissues of DCM mice ($p<0.05$) (Figure 5).

Effect of PIO on Cardiomyocyte Hypertrophy in DCM Mice

Myocardial hypertrophy and cardiac fibrosis are important pathological changes of DCM. Hypertrophic cardiomyocytes can secrete specific cytokines to promote the production of a large number of collagen fibers and phenotypic transformation of myocardial cells *via* paracrine. The results of hematoxylin-eosin (H&E) staining assay (Figure 6) showed that PIO significantly repressed cardiomyocyte hypertrophy and decreased heart weight in DCM mice ($p<0.05$).

Regulation of PIO on Gene of Phosphate and Tension Homology Deleted on Chromsome Ten (PTEN)/Protein Kinase B (AKT)/Focal Adhesion Kinase (FAK) Pathway in the Myocardium of DCM Mice

Previous studies have manifested that PPARγ relieves cardiac fibrosis by suppressing AKT activation that is negatively regulated by PTEN. The results (Figure 7) revealed that the activation of PPARγ notably inhibited PTEN expression in

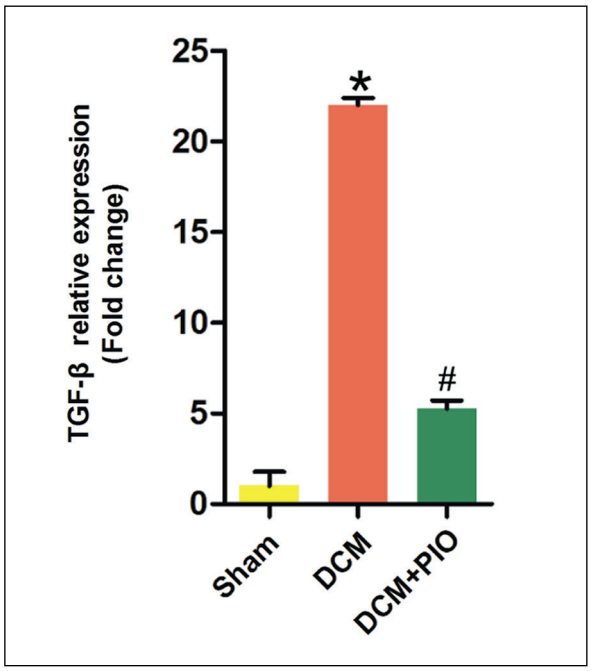

Figure 5. Effect of PIO on TGF-β expression in the myocardium of DCM mice. Sham: Sham group, DCM: DCM group, and DCM + PIO: DCM + PIO group. *p<0.05 *vs.* Sham group, #p<0.05 *vs.* DCM group.

myocardial tissues of DCM mice (p<0.05). In addition, the phosphorylation of AKT/FAK was remarkably activated (p<0.05).

Discussion

DCM is a common disease with abnormal myocardial structure and function in patients with diabetes. It often occurs independently from coronary arteriosclerosis, valvar heart disease, hypertension and other congenital heart diseases. DCM is manifested as myocardial hypertrophy, fibrosis, cardiomyocyte apoptosis, and cardiac diastolic and systolic dysfunction[10]. During the development and progression of DCM, left ventricular remodeling will approach inflammation-related and fibrosis-related signaling pathways including nuclear factor-kappa B (NF-κB), mitogen-activated protein kinase (MAPK) and TGF-β[11,12]. Cardiac fibrosis is an important pathological change of myocardial diseases, which refers to the abnormal deposition of collagen, fibronectin, and other ECM. Eventually, it can lead to increased myocardial bluntness, cardiac dysfunction, and even heart failure. Mice with STZ-induced diabetic have such characteristics as impaired diastolic function, heart failure, myocardial hypertrophy, myofibril depletion, and interstitial fibrosis. Therefore, they are often used as research subjects in the exploration of the pathogenesis of DCM[13]. In this study, the model of DCM was successfully constructed in mice through the induction with STZ. Moreover, the effect of PPARγ, one of the potential targets affecting the development and progression of DCM, was revealed.

PPARγ is a ligand-dependent nuclear receptor transcription factor, which is able to regulate glucose, fat and energy homeostasis in cells. In addition, it is capable of modulating the proliferation, differentiation and apoptosis of various human tumor cells[14,15]. Moreover, PPARγ can attenuate myocardial injury and inflammation induced by local myocardial ischemia-reperfusion injury in rabbits and rats with cardiovascular diseases[16,17]. Yasuda et al[18] have shown that the anti-myocardial infarction effect of PPARγ may be correlated with the activation of the PI3K/AKT/eNOS signaling pathway. Yasuda et al[18] have indicated that the activation of PPARγ is also able to improve the cardiac function and increase the survival rate of mice with septic cardiomyopathy. In fact,

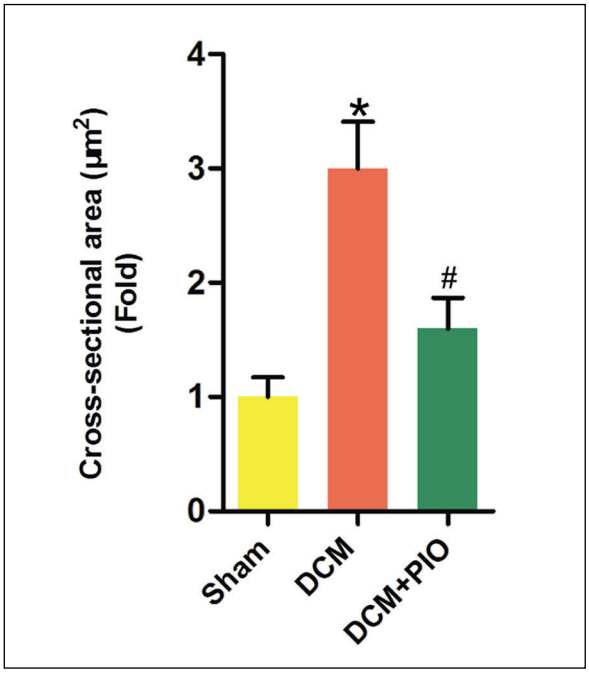

Figure 6. Effect of PIO on cardiomyocyte hypertrophy in DCM mice. Sham: Sham group, DCM: DCM group, and DCM + PIO: DCM + PIO group. *p<0.05 *vs.* Sham group, #p<0.05 *vs.* DCM group.

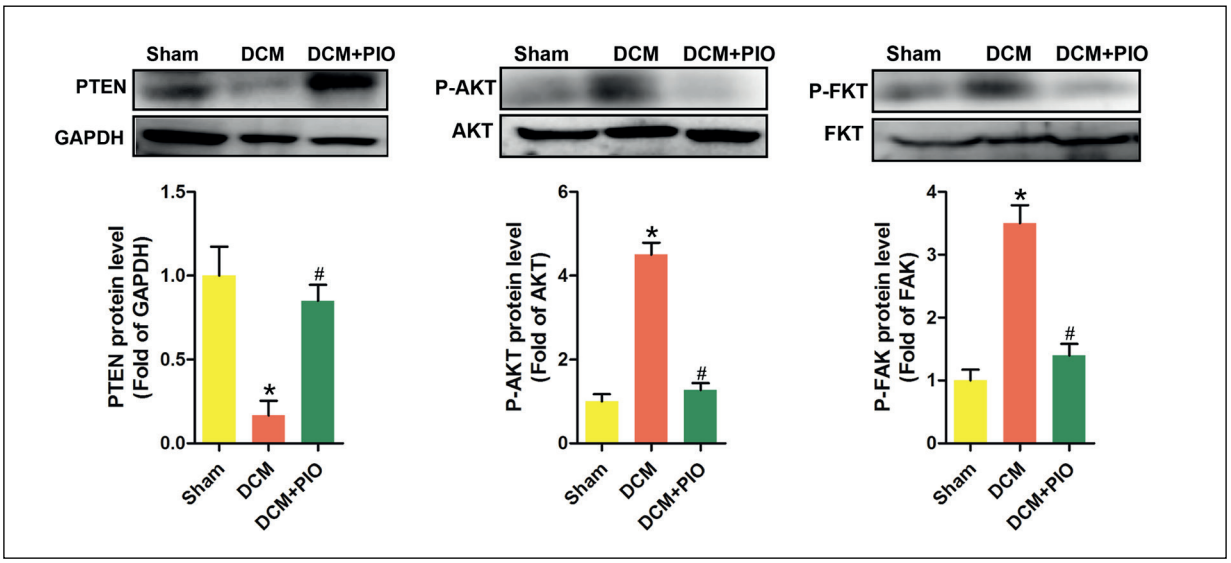

Figure 7. Regulation of PIO on PTEN/AKT/FAK pathway in the myocardium of DCM mice. Sham: Sham group, DCM: DCM group, and DCM + PIO: DCM + PIO group. *$p<0.05$ *vs.* Sham group, $^{\#}p<0.05$ *vs.* DCM group.

PPARγ plays a crucial role in inhibiting cardiac fibrosis. Mohamad et al[19] have reported that coexistence of diabetes and hypertension can induce cardiomyopathy and cause cardiac fibrosis in rats. It is interesting that rosiglitazone combined with felodipine significantly alleviate metabolic abnormalities and cardiac fibrosis in rats. However, rosiglitazone alone achieves unobvious effects in protecting the heart and mitigating hypertension in rats. In the present study, it was found that PIO remarkably reduced STZ-induced collagen synthesis in the myocardium and TGF-β expression, impeded cardiomyocyte hypertrophy, and improved cardiac function in DCM mice.

AKT is an important player in cardiac fibrosis, whose phosphorylation can be promoted by TGF-β through activation of PI3K. AKT inhibition is able to evidently ameliorate stress-induced and diabetes-induced cardiac fibrosis. The activation of PPARγ clearly inhibits the phosphorylation of AKT[20,21]. Burgess et al[22] have revealed that the electrophilic center of PPARγ ligands is a vital factor inhibiting the phosphorylation of AKT. PPARγ can bind to the PTEN promoter to transcriptionally activate PTEN, thereby repressing the activation of AKT[23]. FAK is a non-receptor tyrosine kinase with a molecular weight of 125-kDa. Zhang et al[24] have pointed out that targeted inhibition of FAK relieves cardiac fibrosis and improves cardiac function. Meanwhile, the phosphorylation of mTOR, AKT, P70S6K and ERK1/2 is inhibited after inhibiting FAK with drugs. All their findings imply that the activation of AKT is also positively regulated by FAK. In the current study, it was uncovered that PIO prominently activated PPARγ expression and inhibited FAK phosphorylation, ultimately leading to the inhibition of AKT phosphorylation and alleviating cardiac fibrosis in DCM mice.

Conclusions

Altogether, PPARγ was lowly expressed in myocardial tissues of DCM mice. Moreover, PPARγ agonist PIO was able to distinctly attenuate cardiac fibrosis and hypertrophy in DCM mice. The possible underlying mechanism might by related to the activation of PPARγ and the inhibition of FAK to suppress the phosphorylation of AKT.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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