

Original Article

Qiliqiangxin attenuates isoproterenol-induced cardiac remodeling in mice

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Abstract: Background: To further explore the role of PPAR γ in QL treatment, ISO-induced mice model and following methods were established. Methods: Cardiac remodeling on mice model was induced by isoproterenol (ISO) infusion or saline infusion as control for two weeks then divided into 4 groups, after that divided in 5 different treatment methods to investigate the role of PPAR γ in QL therapy. Echocardiography and Masson's trichrome staining were respectively used to determine cardiac function and fibrosis. Immunoblotting was applied to evaluate the expression levels of proliferator-activated receptor- γ (PPAR γ), Bax, Bcl, phospho-Akt (Ser473), Akt, phospho-P38 and P38, phospho-ERK and ERK. Results: QL treatment improved left ventricular function, decreased apoptosis, and prevented myocardial fibrosis at the same time. Meanwhile, the PPAR γ level was elevated with QL treatment in ISO-injected mice hearts. Inhibition of PPAR γ activity blocked the protective effects of QL, while the activator of PPAR γ did not provide additional benefit. Specifically, the results indicated a decline in PPAR γ in ISO-infused mice and QL decreased the toxicity of ISO by improving the level of PPAR γ . Conclusions: Our study demonstrated that QL treatment provided cardioprotection against ISO-induced cardiac remodeling by improving PPAR γ level, which could be as the potential therapeutic target in reversing cardiac remodeling and heart failure.

Keywords: Qiliqiangxin, heart failure, PPAR γ , cardiac dysfunction, isoproterenol, cardiac fibrosis

Introduction

Cardiac remodeling is defined as changes in size, shape and function of the heart resulting from cardiac load or injury, which is regarded as a determinant of the clinical course of heart failure (HF) [1]. Whereas patients with major remodeling undergo progressive worsening of cardiac function, slowing or preventing cardiac remodeling has become a new goal of heart failure therapy [2, 3]. One of the major components of cardiac remodeling is due to the neurohormonal over-activation. The therapeutic antagonism of neurohormonal systems, as we all know, β -adrenergic blocking agents, angiotensin converting enzyme inhibitors (ACEIs), angiotensin receptor blockers (ARBs), and mineralocorticoid-receptor antagonists (MRAs), has become the cornerstone of contemporary pharmacotherapy for heart failure [1, 4, 5]. Isoproterenol-induced cardiac injury includes

activation of inflammation, necrosis of myocardium, and disarrangement of energy reserves in cardiomyocytes, interstitial fibrosis and cardiac remodeling occur, and eventually caused cardiac dysfunction [6, 7]. Qiliqiangxin (QL), a traditional Chinese medicine (TCM), is an extract of 11 herbs. Our previous study showed reducing of plasma N-terminal pro-B-type natriuretic peptide (NT-proBNP) level after treatment with QL in 512 patients with chronic heart failure, among those 20% of the patients have hypertension [8]. Our group previously reported that QL protected against cardiac remodeling after experimental acute myocardial infarction (AMI) in animal models [9], and enhanced metabolism in cardiomyocytes by increasing mitochondrial content and biogenesis [10]. Although the evidence of beneficial effects with QL on chronic heart failure is established, the underlying mechanism remains unclear.

Materials and methods

Animals

C57/BL6 male mice, 8-10 weeks old, weighing 22-28 g, were obtained from Nanjing University. Mice maintained in specific pathogen-free (SPF) conditions under a 12 h light/dark cycle, and fed ad libitum on a standard rodent diet. The study was approved by the ethical committees of the Nanjing Medical University and all animal experiments were conducted under the guidelines on humane use and care of laboratory animals for biomedical research published by National Institutes of Health (No. 85-23, revised 1996). ISO was dissolved in sterile saline and was intraperitoneally injected (60 mg/kg/day) once daily for 14 consecutive days. The day after final injection, cardiac function were measured by echocardiography.

Echocardiography

The systolic heart function of the mice was measured by echocardiography. Mice were anesthetized with 2% isoflurane, maintained in the decubitus position and were allowed to breathe spontaneously during the procedure. Echocardiography was performed with a 35-MHz phased-array ultrasound system Vevo 2100 (Visual Sonics Inc, Toronto, Ontario, Canada). The following parameters were measured from M-mode images taken from the parasternal short-axis view at papillary muscle level: interventricular septum (IVS), left ventricular internal dimension (LVID), Left ventricular volume (LV vol), left ventricle mass (LV mass), left ventricular fractional shortening (FS) and left ventricular ejection fraction (EF).

Animal groups

QL was provided by Shijiazhuang Yiling Pharmaceutical Co., Ltd. (Shijiazhuang, Hebei, China). QL were given intragastrically after ISO-infusion to the mice with the optimum dose (0.5 g/kg/d) found in our previous study [9]. Mice were randomly divided into four groups: (1) saline; (2) Saline+QL; (3) ISO; (4) ISO+QL.

To prove whether PPAR γ provide beneficial effects on ISO-induced heart failure, mice were randomly divided into five groups and were treated as follows: (1) saline; (2) ISO; (3) ISO+QL;

(4) ISO+QL+PPAR γ activator (Rosiglitazone, 1 mg/kg/d); (5) ISO+QL+PPAR γ inhibitor (T007-0907, 1 mg/kg/d), PPAR γ activator and inhibitor were intraperitoneally injected just after ISO injection.

Immunohistochemistry

To assess the degree of fibrosis, sections were stained with Masson-Trichrome and scanned with computer-assisted video densitometry; the fibrotic fraction was obtained by calculating the ratio of blue (fibrotic) to total myocardial area using Image J (NIH).

Immunoblotting

Heart tissues were lysed in RIPA buffer (P0013C, Beyondbtime) supplemented with 1 mM PMSF (ST505, Beyondbtime). 30 μ g of total protein was subjected to electrophoresis on 10% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes which incubated with 5% milk and with primary antibody at 4°C overnight, and then washed three times with TBST buffer before adding secondary antibody. Specific protein bands were visualized by using ECL (Invitrogen) reagents.

Primary antibodies as follows: transforming growth factor beta (TGF- β , 1:1000 dilution, Cell Signaling Technology), metalloproteinase-2 (MMP-2, 1:1000 dilution, Abcam), matrix metalloproteinase 9 (MMP-9, 1:1000 dilution, Abcam), B-cell lymphoma 2 (Bcl-2, 1:1000 dilution, Cell Signaling Technology), Bcl-2-associated X protein (Bax, 1:1000 dilution, Cell Signaling Technology), proliferator-activated receptor- γ (PPAR γ , 1:500 dilution, Abcam), peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1- α , 1:1000 dilution, NOVUS), protein kinase B (Akt, 1:1000 dilution, Cell Signaling Technology), phospho-Akt (Ser-473, 1:1000 dilution, Cell Signaling Technology), extracellular signal regulated kinases (ERK, 1:1000 dilution, Cell Signaling Technology) and phospho-ERK (Thr202/Tyr204, 1:1000 dilution, Cell Signaling Technology), phospho-P38 MAPK (p-P38, 1:1000 dilution, Cell Signaling Technology), protein 38 mitogen activated protein kinases (P38, 1:1000 dilution, Cell Signaling Technology), Glyceraldehyde 3-phosphate dehydrogenase antibody (GAPDH, 1:1000 dilution, Kangchen, Shanghai, China).

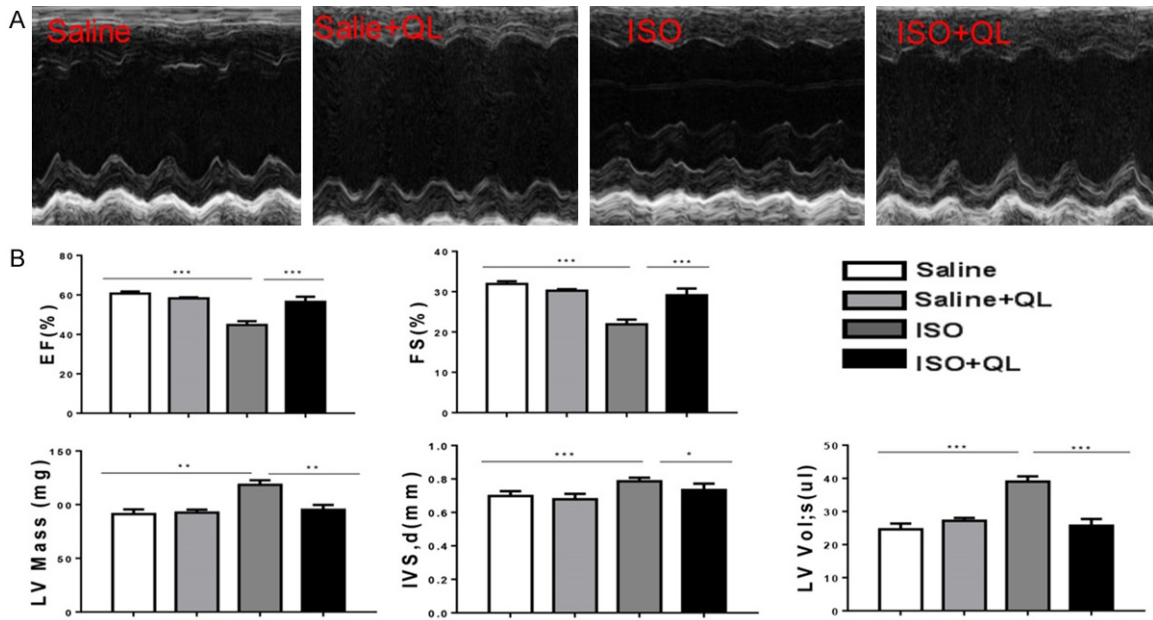


Figure 1. QL attenuates cardiac remodeling after ISO infusion. A. Left ventricular fractional shortening (%) (EF) and ejection fraction (%) (FS) as measured by echocardiography. B. Echocardiography revealed significant impairment in LV function after ISO infusion, including EF and FS, QL treatment improved cardiac function in EF and FS. Data were analyzed by one-way ANOVA followed by Bonferroni's post-hoc test and expressed as the mean \pm SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. $n = 6$ per group.

Quantitative real time polymerase chain reaction (qRT-PCR)

Total RNAs were isolated from heart tissues using TRIZOL RNA extraction kit (Invitrogen, USA) and reverse transcribed to cDNAs using iScript™ cDNA Synthesis Kit (Bio-Rad, USA) according to the manufacturer's instructions. Real-Time PCRs were used to determine gene expression levels by using an ABI 7900HT fast Real-Time PCR System (Applied Biosystems) with SYBR-Green supermix Kit (Bio-Rad).

The primer sequences (forward and reverse) used in this study are listed as follows: atrial natriuretic peptide (ANP): CTC CCA GGC CAT ATT GGAG, TCC AGG TGG TCT AGC AGGTT; brain natriuretic peptide (BNP): TGG GAA GTC CTA GCC AGT CTC, TCT GAG CCA TTT CCT CTGAC. Relative mRNA expression was presented using the $2^{-\Delta\Delta ct}$ method.

Statistical analysis

Results were presented as mean \pm SEM. Independent-samples t-test was used for comparisons between two groups. One-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc test were used to compare among

three (or more) groups. A value of $p < 0.05$ was considered statistically significant. The data were analyzed by GraphPad Prism 7 software.

Results

QL attenuates cardiac remodeling in ISO-induced cardiac dysfunction mice

Cardiac dysfunction was induced by ISO (60 mg/Kg/d) intraperitoneal injection, and then QL or saline administered intragastrically for 2 weeks, following echocardiography was performed for evaluating cardiac function. The echocardiography revealed significant impairment in LV function after ISO infusion, including EF and FS (**Figure 1A**).

On the other side, two consecutive weeks apply of ISO in mice displayed a marked increase in heart size, such as interventricular septum (IVS), left ventricular volume (LV vol), left ventricle mass (LV mass) (**Figure 1B**), and also increased expressions of atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) showed in real-time qPCR (**Figure 2A**), which these physiological changes were prevented by QL treatment.

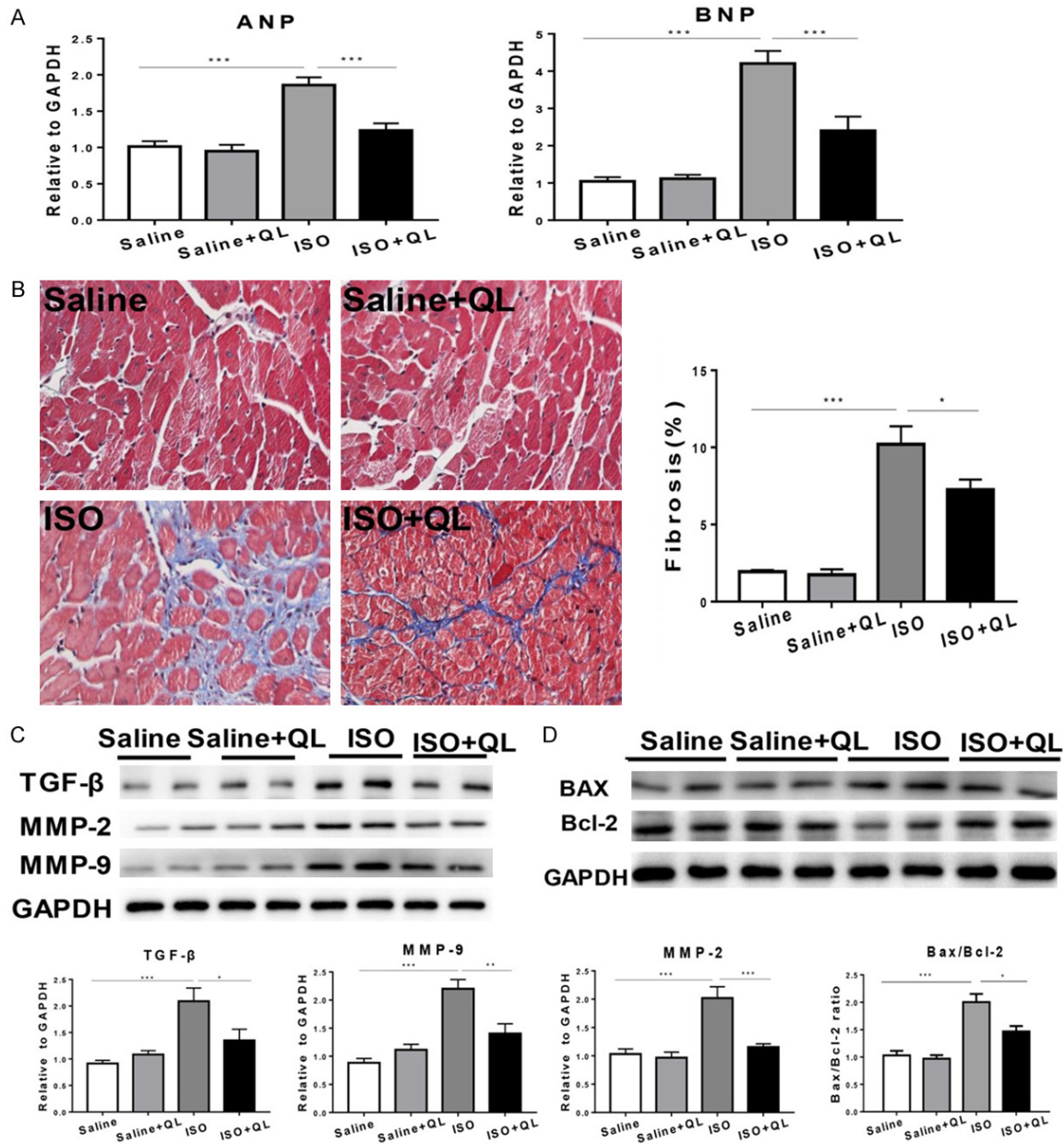


Figure 2. QL treatment diminishes isoproterenol (ISO)-induced cardiac fibrosis and cell death. A. Atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) were increased in the ISO-induced mice which were reversed by QL treatment. B. Effects of QL on interstitial fibrosis assessed by masson trichrome staining, 400× magnifications. C. Western blotting analysis showed protein level of TGF-β, MMP-2 and MMP-9 was decreased by QL treatment. D. Western blotting analysis showed the Bcl-2/Bax ratio was decreased by QL in the ISO-induced mice. Data were analyzed by one-way ANOVA followed by Bonferroni's post-hoc test and expressed as the mean ± SEM. *P<0.05, **P<0.01, ***P<0.001. n=6 per group.

QL reduces ISO-induced cardiac apoptosis and fibrosis

Apoptosis and fibrosis are considered to be the main elements for cardiac remodeling [11, 12]. The application of ISO increased the amount of apoptosis and fibrosis. Western blot indicated

that the ratio of pro-apoptotic molecule Bax to anti-apoptotic molecule Bcl-2 was decreased with QL treatment (Figure 2D and Supplementary Figure 1A). Masson trichrome staining showed collagen deposition in left ventricle section was decreased with QL treatment compared to ISO infusion (Figure 2B). The

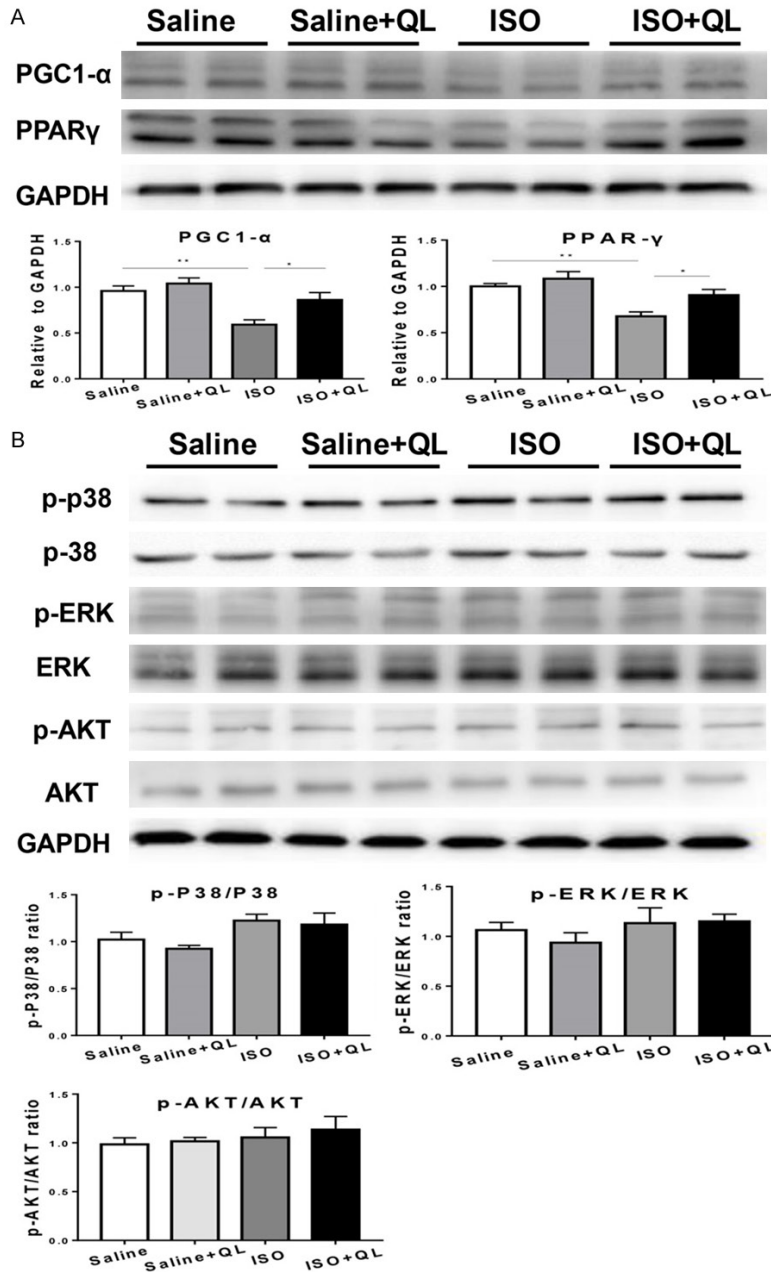


Figure 3. Expression of PPAR γ , p-AKT, AKT, p-ERK, ERK, p-P38, P38 in western blotting. A. The protein level of PPAR γ and PGC1- α was down-regulated in the ISO-induced mice, which was reversed by QLQX treatment. B. QL has no effect on AKT, P-38 and ERK pathways. Data were analyzed by one-way ANOVA followed by Bonferroni's post-hoc test and expressed as the mean \pm SEM. *P<0.05, **P<0.01, ***P<0.001. n=6 per group.

developmental markers of cardiac remodeling, TGF- β , MMP-9 and MMP-2 levels were markedly improved by ISO-infusion; on the contrary, QL treatment significantly reduced ISO-induced improvements of TGF- β , MMP-9 and MMP-2 levels (Figure 2C and Supplementary Figure 1B).

Increased PPAR γ and PGC1- α with QL treatment in ISO-induced cardiac dysfunction mice

The mechanism of the protective effect of QL treatment in ISO-induced cardiac dysfunction mice was further explored. According to our previous research, PPAR γ plays an important role in QL treatment after AMI in mice. This study was conducted to explore whether PPAR γ is involved in sustained ISO activation with QL treatment.

Based on the immunoblotting analysis, decreased level of PPAR γ and PGC1- α were found in ISO-induced mice with vehicle-treated by QL treatment (Figure 3A and Supplementary Figure 1C). However, QL treatment did not alter the expression of AKT, p-ERK1/2/ERK1/2 and p-P38/P38 in ISO-induced cardiac dysfunction, suggesting these signaling pathways were not participated in QL-mediated improvement in cardiac remodeling in ISO-induced heart failure (Figure 3B and Supplementary Figure 1C).

QL attenuates cardiac remodeling in sustained ISO infusion via PPAR γ

To further explore the contribution of PPAR γ to the beneficial effects of QL in ISO infusion, we treated mice with T0070907, an inhibitor of PPAR γ (1 mg/kg/d), combined with QL and ISO. The effects of PPAR γ inhibitor or PPAR γ activator were detected by the decreased or raised PPAR γ expression levels (Figure 5A and Supplementary Figure 1D). Consequently, PPAR γ inhibitor prevented the beneficial effects of QL on cardiac function (evaluated by EF and FS) after ISO infusion

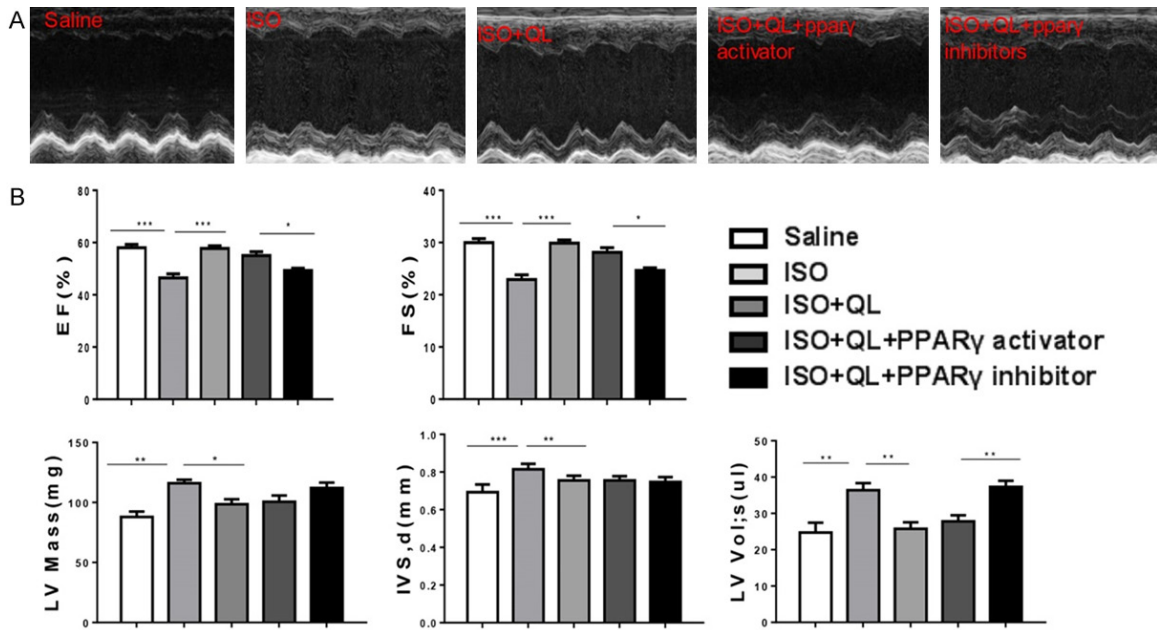


Figure 4. QL improves cardiac function via increasing PPAR γ in ISO-induced mice. A. Left ventricular fractional shortening (%) (EF) and ejection fraction (%) (FS) as measured by echocardiography. B. Echocardiography parameters show that PPAR γ inhibitor abolish the beneficial effects of QL on improving cardiac function, while PPAR γ activator failed to provide any further improvement in cardiac function in the presence of QL. Data were analyzed by one-way ANOVA followed by Bonferroni's post-hoc test and expressed as the mean \pm SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. $n = 6$ per group.

(Figure 4A, 4B). Moreover, PPAR γ activator failed to provide extra profits in cardiac function in the presence of QL (Figure 4A, 4B), suggesting that PPAR γ activation is necessary for its beneficial effects in attenuating cardiac remodeling after ISO infusion. Meanwhile, inhibition of PPAR γ activity potently blocked the protective effects of QL against ISO-induced cardiac dysfunction, which revealed in cardiac fibrosis determined by Masson trichrome staining (Figure 5B). PPAR γ inhibitor abolished the effects of QL in decreasing ISO-induced apoptosis which indicated by the ratio of Bax/Bcl-2 (Figure 5C and Supplementary Figure 1E). Consequently, these data suggested that the increased levels of PPAR γ and PGC1- α could contribute to the beneficial effects of QL in attenuating cardiac remodeling after sustained ISO activation.

Discussion

With use of QL in ISO-induced cardiac remodeling, this study found that QL-mediated cardiac protection in isoproterenol-induced cardiac remodeling by inducing PPAR γ , decreasing apoptosis and reducing cardiac fibrosis are

involved in the pharmacologic effects. The peroxisome proliferation-activated receptor γ (PPAR γ) is from the nuclear receptor superfamily of ligand-inducible transcription factor family [13]. Study showed PPAR γ activation can prevent cardiac hypertrophy, inhibit cardiac remodeling, and reduce infarct size in several animal models; the underlying protective effects of PPAR γ activation have been attributed to reducing inflammation, oxidative stress and cell death [14-16]. However, the limitation of PPAR γ as a therapeutic measure in cardiovascular disease attributes to the risk for fluid retention, weight gain, bone loss and congestive heart failure [17]. Therefore, some aspects of PPAR γ function are poorly understood. Designing or identifying the selective PPAR γ modulators with respect to therapeutic applications is highly desirable and is a major challenge to date [18]. Only parts of the cardioprotective mechanism of QL on variety of pathological animal models have been demonstrated in the line of the previous studies [10, 19-21]. As increased sympathetic nerve activity in the myocardium is a central feature of patients with heart failure [4, 22]. Therefore, ISO-infused mice were established to develop cardiac dysfunction. Following with

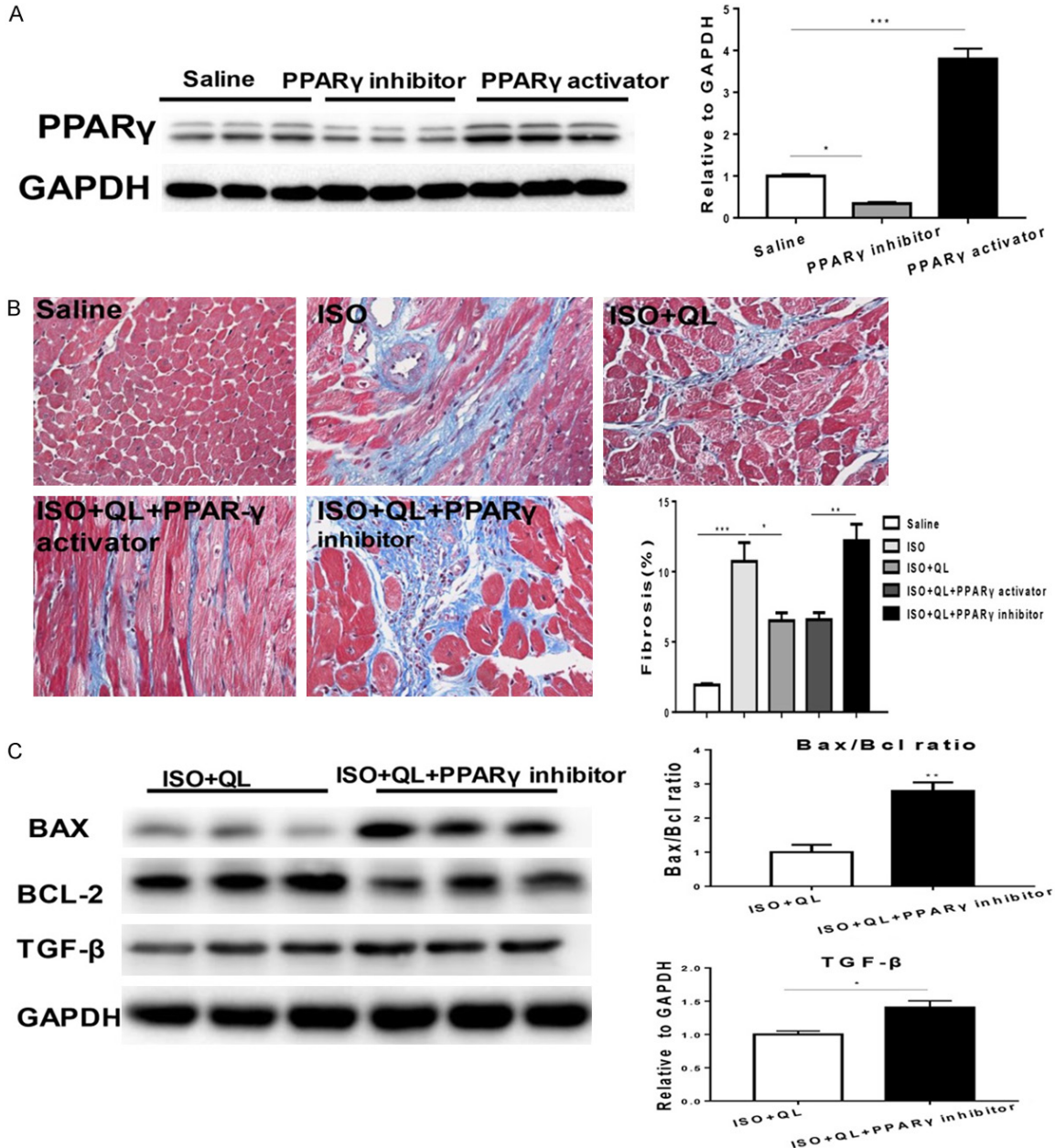


Figure 5. QL attenuates cardiac fibrosis and apoptosis via PPAR γ . (A) PPAR γ inhibitor abolishes the effects of QL in decreasing cardiac fibrosis. (B) PPAR γ inhibitor decreases the expression of PPAR γ and PPAR γ activator increases the expression of PPAR γ . (B) Masson trichrome staining for cardiac fibrosis, 400 \times magnification. (C) PPAR γ inhibitor abolishes the effects of QL in decreasing apoptosis as indicated by the ratio of Bax/Bcl-2. Data were analyzed by one-way ANOVA followed by Bonferroni's post-hoc test (A and B), independent-samples t-test was used for comparisons between two groups (C) and expressed as the mean \pm SEM. * P <0.05, ** P <0.01, *** P <0.001. n =3 per group.

echocardiographic parameters revealed significant impairment in cardiac function; immunohistochemical staining indicated that myocardial fibrosis was significantly improved in ISO group. However, QL-treated mice significantly improved cardiac function, decreased myocardial fibrosis and apoptosis. Based on the immu-

noblotting analysis, the PPAR γ and PGC1- α levels were shown to be decreased in ISO-induced mice compared to vehicle-treated mice, and these decreases were prevented by QL treatment. In addition, inhibition of PPAR γ activity potently prevented the protective effects of QL against ISO-induced cardiac dysfunction.

Moreover, PPAR γ activator was unable to provide additional profits in cardiac function with QL treatment, suggesting that PPAR γ and QL function predominantly through the same pathway, which is fully activated with either treatment alone. Fluid retention may be the major contributor to the negative impact of PPAR γ agonist thiazolidinediones in heart and the underlying mechanism has not been fully elucidated [23]. While some studies reported that some new PPAR γ activators showed a promising therapeutic effect with lesser adverse effects such as fluid retention and heart failure [24, 25]. Non-genomic and genomic mechanisms have been crucial in helping to analyze the relative contributions of PPAR γ activation in the therapeutic effects of QL, as well as determining which cell types in myocardial tissue are involved in PPAR γ transformation and would be expected to have a role in the cardioprotective effect of PPAR γ on the heart [23]. Further studies are needed to clarify the role of PPAR γ in heart and to understand the molecular mechanism of PPAR γ -dependent and PPAR γ -independent pathways [17]. It is also important to identify the exact active ingredients of QL responsible for the therapeutic effects of QL in the future [26].

In conclusion, the present study showed that QL administration offers notable benefits on cardiac function and attenuates the progression of heart remodeling induced by ISO, mediating in activation of PPAR γ and PGC-1 α , which could provide favorable evidence of clinical therapeutic effects on reversing cardiac remodeling and heart failure

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Disclosure of conflict of interest

None.

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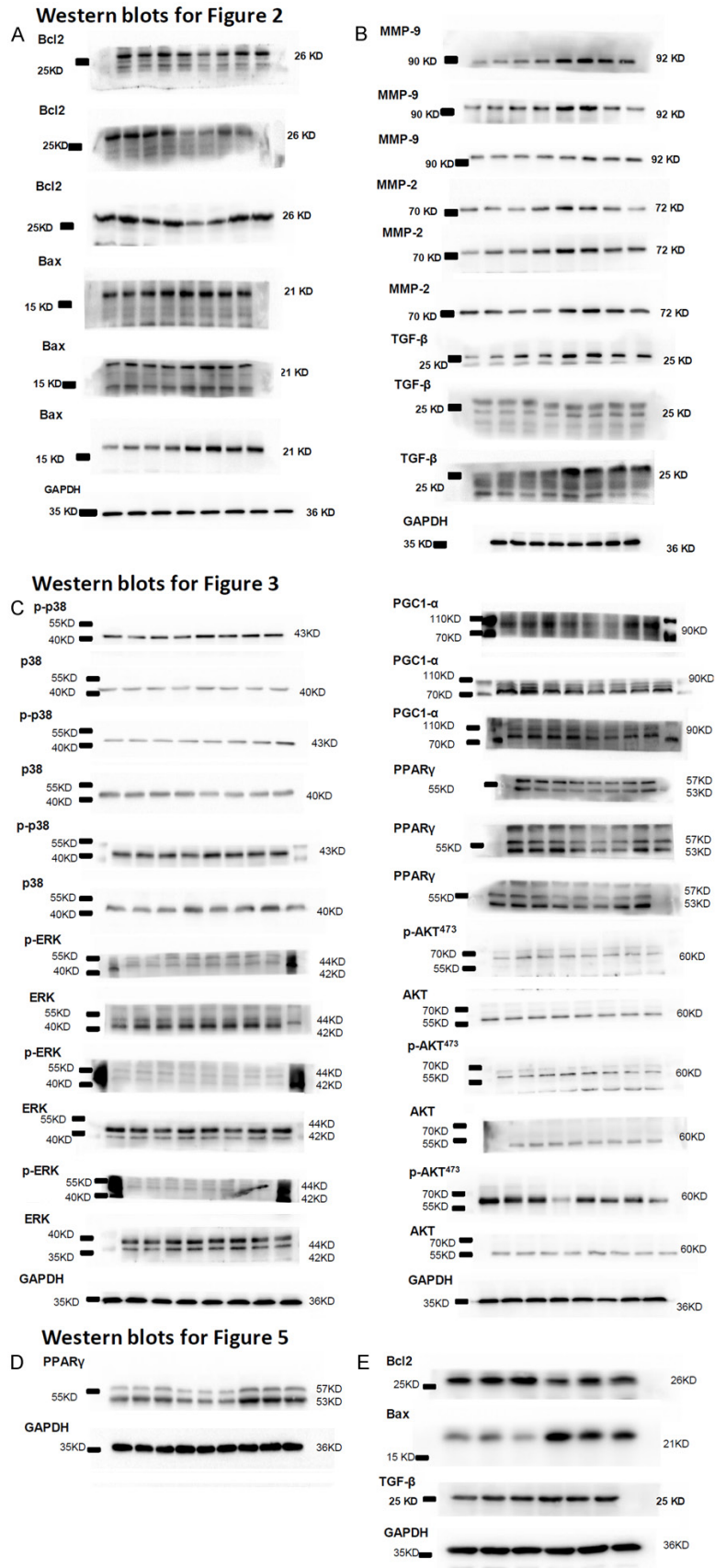
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Supplementary Figure 1. Uncropped scans of Western blots for all relevant western bands.