

Traditional Chinese medicine Qiliqiangxin attenuates phenylephrine-induced cardiac hypertrophy via upregulating PPAR γ and PGC-1 α

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Background: Clinical study has demonstrated that the traditional Chinese medicine Qiliqiangxin (QLQX) has protective effects on heart failure. Phenylephrine (PE) is an important inducing factor for cardiac hypertrophy and our previous studies have showed that QLQX attenuates PE-induced cardiac hypertrophy. Besides, QLQX protects against cardiac remodeling after myocardial infarction via activating PPAR γ . However, whether QLQX prevents PE-induced cardiac hypertrophy through PPAR γ and its coactivator PGC-1 α is still unknown.

Methods: The effects of QLQX were investigated based on PE-induced cardiac hypertrophy mouse models. Echocardiography and hematoxylin-eosin (HE) staining were used to determine cardiac function and cross-sectional area, respectively. Quantitative real time PCR (qRT-PCR) was used to determine ANP and BNP expressions. Based on primary neonatal rat ventricular cardiomyocytes (NRVMs) treated with PE, the cell size and expressions of ANP and BNP were determined by immunofluorescent staining and qRT-PCR, respectively. In addition, western blot was used to determine PPAR γ and PGC-1 α expressions.

Results: In present study, we confirmed that QLQX could significantly attenuate cardiac hypertrophy in mice treated with PE. Then we showed that PPAR γ and PGC-1 α were downregulated in PE-induced cardiac hypertrophy, and QLQX could block the decrease of PPAR γ and PGC-1 α both *in vitro* and *in vivo*. Importantly, we found that PPAR γ inhibitors or PGC-1 α siRNAs eliminated the protective effects of QLQX on PE-induced cardiac hypertrophy.

Conclusions: Our study suggested that QLQX prevents from PE-induced cardiac hypertrophy by activating PPAR γ and its coactivator PGC-1 α .

Keywords: Cardiac hypertrophy; PPAR γ ; PGC-1 α ; Qiliqiangxin (QLQX)

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Introduction

Cardiac hypertrophy is a great risk factor in many cardiovascular diseases including heart failure, which is an intractable disease with high morbidity and mortality worldwide (1). Although the initial stage of cardiac hypertrophy is an adaptive response to increased workload (2-4), it eventually turns into maladaptive hypertrophy and causes cardiac dysfunction, such as diastolic dysfunction along with selective reduction in substance oxidation (5). In hypertrophic myocardium, the energy resource transits from fatty acid to glucose (5,6) and the efficiency of mitochondrial metabolism is also decreased which leads to less ATP production (7,8). The possible signaling pathways or alteration in gene expression has been investigated in detail in various cardiac diseases (8-10). But there is still no available strategy to terminate or reverse the pathological process.

Peroxisome proliferator-activated receptors (PPARs) play vital roles in regulating the fatty acid and glucose metabolism. Among the PPARs, PPAR γ is lowly expressed in heart but occupies a vital role in cardiac development and heart diseases (8,9,11). Besides, previous studies reported that PPAR γ was downregulated during cardiac hypertrophy and the cardiomyocytes-restricted PPAR γ knockout mice developed spontaneous cardiac hypertrophy (12,13). Furthermore, PPAR γ activation inhibits hypertrophic signals of cardiomyocytes in response to various pathological stimuli (14).

Apart from PPAR γ , its coactivator PGC-1 α also regulates the energy metabolic process. PGC-1 α is highly expressed in heart tissue under physiological condition, while PGC-1 α is decreased and the mitochondrial function is also reduced in pressure overload-induced hypertrophy (8). PGC-1 α activation increased the mitochondrial number and oxidative capacity (15) through activating PPAR γ in cardiomyocytes (10). Furthermore, mice with knockdown of PGC-1 α developed into cardiac dysfunction with significantly impaired mitochondrial dysfunction (9,16).

A double-blind clinical study in patients with chronic heart failure has demonstrated that Chinese medicine Qiliqiangxin (QLQX) have beneficial effects on cardiac function, exercise tolerance and patient quality of life (17). And our previous studies have showed that the QLQX attenuates cardiac hypertrophy (18). Besides, we previously demonstrated that PPAR γ was crucial for the protective effects of QLQX in cardiac remodeling after myocardial

infarction (19). And we have identified that QLQX treatment improved mitochondrial biogenesis and increased mitochondrial metabolism by inducing the expression of PGC-1 α in H9C2 cells under physiological condition (20). However, whether QLQX prevents cardiomyocytes from cardiac hypertrophy through regulating PGC-1 α /PPAR γ is still unclear.

Here we used phenylephrine (PE) to induce cardiac hypertrophy, and found that QLQX could prevent the cardiac hypertrophy. Moreover, we found that PPAR γ and its coactivator PGC-1 α were both downregulated during cardiac hypertrophy. Ultimately, we revealed that QLQX could prevent the cardiac hypertrophy through upregulating PGC-1 α and PPAR γ expression.

Methods

Animal experiments

This study was 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). All animal experiments were approved by the ethical committees of the Nanjing Medical University.

Male C57BL/6 mice aged 7–8 weeks were purchased from Beijing Vital River Laboratory Animal Technology Corporation and housed in a room with normal receiving 12-hour dark/light cycle and free access to food. Osmotic mini-pumps (model 2004; Alzet Corp, Palo Alto, CA, USA) were implanted subcutaneously at the dorsum of the neck to infuse a pressor dose of PE (40 mg/kg/d) for 3 weeks. The control mice were treated with the same procedure except their pumps were filled with vehicle. At the same time, the Chinese medicine QLQX was administrated at a dose of 0.5 g/kg/d for 3 weeks.

Echocardiography

Echocardiography was performed on mice anesthetized with 2% isoflurane using a Vevo 2100 (Visual Sonics Inc., Toronto, Ontario, Canada). The following parameters were performed: ejection fraction% (EF%), interventricular septal (IVS) and left ventricular posterior wall (LVPW).

Hematoxylin-Eosin (HE) staining

The isolated hearts were fixed with 4% paraformaldehyde

and then performed the HE staining. We used the Nikon Eclipse microscope with NIS Elements software to capture the images. Image J software was used to evaluate the cross-sectional area.

Neonatal rat ventricular cardiomyocytes (NRVMs) isolation, culture and treatment

The primary NRVMs were isolated and digested as previous described (18). After the digestion, the cell suspension was centrifuged and purified by Percoll gradient centrifugation. Subsequently, the NRVMs were re-suspended in high-glucose Dulbecco's modified Eagle's medium (DMEM) (Gibco, Pasadena, CA, USA) with 5% fetal bovine serum (FBS) (Gibco, Pasadena, CA, USA), 10% horse serum (HS) (Gibco, Pasadena, CA, USA), 1% penicillin-streptomycin and then plated to different culture dishes.

To induce the hypertrophy, NRVMs are firstly starved for 8 h in serum-free DMEM and exposed to 50 μ M PE (Sigma, St. Louis, MO, USA) for 48 h. To investigate the roles of QLQX in PE-induced cardiac hypertrophy, NRVMs were pretreated with QLQX (0.5 μ g/mL) (Shijiazhuang Yiling Pharmaceutical Co., Hebei, China) for 48 h before the PE stimulation.

To investigate whether QLQX protects NRVMs from cardiac hypertrophy via PPAR γ and its coactivator PGC-1 α , the NRVMs were treated with QLQX together with 1 μ M PPAR γ agonist (Rosiglitazone) or 1 μ M PPAR γ inhibitor (T0070907) for 48 h, followed by the exposure to PE. Similarly, after NRVMs treated with QLQX, PGC-1 α siRNAs (75 nM) were transfected using Lipofectamine 3000 (Invitrogen, MA, USA). Six to eight hours after the siRNA transfection, NRVMs were treated with 50 μ M PE for the next 48 h.

Immunofluorescent staining for α -actinin and cell size analysis

The NRVMs were respectively fixed in 4% paraformaldehyde and permeabilized with 0.2% Triton X-100 for 20 min at room temperature. Then the cells were blocked with 10% goat serum in PBS for 1 h at room temperature and incubated with primary antibody α -actinin overnight (1:200) (Sigma-Aldrich, St. MO, USA). Subsequently, the cells were washed three times for 5 min each and incubated with secondary antibody for 2 h at room temperature. Finally, the cells were stained with DAPI and the images were captured

by the inverted fluorescent microscope (Carl Zeiss).

Western blot analysis

The NRVMs or heart samples were lysed using RIPA buffer (Beyotime, Nantong, China), and the concentration of protein samples was evaluated by BCA protein assay kit (Beyotime, Nantong, China). Equal amounts of protein were separated in SDS-PAGE and transferred into PVDF membranes. The primary antibodies were used as follow: PPAR γ (1:1,000, Cell Signaling Technology), PGC-1 α (1:1,000, NOVUS), and GAPDH (1:5,000, Santacruz) was used as a loading control.

Quantitative real time PCR (qRT-PCR)

Total RNA was extracted from NRVMs or heart samples by using TRIZOL RNA extraction kit (Invitrogen). The reverse transcription was carried out using Bio-Rad iScriptTM cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) according to the quantity (400 ng). The expression of related genes was detected by using Bio-Rad SYBR qPCR (Bio-Rad, Hercules, CA, USA) on ABI-7900 Real-Time PCR Detection System (7900HT, Applied Biosystems, CA, USA). GAPDH was used as an internal control. The primers for qRT-PCR were listed in *Table 1*.

Statistical analysis

All data were expressed as mean \pm SEM. The statistical significance was assessed using an independent-sample *t*-test for two-group comparison or one-way ANOVA for multiple-group comparison followed by Bonferroni's post hoc test. A value of $P < 0.05$ was considered significant.

Results

QLQX attenuates cardiac hypertrophy in mice

Continuous 3-week infusion of PE caused cardiac hypertrophy in mice, revealed by the echocardiography examination (*Figure 1A,B*), HW/BW ratio (*Figure 1C*), HE staining (*Figure 1D*), ANP and BNP expression (*Figure 1E*). QLQX treatment attenuated cardiac hypertrophy and the increased expression of ANP, BNP induced by PE (*Figure 1A,B,C,D,E*). These data indicated that QLQX attenuates cardiac hypertrophy in PE-treated mice.

Table 1 List for primers used for qRT-PCR

Gene	Species	Forward primer	Reverse primer
ANP	Rat	GAGCAAATCCCGTATACAGTGC	ATCTTCTACCGGCATCTTCTCC
BNP	Rat	GCTGCTGGAGCTGATAAGAGAA	GTTCTTTTGTAGGGCCTTGGTC
GAPDH	Rat	AAGCTCACTGGCATGGCCTT	CGGCATGTCAGATCCACAAC
ANP	Mouse	AGGCAGTTCGATTCTGCTT	CGTGATAGATGAAGGCAGGAAG
BNP	Mouse	TAGCCAGTCTCCAGAGCAATTC	TTGGTCCTTCAAGAGCTGTCTC
LC3	Mouse	GGCTACGGCTACTATCGCAC	AGGAGGGCATGACAAAGGAGA
Beclin1	Mouse	ATGGAGGGGTCTAAGGCGTC	TCCTCTCCTGAGTTAGCCTCT
ATG4	Mouse	GTCAAGTATGGTTGGGCAGTT	TGTCACCCTCTCCCTCGAAAT
ATG7	Mouse	GTTCCGCCCTTTAATAGTGC	TGAACTCCAACGTCAAGCGG
GAPDH	Mouse	CCTTCGGTGTCTACCCC	GCCCAAGATGCCCTTCAGT

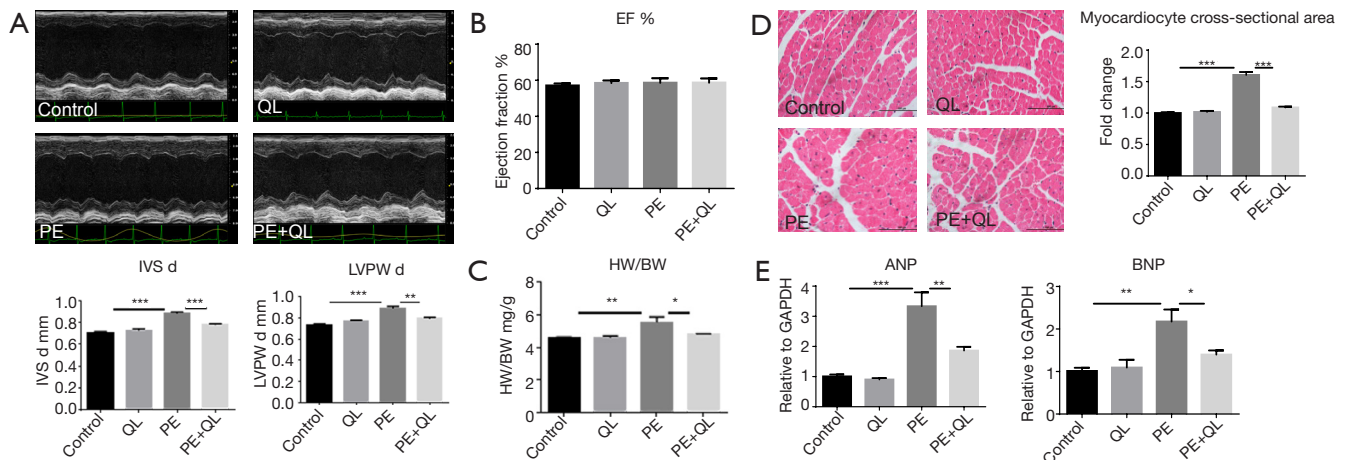


Figure 1 QLQX prevents the cardiac hypertrophy in mice induced by PE infusion. (A-C) Wall thickness (A) and EF (B) were evaluated and HW/BW ratio (C) were measured after 3-week infusion of PE in mice (n=6 or 7); (D) HE staining for evaluating myocardial cross-sectional area *in vivo*. Scale bars, 100 μ m (n=6); (E) qRT-PCR analysis of ANP and BNP expression with or without QLQX treatment (n=6). *, P<0.05; **, P<0.01; ***, P<0.001. QLQX, Qiliqiangxin; PE, phenylephrine; EF, ejection fraction; HE, Hematoxylin-Eosin; HW/BW, heart weight/body weight.

Both PPAR γ and PGC1- α are decreased in PE-induced hypertrophy in NRVMs

In addition, the existing pressure overload induced by PE increases the cardiac workload, which leads to the imbalance in energy supply and demand. At present, improving the energy metabolism has been a new strategy to treat heart diseases. PPAR γ and PGC-1 α have been reported to be crucial for energy metabolism (21,22). Thus, we detected

the expression changes during cardiac hypertrophy. NRVMs were exposed to 50 μ M PE to induce cardiomyocytes hypertrophy. PE treatment increased the expression of ANP and BNP (Figure 2A), as well as cardiomyocytes size (Figure 2B). Subsequently, we detected the expression of PPAR γ and PGC-1 α by western blot and the results showed the decrease of PPAR γ and PGC-1 α during PE-induced cardiac hypertrophy (Figure 2C), indicating that PPAR γ and PGC-1 α might suppress cardiac hypertrophy.

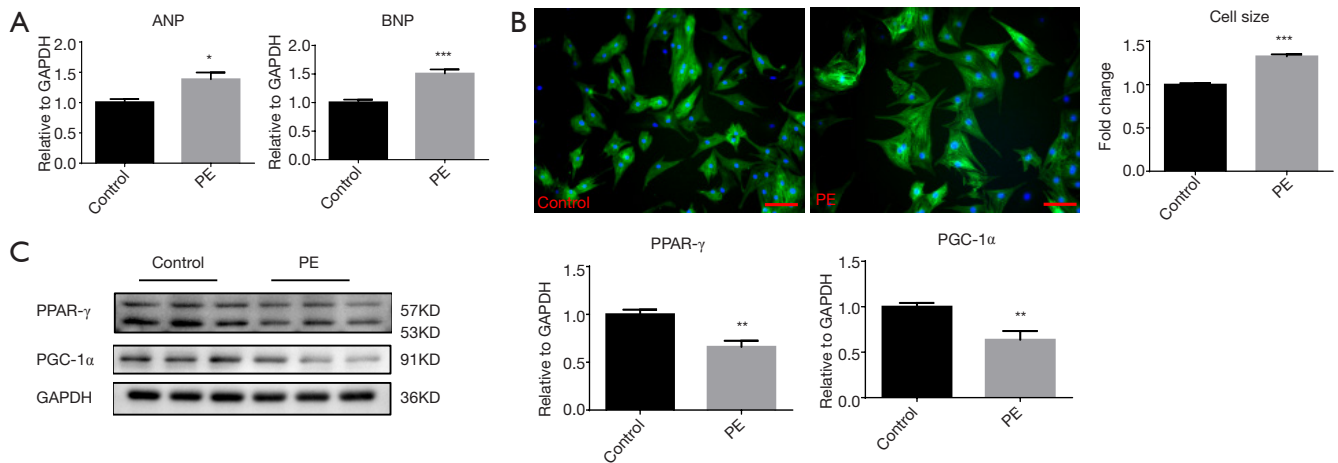


Figure 2 Cardiomyocytes hypertrophy induces downregulation of PPAR γ and its coactivator PGC-1 α . (A) qRT-PCR for ANP, BNP mRNA levels in cardiomyocytes after 50 μ M PE (phenylephrine) treatment (n=6); (B) immunofluorescent staining for α -actinin (green) to qualify the cardiomyocytes size, nuclei were counterstained with DAPI (blue), scale bars, 100 μ m (n=4); (C) western blot to detect the expression of PPAR γ and PGC-1 α in hypertrophic cardiomyocytes (n=6). *, P<0.05; **, P<0.01; ***, P<0.001. PE, phenylephrine.

QLQX prevents cardiomyocytes hypertrophy and activates PPAR γ and PGC-1 α

Clinical study has proved that QLQX has a protective effect on heart failure (17). In our study, we showed QLQX protected the cardiomyocytes against the increase of ANP and BNP expression and cell size induced by PE (Figure 3A,B). Importantly, we identified that OLQX increased the expression of PPAR γ and PGC-1 α in NRVMs during PE stimulation (Figure 3C). Moreover, we also demonstrated that OLQX increased PPAR γ and PGC-1 α expression in the mice model of cardiac hypertrophy (Figure 3D). These results inspired us that PPAR γ and PGC-1 α might be the targets of QLQX in hypertrophic progression.

QLQX attenuates the cardiomyocytes hypertrophy via increasing PPAR γ and PGC-1 α expression.

To further confirm that QLQX can prevent hypertrophy via PPAR γ and PGC-1 α , we performed rescue experiments. Firstly, we efficiently repressed the expression of PGC-1 α (Figure 4A) and found that knockdown of PGC-1 α blocked the protective effects of QLQX in NRVMs stimulated by PE (Figure 4B,C,D). Secondly, we exposed the cells to PPAR γ inhibitor (T0070907) or PPAR γ agonist (Rosiglitazone). The protective effects of QLQX on hypertrophic cardiomyocytes were also abrogated by PPAR γ inhibitors (Figure 4E,F,G,H). In addition, PPAR γ agonist

rosiglitazone did not further aggravate the protective effects of QLQX (Figure 4E,F,G,H). Taken together, our results revealed that QLQX prevents cardiomyocytes hypertrophy via increasing PGC-1 α and PPAR γ expression.

Discussion

Hypertrophy is a compensative response to increased cardiac workload, mainly resulting in cardiomegaly and increased protein content (2,4,23). Hypertrophy is classified as physiological or pathological, which are induced by different stimuli (24,25). In abnormal condition, hypertrophy induces downregulation of PGC-1 α signaling and the cognate target PPARs, causing the shift of energy metabolism to resembling the fetal heart metabolism (26,27). Our study demonstrated that both PPAR γ and PGC-1 α were downregulated in PE-induced hypertrophy, and the Chinese medicine QLQX could attenuate cardiac hypertrophy through preventing the decrease of PPAR γ and PGC-1 α expression.

PGC-1 α , firstly identified as a coactivator of PPAR γ , serves as an activator in FA metabolism through PPARs. Activation of PGC-1 α promoted the mitochondrial biogenesis and prevented the diabetic cardiomyopathy progression (28). Furthermore, the greater LV hypertrophy and dysfunction were presented in PGC-1 α ^{-/-} mice versus the wild type (9,14,26). Increase of PGC-1 α has been shown in QLQX-treated spontaneously hypertensive rats (29)

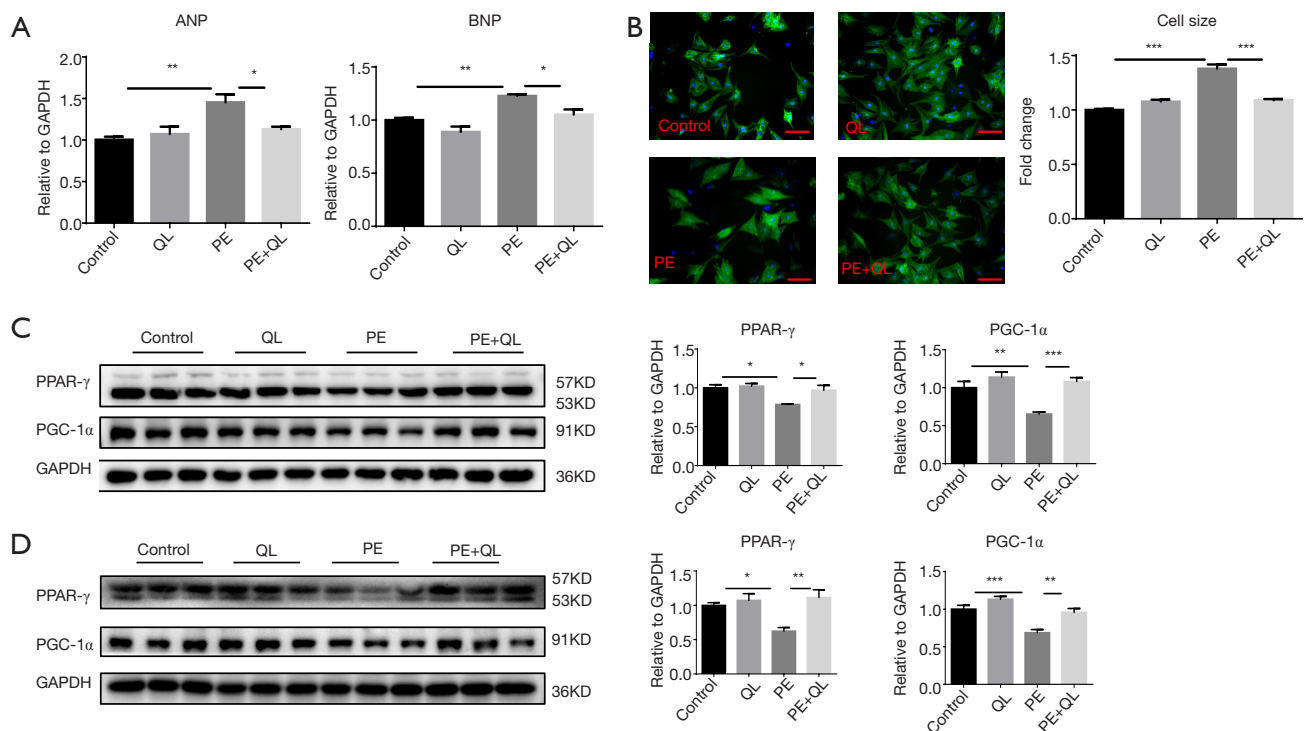


Figure 3 QLQX prevents cardiac hypertrophy stimulated by PE and upregulates the expression of PPAR γ and PGC-1 α . (A) QLQX prevented the increased expression of ANP, BNP by qRT-PCR analysis (n=6); (B) immunofluorescent staining showed that increased cardiomyocytes size was reduced by QLQX. Cell membrane was stained for α -actinin (green) and nuclei were counterstained with DAPI (blue). Scale bars, 100 μ m (n=3); (C) expression of PPAR γ and PGC-1 α was determined by western blot in NRVMs treated with PE and/or QLQX (n=6); (D) expression of PPAR γ and PGC-1 α were determined by western blot in mice treated with PE and/or QLQX (n=6). *, P<0.05; **, P<0.01; ***, P<0.001. QLQX, Qiliqiangxin; PE, phenylephrine.

and pressure overload heart failure rats (30). In addition, QLQX induced PGC-1 α to promote the mitochondrial biogenesis and mtDNA transcription and replication in H9C2 cardiomyocytes (20). However, during PE-induced cardiomyocytes hypertrophy, the relationship between QLQX and PGC1- α is still unclear. Our results clearly showed that QLQX attenuates PE-induced cardiomyocytes hypertrophy through activating PGC1- α .

PPAR γ is also a well-known crucial regulator during cardiac hypertrophy. And PPAR γ can interact specifically with PGC-1 α by forming a complex (8-10). It has been reported that PPAR γ agonists suppress the hypertrophy of cultured NRVMs induced by mechanical stress (31). Importantly, pressure overload-induced cardiac hypertrophy in heterozygous PPAR γ -deficient mice is slightly more pronounced than that in wild-type mice (13). Moreover, cardiomyocytes-specific deletion of PPAR γ in mice ultimately developed into cardiac hypertrophy in mice (12). Mechanically, deletion of PPAR γ increased the expression of

cardiac embryonic genes (atrial natriuretic peptide and beta-myosin heavy chain), elevated the nuclear factor kappaB activity while suppressed in the heart (12). Consistent with previous studies, our work also showed that PPAR γ acted as a suppressor for cardiomyocytes hypertrophy. PPAR γ plays a key role in the protective effects of QLQX during cardiac remodeling after acute myocardial infarction (19,32). To date, little is known about whether PPAR γ mediated the protective roles of QLQX in protecting against cardiac hypertrophy. Importantly, by performing rescue experiments, our results demonstrated for the first time that QLQX protects against PE-induced cardiac hypertrophy via increasing PPAR γ .

Multiple studies have shown that QLQX attenuates pressure overload-induced cardiac hypertrophy through increasing the expression of ErbB2 and CBP/p300-interacting transactivator (33). Besides, QLQX protects against PE-induced cardiac hypertrophy via reducing microRNA-199a-5p expression (18). The important roles

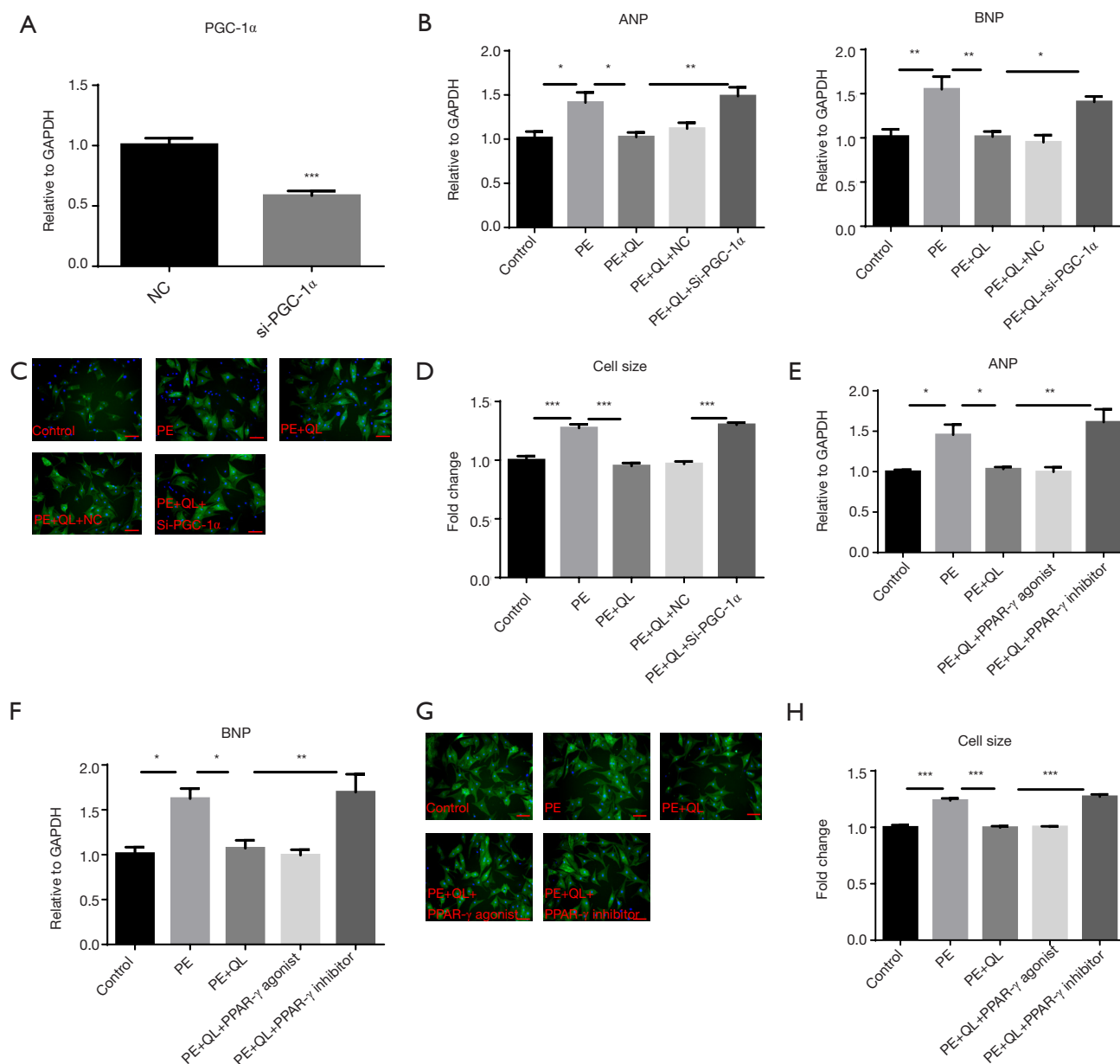


Figure 4 PGC-1 α siRNAs or PPAR γ inhibitors eliminate the protective effects of QLQX on cardiomyocytes hypertrophy, and PPAR γ agonists do not further increase the protective effects of QLQX. (A) PGC-1 α siRNAs significantly reduced the PGC-1 α expression in NRVMs. n=4; (B) qRT-PCR analysis of ANP and BNP expression in NRVMs treated with PGC-1 α siRNAs, PE and/or QLQX (n=6); (C,D). cell size was qualified using immunofluorescent staining for α -actinin (green), nuclei were counterstained with DAPI (blue). Scale bars, 100 μ m; n=3; (E,F) expression of ANP, BNP was determined by qRT-PCR analysis after the treatment (n=6); (G,H) using immunofluorescent staining for α -actinin (green) to evaluate the cardiomyocytes size, nuclei were counterstained with DAPI (blue). Scale bars, 100 μ m (n=3). *, P<0.05; **, P<0.01; ***, P<0.001. QLQX, Qiliqiangxin.

of PGC-1 α and PPAR γ have been seen in various models of cardiac hypertrophy and heart failure (10). Present study provides PGC-1 α and PPAR γ as novel downstream genes of QLQX in preventing from cardiac hypertrophy.

However, there are still limitations in our study. We investigated the QLQX prevented the cardiac hypertrophy by activating PPAR γ and PGC-1 α . Owing to the complexity in cardiac remodeling, the apoptosis, fibrosis and the oxidative stress were not taken into research though pressure overload often caused. The relationship was also not considered in detail between PPAR γ and PGC-1 α in our research, although their connection has been reported clearly (8-10,27,34). Based on these findings, further investigations are highly required to demonstrate the relationship between PPAR γ and PGC-1 α , as well as the detailed mechanisms of QLQX in preventing the hypertrophy.

Conclusions

In conclusion, the present study reveals that QLQX prevents the PE-induced cardiomyocytes hypertrophy. PPAR γ and PGC-1 α are decreased in cardiomyocytes hypertrophy, whereas increased by QLQX treatment. PPAR γ and PGC-1 α activation are required for the protective roles of QLQX against cardiomyocytes hypertrophy. The novel mechanism for the protective effects of QLQX against cardiomyocytes hypertrophy sheds light on further investigation, and provides a promising prescription for the treatment of cardiac hypertrophy and dysfunction.

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Footnote

Conflicts of Interest: Dr. XL received research grants from Shijiazhuang Yiling Pharmaceutical Co., Ltd. The other authors have no conflicts of interest to declare.

Ethical Statement: This study was 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). All animal experiments were approved by the ethical committees of the Nanjing Medical University.

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