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Original Article

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AdipoRon prevents L-thyroxine or isoproterenol-induced cardiac hypertrophy through regulating the AMPK-related pathway

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Abstract

Cardiac hypertrophy is a risk factor which can intrigue heart failure. In the present study, we explored whether AdipoRon attenuates isoprenaline (ISO) or L-thyroxine-induced cardiac hypertrophy in Sprague–Dawley (SD) rats and whether the anti-hypertrophy effect is mediated by AMPK-related pathway. Here, cardiac hypertrophy was induced by injection of L-thyroxine or ISO in SD rats. In the treatment group, AdipoRon was co-administered. We examined the effects of AdipoRon on cardiac hypertrophy and hypertrophy signaling pathway. The weight of SD rats was recorded every day. Rats were killed for collection of blood and heart under anesthesia. The left heart weight and heart weight were weighed. Paraffin-embedded heart tissue regions (4 μm) were stained with hematoxylin and eosin or Masson to detect left heart hypertrophy and myocardial fibrosis. The serum BNP levels were determined by using an enzyme-linked immunosorbent assay. The mRNA levels of ANP, BNP, PGC-1α, and ERRα were evaluated by real-time PCR analysis. The protein expression levels of PGC-1α, ERRα, and pAMPK/AMPK were determined by western blot analysis. The results showed that AdipoRon significantly reversed heart weight (HW)/body weight (BW) ratio, left ventricular (LV)/BW ratio, serum BNP level and the mRNA level of ANP and BNP induced by ISO or L-thyroxine. ISO or L-thyroxine reduced both the mRNA level and protein level of ERRα and PGC-1α, and also reduced the protein level of pAMPK/AMPK. However, AdipoRon reversed ISO or L-thyroxine-induced changes of pAMPK/AMPK, ERRα, and PGC-1α. Our data indicated that the effects of AdipoRon are mediated partly by activating AMPK-related pathway, and AdipoRon plays a potential role in the prevention of cardiac hypertrophy.

Key words: AdipoRon, cardiac hypertrophy, PGC-1α, AMPK-related pathway

Introduction

Cardiac hypertrophy is usually characterized by an increase in cardiomyocyte size and thickening of ventricular walls. Initially, such growth is an adaptive response to maintain cardiac function; as time progresses, this process develops to maladaptive remodeling and ultimately heart failure. Judging from this, cardiac hypertrophy can be an important risk factor for heart failure [1]. The progression of cardiac hypertrophy is often associated with increased interstitial fibrosis, cell death, and cardiac dysfunction [2]. Administration of isoprenaline (ISO), which is a β-adrenoreceptor agonist, contributes to left ventricular (LV) hypertrophy, which is an independent risk factor for cardiovascular mortality and morbidity [3].

Adenosine 5′-monophosphate (AMP)-activated protein kinase (AMPK) is a heterotrimeric complex composed of α-catalytic subunit, β-regulatory subunit, and γ-regulatory subunit. The combination of AMPK subunits produces a variety of AMPK holoenzymes. Phosphorylation of threonine 172 in the catalytic domain of the α subunit is required for AMPK activation [4]. AMPK is expressed in cardiomyocytes, cardiac fibroblasts, endothelial cells, and smooth muscle cells. AMPK is important for the maintenance of endothelial cell function [5–7]. Studies have shown that AMPK activates endothelial NO synthase (eNOS) to improve myocardial ischemia [8,9]. Activation of AMPK in cardiac fibroblasts protects the heart [10].

Studies have shown that mitochondrial energy metabolism disorders can lead to cardiac dysfunction [11]. Peroxisome proliferator-activated receptor (PPAR) γ coactivator-1α (PGC-1α), which is one of the important transcriptional regulators, is also a protein that induces brown adipose tissue enrichment and induces mitochondrial biosynthesis and uncoupled respiration in brown adipocytes. PGC-1α induces high levels of respiratory coupling in mitochondria, producing sufficient ATP in cardiomyocytes [12]. PGC-1α is the most widely studied family of PGC-1 proteins in the heart. Since the PGC-1 protein family cannot directly bind to DNA, these coactivators act by binding to specific DNA elements in the promoter region of the gene. In the heart, PGC-1α has three co-transcription factors: PPARα, PPARγ, PPARβ [13–15]. PPARs are enriched in the heart muscle and play an important role in regulating cardiac fatty acid uptake and oxidation [5,16]. Rosiglitazone, a PPARγ ligand, protects the heart by stimulating adiponectin production [17]. The estrogen receptor-related receptor (ERR) family ERRs (ERRα,β,γ) of the myocardium is another target of PGC-1α. In the presence of PGC-1α, overexpression of ERRα significantly enhances the expression of the gene encoding the fatty acid oxidation and the OXPHOS enzyme gene [18–20]. ERRα mediates the regulation of fatty acid oxidation by PGC-1α through partially activating the expression of PPARα. The PGC-1α/ERRα complex is directly involved in the regulation of glucose oxidation-related gene expression [21]. In primary cardiomyocyte hypertrophy, ERRα, PPARγ, and PGC-1α expression and activity are often down-regulated [22–24].

A variety of myocardial mitochondrial enzymes are involved in the development of heart failure, and are important to facilitate responses to the stress and then maintain the cardiac energy reservation. Myocardial ERRα is significantly decreased in congestive heart failure, and ERRα is regulated by the metabolic sensor AMPK [25]. The down-regulation of ERRα and PGC-1α target gene is a signature of the failing human heart [26]. In the adiponectin-deficient, wild-type and diabetic db/db mice, adiponectin attenuated cardiac hypertrophy in response to pressure overload [27]. Adiponectin was found to promote metabolic function in the heart and activate the AMPK/PGC-1α signaling pathway to regulate cardiac mitochondrial function, showing a cardio-protective effect [28]. Adiponectin exerts effects mostly via activation of AdipoRs. In mice hearts lacking AdiopR1, activities of mitochondrial function is impaired, accompanied by reduced phosphorylation of AMPK, OXPHOS protein level, PGC-1α, and sirtuin 1 activity (SIRT1), while AdipoR2 deficiency does not impair mitochondrial coupling and function in the heart [29]. Molecules bind to and activate AdipoR1 and AdipoR2 showed good effects on obesity-related diseases.

AdipoRon, a small molecule adiponectin receptor agonist (**Fig. 1**), can bind to and activate AdipoR1 and AdipoR2, and ameliorate obesity-related diseases such as type 2 diabetes. AdipoRon shows a very similar effect to adiponectin in muscle and liver. AdipoRon activates AMPK and PPARα pathways, and ameliorates insulin resistance and glucose intolerance in mice treated with high-fat diet [30]. AdipoRon attenuates postischemic myocardial apoptosis through both AMPK-independent and AMPK-mediated signaling [31]. AdipoRon attenuates PDGF-induced VSMC proliferation through inhibition of mTOR signaling independent of AMPK [32]. AdipoRon can protect and reverse diabetic nephropathy by the activation of AdipoRs and downstream targets through increased intracellular Ca^{2+}/AMPK-LKB1/PPARα pathway [33].

Whether AdipoRon can ameliorate cardiac hypertrophy induced by L-thyroxine or β-adrenoreceptor activation is still unexplored. In this study, we applied L-thyroxine or ISO-induced cardiac hypertrophy animal model to study the cardiac protection function of AdipoRon. We found that AdipoRon ameliorates L-thyroxine or ISO-induced cardiac hypertrophy and exerts its effects partly by activating the AMPK-related pathway.

Materials and Methods

Experimental animals

Male Sprague–Dawley (SD) rats weighing 250–300 g were purchased from Shanghai Sippr-BK Laboratory Animal Co., Ltd (Shanghai, China). Animals were allowed free access to food and water throughout the acclimatization and experimental periods. They were housed in a room with a 12:12 h light:dark cycle at an ambient temperature of 25°C and 60% humidity. Rats used in this study were maintained in accordance with the guide for care and use of Laboratory Animal published by the National Institutes of Health of USA (NIH publication No. 85-23, revised 1996) and the experiment was approved by the Animal Ethics Committee of China Pharmaceutical University (Nanjing, China).

Chemicals and reagents

AdipoRon [9] was obtained from the Center for Drug Discovery at China Pharmaceutical University (Nanjing, China) and was dissolved in 0.5% carboxymethylcellulose sodium (CMC-Na) solution. L-thyroxine or ISO was purchased from Sigma-Aldrich Chemical Co. (St Louis, USA). Real-time polymerase chain reaction (PCR) primers were synthesized by Shanghai Generay Biotech (Shanghai, China). PrimeScripRT Master Mix and SYBR Premix Ex Taq were purchased from Takara (Takara, Dalian, China). And 96-well optical reaction plates with optical adhesive films were purchased from Applied Biosystems (Foster City, USA). Acrylamide, *N*′,*N*′-bis-methylene-acrylamide, β-mercaptoethanol, ammonium persulfate, glycine, pure nitrocellulose membrane (0.2-μm), and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) were purchased from Sangon Biotech (Shanghai, China). Chemiluminescent western blot detection reagents were purchased from Tanon Biotech (Shanghai, China). Rabbit polyclonal antibodies against AdipoR1, ERRα, and PGC-1α were purchased from Abcam (Cambridge, USA). Rabbit polyclonal antibodies against AMPK and pAMPK were purchased from Cell Signaling Technology (Beverly, USA). Anti-GAPDH antibody was purchased from Arigo (Hsinchu, China). Other chemicals were purchased from Sangon Biotech.

Experimental design

Two models of cardiac hypertrophy were set up in this study. In one model, rats were injected i.p. with ISO dissolved in saline (0.25 ml per rat) at 2.5 mg/kg/day for 30 days. These rats were named as the ISO model. In another model, male SD rats (250–300 g) were injected i.p. with L-thyroxine dissolved in saline (0.25 ml per rat) at

1 mg/kg/day for 10 days. These rats were named as the L-thyroxine model.

In the ISO model, rats were randomly divided into three groups. The control group (n = 10) received saline (1 mg/kg/day; i.p.) plus 0.5% CMC-Na (1 mg/kg/day; i.g.). The model group (n = 15) received ISO (2.5 mg/kg/day; i.p.) plus 0.5% CMC-Na (1 mg/kg/day; i.g.). The AdipoRon group (n = 15) received ISO (2.5 mg/kg/day; i.p.) plus AdipoRon (50 mg/kg/day; dissolved in 0.5% CMC-Na; final volume = 1.0 ml per rat; i.g.). The rats were subject to the corresponding treatment for 30 days.

In the L-thyroxine model, rats were randomly divided into four groups. The control group (n = 10) received saline (1 mg/kg/day; i.p.) plus 0.5% CMC-Na (1 mg/kg/day; i.g.). The model group (n = 12) received L-thyroxine (1 mg/kg/day; i.p.) plus 0.5% CMC-Na (1 mg/kg/day; i.g.). The AdipoRon group (n = 12) received L-thyroxine (1 mg/kg/day; i.p.) plus AdipoRon (50 mg/kg/day or 100 mg/kg/day; dissolved in 0.5% CMC-Na; final volume = 1.0 ml per rat; i.g.). The rats were subject to the corresponding treatment for 10 days.

Echocardiography measurement

Following 30 days of ISO treatment or 10 days of L-thyroxine treatment, rats (n = 6 per group) underwent two-dimensional guided short-axis M-mode echocardiography (Vevo 2100; VisualSonics, Toronto, Canada). General anesthesia was maintained with isoflurane (1.5%) vaporized in medical O_2. Heart rate was monitored during examination to minimize cardio-depression-related effects. The heart was examined from base to apex using a parasternal short-axis view, and echocardiographic data were acquired at the level of the papillary muscle in order to assure reproducibility. Images were analyzed by an investigator blinded to treatment. Echocardiographic measurements were obtained from the parasternal long-axis view from M- and B-mode images. Conventional measurements of the left ventricle included the following: interventricular septum thickness (IVS) in systole and diastole, left ventricular internal diameter (LVID) in systole and diastole, left ventricular posterior wall thickness (LVPW) in systole and diastole, fractional shortening (FS), and ejection fraction (EF). LV mass and volume were evaluated.

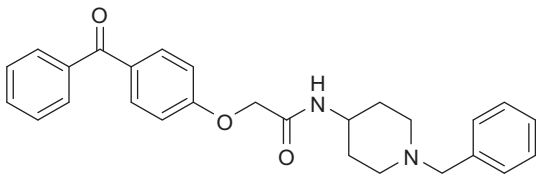

Figure 1. Molecular structure of AdipoRon

Histopathology analysis

Rat liver specimens from different groups were collected and fixed with 4% paraformaldehyde in 0.1 M PBS. Paraffin-embedded tissue regions (4 μm) were stained with hematoxylin and eosin (H&E) according to standard techniques. Samples were examined blindly by a professional pathologist to evaluate steatosis, inflammation, and fibrosis.

RNA extraction and real-time PCR

Total RNA was extracted from whole biopsies of the LV using TRIzol reagent. The RNA was transcribed to cDNA with PrimeScripRT Master Mix (Takara) according to the manufacturer's instructions. The mRNA levels of ANP, BNP, ERRα, and PGC-1α were measured by real-time quantitative PCR analysis using the ABI Step One Plus sequence detection system (Applied Biosystems). Quantitative real-time PCR was performed by using a SYBR Premix Ex Taq (Takara) according to the manufacturer's instructions. The specific primers are shown in **Table 1**. The expression levels of each gene were normalized against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) using the comparative $2^{-\Delta\Delta CT}$ method and the results were obtained from three independent experiments according to the manufacturer's protocols.

Western blot analysis

The LV tissues were lysed in an RIPA buffer (Beyotime Institute of Biotechnology, Shanghai, China), homogenates were centrifuged at 10,000 g for 10 min (4°C), and protein concentration in the supernatant was quantified using the BCA Protein Assay Reagent Kit (Beyotime Institute of Biotechnology). Whole cell lysate was denatured by boiling for 10 min in 4× SDS loading buffer (Bio-Rad, Hercules, USA). And 20 μg of cell lysate protein was subject to 10% SDS-PAGE and then transferred to PVDF membrane (Bio-Rad, Hercules, USA). The membrane was blocked with 5% BSA in 0.1% TBST for 1 h at room temperature and then incubated overnight at 4°C with primary antibodies against AMPK, ERRα, and PGC-1α at 1:1000 dilution, followed by incubation with HRP-conjugated secondary antibody (Beyotime Institute of Biotechnology). Signals were detected using ECL kit (Tanon Biotech). Immunoreactivity was quantified by scanning densitometry using Tanon GIS system (Tanon Biotech). All western blotting results are representative of at least two independent experiments.

Enzyme-linked immunosorbent assay

The blood samples were clotted for 2 h at room temperature before centrifugation for 20 min at approximately 1000 g. Serum samples were assayed immediately or stored at −80°C, avoiding repeated freeze-thaw cycles. The serum BNP level was determined by using enzyme-linked immunosorbent assay (ELISA) following the protocol provided in the kit (Elabscience Biotechnology, Wuhan, China).

Table 1. Sequence of primers used in the study

Gene	Forward primer (5′→3′)	Reverse primer (5′→3′)
BNP	GACTCCGGCTTCTGACTCTG	CACTGTGGCAAGTTTGTGCT
ANP	CCGAGACAGCAAACATCAGA	CCTCATCTTCTACCGGCATC
PGC-1α	AAGACTAAACGGCCCAGTCTAC	TCTGGTGCTGCAAGGAGA
ERRα	ACAGTCCAAAGGGTTCCTCAG	ACAGGATGCCACACCGTAGT
GAPDH	CCTTCATTGACCTCAACTACATG	CTTCTCCATGGTGGTGAAGAC

Statistical analysis

Data were expressed as the mean ± standard deviation (SD), unless indicated otherwise. Unpaired Student's *t*-test was performed to determine statistically significant differences. A value of $P < 0.05$ was considered significant at the 95% confidence level.

Results

Effect of AdipoRon on HW/BW ratio and LV/BW ratio

To investigate the effect of AdipoRon on body weight and hypertrophy, the rats were weighed daily. The heart weight/body weight (HW/BW) ratio and LV weight/body weight (LV/BW) ratio were applied to assess cardiac hypertrophy. As compared to the control group, ISO or L-thyroxine-treated group showed a significant increase in HW/BW and LV/BW, while AdipoRon reversed this increase (**Fig. 2**). These data indicated that AdipoRon obviously ameliorates cardiac hypertrophy.

Effect of AdipoRon on echocardiographic parameters

To investigate the inhibition effect of AdipoRon on cardiac hypertrophy, an M-mode echocardiography was performed before the rats were killed. As shown by the echocardiographic parameters, ISO-treated rats had increased IVS and LVPW, as compared to control rats (**Table 2**). The LVPW was increased markedly in the ISO group compared to the control group, indicating severe concentric hypertrophy, while AdipoRon group showed a reduced LV hypertrophy (**Fig. 3A** and **Table 2**). The echocardiographic parameters indicated that L-thyroxine-treated rats exhibited increased IVS, LVPW, LVID, EF, FS, LV Vol;d, and LV Vol;s as compared to control rats. However, the LV mass had no significant change. AdipoRon high-dose group showed a reduced LVPW;s (**Fig. 3B** and **Table 3**). These data indicated that AdipoRon can significantly attenuate L-thyroxine and ISO-induced cardiac hypertrophy.

Effects of AdipoRon on serum BNP level

BNP belongs to the natriuretic peptide (NP) family. In the cardiac hypertrophy model, the secretion of NPs increases in the heart, resulting in an increased BNP level in the blood. ELISA was used to determine the serum BNP level following the protocol provided in the kit. In the ISO group, 30 days after drug administration, the ISO-treated group showed significant increase in serum BNP compared with the control group, while AdipoRon could decrease the BNP level induced by ISO-treatment (**Fig. 4**). These data showed

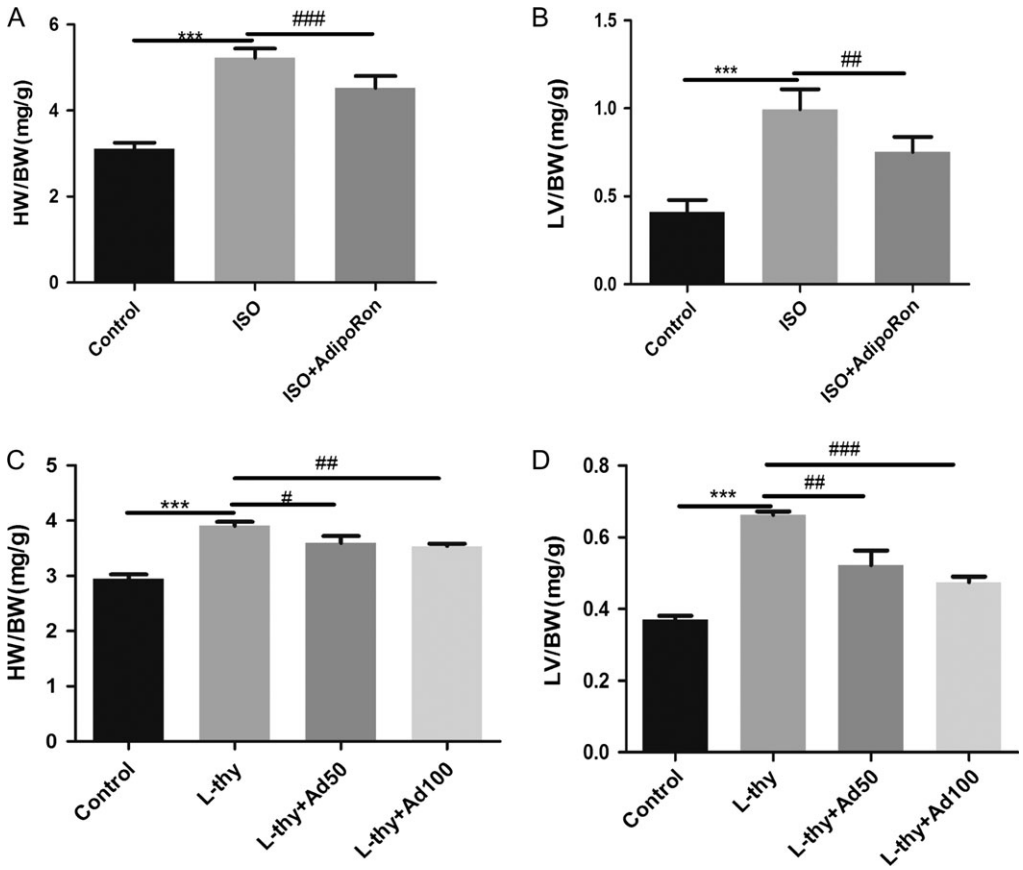

Figure 2. Effect of AdipoRon on cardiac hypertrophy induced by ISO or L-thyroxine (A) Heart weight to body weight ratio (HW/BW; mg/g) and (B) left ventricular weight to body weight ratio (LV/BW; mg/g) were determined in Sprague–Dawley rats after 30 days of treatment with ISO. (C) Heart weight to body weight ratio (HW/BW; mg/g) and (D) left ventricular weight to body weight ratio (LV/BW; mg/g) were determined in Sprague–Dawley rats after 10 days of treatment with L-thyroxine. Duplicate reactions were performed for each experiment, and the results are presented as the mean ± SD of six independent experiments. $n \geq 6$. $^{***}P < 0.001$ compared with control group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared with ISO group or L-thyroxine group. L-thy: L-thyroxine; Ad50: AdipoRon 50 mg/kg/day; Ad100: AdipoRon 100 mg/kg/day.

Table 2. Echocardiography analysis of the left ventricular in ISO-treated rats

	Control	ISO	ISO+AdipoRon
IVS;d (mm)	1.64 ± 0.01	2.33 ± 0.05***	1.78 ± 0.10##
IVS;s (mm)	2.80 ± 0.07	3.75 ± 0.02 ***	2.88 ± 0.24#
LVID;d (mm)	7.19 ± 0.55	7.25 ± 0.21	7.81 ± 0.42
LVID;s (mm)	4.10 ± 0.51	4.00 ± 0.14	4.87 ± 0.18
LVPW;d (mm)	1.71 ± 0.09	2.60 ± 0.10**	2.20 ± 0.14
LVPW;s (mm)	2.65 ± 0.18	3.80 ± 0.22*	3.25 ± 0.09
EF (%)	72.76 ± 3.90	74.70 ± 0.87	65.54 ± 3.00#
FS (%)	43.29 ± 3.18	44.86 ± 0.79	37.44 ± 2.46
LV mass (mg)	855.00 ± 125.60	1506.00 ± 109.50	1240.00 ± 162.70
LV mass (Corrected, mg)	684.00 ± 100.50	1205.00 ± 87.56	991.60 ± 130.20
LV Vol;d (μl)	275.40 ± 45.65	276.90 ± 18.07	329.40 ± 40.15
LV Vol;s (μl)	77.79 ± 22.94	70.13 ± 5.81	111.90 ± 9.57

Data are expressed as the mean ± SD. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with control group. #$P < 0.05$, ##$P < 0.01$ compared with ISO group.

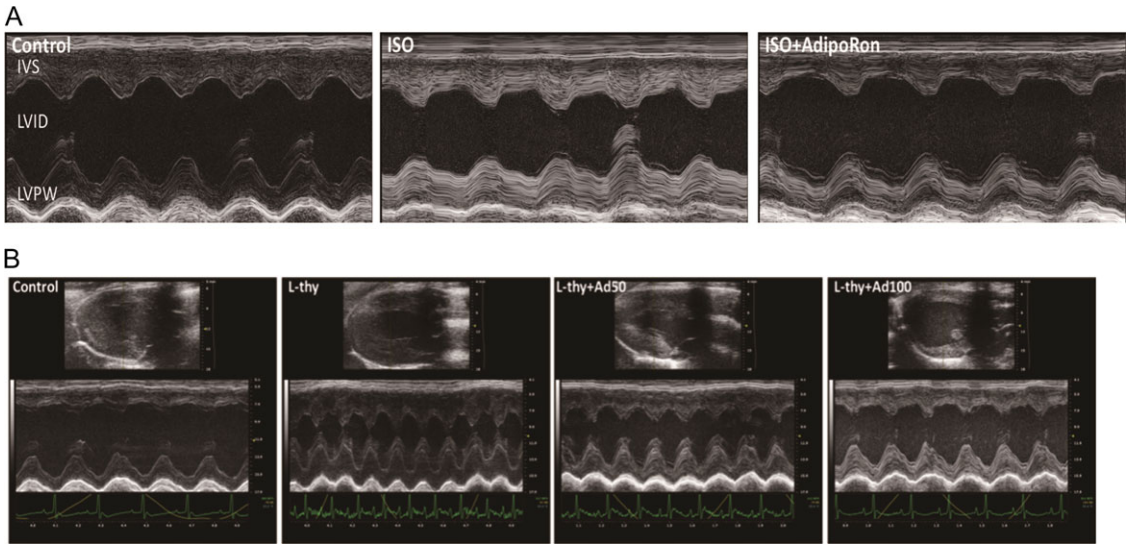

Figure 3. Photograph of the heart by CDUS examination (A) Representative photomicrographs of the color Doppler ultrasound (CDUS) from ISO-induced Sprague–Dawley rats. (B) Representative photomicrographs of the color Doppler ultrasound (CDUS) from L-thyroxine-induced Sprague–Dawley rats. LVID: left ventricular internal diameter; IVS: interventricular septum; LVPW: left ventricular posterior wall thickness. L-thy: L-thyroxine; Ad50: AdipoRon 50 mg/kg/day; Ad100: AdipoRon 100 mg/kg/day.

Table 3. Echocardiography analysis of the left ventricular in L-thyroxine-treated rats

	Control	L-thy	L-thy+Ad50	L-thy+Ad100
IVS;d (mm)	1.64 ± 0.03	2.14 ± 0.09**	2.07 ± 0.07	2.10 ± 0.08
IVS;s (mm)	2.70 ± 0.10	3.65 ± 0.11***	3.53 ± 0.06	3.51 ± 0.12
LVID;d (mm)	8.10 ± 0.24	6.85 ± 0.14**	6.93 ± 0.22	6.76 ± 0.48
LVID;s (mm)	4.70 ± 0.24	3.12 ± 0.17**	3.17 ± 0.17	3.23 ± 0.39
LVPW;d (mm)	1.76 ± 0.06	2.29 ± 0.02***	2.09 ± 0.12	2.16 ± 0.05
LVPW;s (mm)	3.04 ± 0.05	3.61 ± 0.04**	3.46 ± 0.20	3.39 ± 0.04##
EF (%)	71.02 ± 1.87	84.02 ± 2.10**	84.00 ± 1.09	82.48 ± 2.5
FS (%)	41.97 ± 1.56	54.53 ± 2.40**	54.34 ± 1.20	52.79 ± 2.72
LV mass;d (mg)	1044.00 ± 50.20	1174.00 ± 20.02	1081.00 ± 29.49#	1086.00 ± 81.94
LV mass;s (mg)	834.80 ± 40.16	939.50 ± 16.02	865.10 ± 23.59#	868.90 ± 65.55
LV Vol;d (μl)	355.10 ± 23.94	243.70 ± 10.78**	250.10 ± 17.89	240.60 ± 37.37
LV Vol;s (μl)	103.70 ± 12.05	38.93 ± 5.12**	40.31 ± 5.32	44.74 ± 11.98

Data are expressed as the mean ± SD. **$P < 0.01$, ***$P < 0.001$ compared with control group. #$P < 0.05$, ##$P < 0.01$ compared with L-thyroxine group.

that AdipoRon can reduce serum BNP level and attenuate left heart hypertrophy.

Effects of AdipoRon on cardiac hypertrophy and cardiac fibrosis

The anti-fibrotic and anti-hypertrophic effects of AdipoRon were investigated using H&E staining. H&E staining results revealed that the myocardial cells were significantly enlarged and irregularly arranged in the ISO or L-thyroxine-treated rats, while in the AdipoRon group, the myocardial cells were similar to those of the control group (**Fig. 5A**, upper panel and **Fig. 5B**). To further determine the effect of AdipoRon on maladaptive cardiac remodeling, we examined cardiac fibrosis, a classical feature of developing pathological cardiac hypertrophy, in this pressure-overloading heart. Masson staining of the heart tissue indicated that left ventricular fibrosis occurred in ISO-treated rats, but not in the control rats; and the fibrosis is reversed in AdipoRon-treated rats (**Fig. 5A**, lower pannel). These data indicated that AdipoRon can attenuate myocardial hypertrophy.

Effects of AdipoRon on the gene expressions of ANP and BNP

To study the effects of AdipoRon on cardiac hypertrophy, we examined the gene expressions of ANP and BNP. The ISO-treatment was found to increase the expressions of ANP and BNP, while AdipoRon is able to reduce the gene expressions of ANP and BNP (**Fig. 6A,B**). These data indicated that AdipoRon can attenuate ISO-induced cardiac hypertrophy.

AdipoRon regulates AMPK-related pathway in hypertrophic hearts

To explore the possible mechanism of the effects of AdipoRon on cardiac hypertrophy, the gene and protein expressions of ERRα, PGC-1α, pAMPK, and AMPK were measured. The results showed that the gene and protein expressions of ERRα and PGC-1α, as well as the protein expressions of pAMPK and AMPK were decreased after L-thyroxine or ISO-treatment, while AdipoRon could reversed the effects of L-thyroxine or ISO (**Figs. 6C-F** and **7**). These data indicated that AdipoRon ameliorates myocardial hypertrophy possibly by AMPK-related pathway (**Fig. 8**).

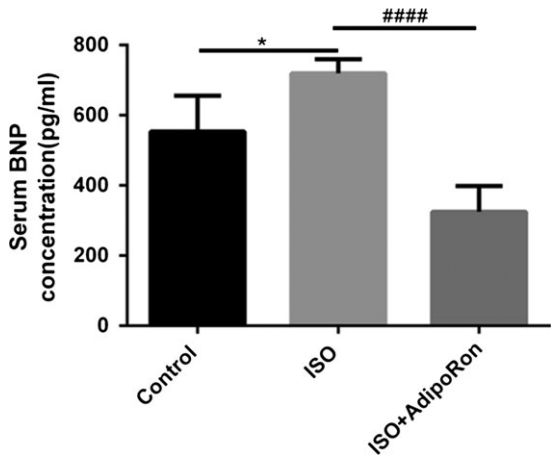

Figure 4. Serum BNP measured by enzyme-linked immunosorbent assay
Concentration of serum BNP level in ISO-induced rats. Data are presented as the mean±SD. $^{*}P < 0.05$ compared with control group. $^{\#\#\#\#}P < 0.0001$ compared with ISO.

Discussion

Cardiovascular disease is the main cause of mortality in the Western country. Under most hypertrophic growth conditions, the heart enlarges its myocytes to decrease ventricular wall tension and increase cardiac pump function. That process is often compensatory initially, and as time goes by these changes become maladaptive, eventually leading to heart failure [34]. There are different types of cardiac hypertrophy. Physiological cardiac hypertrophy is induced by physical exercise and pregnancy, cardiac hypertrophy like this kind often goes along with normal or enhanced contractile function, which is reversible [35]. Pathological cardiac hypertrophy is the result of chronic pressure or volume overload under certain disease conditions, valvular heart disease, hypertension, and coronary artery disease [35,36]. Sustained β-adrenoreceptor (β-AR) activation contributes to pathological cardiac hypertrophy. The injection of ISO, a β-adrenoreceptor agonist, leads to left ventricular (LV) hypertrophy. ISO-induced cardiac dilation is often associated with LV hypertrophy, elevated LV end-diastolic pressure, reduced ventricular systolic function, and global diastolic wall stress [3,37]. L-thyroxine is hormone secreted by the thyroid gland. Hyperthyroidism can lead to increased heart rate and increased myocardial contraction, eventually leading to compensatory cardiac hypertrophy. In this study, we established a model of cardiac hypertrophy by intraperitoneal injection of L-thyroxine or ISO, and investigated the protective effects and mechanisms of AdipoRon on the heart. The results of HW/BW and LV/BW showed that ISO- or L-thyroxine-treated group had a significant increase in heart weight and left ventricular weight compared to the control group, while AdipoRon obviously ameliorated cardiac hypertrophy. At the same time, echocardiographic results showed that the end-diastolic interventricular septal thickness (IVS;d) and end-diastolic left ventricular posterior wall thickness (LVPW;d) were significantly increased in the L-thyroxine and ISO-treated groups, while AdipoRon significantly reduced the above indicators. These results indicate that AdipoRon can significantly improve L-thyroxine and ISO-induced cardiac hypertrophy.

Cardiomyocytes are the main cells of cardiac contraction. Shortly after mammals are born, cardiomyocytes stop differentiation and lose most of their proliferative capacity [38]. Thus, despite fibroblast proliferation, increased progenitor cell activity, and cardiomyocyte turnover can increase heart weight, and the increase of cardiac mass is primarily due to cardiomyocyte hypertrophy. H&E staining showed that L-thyroxine and ISO-induced cardiomyocyte hypertrophy and abnormal cardiac structure, while AdipoRon ameliorated the aberrant tissue architecture induced by ISO or L-thyroxine treatment. Myocardial fibrosis is a pathological process of remodeling of extracellular matrix (ECM), which usually leads to increased hardness of the myocardial wall and significant change in cardiac function. The main feature of myocardial fibrosis is the accumulation of fibroblasts and the secretion of ECM, which ultimately leads to changes in myocardial structure. Myocardial fibrosis is most common in ischemic cardiomyopathy, but myocardial fibrosis also occurs in other diseases, such as hypertension, valvular heart disease, diabetic cardiomyopathy, hypertrophic cardiomyopathy, and special dilated cardiomyopathy [39]. Masson staining results showed myocardial fibrosis in ISO-treated rats, indicating that cardiac hypertrophy turn worse when fibrosis occurs. AdipoRon ameliorates the cardiac damage caused by

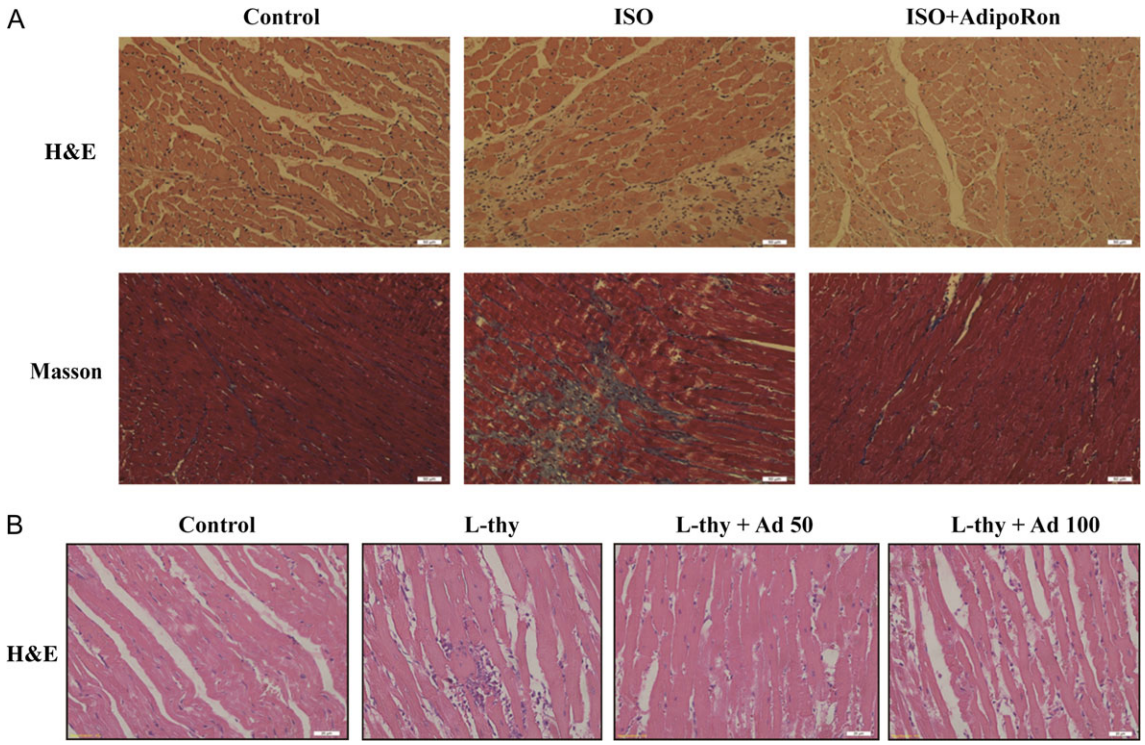

Figure 5. Effects of AdipoRon on cardiac hypertrophy and fibrosis (A) Cardiac hypertrophy was shown by H&E staining (original magnification, ×200) and Masson's trichrome staining (original magnification, ×200) in fibrotic tissues in the ISO group. (B) Cardiac hypertrophy was shown by H&E staining (original magnification, ×200) in the L-thyroxine group.

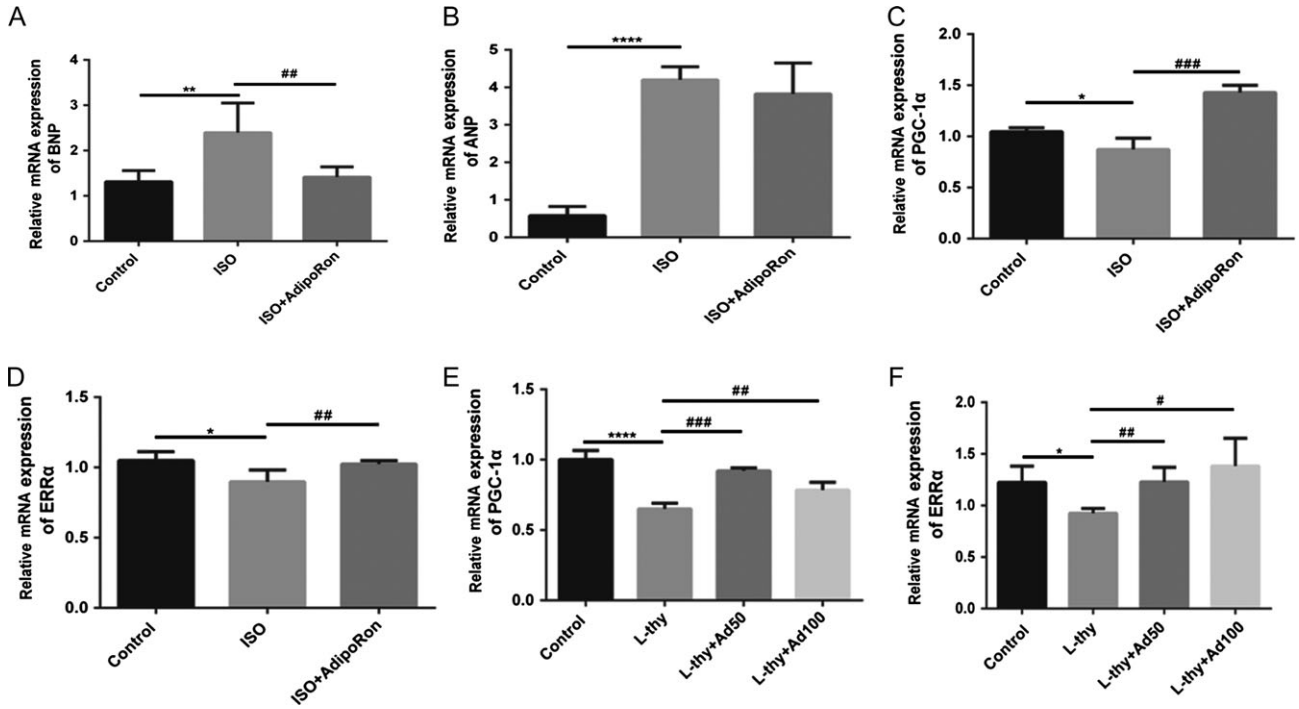

Figure 6. mRNA level of cardiac hypertrophy genes The mRNA expressions of BNP (A), ANP (B), PGC-1α (C), and ERRα (D) were analyzed by qRT-PCR in the ISO-induced rat heart. The mRNA expressions of PGC-1α (E) and ERRα (F) were analyzed by qRT-PCR in the L-thyroxine-induced rat heart. Results are presented as the mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$ compared with the control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared with the ISO group or L-thyroxine group.

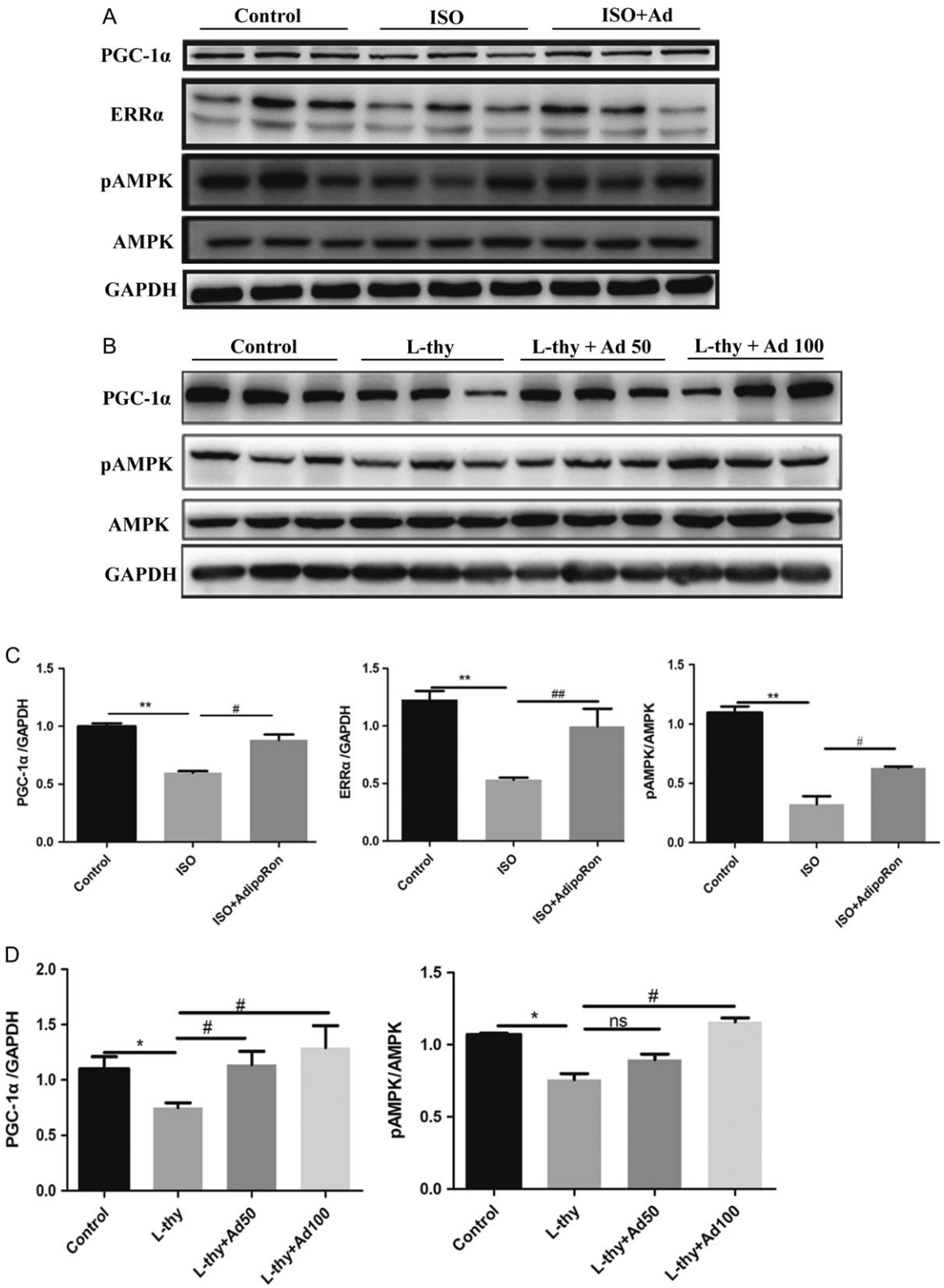

Figure 7. Effect of AdipoRon on protein expression (A) pAMPK, AMPK, PGC-1α, and ERRα proteins were detected by western blot analysis in ISO-induced rats. (B) pAMPK, AMPK, and PGC-1α proteins were detected by western blot analysis in L-thyroxine-induced rats. The fold change was calculated based on the densitometric analysis of band intensities in (C) ISO-induced or (D) L-thyroxine-induced rats. GAPDH was used as an internal control. Data are shown as the mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with the ISO group or L-thyroxine group.

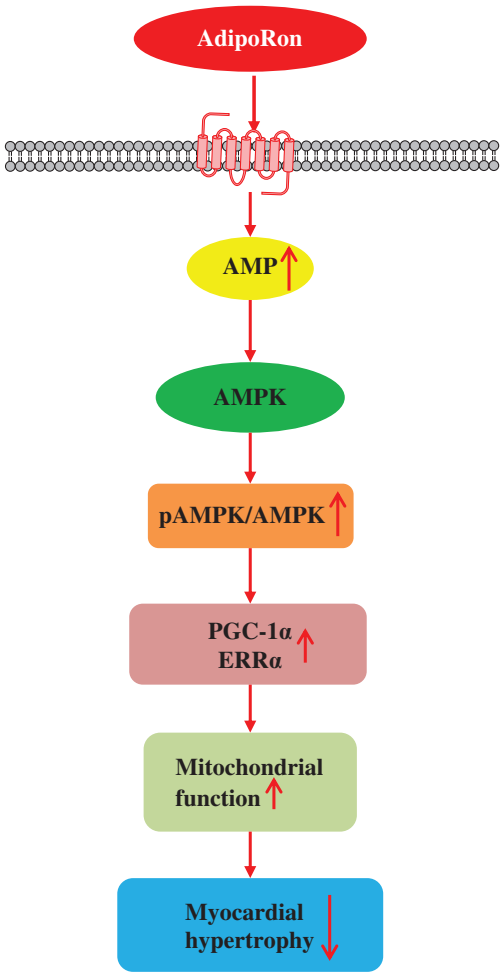

Figure 8. Signaling pathway of AdipoRon ameliorates myocardial hypertrophy

ISO. However, additional studies are needed to further our understanding of this signaling pathway.

Fetal gene expression has been extensively investigated for many years in pathological cardiac hypertrophy [40,41]. The well-studied and well-characterized fetal genes, whose expressions are significantly up-regulated in myocardial pathogenesis, include B-type natriuretic peptide (BNP) and atrial natriuretic peptide (ANP) [42,43]. In the present study, ISO-treatment was found to increase the expressions of ANP and BNP, while AdipoRon is able to reduce the expressions of ANP and BNP.

Previous studies have identified several signaling pathways mediating the process of hypertrophy, such as the VEGF-VEGF receptor-1 pathway, PKG-1, AMPK, and HIF-1 [2,44]. Effective treatment of these signaling pathways might provide promising approaches for treating cardiac hypertrophy and heart failure. Therefore, the current challenge will be to find promising pharmacological drugs that can prevent pathological cardiac hypertrophy and selectively modulate the specific signaling pathways. Unfortunately, so far no effective agents targeting the molecular pathways involved in cardiac hypertrophy have been found.

AMP binds to Thr172 in the α subunit, increases the activity of AMPK and induces a conformational change. The cellular ratio of AMP/ATP determines the activity of AMPK. Cardiomyopathy is characterized by glycogen storage, fibrosis, increased protein synthesis, mitochondrial dysfunction, and even decreased cardiac contraction, whereas these processes can be attenuated or delayed by the activation of AMPK. In response to increased workload, AdipoRon increases the phosphorylation of AMPK, which inhibited by ISO or L-thyroxine treatment. Cardiomyopathy is the reflection of myocardial decompensation [44]. In this study, we found that protein of pAMPK/AMPK was significantly decreased in ISO or L-thyroxine-treated groups, and AdipoRon could significantly reverse the ISO or L-thyroxine-mediated effect.

Fatty acids are the main mitochondrial energy substrate in the heart, comparing with other substrates such as lactate or glucose, they provide the highest yield of ATP per mole. Fatty acids are catabolized in mitochondria via the fatty acid β-oxidation (FAO) pathway, leading to reduced equivalents for acetyl-CoA and the electron transport chain, a substrate for further oxidation in the tricarboxylic acid cycle. PGC-1 is capable of co-activating PPARα, a cardiac-enriched member of the PPAR family known to control mitochondrial FAO enzyme expression [11]. PGC-1α is an inducible brown adipose tissue-enriched protein which drives uncoupled respiration and mitochondrial biogenesis in brown adipocytes. PGC-1α was shown to induce the production of mitochondria poised for ATP production and high-level coupled respiration in cardiac myocytes [12]. Cardiac metabolism is important for energy supply. PGC-1α activates ERRα expression to protect cardiac mitochondrial function. We found that AdipoRon could promote the protein expressions of PGC-1α and ERRα which were decreased by ISO or L-thyroxine.

In summary, in this study we established a cardiac hypertrophy model and found that AdipoRon prevents myocardial hypertrophy and fibrosis. Furthermore, we demonstrated that AdipoRon ameliorates cardiac hypertrophy partially through the regulation of AMPK-related pathway. So far as we know, it is the first report showing that AdipoRon can ameliorate cardiac hypertrophy induced by ISO or L-thyroxine and regulate myocardial mitochondrial energy metabolism pathways. Future studies should focus on how AdipoRon exerts its effects and whether there are other pathways involved is in this process.

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