Continuous activation of β1-AR/CaMKII pathway is participated in HHcy-induced myocardial injury

Jiayin Chai
Capital Medical University School of Basic Medical Sciences
Yuhui Zhao
Capital Medical University School of Basic Medical Sciences
Wenjing Yan
Capital Medical University School of Basic Medical Sciences
Ke Xue
Capital Medical University School of Basic Medical Sciences
Shuai Chen
Capital Medical University School of Basic Medical Sciences
Xinyu Zhu
Capital Medical University School of Basic Medical Sciences
Yan Li
the people's Hospital of Jizhou District, Tianjin, China
Wen Wang (wangwen@ccmu.edu.cn)
Capital Medical University https://orcid.org/0000-0003-4120-0773

Research Article

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Abstract

Purpose Although hyperhomocysteinemia（HHcy）is regarded as an independent risk factor for cardiovascular diseases, the role of HHcy in the development of heart failure has not been clarified yet. During the occurrence and progression of heart failure, usually the β_1-adrenergic receptor（β_1-AR）is activated continuously. Here we aim to evaluate the association of hyperhomocysteinemia with continuous activation of β_1-AR in heart.

Methods HHcy models were established by feeding mice with 2.5% methionine diet for 7 months, and HHcy+metoprolol group was established by feeding mice with 2.5% methionine diet and treating with metoprolol (15mg/Kg) through intragastric administration. Echocardiography was used to evaluate the cardiac function after 2, 6, and 7 months feeding. Myocardial injury was assessed by serum myocardial enzyme level. Myocardial ultrastructure was assessed by transmission electron microscopy. The activation level of β_1-AR signal pathway was analyzed using immunoprecipitation and western blot. H9c2 cardiomyocytes and neonatal rat cardiomyocytes were treated with Hcy (500 μM) or metoprolol (100 μM) for 5-60 minutes or 48 hours separately.

Results In HHcy mice, the cardiac function was decreased, the serum myocardial enzyme level was increased and the myocardial ultrastructure was destroyed, which could be alleviated by β_1-AR receptor blocker metoprolol. Hcy activated the cardiac β_1-AR signaling pathway, the downstream PKA signaling pathway was activated by Hcy stimulation within 5-60 min, and the CaMKII signaling pathway could be activated at 48h.

Conclusion Continuous activation of β_1-AR/CaMKII pathway is participated in HHcy-induced myocardial injury, which can be alleviated by β_1-AR receptor blocker.

1. Introduction

As a globally prevalent disease, the number of patients with heart failure is increasing every year, which produces immense health and economic burdens globally[1]. Heart failure is the terminal stage of many heart diseases which with a 5-year survival rate of 25% after hospitalization[2]. However, the main reason caused heart failure is myocardial injury, which is reflected in the decrease of cardiac function, the increase of serum myocardial enzyme level and the destruction of myocardial ultrastructure.

β-Adrenergic receptors (β-AR) are important components of the sympathetic system that regulate the contractile function of the heart. Among all the β-AR, β_1-adrenergic receptor (β_1-AR) is the most important β-AR subtype in the human heart, accounting for about 70% of all β-AR, which is involved in regulating the contractile function of the heart[3]. In the physiological state, faced with external stimuli, β_1 -AR is activated rapidly, then the positive timing, force, and conduction of the heart are rapidly increased by activating the PKA signaling pathway to maintain the body's needs[4]. However, sustained β1-AR activation has a toxic effect on the heart, mediating cardiac hypertrophy and apoptosis through the

activation of the CaMKII signaling pathway[5–7]. As heart failure is often accompanied by continuous activation of β_1 -AR, β_1 -AR blockers are commonly used in the prevention and treatment of heart failure in clinics[8].

Hyperhomocysteinemia (HHcy) is one of the independent risk factors of heart diseases, and the occurrence and development of heart disease are related to the concentration of plasma Hcy[9]. HHcy is caused mainly due to impairment of metabolic enzymes involved in either remethylation (methyltetrahydrofolate reductase –MTHFR) or transsulfuration (cystathionine beta-synthase -CBS and cystathionine gamma-lyase -CTH) pathways[10; 11]. However, there is no particularly effective way to treat HHcy in the clinic at present. Whether the β_1 -AR signal pathway is involved in myocardial injury induced by HHcy and whether β_1 -AR blocker metoprolol can alleviate myocardial injury remains unclear. In this study, we established both *in vivo* and *in vitro* models to explore the role of β_1-AR activation in HHcy-induced myocardial injury and observe the therapeutic effect of metoprolol on it.

2. Methods

2.1 Animals and cells

All animal procedures were in accordance with the “Guiding Principles in the Use and Care of Animals” published by the National Institutes of Health (NIH Publication No. 85 – 23, Revised 1996) and approved by the Institutional Animal Care and Use Committee of Capital Medical University. Male C57BL/6J mice (2-month-old, n = 30) were randomly divided into three groups, receiving normal diet (Control group) 2.5% methionine diet (HHcy group) 2.5% methionine diet and 15 mg/Kg/d metoprolol intragastric administration (HHcy + metoprolol group) for 28 weeks. The cardiac function was monitored at 8th week 24th week and 28th week. All mice had free access to water.

0–3 day-old newborn SD rats purchased from Vital River Laboratories (Beijing, China) were used to obtain neonatal rat cardiomyocytes (NRCMs). NRCMs were cultured in DMEM media (Hyclone, Logan, UT) supplemented with 10% fetal bovine serum, and were incubated in 5% CO_2/95% O_2 at 37°C. Once the cells reached out 60–70% confluence, the cells would be treated with 500 μM Hcy prepared with DMEM for 5–60 minutes in the acute experiments and 500 μM Hcy for 48 hours in the chronic experiments..H9c2 cardiac myoblast cells were cultured in DMEM media (Hyclone, Logan, UT) with 10% fetal bovine serum. Cells were passaged when they reached 80–90% confluence.

2.2 Biochemical analysis

The blood samples of each group of mice were collected from the abdominal aorta and were centrifuged to obtain serum at 3000 rpm, 4 ℃ for 15 min. The serum samples were loaded to an automatic biochemical analyzer (Hitachi 7020, Tokyo, Japan) to detect the levels of Hcy, Creatine kinase isoenzymes-MB(CK-MB), and Lactate dehydrogenase (LDH).

2.3 Measurements of cardiac function

The cardiac ultrasound was used to monitor the cardiac functions of each group of mice. 1% isoflurane was used to anesthetize mice by inhalation. Then Vevo 2100 system (Visual Sonics Inc., Canada) was performed to record ejection fraction (EF), fractional shortening (FS), and left ventricular end-systolic volume (LV Vol; s).

2.4 Transmission electron microscopy (TEM)

In transmission electron microscopy (TEM) observation, the tip of heart tissue was fixed with 3% glutaraldehyde and incubated with 2% OsO4, and dehydrated in ethanol. Then the heart tissues were critical-point dried, sputter-coated with gold, and observed using a JSM-6360LV scanning electron microscopy (JEOL, Tokyo, Japan).

2.5 Western blot analysis

Total proteins were obtained from heart tissues using RIPA protein lysis buffer (APPLYGEN, Beijing, China, 100 mg/mL) with a 1% proteinase inhibitor. BCA assay (Thermo Scientific, America) was performed to determine the protein concentrations. 20 μg proteins of heart tissues or 10 μg proteins of cells were loaded and subjected to 10% SDS-PAGE and then transferred to a polyvinylidene difluoride (PVDF) membrane. After being blocked with 5% non-fat milk for 1 h at room temperature, the blots were probed with the rabbit anti-CaMKII antibody (Abcam, USA, 1:1000), the rabbit anti-p-CaMKII antibody (Abcam, USA, 1:1000), rabbit anti-PKA antibody (Abcam, USA, 1:1000), the rabbit anti-p-PKA antibody (Abcam, USA, 1:1000) at 4℃ overnight. After being washed with TBST three times for 10 minutes each, the membranes were then incubated with either horseradish peroxidase-conjugated goat anti-rabbit IgG (1: 4000, Zhongshan Golden Bridge Biotechnology, China) for 1 h at room temperature. After washing with TBST three times for 15 minutes each, ECL Plus substrate (Thermo Scientific, Massachusetts, USA) was applied to the blot. The images were captured by the gel documentation system (ChemiDoc XRS + system, Bio-Rad, USA), and the optical density of protein bands was analyzed using gel software Image Lab 3.0.

2.6 Immunoprecipitation

For immunoprecipitation assay, HEK293 cells were homogenized in lysis buffer (Tris-HCl 20 mM, Triton 0.1%, NaCl 100 mM, PMSF 100 μM), then centrifuged at 12,000 rpm for 15 min at 4℃. Cell lysis (500 μg total protein) were incubated with 20 μl Protein A/G PLUS-Agarose (Santa Cruz) by occasional gentle mixing for 6 h at 4℃, then centrifuged at 2500 g for 3 min at 4℃. The supernatant was incubated with 2 μg anti-β1-AR antibody (Abcam) with occasional gentle mixing for 8 h or overnight at 4◦C, then added 20 μl Protein A/G PLUS-Agarose (Santa Cruz) and co-incubated for 4 h. Beads were pelleted by centrifuging at 2500 g for 3 min at 4℃, washed with washing buffer (Tris-HCl 20 mM, Triton 0.1%, NaCl 300 mM) 3 times for 10 min each and finally added with 20 μl 2 × SDS-PAGE buffer and boiled at 99℃ for 10min followed by gel electrophoresis. Gels were transferred to the PVDF membrane (Millipore). The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween (TBST, pH 7.6). The primary antibodies used for immunoblotting were anti-Phospho-(Ser/Thr) phe antibody (1:1000, CST).

Membranes were then washed with TBST (3 times for 10 min each), incubated with secondary antibodies coupled to horseradish peroxidase, washed again with TBST (3 times for 10 min each). ECL Plus substrate (Millipore) was applied to the imaging, and images were captured in a gel documentation system (Bio-Rad). The relative optical density of protein bands was analyzed using gel software Image Lab 3.0. Then regenerated the member with Western Stripper (Pulilai, Beijing, China), washed it with TBST and detected loading protein with anti-β1-AR antibody (1:1000, Abcam).

2.7 Statistical Analysis

The results are presented as the means ± standard deviation (SD). Comparisons between two groups were conducted with the unpaired Student t-test. Groups of three or more were analyzed with one-way analysis of variance (ANOVA). Differences were considered statistically significant when $P < 0.05$. Statistical analyses were performed using SPSS v13.0.

3. Results

3.1 HHcy induced myocardial injury in C57BL/6J mice

First, we constructed a hyperhomocysteinemia C57BL/6J mice model through a high methionine diet (Fig. 1A). After 7 months of feeding, the serum Hcy concentration of mice in the HHcy group was significantly higher than that in the control group (Fig. 1B), indicating that the HHcy mouse model was successfully established. Cardiac echocardiography showed that the systolic ability of HHcy mice was decreased compared with the Control group (Fig. 1C), and serum lactate dehydrogenase(LDH) and creatine kinase isoenzymes-MB (CK-MB) were increased compared with the Control group (Fig. 1D-E). Transmission electron microscopy observation showed that the myocardial ultrastructure of HHcy mice was damaged, which was reflected in sarcomere fracture, mitochondrial swelling, and crest disappearance (Fig. 1F). All the above results confirmed that HHcy induced myocardial injury in C57BL/6J mice.

3.2 HHcy promoted the activation of β_1-AR in myocardium

To explore the specific mechanism of HHcy inducing myocardial injury, genome-wide expression analysis was performed in the myocardial tissues of normal and HHcy mice. KEGG analysis results showed that, compared with normal mice, the β_1-AR-related signaling pathway was significantly enriched in myocardial tissues of HHcy mice (Fig. 2A), suggesting that the β_1-AR signaling pathway may be involved in HHcy-induced myocardial injury. The activation form of β_1-AR is phosphorylation. Due to the numerous phosphorylation sites of β_1-AR, there is no commercial p-β_1-AR antibody, so we detected the phosphorylation level of β_1-AR by immunoprecipitation(IP). To avoid the interference of IgG heavy chain (55KD), we constructed the GFP-β_1-AR fusion plasmid (Fig. 2B). Having been transfected into HEK293 cells (Fig. 2C), the molecular weight of exogenous GFP-β_1-AR was increased to 77KD, and the phosphorylation level of β_1-AR was detected by IP. The results showed that Hcy significantly increased

the phosphorylation level of β_1-AR, and the activation effect of Hcy on β_1-AR was inhibited by the β_1-AR blocker metoprolol (Fig. 2D-E).

3.3 Hcy increased myocardial contractility by activating the PKA signaling pathway in the short term and induced myocardial injury by activating the CaMKII signaling pathway in a chronic manner

To further clarify the role of HHcy in activation of the β_1-AR signaling pathway, we did a cardiac ultrasound detection at different time points to observe the effects of HHcy on cardiac function. The results showed that at the 8th week of high methionine feeding, the value of Ejection Fraction (EF) and Fraction shortening (FS) in HHcy mice were higher than those in normal mice. However, after feeding high-methionine diet for 24 weeks, the EF value of HHcy mice began to be lower than that of normal mice, and when feeding high-methionine diet for 28 weeks, the value of EF、FS and systolic left ventricular volume (LEVSV) of HHcy mice were significantly lower than these of control group (Fig. 3A-C). These results suggested that HHcy improved myocardial contractility in the short term, but ultimately induced cardiac function decline in the long term, which was the same as the process of cardiac changes after β_1-AR activation. Acute sympathetic stimulation of cardiac β_1-AR through the PKA signaling pathway to induce positive inotropic and chronotropic effects, the most effective mechanism to acutely increase the output of the heart, while the long-term activation of β_1-AR can be shown as desensitization of the PKA signaling pathway and activation of CaMKII signaling pathway playing a role in myocardial injury. Furthermore, we extracted NRCMs and observed the beat frequency after the addition of Hcy. We found that Hcy accelerated the beating frequency of NRCMs, meanwhile this effect was effectively reduced by β_1-AR blocker metoprolol (Fig. 3D). Then we established 0-60min observation points in Hcy challenged NRCMs and observed that the PKA was significantly activated 5 min after the addition of Hcy and maintained for at least 1 hour, which could be inhibited by β_1-AR blocker metoprolol (Fig. 3E). We then stimulated H9c2 cardiomyocytes with Hcy for a 48 hours, and the results showed that the phosphorylation level of CaMKII was significantly increased in the Hcy group, which was inhibited by both β_1-AR inhibitors metoprolol, bisoprolol, atenolol, and CaMKII blocker KN93 (Fig. 3F).

3.4 Metoprolol significantly alleviated HHcy-induced myocardial injury in C57BL/6J mice

To further verify the role of the β_1-AR signaling pathway in HHcy induced myocardial injury, we established the Hcy + Metoprolol group in C57BL/6J mice by simultaneously feeding the mice with a high methionine diet and metoprolol (Fig. 4A). The results showed that the model was successfully established (Fig. 4B). The phosphorylation level of CaMKII protein in the myocardial tissue of mice in the HHcy + Metoprolol group was lower than that in the HHcy group (Fig. 4C), and the serum concentration of LDH was also lower than that in the HHcy group (Fig. 4D). Meanwhile, the cardiac contractility of the mice in this group was stronger than in the HHcy group (Fig. 4E) and ultrastructural damage was also lighter than that in the HHcy group (Fig. 4F). These results all confirmed that the β_1-AR signaling pathway

was involved in HHcy induced myocardial injury, and β_1-AR blocker metoprolol alleviated the myocardial injury induced by HHcy.

4. Discussion

β_1-AR metoprolol is one of the most commonly used drugs to treat heart failure in clinic[12; 13]. Our study demonstrates that metoprolol can improve cardiac function in HHcy mice. Cardiac ultrasound showed that treatment with metoprolol improved left ventricular systolic function decline in HHcy mice. The biochemical detection of plasma showed that metoprolol decreased the plasma concentration of LDH in HHcy mice, Transmission electron microscope showed that metoprolol reversed myocardial ultrastructural injury in HHcy mice. This is related to metoprolol inhibiting the activation of β_1-AR/CaMKII pathway induced by Hcy. Therefore, we first proposed that metoprolol reduces myocardial injury in HHcy mice through inhibiting β_1-AR/CaMKII signal pathway.

Hyperhomocysteinemia (HHcy) is one of the independent risk factors of heart diseases, It has been reported that the risk of heart disease will increase by 10% when the concentration of plasma Hcy increases by 10μM[14]. Folic acid is an important determinant of the Hcy metabolic pathway which can reduce the production of Hcy, therefore, exogenous folic acid supplementation is effective for some patients[15; 16]. However, clinical investigation shows that about 40% of HHcy patients do not have a satisfactory effect after taking folic acid treatment[17; 18]. This may be related to the mutation of methylene reductase, therefore it is of great significance to clarify the mechanism involved in HHcy-induced myocardial injury and find new therapeutic targets .

we constructed a HHcy model by feeding C57BL / 6J mice with 2.5% methionine diet[19]. After 28 weeks of feeding, a significant increase in serum Hcy concentration was detected, suggesting that the HHcy model was established successfully and can be used for subsequent experiments. Compared with normal mice, echocardiography showed that cardiac contractility was decreased in HHcy mice, and the serum LDH and CK-MB concentrations were increased, meanwhile the disrupted myocardial ultrastructure, disorganized sarcomeres, and swollen mitochondria, indicating that chronically suffering from HHcy induces cardiac damage.

To further explore the mechanism of HHcy-induced myocardial injury, genome-wide expression analysis was performed on the myocardium of normal mice and HHcy mice, and KEGG analysis showed that the adrenalin signaling pathway and cAMP signaling pathway were significantly enriched in the myocardium of HHcy mice compared with the Control group. Since β-AR is the main AR expressed in the heart, and the β_1-AR is the most widely expressed β-AR in the myocardial cells. β_1-AR plays an important role in the sympathetic regulation of the heart and is highly conserved during evolution[3]. Meanwhile, the expression of β_1-AR changes in myocardial cells of heart failure patients. We wonder whether HHcy can activate β_1-AR? The activation form of β_1-AR is phosphorylation. There are many phosphorylation sites in β_1-AR[20], at present there is no commercial antibody that can detect all the phosphorylation sites of β_1-AR.

Therefore, we detected the phosphorylation level of β_1-AR by immunoprecipitation. The molecular weight of β_1-AR is about 50KD, it is difficult to distinguish the target band from the IgG heavy chain. Therefore, we constructed the β_1-AR-GFP fusion plasmid, whose molecular weight is about 77KD. We transfected the plasmid into HEK293 cells and found that Hcy increased the phosphorylation level of β_1-AR.

β_1-AR activation can be divided into two types: short-term stimulation of β_1-AR activates the PKA signaling pathway and induces positive inotropic an chronotropic effects which are the most effective mechanisms for rapidly increasing cardiac output[21], while chronic activation of β_1-AR activates CaMKII which is harmful to the heart[22; 23]. Therefore, in this study different time points were established at 8 weeks, 24 weeks, and 28 weeks to monitor the effects of Hcy on the cardiac function of C57BL/6J mice. The results showed that after 8 weeks of high methionine diet, the myocardial contractility of HHcy mice was significantly stronger than that of the normal mice. However at the 24th week, it's lower than the Control group, and the same trend remained until 28 weeks, consistent with the trend of cardiac function changes after cardiac β_1-AR activation. Then we extracted NRCMs and observed that the beat frequency of NRCMs was significantly increased after the addition of Hcy for 10 minutes, which was effectively reduced by β_1-AR blocker metoprolol. To further discuss whether Hcy can activate the β_1-AR downstream signaling pathway, we stimulated cardiomyocytes with Hcy for a short term (5–60 minutes), and the results showed that the activation level of PKA was increased in cardiomyocytes. Then, we stimulated cardiomyocytes with Hcy for a long term (48 hours) and found that the phosphorylation level of CaMKII was increased. All of these effects were inhibited by β_1-AR blocker metoprolol. Finally, we constructed a mouse model fed with a high methionine diet and treated with metoprolol. After 28 weeks, the serum Hcy concentration showed that the model was successfully established. Compared with HHcy mice, mice treated with metoprolol showed reduced activation levels of CaMKII in myocardial tissue, lower serum LDH concentration, stronger myocardial contractility, and less damage in cardiac ultrastructure. These results suggest that Hcy enhances myocardial contractility through activation of the β_1-AR/PKA signaling pathway in the short term, while chronic Hcy stimulation activates the β_1-AR/CAMKII signaling pathway and plays a role in myocardial injury.

Our study showed that β_1-AR blocker metoprolol is a potential drug for the treatment of myocardial injury induced by HHcy, For HHcy patients with increased myocardial contractility, early administration of metoprolol may have a good effect on alleviating HHcy induced myocardial disease. There's limitation to this study. Due to technical limitations, at present β_1-AR protein cannot be purified. Therefore, whether Hcy can directly bind to β_1-AR as a ligand to regulate its conformation still remain unclear.

Conclusion

Based on the above experimental results, we proposed that HHcy can induce myocardial injury through continuous activation of cardiac β_1-AR, which is reflected in the temporary increase of myocardial

contractility in the early stage, and then continuously decreased after chronic stimulation. β_1-AR receptor blockers have therapeutic significance for HHcy-induced cardiomyopathy.

Declarations

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Conflicts of Interest The authors declare that they have no conflict of interest.

Authors' Contributions Jiayin Chai, Wen Wang and Yan Li designed the experiments. Yuhui Zhao and Yan Li provided materials, instruments and technical support. Jiayin Chai established the vivo and vitro models, performed experiments and collected samples with the help of Yuhui Zhao, Wenjing Yan, Ke Xue, Shuai Chen Xinyu Zhu and Yan Li. Jiayin Chai and Wen Wang wrote the manuscript with modification from other authors.

Date Availability The use and/or analyzed datasets are available from the corresponding author (Wen Wang) on reasonable request.

Ethics Approval The protocols used for all animal studies were approved by the Capital Medical University Animal Policy (Ethics number [2018]003) and Welfare Committee and complied with the NIH guidelines (Guide for the Care and Use of Laboratory Animals).

Consent to Participate This research does not involve human experiments.

Consent for Publication All authors agree to publish in Cardiovascular Drugs and Therapy.

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Figures

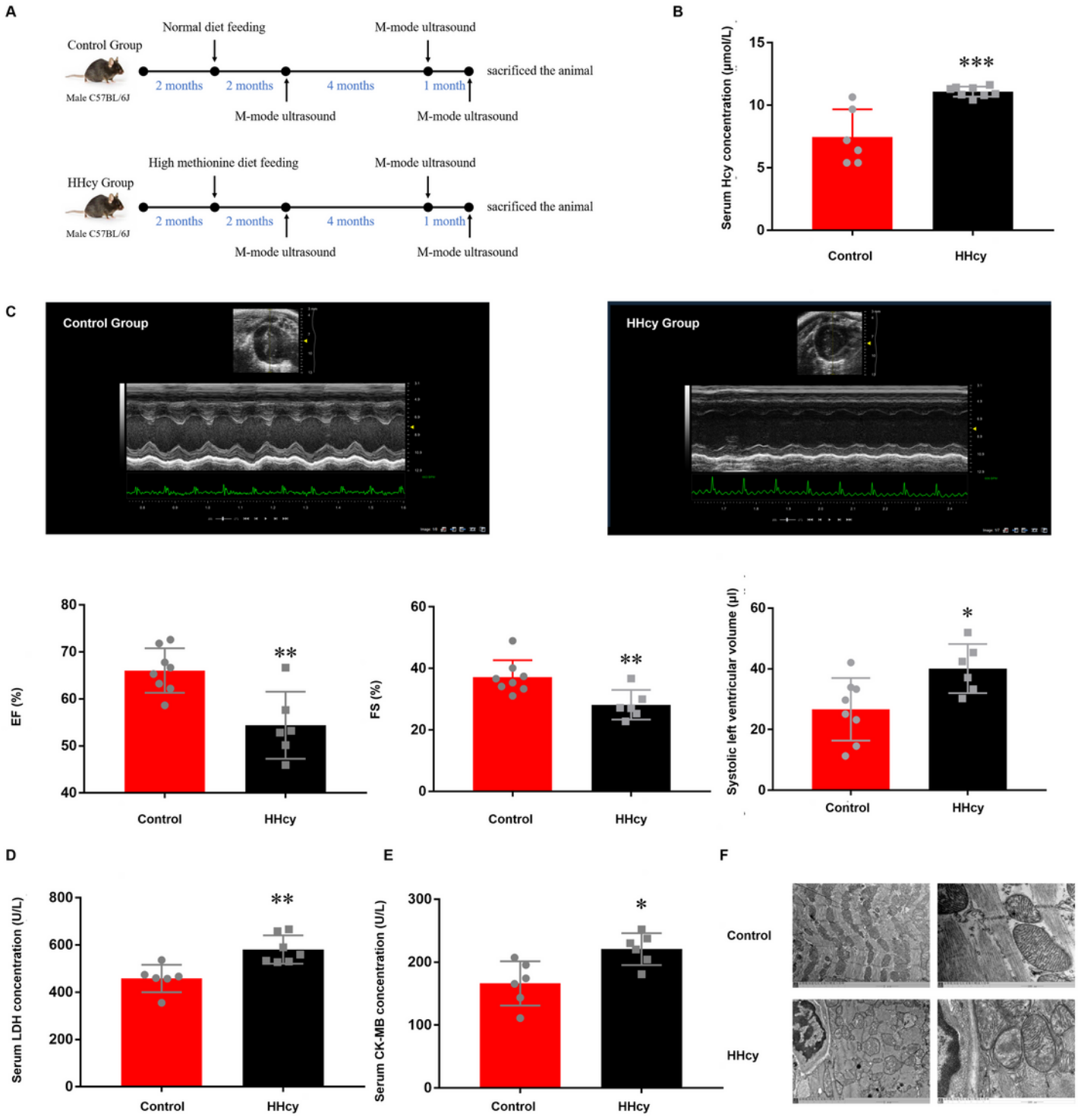

Figure 1

HHcy induced myocardial injury in C56BL/6J mice **A.** Schematic diagram of constructing hyperhomocysteine C57BL/6J mice model. **B.** The serum Hcy concentration of two groups of mice (n=6-8). Data were expressed as mean ± SD. $^{***}P$<0.001 vs. Control group. **C.** The typical images of EF、FS、Vol which were measured by M-mode ultrasound（n=6-8 for each）. Data were expressed as mean ± SD. $^{*}P$<0.05 vs. Control group. $^{**}P$<0.01 vs. Control group. **D-E.** The concentration of serum myocardial enzyme

LDH and CK-MK was detected by an automatic biochemical detector（n=6-7 for each）. Data were expressed as mean ± SD. $^{*}P$＜0.01 vs. Control group. $^{**}P$＜0.01 vs. Control group. **F.** The change in the ultrastructure of heart tissue is shown by TEM.

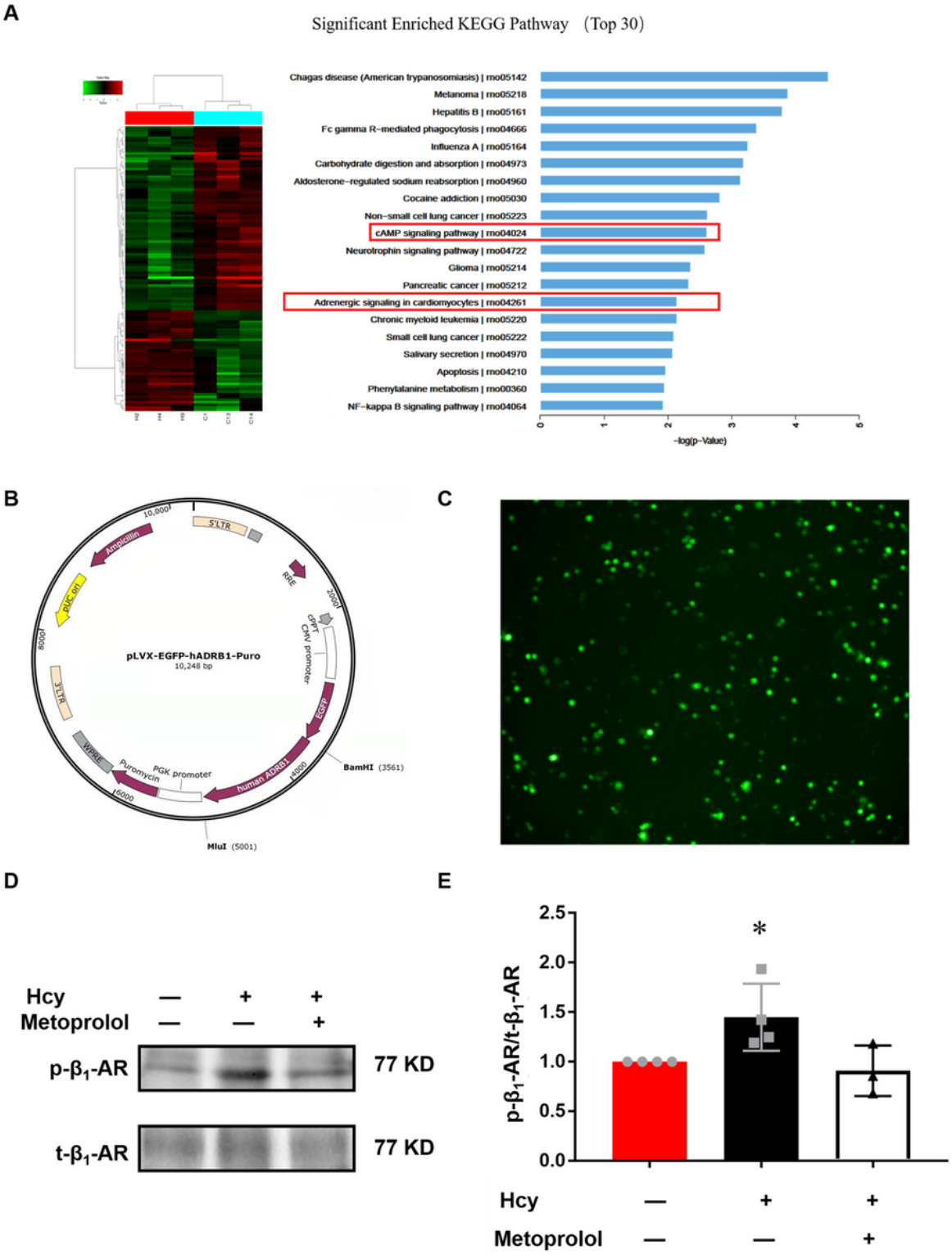

Figure 2

HHcy promoted the activation of β_1-AR in myocardium **A.** Genome-wide expression microarray results showed that the Adrenergic-related signaling pathway and cAMP signaling pathway were significantly up-regulated in HHcy mice. **B.** Construction of β_1-AR-GFP fusion plasmid. **C.** The plasmid was successfully transfected into HEK293 cells. **D-E.** Hcy increased the phosphorylation level of exogenous β_1-AR, and this effect was inhibited by β_1-AR blocker metoprolol (n=3-4). Data were expressed as mean ± SD. $^*P$<0.05 vs. Control group.

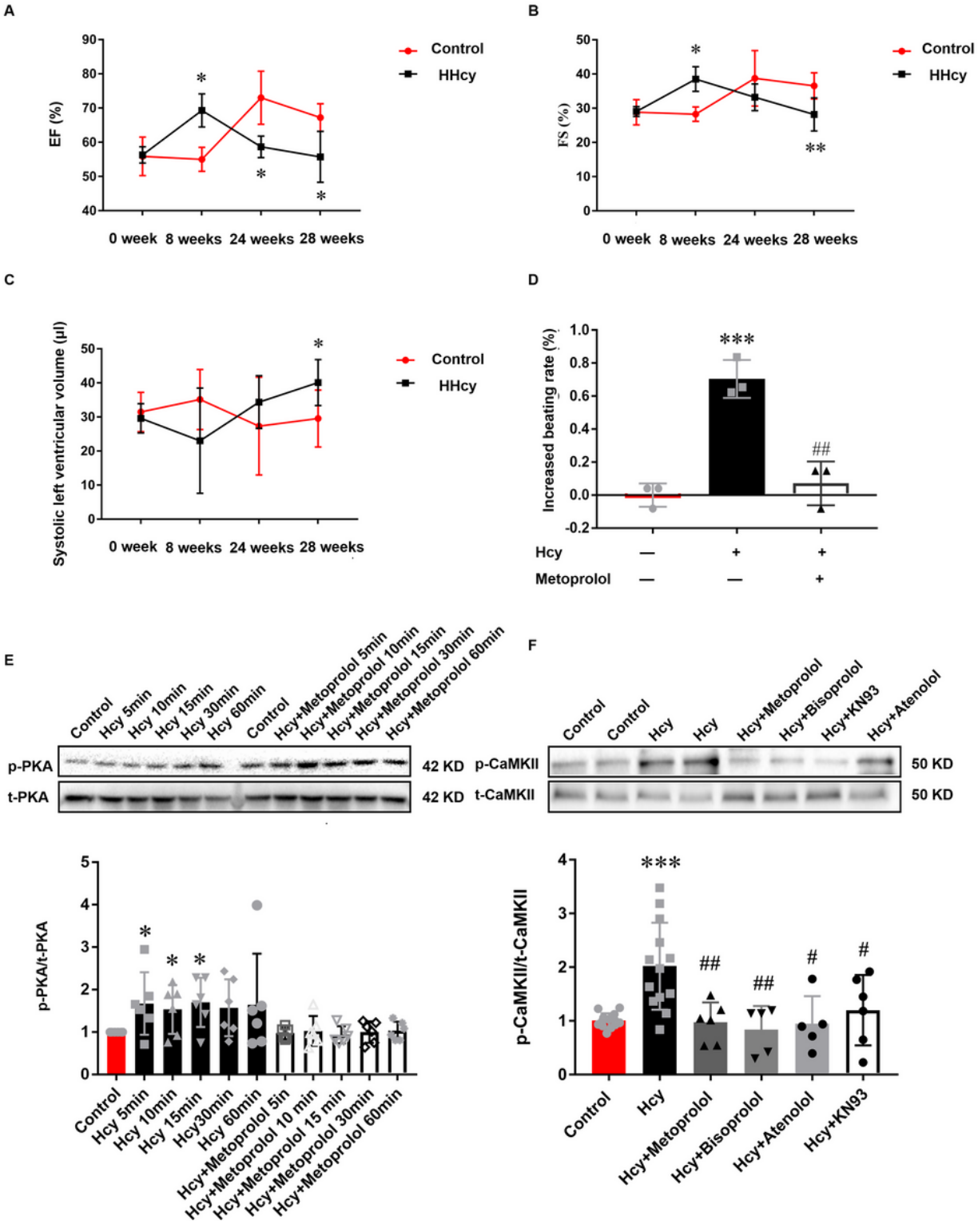

Figure 3

Hcy active PKA signaling pathway and CaMKII signaling pathway **A-C.** the graphs of M-mode ultrasound of EF□%□□FS□%□□systolic left ventricular volume □μl□at different time points, respectively (n=3-6 for each). Data were expressed as mean ± SD. $^{*}P$□0.05 vs. Control group, $^{**}P$□0.01 vs. Control group **. D.** the statistical graph of NRCMs beat frequency (n=3). Data were expressed as mean ± SD. $^{***}P$□0.001 vs. Control group, $^{\#\#}P$□0.01 vs. Hcy group. **E.** the graph shows the ratio of phosphorylated PKA to total PKA in H9c2 cells (n=6). Data were expressed as mean ± SD. $^{*}P$□0.05 vs. Control group. **F.** the graph shows the ratio of phosphorylated CaMKII to total CaMKII in H9c2 cells (n=5-14). Data were expressed as mean ± SD. $^{***}P$□0.001 vs. Control group, $^{\#}P$□0.05 vs. Hcy group, $^{\#\#}P$□0.01 vs. Hcy group.

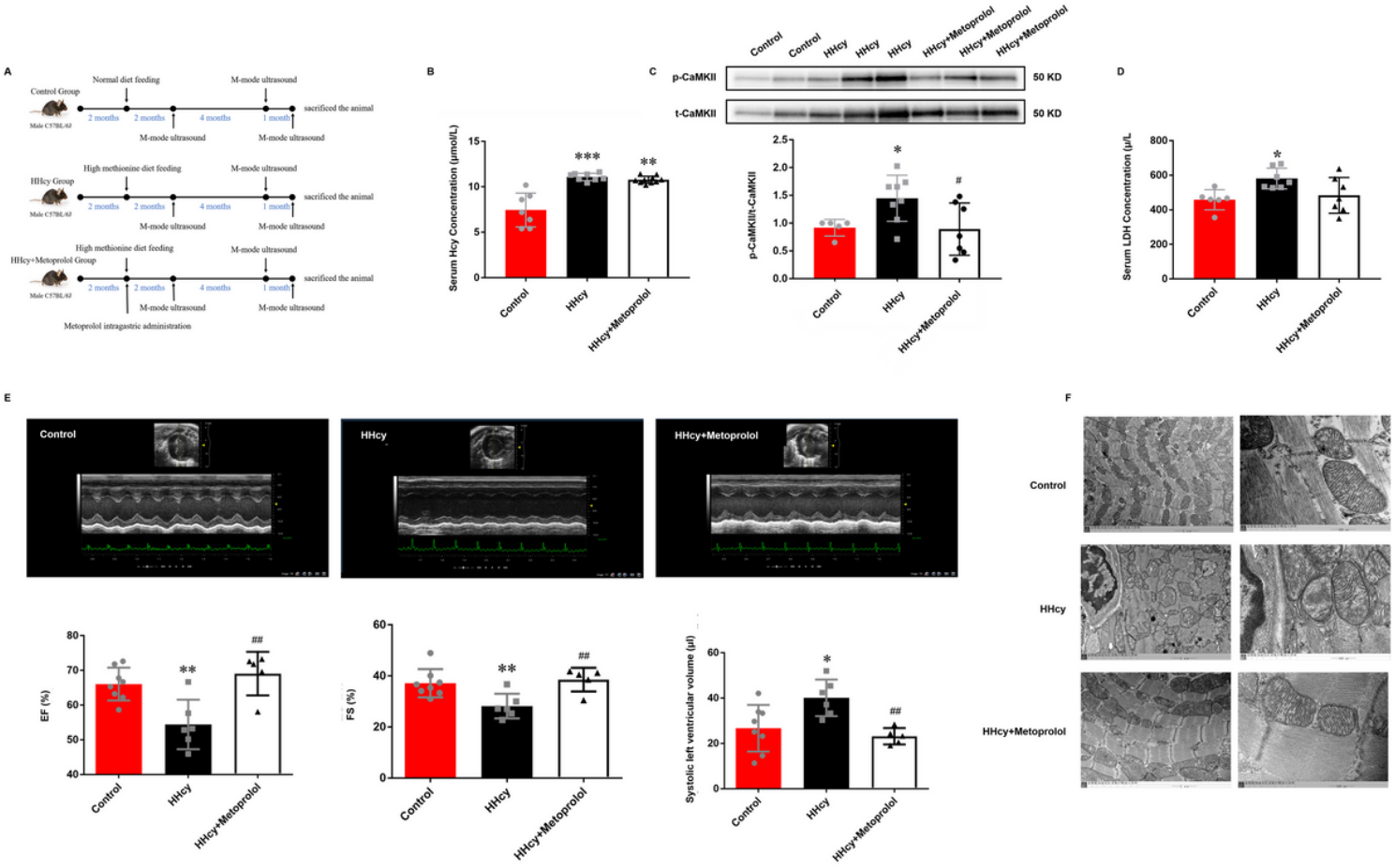

Figure 4

Metoprolol significantly alleviated HHcy-induced myocardial injury in C57BL/6J mice **A.** The schematic diagram of C57BL/6J mice model of HHcy treated with metoprolol. **B.** The serum Hcy concentration of three groups of mice (n=7-10). Data were expressed as mean ± SD. $^{**}P$□0.001 vs. Control group, $^{***}P$□0.001 vs. Control group. **C.** the graph shows the ratio of phosphorylated CaMKII to total CaMKII in the myocardium of three groups of mice (n=5-8). Data were expressed as mean ± SD. $^{*}P$□0.05 vs. Control group, $^{\#}P$□0.05 vs. HHcy group. **D.** The expression of serum myocardial enzyme LDH was detected by an

automatic biochemical detector（n=6-7 for each）. Data were expressed as mean ± SD. $^{*}P$＜0.01 vs. Control group. **E.** the images of M-mode ultrasound of EF（%）、FS（%）、systolic left ventricular volume （μl） representatively（n=5-8 for each）. Data were expressed as mean ± SD. $^{*}P$＜0.05 vs. Control group. $^{**}P$＜0.01 vs. Control group, $^{\#\#}P$＜0.01 vs. HHcy group. **F.** The images of TEM show the degree of mitochondrial damage to heart tissue.

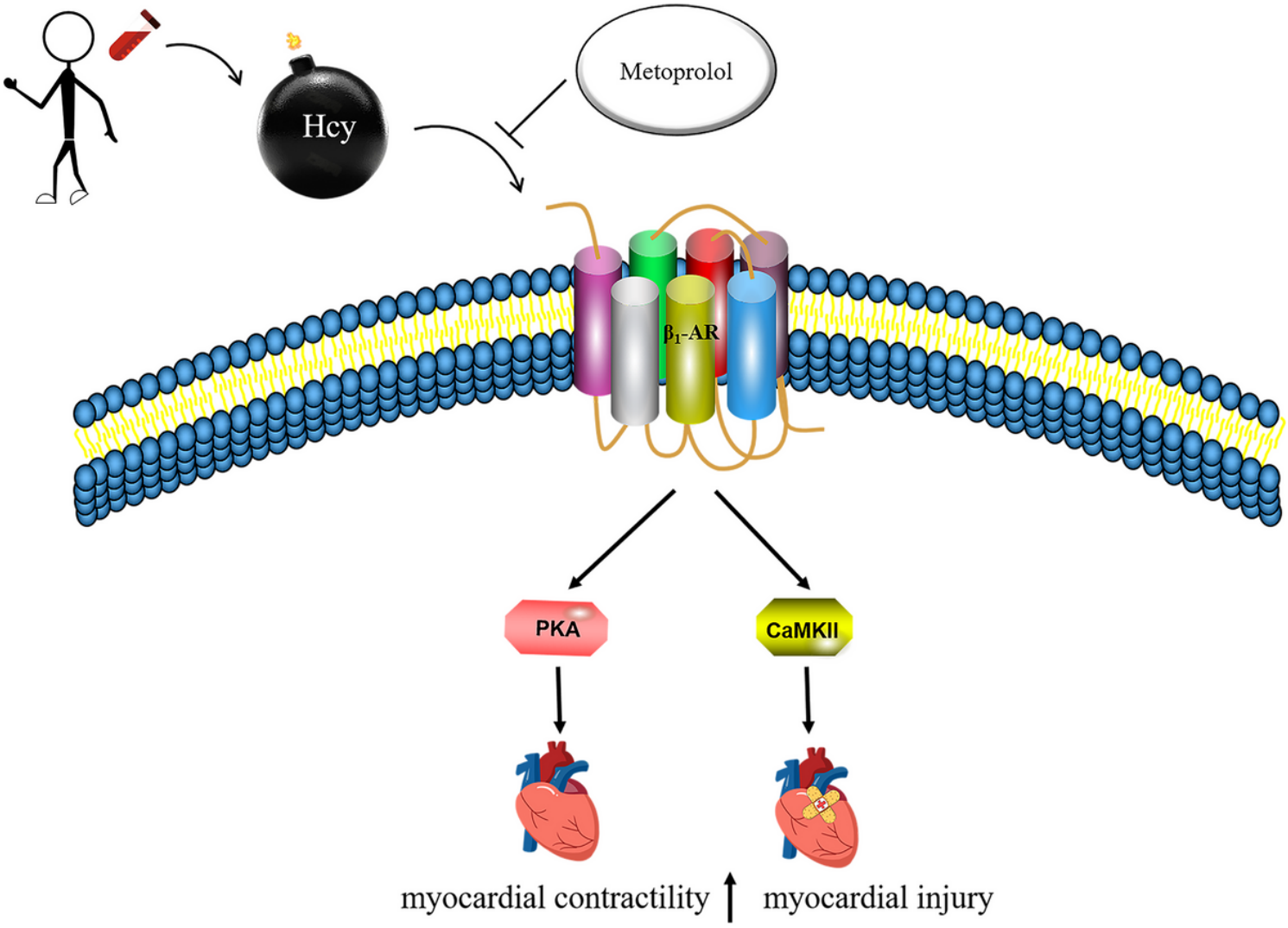

Figure 5

HHcy can induce myocardial injury through continuous activation of cardiac β_1-AR, which is reflected in the temporary increase of myocardial contractility in the early stage, and then continuously decreased after chronic stimulation. β_1-AR receptor blockers can relieve the myocardial injury induced by HHcy.