Plantamajoside attenuates isoproterenol-induced cardiac hypertrophy associated with the HDAC2 and AKT/ GSK-3β signaling pathway

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A B S T R A C T

As a compensatory response to cardiac overload, cardiac hypertrophy is closely associated with the occurrence and development of a variety of cardiovascular diseases, in which histone deacetylase 2 (HDAC2) has been reported to play an important role. Plantamajoside (PMS) is an active component extracted from *Herba Plantaginis*, which is a traditional Chinese medicine, and many biological activities of PMS have been reported. Here, we investigated the effects and mechanism of PMS on isoproterenol (ISO)-induced cardiac hypertrophy. ISO at 10 μmol/L was used *in vitro* to induce H9c2 cardiomyocyte hypertrophy. Cell viability and cell surface area were determined by MTT assay and immunocytochemistry, respectively. Furthermore, an *in vivo*, cardiac hypertrophy model was established by subcutaneous injection of ISO. Pathological alterations and fibrosis in the myocardium were studied by H&E and Masson's trichrome staining, respectively. Myocardial injury-related genes and proteins were detected by real-time PCR and western blotting. HDAC2 and its downstream proteins, AKT and GSK3β, were analyzed by western blotting. Our results showed that, *in vitro*, PMS inhibited the ISO-induced increase in H9c2 cell surface area and the mRNA expression of ANP, BNP and Myh7. In vivo, PMS improved the ISO-induced decrease in cardiac function, inhibited the increase in cardiac anatomical parameters and alleviated the histopathological changes in cardiac tissues. Moreover, PMS inhibited the mRNA and protein expression of ANP, BNP, Myh7, COL1 and COL3. Furthermore, PMS suppressed the activity of HDAC2 and down-regulated the expression of the downstream proteins *p*-AKT and *p*-GSK3β both *in vitro* and *in vivo*. Overall, our results indicated that PMS exerts significant cardioprotective effects against ISO-induced cardiac hypertrophy, and this protective effect may be mediated by inhibition of the HDAC2 and AKT/GSK-3β signaling pathway.

1. Introduction

Physiological cardiac hypertrophy is characterized by an increase in the total amount of cardiac muscle mass and myocardial contractility. Although cardiac hypertrophy may initially be a compensatory mechanism to maintain normal blood circulation, continuous hypertrophy will develop into pathological cardiac hypertrophy [1], which is accompanied by obvious disorder of the cardiac myocytes arrangement, fibrosis, myocytes apoptosis, and re-expression of embryonic genes (ANP, BNP, β-MHC, α-skeletal actin) [2], and persistent cardiac hypertrophy may lead to myocardial infarction, arrhythmia, heart failure and sudden death [3,4].

Although cardiac hypertrophy has been known for more than a century, its underlying mechanism is still not fully understood [5], and a multitude of extracellular factors and signaling pathways are involved according to previous studies [6]. Histone deacetylases (HDACs) are important epigenetic regulators of gene transcription through the removal of acetyl groups from substrate lysines [7]. It has been reported that HDACs dysfunction contributes to a variety of heart diseases, including cardiac hypertrophy, heart failure, acute coronary syndromes and arrhythmia [8]. HDACs inhibitors attenuated cardiac hypertrophy induced by pressure overload or infusion of isoproterenol (ISO) [9–11]. There are four major classes of HDACs in mammals, class I - IV; HDAC2 belongs to class I, and its phosphorylation at serine 394 plays an

Abbreviations

PMS	Plantamajoside
ISO	isoproterenol
HDACs	Histone deacetylases
AKT	Protein Kinase B
GSK-3β	Glycogen synthase kinase 3β
FBS	Fetal Bovine Serum
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
H&E	hematoxylin and eosin

LVPW	left ventricular posterior wall thickness
LVAW	left ventricular anterior wall thickness
LVID	left ventricular interior diameter
LV Vol	left ventricular volume
EF	ejection fraction
ANP	atrial natriuretic peptide
BNP	B-type natriuretic peptide
COL1	collagen I
COL3	collagen III
Myh7	β-myosin heavy chain

important role in cardiac hypertrophy [8,12]. Moreover, evidence exist that HDAC2 and protein kinase B (AKT)/glycogen synthase kinase 3β (GSK-3β) signaling pathway played vital roles in cardiac hypertrophy [13].

As a traditional Chinese medicine, *Herba Plantaginis* the dried whole grass of *Plantagoasiatica* L. has been used as an antipyretic, antitussive and diuretic agent, and for promoting wound healing [14,15]. One of its active components is Plantamajoside (PMS) (Fig. 1A shows its chemical structure) [16–18]. PMS has been reported to have many biological activities, such as anti-oxidation, anti-inflammation, anti-tumorigenesis and inhibition of the formation of glycosyl end products [19–22]. Using computer-aided drug design technology and molecular docking software together with HDAC2 and more than 30000 compounds in the Chinese traditional medicine database, we found that PMS has a strong binding affinity to HDAC2; the general overview of the PMS docking pose at the HDAC2 active site, and the 3D and 2D molecular docking models are depicted in Fig. S1. In this study, the

protective effect of PMS on ISO-induced cardiac hypertrophy and its relationship with HDAC2 were investigated.

2. Materials and methods

2.1. Cell culture

H9c2 cells were purchased from iCell Bioscience.Inc (Shanghai, China). Cells were cultured in DMEM supplemented with 10% Fetal Bovine Serum (FBS), Penicillin (100 U/mL) and Streptomycin (100 μg/mL) under a humidified incubator with 5%CO_2 at 37 °C. 10 μmol/L ISO (dissolved in PBS, Sigma Aldrich, USA) was applied to induce H9c2 cardiac hypertrophy based on previous studies and our preliminary experiments [23].
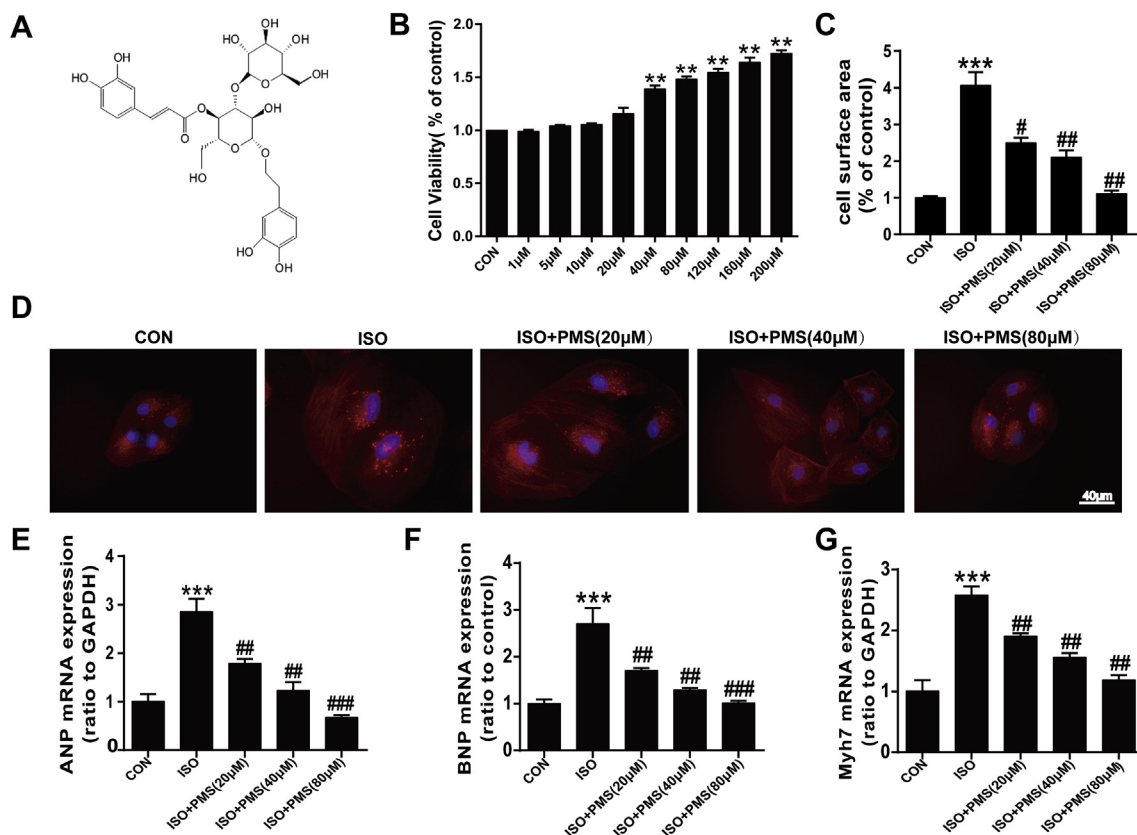

Fig. 1. PMS inhibits ISO-induced cardiac hypertrophy in H9c2 cells. (A) The chemical structure of PMS. (B) PMS effect on cells viability as assessed by MTT assay. (C, D) H9c2 cell area quantification and representative immunofluorescence images (blue is DAPI, red is actin, bar = 40 μm). (E–G) PMS decreased the ISO-induced mRNA expression of ANP, BNP and Myh7 in H9c2 cells.*p < 0.05, **p < 0.01 compared with CON group, #p < 0.05, ##p < 0.01 compared with ISO group. All values are mean ± SD.

2.2. Cell viability measurement

Cell viability was determined by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. 1×10^4 of H9c2 cells were seeded into 96-well plates incubated with different concentrations of PMS (Chengdu Manchester Biotech Co. Ltd, China, purity > 98%) for 24 h, and then added 20 μL 5 mg/mL MTT solution to each well. 4 h later, medium was discarded and 150 μL of dimethylsulfoxide (DMSO) was added to each cell. The absorbance at 490 nm was measured. The percentage of cell viability was calculated by the following formula: cell viability (%) = (mean absorbency in test wells-mean absorbency in blank wells)/(mean absorbency in control wells-mean absorbency in blank wells) × 100%.

2.3. Immunocytochemistry

H9c2 cells were fixed with 4% paraformaldehyde at room temperature for 30 min. Cells were washed three times with ice-cold PBS, permeabilized with 0.1% Triton X-100 for 15 min, then blocked with 1% bovine serum albumin for 30 min, stained with mouse α-actin antibody (Santa Cruz, sc-32251) in blocking medium overnight at 4 °C, and incubated with cy3-conjugated anti-mouse IgG (Yeasen, Shanghai) for 2 h, then cell nucleus were stained with DAPI. Imaging was performed on OLYMPUS DP80 microscope, and used Image-J software for cell surface area data analysis.

2.4. Animals and treatments

Six weeks male BALB/c mice, weighing approximately 18–20 g, were purchased from Liaoning Changsheng Biotechnology Co. Ltd, China. Mice were housed in an environmentally controlled room (24 ± 1 °C, 40%–80% humidity), allowed free access to food and water. The animal protocols were approved by the Animal Care and Use Committee of the General Hospital of Northern theater Command.

In vivo study was conducted by cardiac hypertrophy model, which was established by subcutaneous injection of ISO (5 mg/kg/d, dissolved in saline, Sigma Aldrich, USA) once daily for 9 days, according to the previous studies and our preliminary experiments [24]. Mice were randomly divided into five groups: control group; ISO group; low-, high-dose PMS group (10, 40 mg/kg/d, intravenous injection, respectively) and positive control group (valsartan, 20 mg/kg/d, intragastric administration, Novartis Pharmaceutical Co. Ltd, China). All the administration groups began to inject ISO after 3 days of pretreatment with PMS or valsartan.

2.5. Echocardiography measurement

After removal of the hair of anterior chest, mice were anesthetized with 1.5% isoflurane. Echocardiographic images, left ventricular (LV) internal diameter, ejection fraction, fractional shortening and wall thickness were obtained by a Visualsonics high-resolution Vevo 2100 system with a 10-MHz linear array ultrasound transducer.

2.6. Histopathological studies

Cardiac tissues were excised, fixed in 10% formalin buffer, embedded in paraffin, sectioned at 5 μm of thickness, and stained with hematoxylin and eosin (H&E). Photomicrographs were taken at × 400 magnification by a light microscope (OLYMPUS, CH-20). For the assessment of cardiac fibrosis, sections were stained with Masson's trichrome staining kit (Sigma, USA). Collagen fibers were stained blue, nucleus were stained black and myocardium was stained red. The percentage of the collagen-stained area (blue color) to the total cardiac area was calculated as the collagen volume fraction. Image-J software was used for data analysis.

2.7. Western blot analysis

H9c2 cell proteins and cardiac tissue proteins were extracted by contained protease and phosphatase inhibitors lysis buffer. The concentration was estimated by BCA reagent, and equal amounts of the proteins were separated on SDS-PAGE and transferred to PVDF membranes. After transfer, the membrane was blocked for 1 h at room temperature in 5% nonfat milk, and then incubated with primary antibody such as GAPDH (Santa Cruz, sc-32233), Myh7 (Abcam, ab-50967), ANP (Abcam, ab-76743), HDAC2 (Abcam, ab-32117), BNP (Santa Cruz, sc-18817), COL1 (Santa Cruz, sc-8784), COL3 (Santa Cruz, sc-8781), AKT (CST, 4691S), GSK-3β (Abcam, ab-93926), *p*-HDAC2(Ser394) (Abcam, ab-75602), *p*-AKT (Ser473) (CST, 4051S), *p*-GSK-3β(Ser9) (CST, 14630S) overnight at 4 °C. Membranes were then incubated with corresponding HRP-conjugated secondary antibodies and detected by ECL detection kit (Bio-Rad, USA).

2.8. RNA extraction and real-time PCR

Total RNA was extracted by TRIzol (Takara Biotechnology, Tokyo, Japan), then reverse transcribed into cDNA using the Prime Script RT reagent kit (Takara Shuzo Co. Ltd, Tokyo, Japan). Real-time PCR was performed by an SYBR Green Real-time PCR Master Mix (TIANGEN, Beijing, China). The relative level of mRNA was calculated by the comparative CT method with GAPDH mRNA as the invariant control. The sequences of the primers (Takara Biotechnology, Dalian, China) used *in vitro* and *in vivo* are provided in Table S1.

2.9. Data and statistical analysis

All values were expressed as means ± standard deviation (SD). Statistical significance was defined as a two-sided *p*-value < 0.05. One-way analysis of variance (ANOVA), followed by LSD-t-test, was used to compare each variable for differences among the groups. A *p* value of less than 0.05 was considered to be statistically significant.

3. Results

3.1. Plantamajoside effects on cardiomyocyte viability

Myocardial H9c2 cells were treated with 1–200 μmol/L PMS for 24 h. When the PMS concentration was in the range of 1–20 μmol/L, there was no significant difference in cell viability compared with the control group. However, in the range of 40–200 μmol/L, PMS had a positive effect on cardiomyocyte viability compared with the control group (Fig. 1B). Thus, we inferred that PMS is not toxic for H9c2 cells at 1–200 μmol/L. In the subsequent experiments, 20, 40 and 80 μmol/L were used.

3.2. Plantamajoside effects on ISO-induced cardiomyocyte hypertrophy in vitro

Cardiomyocyte hypertrophy is characterized by increased cell surface area and upregulation of embryonic proteins and genes. H9c2 cell area quantification showed an evident increase in cardiomyocytes area upon ISO treatment compared with the control cells. However, compared with the ISO group, 20, 40 and 80 μmol/L PMS decreased the cell surface area (Fig. 1C and D). In addition, PMS decreased the ISO-induced mRNA expression of ANP, BNP and Myh7 in H9c2 cells in a concentration-dependent manner ($p < 0.05$; Fig. 1E–G).

3.3. Plantamajoside improves cardiac function in ISO-induced mice

The echocardiography parameters, left ventricular posterior wall thickness (LVPW), left ventricular anterior wall thickness (LVAW), left ventricular interior diameter (LVID) and left ventricular volume (LV

Vol), demonstrated hypertrophy of the left ventricle in mice from the ISO group (Table S2). Compared with the ISO group, a low dose of PMS did not exert obvious changes in these values. However, PMS at 40 mg/kg/d significantly reduced the diastolic LVAW, systolic LVAW, diastolic LVPW, systolic LVPW, and diastolic LV Vol. In addition, high-dose PMS improved the values of ejection fraction (EF, 38.7 ± -0.82% in ISO vs. 50.5 ± 6.06% in high dose PMS) and fractional shortening (FS, 28.3 ± 1.66% in ISO vs. 37.0 ± 8.13% in high dose PMS, Fig. 2A–D). In the positive control group, valsartan decreased the hypertrophy of the left ventricle induced by ISO. The various echocardiography measurements revealed, that a high dose of PMS injected intravenously had a stronger effect than 20 mg/kg/d valsartan administered in an intragastric manner.

3.4. Plantamajoside inhibits the increase in cardiac anatomical parameters induced by ISO

Compared with the control group, the ratio of heart weight (HW) to body weight (BW), the ratio of left ventricular weight (LVW) to BW and the ratio of HW to tibia length (TL) were significantly increased in the ISO group ($p < 0.01$; Table S3). Compared with the ISO group, a low dose of PMS had no effect on these ratios, but the high dose of PMS attenuated the increase in the ratio of LVW to BW (3.66 ± 0.12 mg/kg in ISO vs. 3.24 ± 0.20 mg/kg in high dose PMS), the ratio of HW to TL (7.55 ± 0.22 mg/mm in ISO vs. 6.96 ± 0.39 mg/mm in high dose PMS) and the ratio of LVW to TL (5.19 ± 0.16 mg/mm in ISO vs. 4.67 ± 0.20 mg/mm in high dose PMS). Furthermore, there was no significant difference between the positive control group and the high-dose PMS group (Fig. 2E–H).

3.5. Plantamajoside alleviates ISO-induced histopathological changes in cardiac tissues

As demonstrated by the H&E staining, myocardial cells of the

control group were neatly arranged with clear cross-striations. However, in the ISO group, myocardial cells showed irregular morphology, disordered arrangement and obvious fibroplast proliferation. In the low dose PMS group and the positive control group, these effects were reversed. Moreover, in the high-dose PMS group the ISO effects were abolished (Fig. 3A). In the Masson's trichrome staining, under normal conditions collagen fibers were stained blue, while the myocardium stained red. In the ISO group, mice had a large area of collagen fibers between the myocardial cells, but a smaller area was seen in the valsartan and PMS-treated samples (Fig. 3A and B).

3.6. Plantamajoside modulates ISO-induced hypertrophy and fibrosis in vivo

To access the effect of PMS on ISO-induced cardiac hypertrophy and cardiac fibrosis *in vivo*, we investigated the mRNA and protein expression of ANP, BNP, Myh7, COL1 and COL3. Compared with the control group, the protein and mRNA levels of these genes were markedly elevated in the ISO group (Fig. 3C–H and 4). However, treatment with a high dose of PMS significantly decreased the protein and mRNA expression of these markers (Fig. 3C–H and 4). In the positive control, the ANP, BNP, Myh7 and COL1 protein levels, and the Myh7, COL1 and COL3 mRNA levels were also decreased, but to a small degree compared with the high-dose PMS. These findings suggest that PMS protects against ISO-induced cardiac hypertrophy and fibrosis *in vivo*.

3.7. Plantamajoside mediates the protective effect via the inhibition of the HDAC2 and AKT/GSK3β signaling pathway

To determine whether the protective PMS effect is associated with the HDAC2 pathway, we examined the protein expression of HDAC2, AKT and GSK-3β in H9c2 cells and in cardiac tissues by western blot analysis (Fig. 5). *In vitro*, we found that the phosphorylated levels of HDAC2 (Ser394), AKT (Ser473) and GSK-3β (Ser9) were significantly
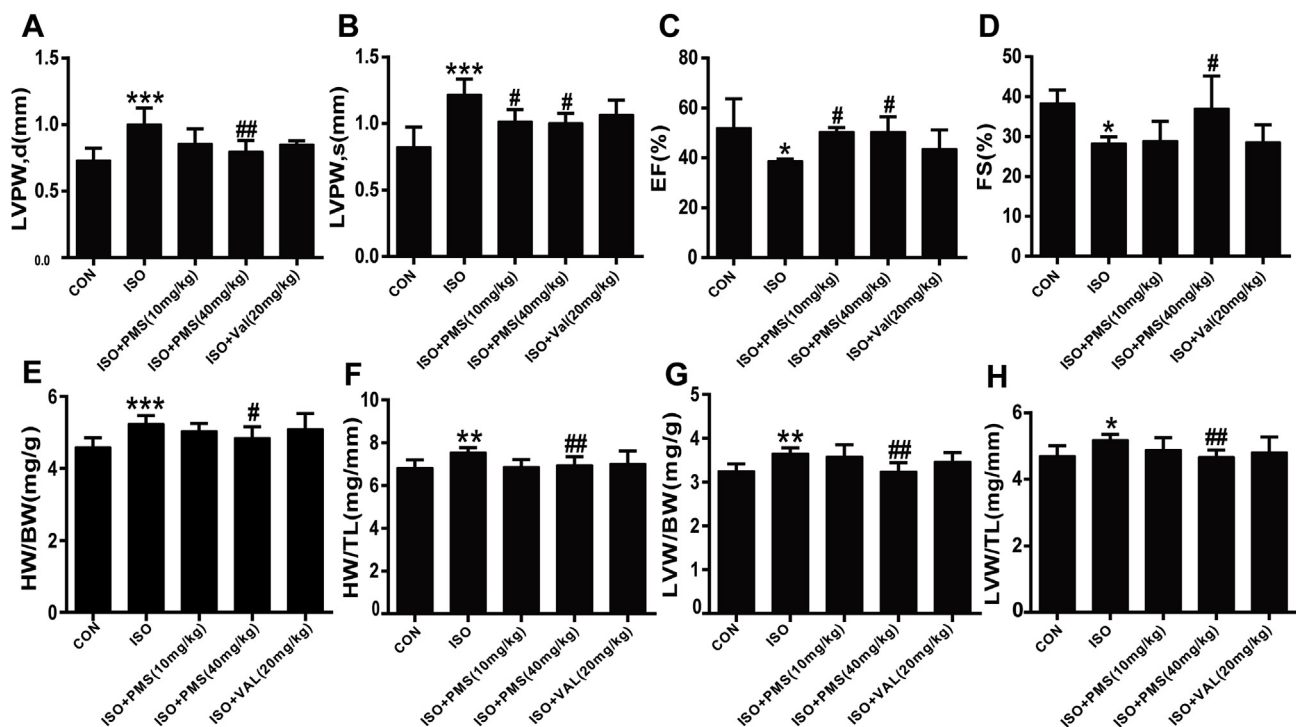

Fig. 2. PMS attenuates ISO-induced cardiac hypertrophy in mice.(A–D) Echocardiographic measurements of left ventricular diastolic posterior wall (LVPW,d), left ventricular systolic posterior wall (LVPW,s), ejection fraction (EF), fractional shortening (FS). (E, G) The ratio of heart weight (HW) and left ventricular weight (LVW) to body weight (BW). (F, H) The ratio of HW and LVW to tibia length (TL). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with CON group, #$p < 0.05$, ##$p < 0.01$ compared with ISO group. All values are mean ± SD.
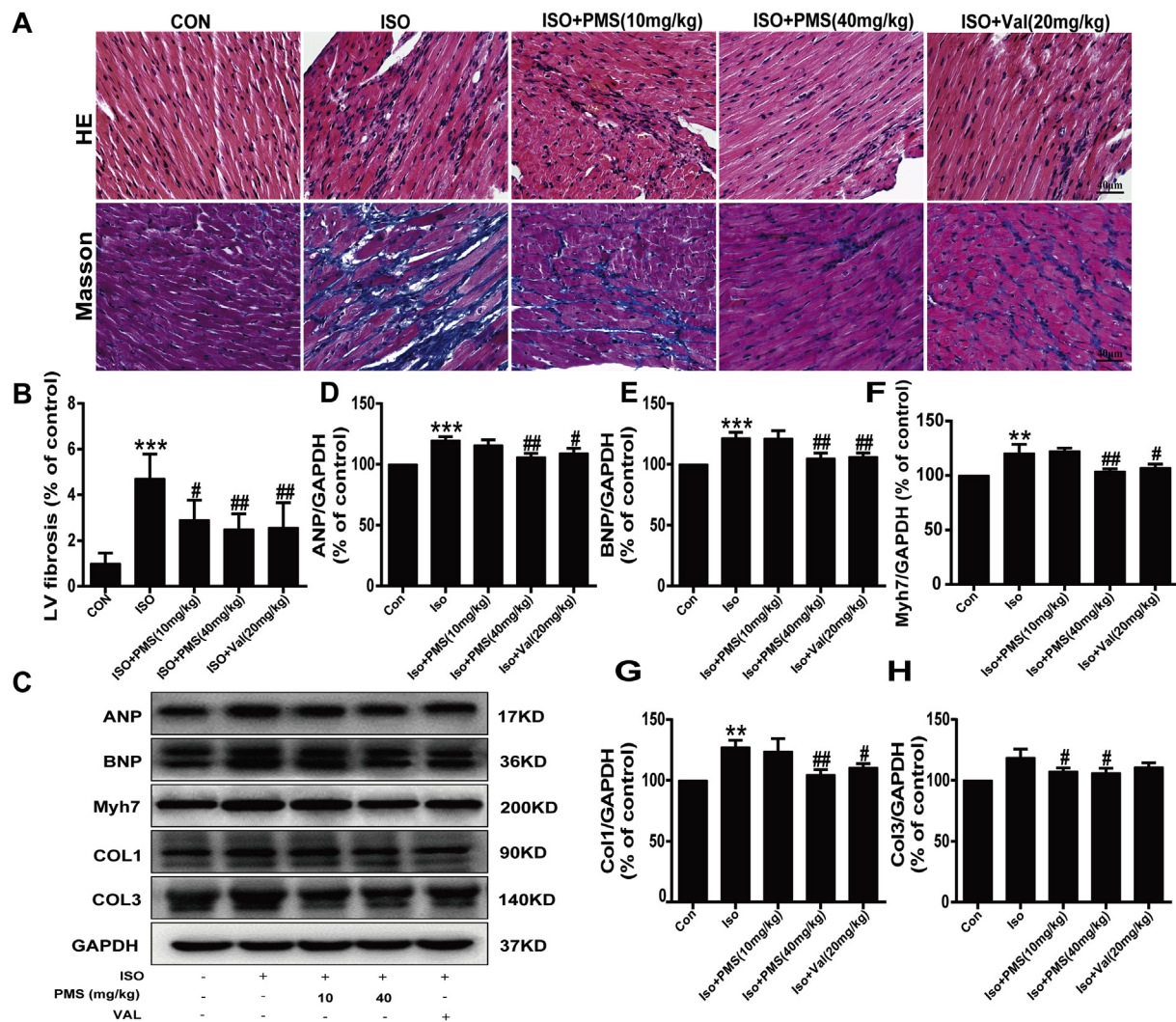
Fig. 3. PMS inhibits ISO-induced mouse cardiac hypertrophy and fibrosis. (A) Left ventricular H&E and Masson's trichrome staining (n = 5, bar = 40 μm). (B) LV collagen volume fraction (% of control) was quantified from the stained sections. (C) Representative western blots of ANP, BNP, Myh7, CoL1, CoL3 levels. (D–H) Quantification of the relative changes in ANP, BNP, Myh7, CoL1 and CoL3 expression. *p < 0.05, **p < 0.01, ***p < 0.001 compared with CON− group, #p < 0.05, ##p < 0.01 compared with ISO group. All values are mean ± SD.

increased in ISO group. However, the ratios of p-HDAC2/HDAC2, p-AKT/AKT and p-GSK-3β/GSK-3β were significantly reduced after treatment with different concentrations of PMS in a dose-dependent manner (Fig. 5A–D). *In vivo*, the low-dose and high-dose PMS inhibited the ISO-induced increase of p-HDAC2, p-AKT, and p-GSK3β. In the positive control group, we also found this positive effect, and there was no significant difference between the high-dose PMS group and the positive control (Fig. 5E–H).

4. Discussion

In the present study, we have demonstrated for the first time that PMS has a cardioprotective effect in ISO-induced cardiac hypertrophy both *in vitro* and *in vivo*. Our mechanism results suggested that the HDAC2 and AKT/GSK-3β signaling pathway may be associated with the cardioprotective effect of PMS. Present results indicated that PMS may be a potential candidate drug against cardiac hypertrophy.

PMS is one of the main bioactive compounds in *Herba Plantaginis* and it has been reported have therapeutic effects in many animal models of diseases, such as lung injury, renal injury, ear edema, breast cancer, and pulmonary metastasis [19–21,25,26]. As far as our knowledge, only one *in vitro* study has indicated PMS have angiotensin-

converting enzyme (ACE) inhibitory activity [27]. Our research may provide the first demonstration of a relationship between PMS and cardiovascular diseases based on cell and animal models. Moreover, it has been demonstrated that long term high dose oral administration of PMS were safe in rat [14]. In consistency with this study, our results in the MTT assay suggested a high dose (up to 200 μmol/L) of PMS is non-toxic to H9c2 cells. These data provide evidence for the safety of PMS *in vitro* and *in vivo*.

Sustained activation of β-adrenoceptors has been reported to induce pathological cardiac hypertrophy, cardiac fibrosis and myocardial ischemia [28–30]. ISO, a powerful synthetic nonselective β-adreno-ceptor agonist, has been widely used to induce models of cardiac hypertrophy [31,32]. In this study, we found significant increase expression of fetal genes and proteins involved in hypertrophy and fibrosis both *in vitro* and *in vivo*, and significant increase of cell surface area in H9c2 cells after ISO treatment. Moreover, our *in vivo* results showed ISO induced obvious pathological changes such as the irregular cell form, disordered arrangement, enlargement of cardiomyocytes, and fibroblast proliferation. With this model, we found PMS treatment effectively inhibited the ISO-induced increase of fetal genes and proteins, cell surface area, and ameliorated the ISO-induced pathological negative changes in the heart. Furthermore, in the present study, we showed that
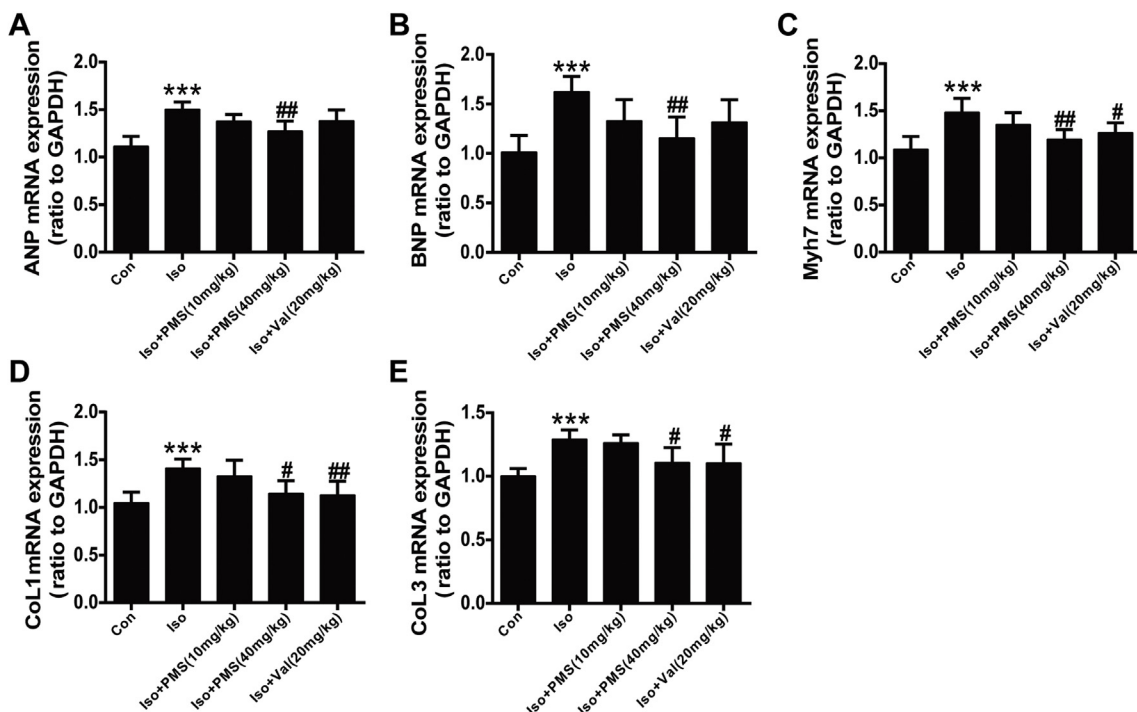
Fig. 4. mRNA expression of ANP, BNP, Myh7, COL1, COL3 in mouse cardiac tissues. ***$p < 0.001$ compared with CON group, #$p < 0.05$, ##$p < 0.01$ compared with ISO group. All values are mean ± SD.
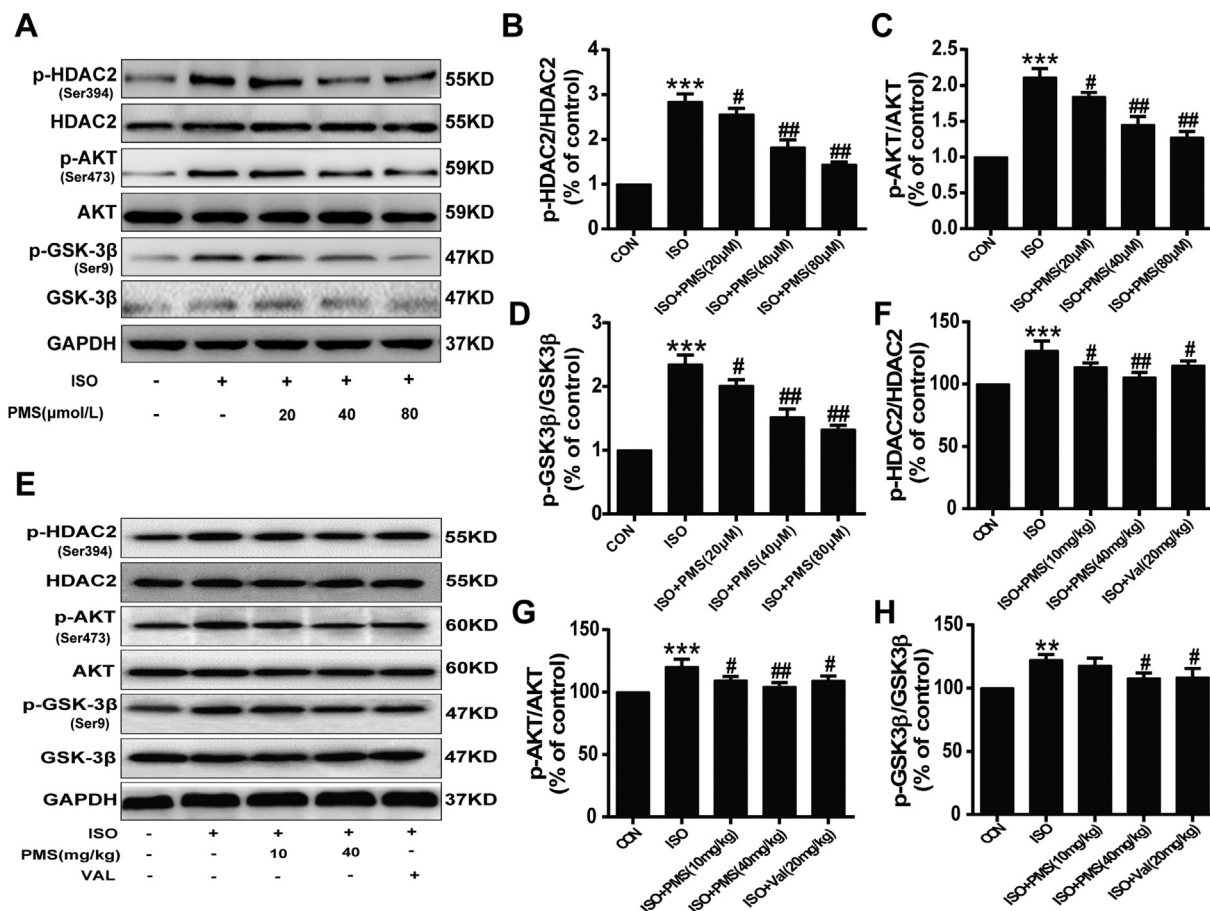

Fig. 5. Effects of PMS on the phosphorylation level of HDAC2, AKT, GSK3β. (A) and (E) Representative western blots of the phosphorylated and the total level of HDAC2, AKT and GSK3β in H9c2 cells and in mouse cardiac tissues, respectively. (B–H) Quantification of the relative changes in phosphorylation of HDAC2, AKT and GSK3β. GAPDH was used as a loading control. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with CON group, #$p < 0.05$, ##$p < 0.01$ compared with ISO group. All values are mean ± SD.

repeated injection of a small dose ISO-induced cardiac hypertrophy and function impairment of the heart, which characterized by significant changes in anatomic data and echocardiography parameters (the increase of HW/BW, LVW/BW, HW/TL, and LVW/TL ratios; the decrease of EF and FS). Interestingly, we found PMS treatment attenuated ISO-induced heart anatomic data increase and impairment function, which suggest that PMS can prevent the heart from cardiac hypertrophy.

Hypertrophic stimulation in cardiomyocytes activated HDAC2 [33], which can inhibit the expression of inositol polyphosphate-5-phosphatase f (Inpp5f). The suppression of Inpp5f can lead to the accumulation of one of its substrate phosphatidylinositol-3, 4, 5-trisphosphate (PIP3) [34], which leading to the AKT activation, then resulting in the phosphorylation of GSK-3β and re-expression of fetal genes. Therefore the phosphorylation of AKT and GSK-3β has been reported involved in the development of cardiac hypertrophy [13,35–37]. Our present western blot results showed the phosphorylation level of HDAC2 (Ser394), AKT (Ser473) and GSK-3β (Ser9) increase after ISO treatment, which is in consistency with previously reported. We used molecular docking software to examine molecular docking based on the binding model of the HDAC2 complex structure (PDB Code: 3MAX). The binding mode of PMS with HDAC2 suggests that hydrogen and Pi bonds are the main bonds involved in their interaction. Specifically, hydrogen bonds are formed at Tyr209, Val399, His183, Asp269, Asp104 and Gly32 of HDAC2. In addition, Pi-bonds are formed at Cys156, Leu144 and Pro34, indicating that PMS has a strong binding affinity to HDAC2. Although Computer-aided drug design and delivery can help to save the time and cost in the process of rational drug development, the docking program only provides the calculated binding affinity, and we do not know whether it is an agonist or inhibitor [38,39]. To check this kind of behavior, further lab experiments should be performed [40]. Therefore, we used lab experiments to verify, and we found the phosphorylation level of HDAC2 significantly and dose dependently decreases after PMS treatment in ISO-induced H9c2 cells and in mice, and thus it may be a potential HDAC2 inhibitor. The same changes were also observed in phosphorylation levels of AKT and GSK-3β. Based on the results in western blot and molecular docking, we propose that the HDAC2 and AKT/GSK-3β signaling pathway may involve in the cardioprotective effect of PMS in cardiac hypertrophy.

The present study has several limitations. First, as a regenerative cell line, H9c2 cells may be unable to fully show the effects of PMS on mammalian cardiomyocytes. By using an ISO-induced mouse model in this study, we reduced the significance of this limitation. In addition, many signaling kinases and transcription factors change when myocardial hypertrophy improves, and it has been reported that PMS has a variety of biological functions, such as antioxidant and anti-inflammatory, and these may be related to the pathogenesis of cardiac hypertrophy. Herein, we investigated the protective effect of PMS on ISO-induced cardiac hypertrophy and found that it is associated with the HDAC2, however we cannot exclude the possibility that other factors besides HDAC2 are associated with the anti-hypertrophy effect of PMS. Further investigations may be needed to explore the specific molecular pathways for cardioprotective of PMS.

In conclusion, this study demonstrated that PMS exerted significant cardioprotective effects against ISO-induced cardiac hypertrophy in H9c2 cells and in mice, and this protective effect may be associated with the phosphorylation level change of HDAC2 and AKT/GSK-3β signaling pathway. These findings suggest that PMS may be one important player that has ability against cardiac hypertrophy, and further research may require explore the specific molecular/causal pathways about how PMS affect cardiac hypertrophy.

Conflict of interest

The authors have no conflicts of interest to report.

Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.cbi.2019.04.024.

Appendix A. Supplementary data

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