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Original Paper

Endoplasmic Reticulum Stress is Involved in DFMO Attenuating Isoproterenol-Induced Cardiac Hypertrophy in Rats

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Key Words
Cardiac hypertrophy • Endoplasmic reticulum stress • Apoptosis • Difluoromethylornithine

Abstract
Background/Aims: Studies performed in experimental animals have shown that polyamines contribute to several physiological and pathological processes, including cardiac hypertrophy. This involves an increase in ornithine decarboxylase (ODC) activity and intracellular polyamines associated with regulation of gene expression. Difluoromethylornithine (DFMO), an irreversible inhibitor of ODC, has attracted considerable interest for its antiproliferative role, which it exerts through inhibition of the polyamine pathway and cell turnover. Whether DFMO attenuates cardiac hypertrophy through endoplasmic reticulum stress (ERS) is unclear. ***Methods:*** Myocardial hypertrophy was simulated by isoproterenol (ISO). Polyamine depletion was achieved using DFMO. Hypertrophy was estimated using the heart/body index and atrial natriuretic peptide (ANP) gene expression. Cardiac fibrosis and apoptosis were measured by Masson and TUNEL staining. Expression of ODC and spermidine/spermine N1-acetyltransferase (SSAT) were analyzed via real-time PCR and Western blot analysis. Protein expression of ERS and apoptosis factors were analyzed using Western blot analysis. ***Results:*** DFMO treatments significantly attenuated hypertrophy and apoptosis induced by ISO in cardiomyocytes. DFMO down-regulated the expression of ODC, glucose-regulated protein 78 (GRP78), C/EBP homologous protein (CHOP), cleaved caspase-12, and Bax and up-regulated the expression of SSAT and Bcl-2. Finally, these changes were partially reversed by the addition of exogenous putrescine. ***Conclusion:*** The data presented here suggest that polyamine depletion could inhibit cardiac hypertrophy and apoptosis, which is closely related to the ERS pathway.

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Introduction

Cardiovascular disease is one of the three major causes of death among worldwide, and its morbidity and mortality have shown an upward trend. Myocardial hypertrophy is generally considered to increase the risk of cardiovascular events, such as cardiac failure, arrhythmia, and coronary heart disease. However, the mechanism by which cardiac hypertrophy remodeling induces cardiac failure remains unclear. In recent years, accumulating evidence has suggested that dysfunction of the endoplasmic reticulum (ER) and apoptosis may be involved in these processes [1, 2].

The endoplasmic reticulum (ER) is involved in the intrinsic pathway of apoptosis and in several important functions, such as the folding of secretory and membrane proteins [3]. Any adverse environmental change, such as the presence of excess reactive oxygen species or calcium overload could affect the normal ER physiological function, which may cause accumulation of incorrectly folded proteins in the lumen of the ER and finally induce ER stress (ERS).

Polyamines, putrescine, spermidine, and spermine are ubiquitous in living cells and essential to eukaryotic cell growth. These polycations interact with negatively charged molecules, such as DNA, RNA, acidic proteins, and phospholipids and modulate various cellular functions including macromolecular synthesis [4]. Increasing evidence indicates that polyamines play a role in various aspects of cardiac disease [5, 6]. Enhanced levels of polyamines and polyamine biosynthetic enzymes, ornithine decarboxylase (ODC) and S-adenosylmethionine decarboxylase (SAMDC) are often associated with hyper-proliferation and cancer. Inhibition of ODC, using the ornithine analog difluoromethylornithine (DFMO), has been proven to be an effective means of depleting cellular polyamines in mammals and is clinically approved for humans [7].

Previous studies have shown that DFMO can attenuate cardiomyocyte hypertrophy by inhibiting the NO/cGMP-dependent protein kinase-1 pathway [8]. However, whether dietary DFMO intake can protect the myocardium from ISO-induced hypertrophy remains unknown. It is here hypothesized that the protective effects of DFMO on cardiomyocytes may be at least partially due to ERS. The purpose of this study was to examine the protective effects of DFMO on cardiac hypertrophy and explore the possible mechanism underlying this.

Materials and Methods

Materials

Isoproterenol (ISO), DFMO, and putrescine (Pu) were purchased from Sigma Chemical Co.(St. Louis, MO, U.S.). ODC, spermidine/spermine N1-acetyltransferase (SSAT), glucose-regulated protein 78 (GRP78), PERK, calreticulin (CRT), caspase-12, Bax, and Bcl-2 primary antibodies were obtained from Santa Cruz Biotechnology Incorporated (Santa Cruz, CA, U.S.). Trizol reagent and a PrimerScript RT Reagent Kit were purchased from TakaRa Biotechnology Incorporated (TakaRa Bio Inc., Japan). The Western blot kit and β-actin antibody were purchased from Boster (Wuhan, China). A TUNEL detection kit was purchased from Roche Corporation (Penzberg, Germany).

Animal protocol

Healthy SD adult male rats (200 – 250 g) were obtained from the Animal Center of Qiqihar Medical University and then housed at 23 ± 1°C and humidity 55–60%. Rats were maintained on a 12 h dark-light artificial cycle with food and water ad libitum. The animal protocol was approved by the Institutional Animal Research Committee. As described previously [9], rats were randomly divided into four groups: Control group (n = 15), ISO group (n = 15), pre-DFMO group (n = 15), and pre-(DFMO+Pu) group (n = 15). The rats from the control group were given subcutaneous injections of 0.9% saline. For the other three groups, ISO (5 mg/kg/day) was subcutaneously injected into the rats once a day for 7 days. The rats in the pre-DFMO group and the pre(DFMO+Pu) group were also given 2% DFMO in their water and 2% (DFMO+Pu) in their water for 4 weeks. Four weeks after treatment, rats in all four groups were killed and ventricular myocardia were collected for further experiments.

Tissue treatment

All rats were killed with a lethal dose of sodium pentobarbitone. The heart was carefully isolated, then blotted slightly and weighed. The ventricles were separated from the atria, and the right ventricle was isolated by dissection along its septal insertion. The degree of ventricular hypertrophy was assessed by measuring the heart-to-body weight (HW/BW) ratio and left ventricle-to-body weight (LVW/BW) ratio. In situ fixed left ventricular tissue slices were embedded in paraffin and stained with hematoxylin and eosin or Masson. Tissue morphometry was performed in a blinded fashion using computerized graphical analysis equipment. Samples on the section were analyzed under a light microscope. The fraction of myocardial volume occupied by fibrillar collagen was calculated as the relative amount of total surface area occupied by the interstitial fibrosis.

TUENL staining

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was used to detect the apoptosis of myocardium from each group by TUNEL detection kit (Roche, Penzberg, Germany) according to the manufacturer's protocol. The sectioned heart tissues (n = 4 for each group) were fixed with 4% paraformaldehyde in PBS for 1 h at room temperature, and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 2 min on ice. After washing in PBS, sections were incubated with the TUNEL reaction mixture for 1 h at 37°C. After washing with PBS, the stained cells were visualized using a fluorescence microscopy (Olympus).

Real time-PCR analysis

Total RNA was extracted from heart tissues using Trizol reagent (Invitrogen, U.S.) according to the manufacturer's instructions. The method of quantifying the level of gene expression was as previously described [10, 11]. The RNA was reverse transcribed with oligo-dT and Superscript First-strand Synthesis System for RT-PCR (TaKaRa), The cDNA was then amplified using an iCycler (ABI) and the Brilliant SYBR Green QPCR master mix (TaKaRa) withβ-actin as an internal control. The nucleotide sequences of the primers used are as follows: (1) ANP: sense 5′-GGG AAG TCA ACC CGT CTCA-3′, antisense 5′-GGG CTC CAA TCC TGT CAAT-3′; (2) ODC: sense 5′-GCT TTC TAT GTT GCG GACCT-3′, antisense 5′-TGC TCA CTA TGG CTC TGCTG-3′, (3) SSAT: sense 5′-GTG AGC ATC CAT TCC AAAGC-3′, antisense 5′-ATT CTG CCT CCA AAC CACAT-3′; β-actin served as an internal control. The relative expression of ANP, ODC and SSAT were quantified using comparative $2^{-\Delta\Delta Ct}$ method.

Western blot analysis

Total proteins were prepared from left ventricle of rats in each group as described previously [12, 13]. For the detection of the expression of ODC, SSAT, GRP78, calreticulin, PERK, caspase-12, Bcl-2, and Bax. Total protein samples (30 μg) were fractionated by SDS-PAGE (10–12% polyacrylamide gels) and transferred to PVDF membrane (Millipore, Bedford, MA, U.S.). Membranes were blocked with 5% skimmed milk in Tris-buffered saline, with 0.1% Tween 20 (TBST) for 1 h at room temperature, and then incubated overnight at 4°C with primary antibody ODC (1:500), SSAT (1:500), GRP78 (1:1000), PERK (1:500), caspase-12 (1:1000), calreticulin (1:500), Bcl-2 (1:500), and Bax (1:500). The membrane was next washed three times in 1×TBST and incubated with anti-IgG antibody conjugated with peroxidase (1:5000) in TBST for 1 h at room temperature. Using an enhanced chemiluminescence detection kit (Pierce Chemical Company, Rockford, IL, U.S.). β-actin expression served as the control.

Statistical analysis

Data are presented as mean ± SEM. Statistical analysis was performed by using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA, U.S.). Two-tailed Student's t-test or Newman-Keuls Multiple Comparison Test were used for comparisons between groups. $P<0.05$ was considered statistically significant.

Results

DFMO inhibited ISO-induced cardiac hypertrophy

First, the effects of DFMO on cardiac hypertrophy induced by ISO were observed. As shown in Fig. 1A, ISO-induced rats showed a significant increase in heart to body weight

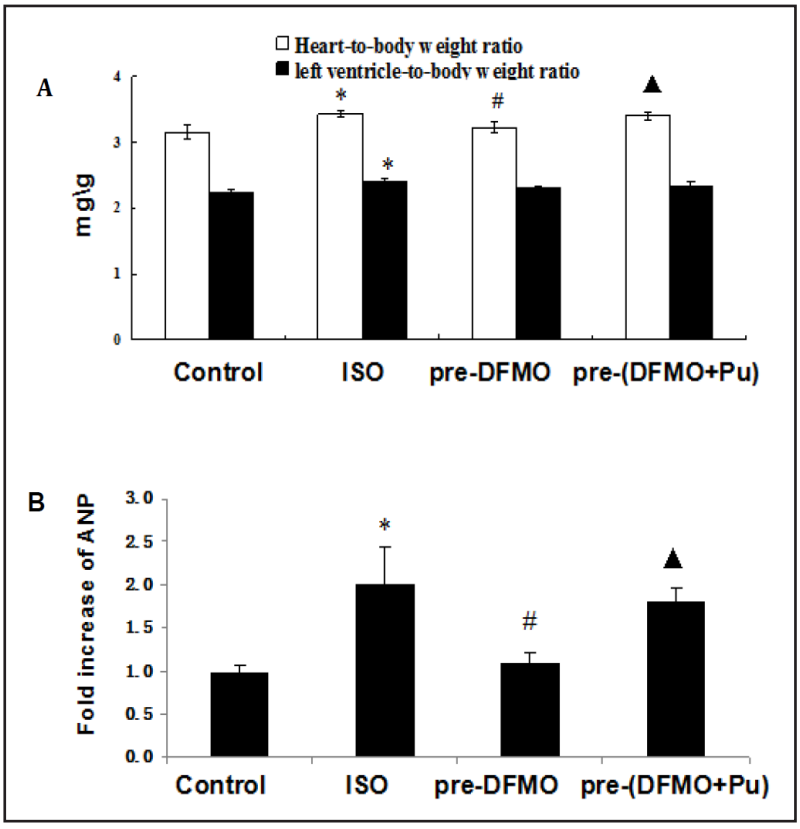

Fig. 1. Hypertrophic state of heart in rats. Data are means ± S.E.M. (n = 8-10 per group). *$P < 0.05$ versus the control group; $^{\#}$ $P < 0.05$ versus the ISO group; $^{\blacktriangle}$ $P < 0.05$ versus the pre-DFMO group. (A) Heart to body weight ratio (HW/BW) and left ventricular to body weight ratio (LVW/BW). (B) The gene expression of ANP was assessed determined by real-time PCR.

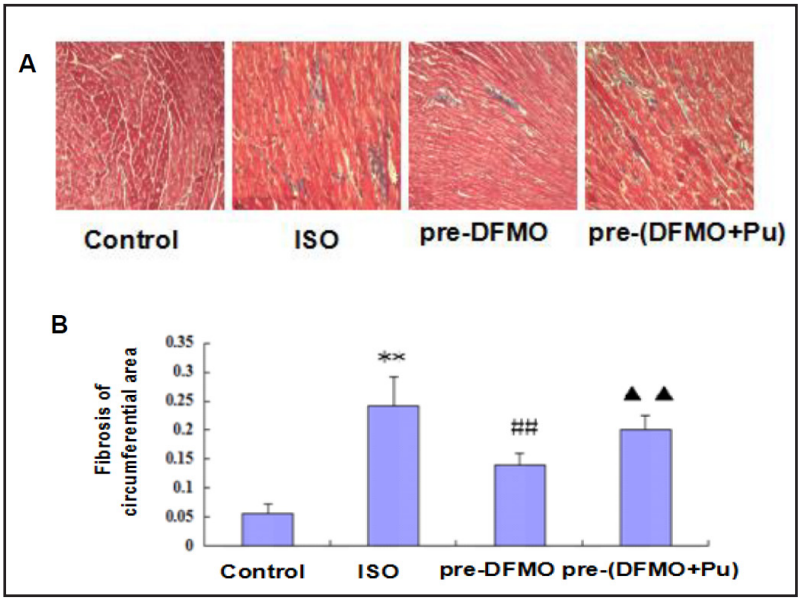

Fig. 2. The degree of interstitial fibrosis as determined by Masson staining. (A) Microscopy of Masson-stained left ventricular tissue. Red staining indicates myocardial cells, and the interstitial fibers are stained as blue. Images are 100× original magnification. (B) Quantification of interstitial fibrosis among the various groups (n = 4-6 per group). ** $P < 0.01$ versus the control group; $^{\#\#}P < 0.01$ versus the ISO group; $^{\blacktriangle\blacktriangle}P < 0.01$ versus the pre-DFMO group.

ratios and left ventricle to body weight ratio compared with control rats ($P < 0.05$). However, 2% DFMO treatment was able to inhibit the increase in heart/body index ($P < 0.05$). The heart-to-body-weight ratio was increased by the addition of exogenous putrescine ($P < 0.05$ versus pre-DFMO group). The atrial natriuretic peptide (ANP) mRNA level was higher in the ISO group than in the control group ($P < 0.05$), DFMO treatment can downregulate the

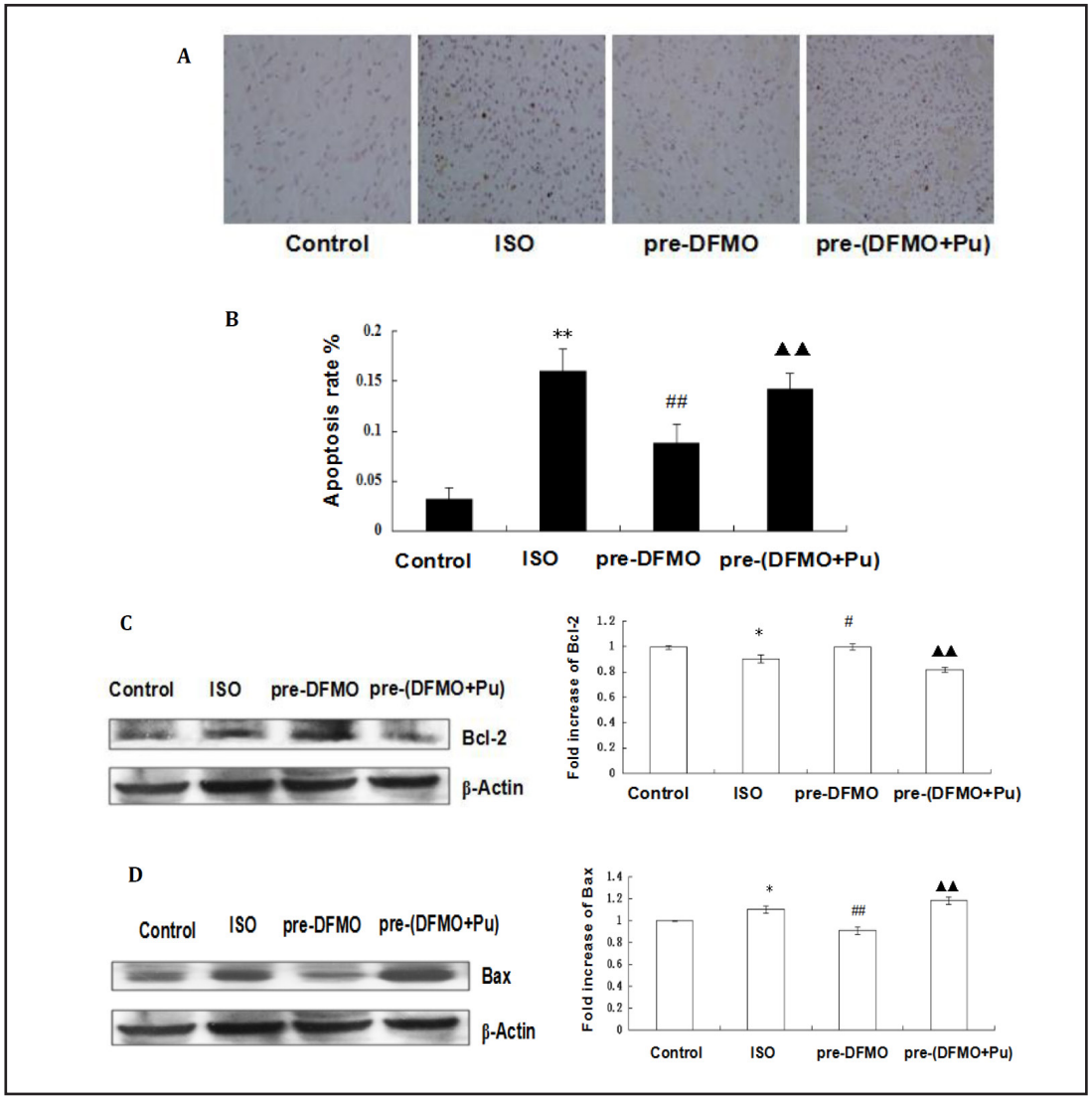

Fig. 3. The effect of DFMO on cardiomyocyte apoptosis induced by ISO. (A) Apoptosis was measured using TUENL staining. (B) Quantification of apoptosis among the various groups. The data are presented as means±S.E.M. (n = 4 per group). (C and D) Bcl-2 and Bax protein levels as shown by Western blot analysis (n = 4 per group).The intensity of each band was quantified by densitometry, and data were normalized to ß-actin. The control group expression levels were considered baseline, and data from other experimental groups are here expressed as fold change from the control group. $^{*}P < 0.05$ or $^{**}P < 0.01$ versus the control group; $^{\#}P < 0.05$ or $^{\#\#}P < 0.01$ versus the ISO group; $^{\blacktriangle\blacktriangle}P < 0.01$ versus the pre-DFMO group.

gene expression of ANP ($P < 0.05$ versus ISO group). There was more hypertrophy in the pretreated (DFMO+Pu) group than in the DFMO treatment group ($P < 0.05$) (Fig. 1B). This suggested that DFMO may inhibit ISO-induced myocardial hypertrophy in rats.

DFMO inhibited ISO-induced alteration of cardiac fibrosis

The effects of DFMO on cardiac fibrosis in rats with ISO-induced cardiac hypertrophy were observed. Masson staining showed that cardiac fibrosis was markedly increased in ISO treated rats as well ($P < 0.01$), DFMO treatment may inhibit cardiac fibrosis in ISO-treated rats ($P < 0.01$). Pretreatment with DFMO and Pu showed an increase in cardiac fibrosis relative to the DFMO treatment group ($P < 0.01$) (Fig. 2A and 2B). All these findings suggest that DFMO can improve cardiac structure in rats subjected to ISO treatment.

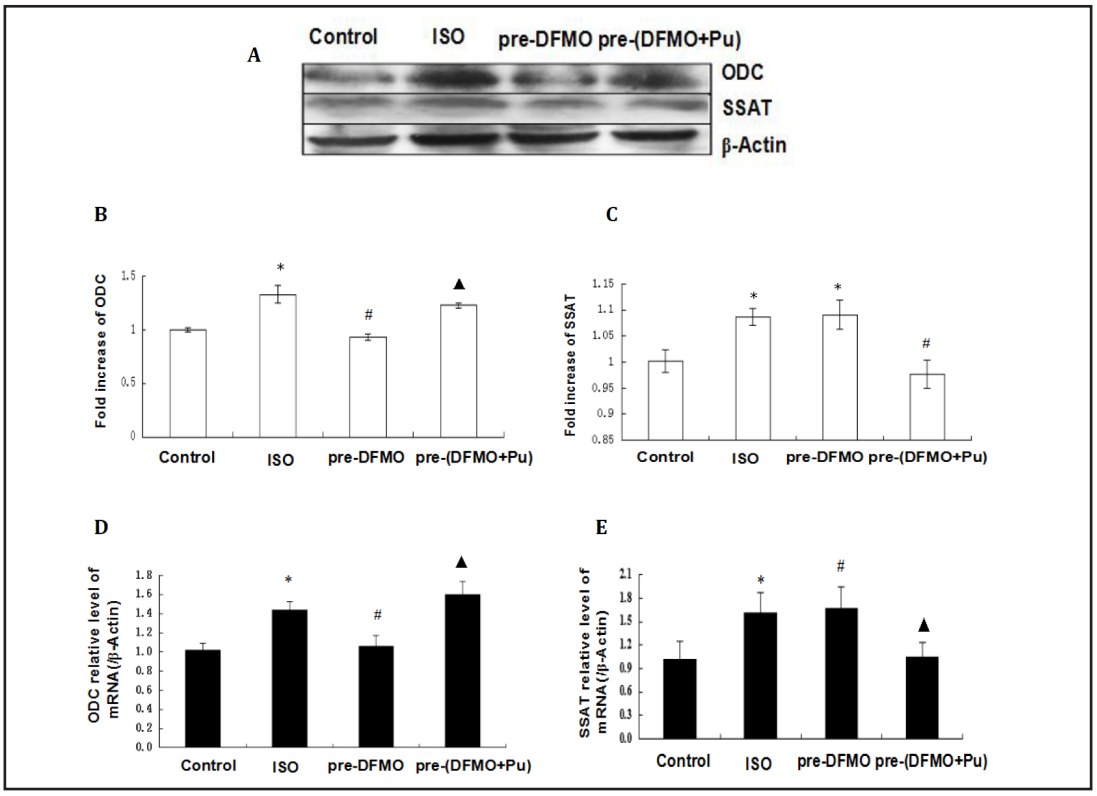

Fig. 4. The effect of DFMO on the gene and protein levels of ODC and SSAT in hearts. (A–C) ODC and SSAT protein levels as shown by Western blot analysis. The intensity of each band was quantified by densitometry, and data were normalized to ß-actin (n = 4 per group). (D and E) ODC and SSAT gene expression as determined by real-time PCR (n = 4 per group). *$P < 0.05$ versus the control group; #$P < 0.05$ versus the ISO group; ▲$P < 0.05$ versus pre-DFMO group.

DFMO suppressed ISO-induced apoptosis of myocardium

As shown in Fig. 3A and 3B, TUNEL staining showed there to be significantly more apoptotic cells in the ISO group ($P < 0.01$ versus control group) and pre-(DFMO + Pu) group ($P < 0.01$ versus DFMO group). DFMO treatment may reduce the percentage of apoptotic cells compared with ISO rats ($P < 0.01$). Furthermore, Western blot confirmed that there was less Bcl-2 protein expression and more Bax protein expression in the ISO group than in the control group. Pretreatment with DFMO up-regulated the expression of Bcl-2 and down-regulated the expression of Bax relative to the ISO group. Pretreatment with DFMO and Pu reversed the patterns of expression of Bcl-2 and Bax relative to the DFMO group ($P < 0.05$ or $P< 0.01$)(Fig. 3C and 3D).

DFMO affected the gene and protein expression of ODC and SSAT

ODC and SSAT are rate-limiting enzymes in polyamine biosynthesis and catabolism respectively. There was significantly more gene and protein expression of ODC and SSAT in the ISO group than in the control group ($P < 0.05$). However, DFMO pretreatment decreased the gene and protein expression of ODC and increased the gene and protein expression of SSAT ($P < 0.05$). These changes were reversed by the addition of exogenous putrescine ($P < 0.05$ versus pre-DFMO group) (Fig. 4A–E).

DFMO affected ERS signal pathway

It is well documented that the ERS signal pathway contributes to myocardial hypertrophy induced by certain stimuli, such as isoproterenol infusion. Whether ERS signals are altered in the ISO-induced hypertrophic myocardium has yet to be elucidated.

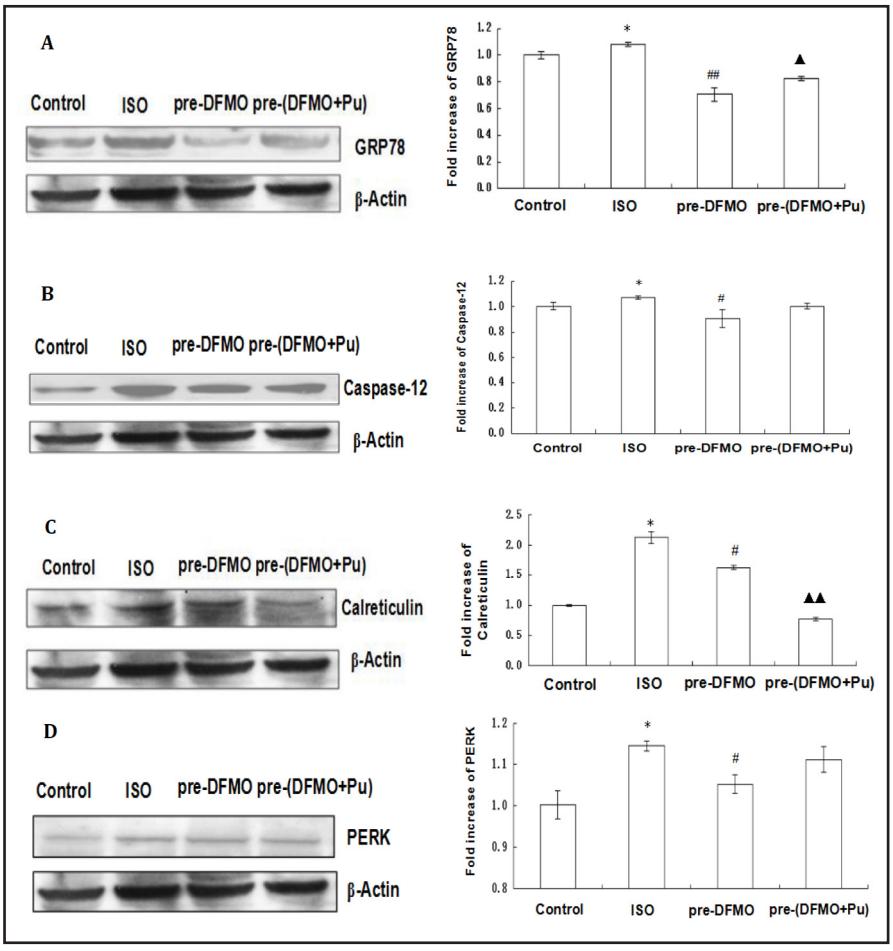

Fig. 5. The effect of DFMO on ERS signal pathway in hearts. (A-D) GRP78, Caspase-12, calreticulin and PERK protein levels as shown by Western blot analysis. The intensity of each band was quantified by densitometry, and data were normolized to ß-actin (n = 4 per group). $^{*}P < 0.05$ versus the control group, $^{\#}P < 0.05$ $^{\#\#}P < 0.01$ versus the ISO group, $^{\blacktriangle}P < 0.05$, $^{\blacktriangle\blacktriangle}P <$ 0.01versus the pre-DFMO group.

In this study, GRP78, PERK, calreticulin, and caspase-12 were tested for involvement in the myocardia of ISO-induced rats. Results showed the expression of GRP78, PERK, calreticulin, and caspase-12 in the ISO group to be significantly higher than in the control group ($P < 0.05$). DFMO treatment visibly decreased the expression of GRP78, PERK, calreticulin, and caspase-12 in rats ($P < 0.05$, $P < 0.01$). Results showed that ERS signal contribute to the prevention of ISO-induced heart hypertrophy by DFMO. (Fig. 5A–D).

Discussion

This is the first work to show that DFMO treatment may inhibit ISO-induced cardiac hypertrophy by suppressing ERS pathway and apoptosis *in vivo*. This study provides insight into molecular mechanisms underlying heart hypertrophy, and results indicated that dietary depletion of polyamine may be a suitable strategy for the prevention and therapy of cardiovascular diseases.

Cardiac hypertrophy is a major risk factor that is associated with adverse cardiovascular outcomes. However, the molecular pathological mechanism underlying cardiac hypertrophy and remodeling has not been fully understood. Several recent studies have demonstrated that growth responses in cardiac tissue that are accompanied by increased polyamine levels and β-adrenoceptor-mediated hypertrophy *in vivo* are attributable to ODC induction. In addition, an irreversible inhibitor of ODC, DFMO, can reduce putrescine levels by inhibiting the activity of ODC [14, 15]. In this study, a relationship between depletion of polyamine and cardiac hypertrophy was observed in rats and the possible mechanism underlying effects of DFMO in cardiac hypertrophy was assessed.

As in previous studies, myocardial remodeling was observed in ISO-induced rats. ISO can induce an increase of heart/body weight index and the expression of ODC relative to the control group. Some studies have shown that transgenic mice overexpressing ODC in the heart experience a much more dramatic left ventricular hypertrophy in response to ISO stimulation than in non-transgenic controls [16]. The left ventricular function and remodeling by transthoracic echocardiography were also found to be valuable indicators of cardiac hypertrophy. Inhibition of polyamine uptake potentiates the anti-proliferative effect of polyamine synthesis inhibition and preserves [17]. Overexpression of ODC was found to decrease ventricular systolic function during cardiac hypertrophy through assessment of the left ventricular ejection fraction (LVEF) and shortening fraction (FS) by echocardiography [18]. Cardiac fibrosis was identified using Masson staining and TUNEL was also used to detect the apoptosis of the myocardium from ISO-treated mice. DFMO can reverse the expression of ODC and SSAT and apoptosis induced by ISO. Some evidence has demonstrated that DFMO depletes the polyamines and protects the cells from ischemia-induced apoptosis in H_9C_2 cardiomyoblasts [6]. Taken together, these findings suggest that pathological hypertrophy was induced by the subcutaneous injection of ISO. DFMO may play an anti-hypertrophic and anti-apoptotic role by inhibiting polyamine biosynthesis.

ERS, which is a subcellular stress response, exerts a protective effect in clearance of impaired organelles and maintaining survival. It is also involved in the pathogenesis of cardiovascular disease [19]. Serious ERS may result in cell apoptosis and necrosis. GRP78 plays an important protective role in ERS, and over-expression of GRP78 often indicates the disturbance of cell homeostasis [20]. In different apoptotic signaling pathways, caspase-12 is an apoptotic signaling molecular and acts as a common downstream regulation factor induced by persistent ERS [21, 22]. Under pathological conditions, the ERS significantly increases the stimulation of calcium overload and accumulation of misfolded proteins. One histological characteristic of failing hearts is morphological development of ER [23].

Results demonstrated a significant reduction in ISO-induced cardiac hypertrophy after oral DFMO treatment, which could also reduce ERS responses. The expression of GRP78, PERK, calreticulin, and caspase-12 were significantly higher in the ISO group than in controls. Calcium overload is known to be widespread in hypertrophied cardiomyocytes, which could result in the upregulation of GPR78, PERK, and calreticulin to degenerate and clear these immature proteins.

However, if the stress is prolonged or overwhelming, the pro-survival effects of ERS switches to proapoptotic signaling, which is mediated primarily by transcriptional induction of CHOP or by activation of the caspase-12-dependent pathway [24]. Previous studies have reported that polyamines are involved in the execution of the death program in cardiomyocytes induced by DFMO interfering with caspase activation [25] and the cardiomyocytes of transgenic mice that over-express cardiac ODC showed more caspase activation [6].

ERS was found to play a role in cardiomyocyte apoptosis, a well-known phenomenon that occurs during the transition from cardiac hypertrophy to heart failure. The data collected here showed that DFMO treatment could completely attenuate the expression of Bax and caspase-12, which are necessary for apoptosis, and increase the expression of Bcl-2 in hypertrophic rats, indicating that DFMO may suppress the ERS-induced cardiac apoptosis involved in the transition of cardiac hypertrophy to heart failure.

In summary, depletion of polyamine by DFMO not only inhibited ISO-induced myocardial hypertrophy but also exerted a preventative effect on apoptosis. This protective effect may be associated with inhibition of ERS signaling. However, further studies are required to determine how different mechanisms of action of DFMO can affect ERS and so distinctly influence cardiac hypertrophy and heart failure. This knowledge may lead to the design of the use of DFMO as a therapeutic agent for cardiac diseases.

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Disclosure Statement

The authors declare no conflict of interest.

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