Inhibition of Cardiomyocytes Hypertrophy by Resveratrol Is Associated with Amelioration of Endoplasmic Reticulum Stress

Yan Lin a Jingbin Zhu d Xiaojie Zhang c Jun Wang a Wei Xiao a Bo Li a Li Jin a Jie Lian b Li Zhou c Jicheng Liu c

a Department of Pathophysiology, b Department of histology and embryology, c Institute of medicine, Qiqihar Medical University, Qiqihar, d Department of Orthopedic Surgery, The First Hospital of Harbin, Harbin, China

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Abstract

Background/Aims: Resveratrol (Res), a polyphenol antioxidant found in red wine, has been shown to play a cardioprotective role. This study was undertaken to investigate whether Res can protect the heart suffering from hypertrophy injuries induced by isoproterenol (ISO), and whether the protective effect is mediated by endoplasmic reticulum (ER) stress. Methods: Cardiomyocytes were randomly assigned to the control group, ISO group (100 nM ISO for 48 h), Res + ISO group (50 μM Res and 100 nM ISO for 48 h) and Res group (50 μM Res for 48 h only). Hypertrophy was estimated by measuring the cell surface area and the atrial natriuretic peptide (ANP) gene expression. Apoptosis was measured using Hoechst 33258 staining and transmission electron microscopy. Protein expression of ER stress and apoptosis factors was analyzed using Western Blot analysis. Results: Res effectively suppress the cardiomyocytes hypertrophy and apoptosis induced by ISO, characterized by the reduction of the myocardial cell surface area, the ANP gene expression, the LDH and MDA leakage amount and the rate of cell apoptosis, while decrease of the protein expression of GRP78, GRP94 and CHOP, and reverse the expression of Bcl-2 and Bax. Conclusion: In summary, Res treatment effectively suppressed myocardial hypertrophy and apoptosis at least partially via inhibiting ER stress.

Introduction
Cardiovascular diseases remain the leading causes of death and disability in the world. During the pathological development of cardiovascular diseases, cardiac hypertrophy plays...
a critical role. Although the cardiac hypertrophy is an adaptive response of the heart that responds to a variety of extrinsic and intrinsic stimuli, prolonged hypertrophy typically culminates in chronic heart failure or sudden cardiac death. Understanding the mechanisms and potential targets underlying cardiac hypertrophy is thus important to the field of cardiovascular biology, and may lead to new strategies for the prevention or treatment of cardiovascular disease [1].

The endoplasmic reticulum (ER) regulates protein synthesis, protein folding, cellular responses to stress and intracellular Ca\(^{2+}\) levels [2-4]. Prolonged ER stress triggers apoptosis in various cell types. A number of studies suggest that ER stress plays a critical role in the pathogenesis of heart failure. Pressure overload induced cardiac myocyte apoptosis is shown to be associated with increased ER stress in the mouse myocardium [5].

Resveratrol (Res) is a polyphenol found in red wine that was initially used to treat cancer. It also shows multiple cardioprotective effects against cardiovascular diseases, such as myocardial ischemia/reperfusion, hypertrophy, and heart failure. The mechanisms involved may include the inhibition of the oxidation of low-density lipoprotein and inhibition of platelet aggregation, alleviation of oxidative stress and so on. Apoptosis of cardiomyocytes was also protected by RES treatment. Several reports have studied the link between RES effects and ER stress related factors as novel molecular targets for the action of polyphenols [6-9].

However, whether Res treatment can protect myocardium against ISO-induced cardiac hypertrophy via inhibiting ER stress remain unknown. Therefore, the present study was designed to clarify this notion.

Materials and Methods

Materials

Isoproterenol (ISO), resveratrol (Res) and trypsinase were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.). High glucose Dulbecco’s modified Eagle’s medium (DMEM) was a product of Gibco BRL (Gaithersburg, MD, U.S.). Glucose-regulated protein 78 (GRP78), glucose-regulated protein 94 (GRP94), C/EBP homologous protein (CHOP), Bax and Bcl-2 primary antibodies were obtained from Santa Cruz Biotechnology Incorporated (Santa Cruz, CA, U.S.). Trizol reagent and the PrimerScript RT reagent kit were purchased from TaKaRa Biotechnology Incorporaed (TaKaRa Bio Inc., Japan). The western blot kit and β-actin antibody were purchased from Boster (Wuhan, China). Hoechst 33258 staining kit was purchased from Beyotime Institute of Biotechnology (Shanghai, China). Assay kits for malondialdehyde (MDA) and lactate dehydrogenase (LDH) were purchased from Jiancheng Bioengineering Institute (Nanjing, China).

Cell culture and treatment

Primary cultures of neonatal rat cardiomyocytes were performed just as previously described [10, 11]. Neonatal rat cardiomyocytes were prepared from 2-3 day old neonatal Wister rats. The rats were handled in accordance with the Guide for the Care and Use of Laboratory Animals published by the China National Institutes of Health. Briefly, the heart were minced and dissociated with 0.25% trypsinase. Dispersed cells were seeded at 2 × 10^5 cells/cm\(^2\) in 60-mm culture dishes with Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and then cultured in a 5% CO\(_2\) incubator at 37°C.

Experimental protocol

Three days after the cells were seeded, the cultured cardiomyocytes were randomly divided into the following four groups: (1) control group: cardiomyocytes continuously cultured for 72 h in DMEM; (2) ISO group: cardiomyocytes were treated with 100 nM ISO for 48 h; (3) Res + ISO group: cardiomyocytes were incubated with 50 μM Res and 100 nM ISO for 48 h; (4) Res group: cardiomyocytes were incubated with 50 μM Res for 48 h. Drugs were dissolved in pre-warmed medium and added directly to the culture. For controls, equivalent volumes of medium were added.
Measurement of the surface area of the cardiomyocytes

After being digested, centrifuged and resuspended in DMEM, the number of the cardiomyocytes was counted for at least three dishes in each group using phase-contrast microscopy. The cellular surface area was measured by the Image Analysis System. Ten fields were randomly chosen for each group and 10 cardiomyocytes determined for each field. The total protein content was measured by Bradford’s method in different groups.

Real-time-PCR analysis of ANP

Total RNA was extracted from the cardiomyocytes using the phenol guanidine isothiocyanate method (Trizol kit, TaKaRa) as per the manufacturer’s instructions. The RNA was reverse transcribed with oligo-dT and Superscript First-Strand Synthesis System for RT-PCR (TaKaRa), according to manufacturer’s instructions. The reverse-transcribed cDNA was then amplified using an iCycler (ABI) with the Brilliant SYBR Green QPCR master mix (TaKaRa). Relative quantification of gene expression was performed using the method with β-actin as the control gene. ANP nucleotide sequence of the primers used was: sense 5′-GGG AAG TCA ACC CGT CTCA-3′, antisense 5′-GGG CTC CAATCC TGT CAAT-3′; β-actin served as an internal control. The relative expression of ANP was quantified using comparative 2−ΔΔCt method.

Hoechst 33258 nuclear staining experiments

Apoptosis was identified by means of Hoechst 33258 staining. The cardiomyocytes were washed three times with PBS and were digested with trypsin and collected. According to the Hoechst staining kit instructions, staining and sheet sealing were performed.

Transmission electron microscopy

Cells were harvested and fixed with 3.0% glutaraldehyde and 1.5% paraldehyde, washed in phosphate buffered saline, and fixed in osmium tetroxide. Then, cells were dehydrated in an ethanol series, embedded in epoxy resin and examined under a transmission electron microscope (Hitachi, TEM-HT7700).

Western blotting analysis of GRP78, GRP94, CHOP, Bcl-2 and Bax

Protein samples were prepared from cultured cardiomyocytes, as described elsewhere [12]. Proteins (30 – 50 μg) were separated by 8% to 12% gradient denaturing SDS-PAGE. After transfer to membranes, immunoblot analysis was performed with the following antibodies: anti-GRP78, anti-GRP94, anti-CHOP, anti-Bcl-2 and anti-Bax. Using an enhanced chemiluminescence detection kit (Pierce Chemical Company, Rockford, IL, U.S.). β-actin expression served as the control.

Measurement of LDH activity and MDA content

The level of malonaldehyde (MDA) in the culture medium was detected with thiobarbituric acid-reactive substances assay and the experiment was carried out with a commercial kit (Jiancheng Bioengineering Institute, Nanjing, China) according to manufacturer's introduction. MDA values were expressed as nmole per gram protein. The activity of lactate dehydrogenase (LDH) in the culture medium, as an indicator of cytotoxicity, was measured spectrophotometrically with a commercially available assay kit (Jiancheng Bioengineering Institute, Nanjing, China).

Statistical analyses

Data were obtained from at least three independent experiments for each condition. Values are means ± S.E.M. Comparisons among the groups were carried out using Kruskal-Wallis one-way ANOVA. P < 0.05 was considered to be statistically significant.

Result

Res inhibited ISO-induced cardiomyocytes hypertrophy

To investigate the effect of Res on cardiomyocytes hypertrophy, cardiomyocytes were treated with 100 nM ISO and 50 μM Res. The parameter of cardiomyocytes hypertrophy, such as the surface area of cardiomyocytes and mRNA expression of ANP (a cardiac
hypertrophy marker gene) were determined. As shown in Fig. 1, the results showed ISO increased the surface area and up-regulated mRNA expression of ANP (P < 0.05 versus the control group). In contrast, after treatment with ISO and Res together, the parameter of cardiomyocytes hypertrophy was significantly decreased (P < 0.05 or P < 0.01 versus ISO group). Pretreatment with 50 μM Res only had not an obvious effect on the parameter of cardiomyocytes hypertrophy.

Res decreased the levels of LDH and MDA

In the ISO group, the membrane permeability increased, resulting in the elevation of the LDH activity in the culture medium. In the Res + ISO group, the activity of LDH was significantly decreased. The content of MDA in the ISO group was significantly higher than the control group, which indicated that ISO induced the lipid peroxidation. Res lower the level of MDA in the Res + ISO group compared with the ISO group, which suggested that Res decreased the lipid peroxidation induced by ISO (Fig. 2).

Res reduced ISO-induced apoptosis of cardiomyocytes

As shown in Fig. 3A, the results of transmission electron microscopy showed that in the control group, structure of nuclear membrane was clear with even distribution of nuclear chromatin, and mitochondria structure was intact. Morphological changes characteristic of apoptosis, including nuclear chromatin margination, aggregation and condensation, and swelling and vacuolisation of mitochondria were observed in the ISO groups. Compared with ISO group, these morphological changes were less severe in the Res + ISO group. Cardiomyocyte apoptosis occurred in the ISO group as evidenced by the increases of Hoechst 33258 staining positive cardiomyocytes. However, pretreatment with 50 μM Res decreased the apoptosis value (P < 0.05 versus the ISO group). In contrast, the Res group couldn’t increase the apoptosis rate (P > 0.05 versus the control group) (Fig. 3B).
Res affected the protein expression of Bcl-2 and Bax

Bcl-2 is an anti-apoptosis factor and is involved in multiple heart diseases by shifting the balance away from cell survival and toward cell apoptosis. The expression of Bcl-2 was
decreased and the expression of Bax was increased markedly in the ISO group compared to the control group \((P < 0.05)\). However, pretreatment with Res reversed the expression of Bcl-2 and Bax \((P < 0.05 \text{ versus ISO group})\) (Fig. 4).

**Res affected ERS signal pathway**

To identify the direct effect of Res on ERS, ER stress marker proteins, GRP78, GRP94 and CHOP were examined (Fig. 5). Compared with the control group, the expression levels of ER stress markers (GRP78, GRP94) and ER-initiated apoptosis markers (CHOP) were significantly increased in the ISO group \((P < 0.05 \text{ or } P < 0.01 \text{ versus the control group})\) and Res reversed these increases \((P < 0.05 \text{ versus the ISO group})\). In contrast, the expression of GRP78, GRP94 and CHOP were not changed obviously in the Res group compared with the control group \((P < 0.05)\).

**Discussion**

Our studies have shown that resveratrol treatment may inhibit ISO-induced cardiomyocytes hypertrophy and apoptosis via suppressing ER stress related factors expression. This study indicates resveratrol treatment as a potential strategy for the prevention and therapy of β-Adrenergic receptors (β-AR)-related cardiovascular diseases.
It has been reported that β-AR plays key roles in the regulation of cardiac function, both under normal conditions and under pathological conditions, such as during heart failure (HF) [13]. Chronic impairment of β-AR signaling contributes to alterations in cardiac structure through increased apoptosis, hypertrophy, fibrosis and steadily decreased contractile function [14, 15]. ISO, an agonist of the adrenergic receptor, induces compromised and unstable cardiac hemodynamics and aggravates cell death, thereby inducing HF [16, 17]. Apoptosis is a critical step in provoking systolic dysfunction and congestive HF [18].

Consistently with previous studies, we also observed myocardial hypertrophy induced by ISO [10]. ISO is able to induce an increase of the surface area of cardiomyocytes and mRNA expression of ANP compared with the control group. Cardiomyocytes apoptosis was also identified with ISO using Hoechst 33258 staining. TEM was also used to detect the ultrastructure of cardiomyocytes. The elevated levels of LDH and MDA further demonstrated that ISO results in the damage of cardiomyocytes associated with lipid peroxidation. These findings suggest that ISO is able to induce the cardiomyocytes hypertrophy and apoptosis.

Res as an important natural substance in red wine is widely found in plants such as grape seed and skin [19, 20]. A large body of evidence has shown that Res may help to prevent the development of certain cancers and cardiovascular diseases due to antiviral, antioxidant and anti-inflammatory actions. A recent study showed that Res may attenuate left ventricular hypertrophy via inhibiting NFAT-dependent transcription and suppress heart failure and reverse myocardial remodeling [21, 22]. Res treatment may inhibit high blood pressure and attenuate apoptosis of ventricular myocytes and improve ventricular function [23, 24]. In this study, we investigated that the effects of Res treatment on myocardial hypertrophy induced by ISO. We found that 50μM Res treatment is enough to inhibit the myocardial hypertrophy and apoptosis. The ultrastructure damage in ISO-induced myocardium was also improved by Res treatment. This result is consistent with the benefits of Res on other heart diseases. For instance, Dolinsky observed that a high dose of RES attenuated high pressure and prevented cardiac hypertrophy in spontaneously hypertensive rats and angiotensin-II infused mice [19]. Preclinical studies showed that pretreatment with RES resulted in a protection against the deleterious effects of myocardial reperfusion after ischemia [25].

Apoptosis is positively and negatively regulated by the Bcl-2 protein family [26, 27]. Apoptosis is mediated by two pathways: the extrinsic pathway and the intrinsic pathway [28]. The extrinsic pathway induces apoptosis through the binding of the ligands to the cell surface receptors, whereas the intrinsic pathway involves the mitochondria and ER. Cell apoptosis of mitochondria pathway is mediated by Bcl-2 family proteins. The Bcl-2 family can be generally divided into two different groups, the anti-apoptotic members such as Bcl-2 and Bcl-xL, and the pro-apoptotic members such as Bax, Bak, Bid and Bad. In normal conditions, most of anti-apoptotic members in Bcl-2 family are isolated as membrane protein of organelle membrane and pro-apoptotic members are distributed in cytoplasm or cytoskeleton in inactivated forms. The present results indicated that ISO could elevate the Bax expression and decrease the Bcl-2 expression, but Res could reverse them dramatically. Bcl-2 is a pivotal molecule in many apoptosis pathways and it exerts inhibitory effect on free radical elevation, calcium overload and lipid peroxidation, and thus the protective role of Res is probably to be achieved by changing the expression of Bcl-2 and Bax. A recent study reported that RES administration could inhibit cold exposure-induced cardiac hypertrophy in mice and had a suppressive action of apoptosis of myocardium via inhibition of Bax and caspase-3 activation [29].

Some studies have already demonstrated that ER stress is involved in the pathogenesis of cardiovascular diseases including myocardial hypertrophy [30]. Moreover, long-term and serious ER stress may result in cell apoptosis and necrosis. GRP78 plays an important protective role in ERS and overexpression of GRP78 often indicates the disturbance of cell homeostasis [31]. CHOP, an apoptotic signaling molecular induced by persistent ERS, can directly regulate target genes in the nucleus to increase the sensitivity to apoptosis [32, 33]. CHOP-deficient cells are resistant to ER-stressed-mediated apoptosis, and the overexpression of CHOP can lead to cell cycle arrest and/or apoptosis [34]. Many studies have demonstrated
that the overexpression of CHOP leads to decreased Bcl-2 protein level and the translocation of Bax protein from the cytosol to the mitochondria. Bcl-2 overexpression or Bax knockout can block CHOP-induced apoptosis.

Our previous work demonstrated that the ER-related apoptosis pathway was involved in the development of cardiac hypertrophy, which indicates that ER stress may be one of the pathogenic factors of cardiac hypertrophy [10]. The present study found that Res significantly down-regulated the ERS-related proteins such as GRP78, GRP94 and CHOP and increased Bcl-2-to-Bax ratio. A recent study showed that Res prevents doxorubicin-induced cardiotoxicity in H9c2 cells and protective function of liver against excessive accumulation of fat through the inhibition of ER stress [35, 36]. So we speculated that Res protected against ISO-induced hypertrophy via inhibiting ERS signal pathway.

In summary, this study confirmed that Res, a natural compound in plants such as grapes, may exert protective effects by inhibiting the ERS pathway and reversing the expression of Bcl-2 and Bax in cardiomyocytes hypertrophy. The present study suggests that Res could protect hearts against β-AR-related cardiovascular diseases.

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Disclosure Statement

The authors declare no conflict of interest.

References


