

Resveratrol protects myocardial apoptosis induced by ischemia-reperfusion in rats with acute myocardial infarction via blocking PI3K/Akt/e-NOS pathway

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Abstract. – **OBJECTIVE:** To elucidate the protective role of resveratrol (RSV) in myocardial apoptosis induced by ischemia-reperfusion injury in rats with acute myocardial infarction (AMI), and to explore its underlying mechanism.

MATERIALS AND METHODS: The AMI rat model was successfully established by ligation of the left anterior descending coronary artery. Rat cardiomyocytes were isolated and cultured. Cells were divided into four groups, including: control group (no specific treatment), AMI group (acute ischemia-reperfusion treatment), AMI+RSV group (RSV pretreatment for 24 h before acute ischemia-reperfusion) and AMI+RSV+LY group (RSV pretreatment combined with 40 $\mu\text{mol/L}$ phosphatidylinositide 3-kinases (PI3K) pathway inhibitor LY294002 for 24 h before acute ischemia-reperfusion). Morphology of apoptotic cardiomyocytes in each group was observed by Hoechst staining. The proliferation, apoptosis and cell cycle progression of cardiomyocytes were determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay, terminal deoxynucleotidyl transferase(TdT)-mediated dUTP nick end labeling (TUNEL) assay and flow cytometry, respectively. Finally, the protein levels of genes relative to PI3K/Akt/eNOS pathway were detected by Western blot.

RESULTS: Hoechst staining showed a large number of necrotic cells, cell retraction, enhanced refractive index and enlarged cell gap in AMI group. A small number of necrotic cells were found in AMI+RSV group, which was significantly fewer than that of AMI group. Meanwhile, remaining cells presented normal morphology. However, a great number of necrotic cells were observed in AMI+RSV+LY group, which was obviously more than that of AMI+RSV group. Compared with control group, cells in AMI group showed significantly decreased proliferative rate, increased early phase, late phase and total

one of apoptosis. In AMI group, the ratio of G0/G1 phase was remarkably increased, whereas those of S and G2/M phases were decreased. Moreover, the expression levels of phosphorylated Akt (p-Akt) and phosphorylated e-NOS (p-eNOS) were significantly downregulated in AMI group. In AMI+RSV group, cell apoptosis, cell cycle progression and levels of p-Akt and p-eNOS showed the opposite trends as those of AMI group. However, LY294002 pretreatment reversed the protective role of RSV in cellular behaviors of cardiomyocytes.

CONCLUSIONS: RSV protects cardiomyocyte apoptosis from ischemia-reperfusion injury through regulating phosphorylation levels of proteins relative to PI3K/Akt/e-NOS pathway.

Key Words

Resveratrol, Ischemia-reperfusion, Cardiomyocytes, PI3K/Akt/eNOS, Apoptosis, Proliferation, Cell cycle progression.

Introduction

Ischemic heart disease is one of the most fatal diseases in the world. It seriously threatens human health, especially in the middle-aged and elderly population¹. The imbalance of coronary blood flow and myocardial demand caused by changes in coronary circulation is the major pathogenic factor of ischemic heart disease². Surgical approaches, including thrombolytic therapy, percutaneous transluminal coronary angioplasty and coronary artery bypass grafting, mainly contribute to the immediate restoration of blood flow in ischemic myocardium³. Hypoxia-reoxygenation injury frequently occurs during the treatment of ischemic heart diseases. The main

characteristics include abnormalities in morphology, structure and function of cardiomyocytes⁴. Therefore, prevention of hypoxia-reoxygenation injury is the key to improve the successful therapeutic rate of ischemic heart disease.

Resveratrol (RSV) is a natural polyphenolic substance extracted from plants. It has strong biological activity, which can prevent free radicals, oxidative stress, and tumor diseases. Meanwhile, it can also enhance immune response in the body. RSV exerts a relatively small side effect, which is frequently applied in clinical treatment⁵. RSV pretreatment has been proved to alleviate hypoxia-reoxygenation injury⁶. It is known to all that hypoxia-reoxygenation injury leads to a cascade of myocardial injury involving multiple factors and pathways⁷. Based on this finding, inhibition or activation of certain pathways is identified to inhibit cell apoptosis, thus efficiently protecting myocardial injury. Previous works have reported that phosphatidylinositol 3-kinases/protein kinase B (PI3K/Akt) pathway is an important intracellular pathway preventing against ischemia-reperfusion injury. This may eventually reverse the occurrence, development and prognosis of hypoxia-reoxygenation injury *via* certain intervention⁸. Various cytokines are produced by the stimulation of myocardial ischemia, hypoxia and reperfusion. Thereafter, this can initiate PI3K/Akt pathway to protect the impaired myocardium⁹. In this study, we investigated the function of RSV-mediated PI3K/Akt/eNOS pathway in rat cardiomyocyte apoptosis after ischemia-reperfusion injury. We aimed to provide a novel reference for the treatment of hypoxia-reoxygenation injury.

Materials and Methods

Experimental Rats and Cardiomyocyte Culture

Thirty-two 10-day-old neonatal Sprague-Dawley (SD) rats weighing 10-20 g were enrolled in this study. The AMI model was successfully established by ligation of the left anterior descending coronary artery¹⁰. In brief, rats were anesthetized by intraperitoneal injection of 1% pentobarbital sodium. Next, the rats were immersed in 75% ethanol for 5 min and transferred to a clean bench. Under sterile conditions, rat heart was harvested to dissect large blood vessels, valves and surrounding connective tissues. Left ventricle was isolated, cut into tissue blocks of about 1 mm³, followed by digestion with 0.25 g/L trypsin at 37°C for 5 min.

After centrifugation at 1000 r/min for 5 min, the precipitate was incubated with 0.2% type II collagenase for 10 min. Subsequently, the precipitate was centrifuged again at 1000 r/min for 5 min. After that, the precipitate was inoculated into a 25 cm² culture bottle and cultured with Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Rockville, MD, USA) containing 15% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA). Non-adherent cells were removed at 12 h. After cell adherence, culture medium was replaced every 1-2 days. This study was approved by the Animal Procedure Ethics Committee of our hospital.

In Vitro Ischemia-Reperfusion in Cardiomyocytes

Cardiomyocytes were cultured in a 6-well plate at a density of 1×10⁶ cells/well for 24 h in a hypoxic-reoxygenation environment. Next, the ischemia-reperfusion model was simulated. Cells were maintained in a three-gas incubator (JVAN. SA) with 94% N₂, 5% CO₂ and 1% O₂ for 24 h. Subsequently, cells were changed to a normal incubator at 5% CO₂ for 24-h reoxygenation.

Experimental Groups

Cells were divided into four groups, including: control group (no specific treatment), AMI group (acute ischemia-reperfusion treatment), AMI+RSV group (RSV pretreatment for 24 h before acute ischemia-reperfusion) and AMI+RSV+LY group (RSV pretreatment combined with 40 μmol/L PI3K pathway inhibitor LY294002 (Beyotime, Shanghai, China) for 24 h before acute ischemia-reperfusion). The dosage of RSV was selected as previous literature reported and was confirmed without any adverse events¹¹. Hoechst staining was performed to observe nucleus of normal and apoptotic cells.

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl Tetrazolium Bromide) Assay

Cardiomyocytes were seeded into 96-well plates at a density of 5×10⁴ cells per well. Marginal wells were added with PBS. 5 replicates were set in each group. After specific treatment, 20 μL of MTT solution (5 mg/mL) (Sigma-Aldrich, St. Louis, MO, USA) were added to each well for 4-h incubation. Subsequently, 150 μL of dimethylsulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) was added to each well, followed by low-speed oscillation for 10 min. Finally, the optical density at the wavelength of 490 nm was detected by a micro-plate reader.

**Terminal Deoxynucleotidyl
Transferase(TdT)-Mediated dUTP
Nick End Labeling (TUNEL) Staining**

According to the instructions of TUNEL Fluorescence Kit (Promega, Madison, WI, USA), fixed cardiomyocytes were immersed in 0.2% Triton X-100 solution for 5 min to enhance cell membrane permeability. Then, the cardiomyocytes were incubated with deoxynucleotide terminal transferase (rTdT) at 37°C for 1 h. Positive cells were stained brown in cell nucleus. 5 fields of vision were randomly selected for each section. Apoptotic rate = number of apoptotic positive cells / total number of cells × 100%.

Flow Cytometry

Flow cytometry (FCM) (BD Biosciences, FranklinLakes, NJ, USA) was used to detect the apoptotic rate using Annexin V-fluorescein isothiocyanate (FITC) / Propidium Iodide (PI) staining. Briefly, cells were first seeded into 6-well plates and digested with 200 µL of Ethylene Diamine Tetraacetic Acid (EDTA)-free trypsin per well (Thermo Fisher Scientific, Waltham, MA, USA). After centrifugation at 1000 r/min for 10 min, the cells were stained with Annexin V-FITC/PI. Finally, the apoptotic rate was calculated. Apoptotic rate = (B2+B4) quadrant cell number / total cell number × 100%. Cells in early phase of apoptosis were located in the lower right quadrant, while those in late phase of apoptosis were located in the upper right quadrant. Viable cells and necrotic cells were located in the lower left quadrant and the upper left quadrant, respectively.

Western Blot

Total protein was first extracted using cell lysate. The concentration of extracted protein sample was quantified by the bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA). Subsequently, protein samples were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel electrophoresis and transferred onto membranes. After blocking with 5% skim milk, the membranes were incubated with primary antibodies (Akt and eNOS; β-actin as the reference). On the next day, the membranes were incubated with corresponding secondary antibodies. Immuno-reactive bands were developed by enhanced chemiluminescence (ECL) method.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 software (IBM, Armonk, NY, USA)

was utilized for all statistical analysis. Quantitative data were represented as mean ± standard deviation ($\bar{x} \pm s$). *t*-test was used to compare the differences between two groups. One-way analysis of variance (ANOVA) was performed to compare the difference among different groups, followed by post-hoc test. *p*<0.05 was considered statistically significant.

Results

**Morphology of Cardiomyocytes
in Each Group**

Hoechst staining showed a large number of necrotic cells, cell retraction, enhanced refractive index and enlarged cell gap in AMI group. A small number of necrotic cells were found in AMI+RSV group, which was significantly fewer than that of AMI group. Meanwhile, remaining cells presented normal morphology. However, a great number of necrotic cells were observed in AMI+RSV+LY group, which was much more than that of AMI+RSV group (Figure 1).

**Effect of RSV on the Proliferative
Potential of Cardiomyocytes
After Ischemia-Reperfusion**

Cell viability was determined by MTT assay. Compared with control group, the proliferative rate of AMI group was markedly decreased (*p*<0.05). The proliferative rate of AMI+RSV group was significantly higher than that of AMI group, which was inhibited in AMI+RSV+LY group (*p*<0.05, Table I). The above data suggested that PI3K inhibitor reversed the protective role of RSV in cardiomyocyte viability.

**Effect of RSV on Cardiomyocyte
Apoptosis After Ischemia-Reperfusion**

TUNEL staining indicated that the apoptotic rate of cardiomyocytes in AMI group was significantly increased when compared with control group. RSV pretreatment markedly reduced apoptotic rate, while was further reversed by LY294002 treatment (*p*<0.05, Figure 2A and 2B). Annexin V-FITC/PI staining demonstrated that there were a great number of necrotic cells in AMI group, showing remarkable cardiomyocyte injury. Moreover, AMI+RSV+LY group showed significantly more apoptotic cells than AMI+RSV group, especially in late phase (*p*<0.05, Figure 3A and 3B).

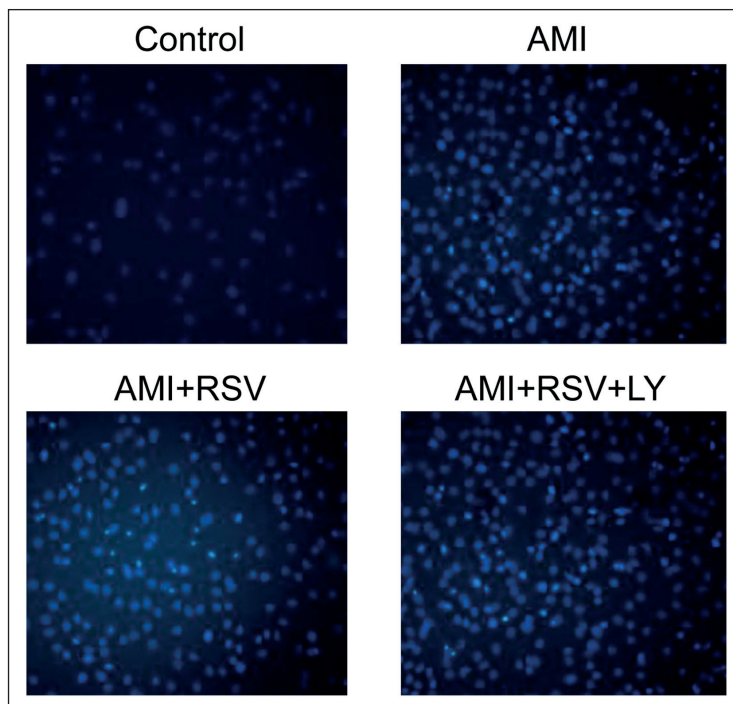


Figure 1. Morphology changes in 4 different group. Control group; AMI group; AMI+RSV group and AMI+RSV+LY group.

Effect of RSV on Cell Cycle Progression of Cardiomyocyte After Ischemia-Reperfusion

Cell cycle progression in each group was determined by FCM. In comparison with control group, cell proportion in G0/G1 phase increased remarkably in AMI group, while those in S phase and G2/M phase decreased. This suggested that cardiomyocytes in AMI group were arrested in G0/G1 phase. RSV pretreatment could significantly alleviate such cell cycle arrest. However, AMI+RSV+LY group showed significantly higher proportion in G0/G1 phase. The proportion of S and G2/M phase was significantly lower than S and G2/M phase (Table II). These results suggested that RSV promoted cell cycle progression, which was arrested after inhibiting PI3K pathway.

Effect of RSV on the Expressions of Relative Genes in PI3K/Akt/e-NOS

Table I. Proliferative rate of cardiomyocytes

Groups	Proliferative rate
Control	0.232±0.034
AMI	0.170±0.058
AMI+RSV	0.228±0.025
AMI+RSV+LY	0.165±0.055

Pathway

No significant changes in the protein expression of Akt and e-NOS were found among different groups. However, the protein expression of phosphorylated Akt (p-Akt) and phosphorylated e-NOS (p-eNOS) in AMI group was markedly downregulated when compared with control group. AMI+RSV group presented significantly higher levels of p-Akt and p-eNOS relative to AMI group, which was then reversed by LY294002 treatment (Figure 4A and 4B). These results indicated that RSV protected cardiomyocyte apoptosis by altering the phosphorylation levels of Akt/e-NOS through PI3K/Akt/e-NOS pathway. However, LY294002 could reverse this protective effect.

Discussion

Due to the extremely high incidence and mortality of ischemic heart disease, we explored whether the protective role of RSV in rat cardiomyocytes induced by hypoxia-reoxygenation injury was achieved *via* activating PI3K/Akt/e-NOS pathway¹². This study aimed to provide references for enhancing the success of surgery for this disease. In the present work, we first observed cardiomyocyte morphology by Hoechst staining. As the results indicated, abundant necrotic cells and abnormal morphol-

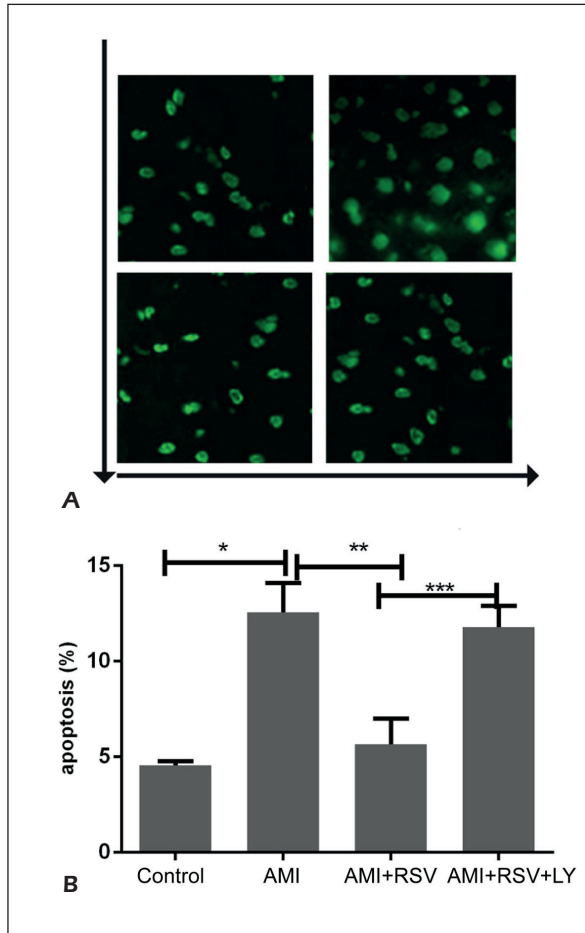


Figure 2. Cell apoptosis in each group determined by TUNEL assay. **A**, TUNEL staining from left to right and upper to bottom: 1. Control group; 2. AMI group; 3. AMI+RSV group; 4. AMI+RSV+LY group. **B**, Apoptotic rate in control group, AMI group, AMI+RSV group and AMI+RSV+LY group. *Control group vs. AMI group; **AMI group vs. AMI+RSV group; ***AMI+RSV group vs. AMI+RSV+LY group. * $p < 0.05$, ** $p < 0.05$ and *** $p < 0.05$.

ogy of cardiomyocytes were observed in AMI group. RSV pretreatment remarkably protected the apoptosis and necrosis of cardiomyocytes, which could be reversed by LY294002 pretreatment. We might conclude that ischemia-reperfusion led to cardiomyocyte injury, which could be alleviated by RSV. However, PI3K pathway blockage aggravated cellular damage. Based on these results, we presumed that the protective role of RSV in ischemic cardiomyocytes might be relied on PI3K/Akt/e-NOS pathway. Subsequently, we highlighted the potential roles of RSV and LY294002 in regulating cell survival, cell cycle progression of ischemic cardiomyocytes and PI3K/Akt/e-NOS pathway involve-

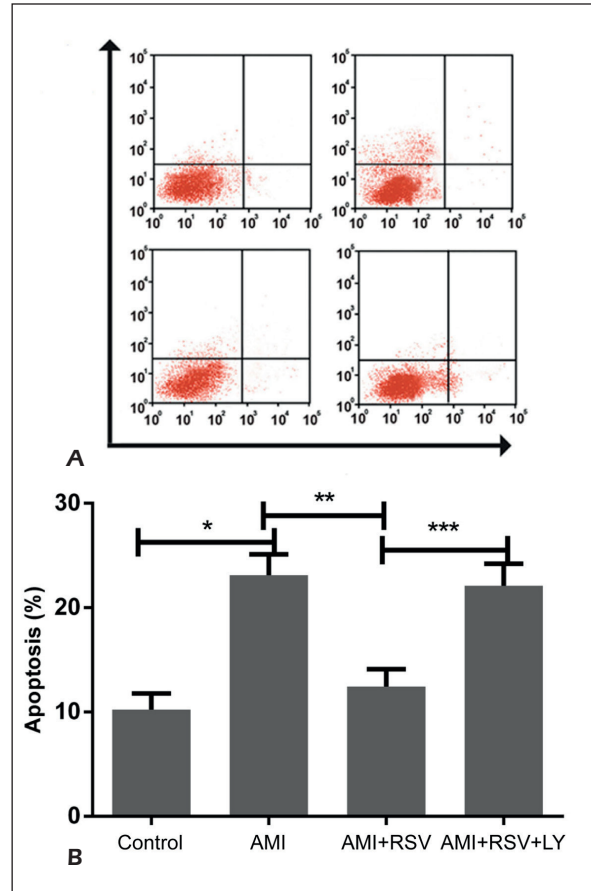


Figure 3. Cell apoptosis in each group determined by FCM. **A**, FCM images from left to right and upper to bottom: 1. Control group; 2. AMI group; 3. AMI+RSV group; 4. AMI+RSV+LY group. **B**, Apoptotic rate in control group, AMI group, AMI+RSV group and AMI+RSV+LY group. *Control group vs. AMI group; **AMI group vs. AMI+RSV group; ***AMI+RSV group vs. AMI+RSV+LY group. * $p < 0.05$, ** $p < 0.05$ and *** $p < 0.05$.

ment. Compared with control group, cells in AMI group showed significantly decreased proliferative rate, increased early phase, late phase and total one of apoptosis. The ratio of cells in G0/G1 phase increased, whereas those in S

Table II. Cell cycle progression of cardiomyocytes

Group	Cell cycle		
	G0/G1	S	G2/M
Control	57.33±3.91	27.10±3.22	10.04±2.93
AMI	76.19±4.18	16.82±2.65	8.82±1.21
AMI+RSV	65.72±3.73	24.41±1.72	17.60±1.62
AMI+RSV+LY	80.90±4.13	15.14±1.66	10.11±1.60

* $p < 0.05$, ** $p < 0.05$ and *** $p < 0.05$.

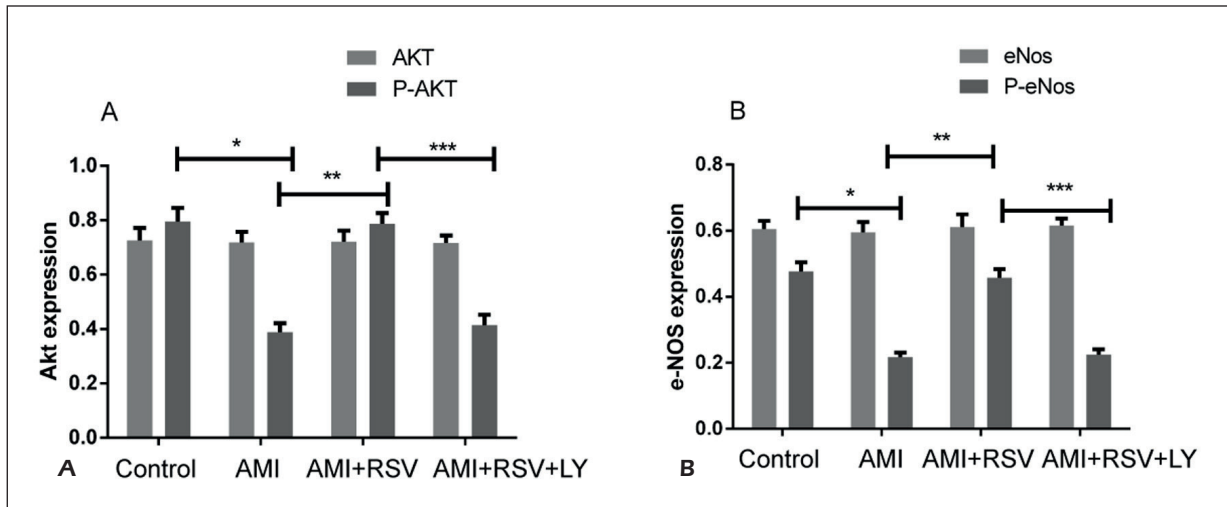


Figure 4. Expression changes of Akt, p-Akt, e-NOS and p-eNOS in each group. **A**, Expression levels of Akt and p-Akt in control group, AMI group, AMI+RSV group and AMI+RSV+LY group. **B**, Expression levels of e-NOS and p-eNOS in control group, AMI group, AMI+RSV group and AMI+RSV+LY group. *Control group vs. AMI group; **AMI group vs. AMI+RSV group; ***AMI+RSV group vs. AMI+RSV+LY group. * $p < 0.05$, ** $p < 0.05$ and *** $p < 0.05$.

and G2/M phases were significantly decreased in AMI group. Moreover, the expression levels of p-Akt and p-eNOS were downregulated in AMI group than control group. However, AMI+RSV group presented the opposite trends to AMI group in cell apoptosis, cell cycle progression and the phosphorylation levels of Akt and e-NOS. However, LY294002 pretreatment could reverse the protective role of RSV in cellular behaviors of cardiomyocytes. Scholars^{13,14} have found that RSV has significant pharmacological effects, such as anti-inflammation, vasodilation promotion, smooth muscle cell proliferation inhibition, lipid metabolism promotion, anti-tumor ability, and oxidative stress inhibition. The inhibitory effect of RSV on abnormal proliferation of vascular smooth muscle cells allows it to be applied in the prevention of cardiovascular diseases, such as coronary heart disease and hypertension¹⁵. In addition, RSV can regulate cell signal transduction, improve endothelial integrity, reduce the expressions of vascular cell adhesion molecules, and inhibit vascular smooth muscle cell hypertrophy by interfering with PI3K/Akt pathway¹⁶. An *in vivo* research has clarified that RSV alleviates hypoxia-reoxygenation injury in rat cardiomyocytes, exerting a protective effect on ischemic and hypoxic myocardium¹⁷. Meanwhile, our findings also indicated that the anti-apoptotic function of RSV might be related to the promotion of RNA replication and protein synthesis in G0/G1

phase, thereby promoting cell mitosis¹⁸. It has been reported that RSV inhibits cell apoptosis through blocking cell cycle progression¹⁹. Moreover, another research has suggested that RSV inhibits cell apoptosis through suppressing the activities of key protein tyrosine kinases and promoting cell proliferation²⁰. The activation of PI3K/Akt/e-NOS pathway regulates the expressions of apoptosis-associated proteins, which in turn mediates cell apoptosis²¹. Activated Akt induces multiple targets in the downstream pathway, which also inhibits the opening of mitochondrial permeability transition pores. Maintaining mitochondrial extracellular stability contributes to reduce the activation of pro-apoptotic factors, thereby impeding cell apoptosis²². Meanwhile, the activation of e-NOS helps to improve vascular function and endothelial function²³. It is believed that RSV protects myocardial function and suppresses apoptosis through PI3K/Akt/e-NOS pathway. As PI3K inhibitor, LY294002 blocks the activation of PI3K/Akt pathway through suppressing the phosphorylation of downstream genes²⁴. Inhibited activities of Akt threonine site and serine site lead to reduction in enzyme activity of e-NOS. This can in turn downregulate the phosphorylated levels of Akt and e-NOS²⁵. PI3K/Akt/e-NOS pathway transmits a variety of pleiotropic effects, such as cell proliferation and apoptosis, hematopoiesis, and immune responses²⁶. Current investigations have showed that RSV can attenuate the apoptosis of rat cardiomyocytes caused by ischemia-reperfusion²⁷. In this study we

observed that RSV reduced cardiomyocyte apoptosis *via* PI3K/Akt/e-NOS pathway. Some limitations should be pointed out here. Firstly, *in vitro* effect of RSV on protecting cardiomyocytes after hypoxia-reoxygenation injury should be further elucidated. Secondly, we only choose a single dose of RSV, and the dosage effect of RSV is required for specific exploration as well.

Conclusions

We found that resveratrol protects cardiomyocyte apoptosis from ischemia-reperfusion injury through regulating phosphorylation levels of proteins relative to PI3K/Akt/e-NOS pathway.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Acknowledgements

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