

Resveratrol alleviates hypoxia/reoxygenation injury-induced mitochondrial oxidative stress in cardiomyocytes

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Abstract. Resveratrol (RES) is a naturally occurring anti-oxidant compound found in red wine. Although it has been demonstrated to have a cardioprotective effect, the mechanism underlying this effect remains to be fully elucidated. The aim of the present study was to determine whether RES exerts a protective effect against mitochondrial oxidative stress and apoptosis in neonatal rat cardiomyocytes (NRCMs) induced by hypoxia/reoxygenation (H/R) injury. Primary cultured NRCMs were used as a model system and were divided into four experimental groups: Control, H/R, H/R + DMSO (H/R with 0.2% DMSO) and H/R + RES (H/R with 100 μ M RES) groups. Mitochondrial oxidative stress was determined by measuring the alteration in the mitochondrial membrane potential ($\Delta\Psi_m$) of NRCMs, the release of lactate dehydrogenase (LDH) and the ratio of B-cell lymphoma 2 (Bcl-2)/Bcl-2-associated X protein (Bax) from NRCMs. Cell apoptosis was assessed by measuring cell apoptotic rates and the activity of caspase 3. In the H/R+RES group, RES significantly alleviated structural impairment, including disordered α -actin and F-actin, in the NRCMs induced by H/R injury. RES attenuated H/R injury-induced mitochondria oxidative stress. RES also attenuated H/R injury-induced cell apoptosis; it decreased the NRCM apoptotic rate from 84.25 \pm 7.41% (H/R) to 46.39 \pm 5.43% (H/R+RES) (P<0.05, n=4), rescued the decrease in the Bcl2/Bax ratio induced by H/R from 0.53 \pm 0.08-fold (H/R) to 0.86 \pm 0.06-fold (H/R+RES) (P<0.05, n=5) and alleviated

the increased activity of caspase 3 induced by H/R from 1.32 \pm 0.06-fold to 1.02 \pm 0.04-fold (P<0.05, n=5). Furthermore, RES significantly attenuated the increment of LDH release induced by H/R injury in NRCMs from 1.41 \pm 0.03-fold (H/R) to 1.02 \pm 0.06-fold (H/R+RES) (P<0.01, n=4) and alleviated the depolarization of $\Delta\Psi_m$ induced by H/R, shifting the ratio of JC-1 monomer from 62.39 \pm 1.82% (H/R) to 35.31 \pm 8.63% (H/R+RES) (P<0.05, n=4). RES alleviated the decrease in sirtuin 1 induced by H/R injury from 0.61 \pm 0.06-fold (H/R) to 1.01 \pm 0.05-fold (H/R+RES) (P<0.05, n=5). In conclusion, the present study is the first, to the best of our knowledge, to demonstrate that RES provides cardioprotection against H/R injury through decreasing mitochondria-mediated oxidative stress injury and structural impairment in NRCMs. These results provide scientific evidence for the clinical application of RES in the treatment of cardiac conditions.

Introduction

Myocardial ischemia/reperfusion (I/R) injury is a major pathophysiological condition associated with cardiopulmonary bypass surgery and various cardiovascular diseases (CVDs), including coronary heart disease and myocardial infarction, both of which have high mortality and morbidity rates (1,2). Mitochondria are the organelles providing the major source of reactive oxygen species (ROS) production and are rich in cardiomyocytes (3,4). Mitochondrial oxidative stress serves a central role in myocardial I/R injury. Hypoxia induces superoxide generation via the electron transport chain. High concentrations of ROS disrupt cell functions, induce apoptosis and cause tissue damage via inducing oxidative stress. Therefore, mitochondria-mediated oxidative stress is the key step in I/R-induced cardiomyocytes injury (5,6). It is also a main target for designing treatment strategies for I/R-induced cardiomyocyte injury.

Resveratrol (RES), a natural compound found primarily in red wine, is a naturally occurring antioxidant. The potential beneficial effects of RES were first observed in major CVDs, including myocardial ischemia, atherosclerosis, hypertension, stroke, aging and heart failure (7). Previous studies have revealed that the cardioprotective effects of RES include anti-oxidative stress, antiplatelet activity, preventing

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endothelial cell damage and inflammation, and increasing the expression of nitric oxide synthase. The biological effects of RES, including chemoprevention, immunomodulatory, antiproliferative and antioxidant effects, have been reported previously (8-11). Recently, it was reported that RES exerts its cardioprotective effect through the AMP-activated protein kinase and phosphoinositide-3-kinase/Akt/Forkhead box O3a signaling pathways (12,13). Additionally, it was reported that pre-treatment with RES may prevent ischemic cerebral damage in rats by activating sirtuin 1 (Sirt1), one of the ubiquitous histone and protein deacetylases (14). RES-mediated autophagy is also involved in cardioprotection (15). However, the mechanisms involved in the cardioprotective effect of RES remain to be fully elucidated. The mitochondria-mediated oxidative stress pathway is one of the major causes of I/R or hypoxia/reoxygenation (H/R) (4,16). ROS are generated in the I/R or H/R myocardium, particularly in mitochondria. I/R injury has been associated with significant increases in ROS, the release of lactate dehydrogenase (LDH) and depolarization of the mitochondrial transmembrane potential ($\Delta\Psi_m$), which are all important indices for reflecting the status of mitochondrial oxidative stress. Enhanced mitochondrial oxidative stress induces cell injury and apoptosis (17,18). Previous findings have also shown that RES has anti-oxidant properties (19). Hypoxia is an important pathophysiological progress and induces cell injury through crosstalk with mitochondria and oxidative stress pathways (20). Sirt1 is also involved in hypoxia-induced pulmonary artery smooth muscle cell injury (21). To gain further insight into the mechanisms underlying the RES-mediated protective effect against I/R injury, an H/R model of cultured NRCMs was established in the present study. Using this model, the cardioprotective effect of RES and the underlying mechanism were examined. The results showed that RES alleviated H/R injury through inhibiting the mitochondria-mediated oxidative stress pathway. This provides a robust scientific basis for the clinical application of RES in the treatment of cardiac conditions.

Materials and methods

Drugs and reagents. RES (molecular formula: $C_{14}H_{12}O_3$; CAS no. 501-36) was purchased from Neptunus Company (Shenzhen, China). A MitoProbe™ JC-1 Assay kit (cat. no. M34152) for measuring the $\Delta\Psi_m$ was obtained from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Dimethyl sulfoxide (DMSO) was purchased from Sigma; Merck KGaA (Darmstadt, Germany). The BCA protein assay (cat. no. 23225) was purchased from Thermo Fisher Scientific, Inc. All other reagents and chemicals were obtained commercially and were of analytical grade.

Cell culture and the treatment. The experimental protocols were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (National Institutes of Health, Bethesda, MD, USA) and with the approval of the Animal Care and Use Committee of the Southwest Medical University (Sichuan, China).

Neonatal rats (age, 2-3 days; weight, 5-8 g; n=150) were purchased from The Animal Center of Southwest Medical University (Luzhou, China). Neonatal rats were maintained at

$22\pm 3^\circ\text{C}$ under a 12-h light/dark cycle. All rats had free access to water and food. Primary cultured neonatal rat cardiomyocytes (NRCMs) were cultured as previously described (22). In brief, neonatal rat ventricles were digested with collagenase II and cardiomyocytes were purified through adherence to culture plastic for different durations. Subsequently, 0.1 mM 5-BrdU was added to DMEM to inhibit the cardiac fibroblasts. The NRCMs were cultured in DMEM (cat. no. SH30021; HyClone; GE Healthcare Life Sciences, Logan, UT, USA) containing 10% FBS (cat. no. 10270; Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin (cat. no. C0222; Beyotime Institute of Biotechnology, Haimen, China) at 37°C in a 5% CO_2 incubator. The NRCMs were randomly divided into four groups: i) Control group (normal DMEM); ii) H/R group: Cells were subjected to a completely enclosed environment containing a Bio-Bag (Thermo Fisher Scientific, Inc.) for 8 h in hypoxic medium and then subjected to reoxygenation under normoxic conditions for another 8 h, following which the hypoxic medium was replaced with fresh medium (containing 10% FBS) upon reoxygenation; iii) DMSO + H/R group: 0.2% DMSO was added to the medium during H/R; iv) RES + H/R group: RES (final concentration of 100 μM) was added to the medium during H/R.

Measurement of LDH release. LDH release was detected using an LDH assay kit (cat. no. A020, Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's protocol. In brief, the NRCMs were subjected to centrifugation at $300 \times g$ for 10 min. Following this, 60 μl of supernatant was added to 30 μl of LDH substrate solution and incubated for 30 min at 37°C . The LDH levels were detected with a microplate reader (Tecan Group, Inc., Mannedorf, Switzerland) at 440 nm.

Measurement of $\Delta\Psi_m$. The $\Delta\Psi_m$ was measured by flow cytometry using the MitoProbe™ JC-1 assay kit. The approximate excitation peak of JC-1 is 488 nm. The approximate emission peaks of monomeric and J-aggregate forms are 529 and 590 nm, respectively. The ratio of monomeric JC-1 represents the level of $\Delta\Psi_m$. An increased ratio represents the depolarization of $\Delta\Psi_m$, whereas a decreased ratio indicates the hyperpolarization of $\Delta\Psi_m$.

Immunofluorescence microscopy. Primary cultured NRCMs were plated on the coverslips at 60-70% confluency and fixed in cold PBS with paraformaldehyde for 15 min, permeabilized with 0.1% triton-X 100 for 15 min and blocked with 5% bovine serum albumin (cat. no. a600332; BBI Life Sciences Corporation, Shanghai, China) for 1 h at room temperature. The NRCMs were then incubated with primary mouse anti- α -actinin 2 antibody (1:100; cat. no. BM4907; Wuhan Boster Biological Technology, Ltd., Wuhan, China) overnight at 4°C and then incubated with the DyLight® 488-conjugated donkey anti-mouse secondary antibody (1:200; cat. no. ab96875; Abcam, Cambridge, UK) for 1 h at room temperature. In addition, for the staining of F-actin, the NRCMs were treated with 100 nM rhodamine phalloidin (cat. no. PHDR1; Cytoskeleton, Inc., Denver, CO, USA) for 30 min at room temperature. The nuclei were stained with DAPI. The cells were mounted and images were captured using an Olympus fluorescence

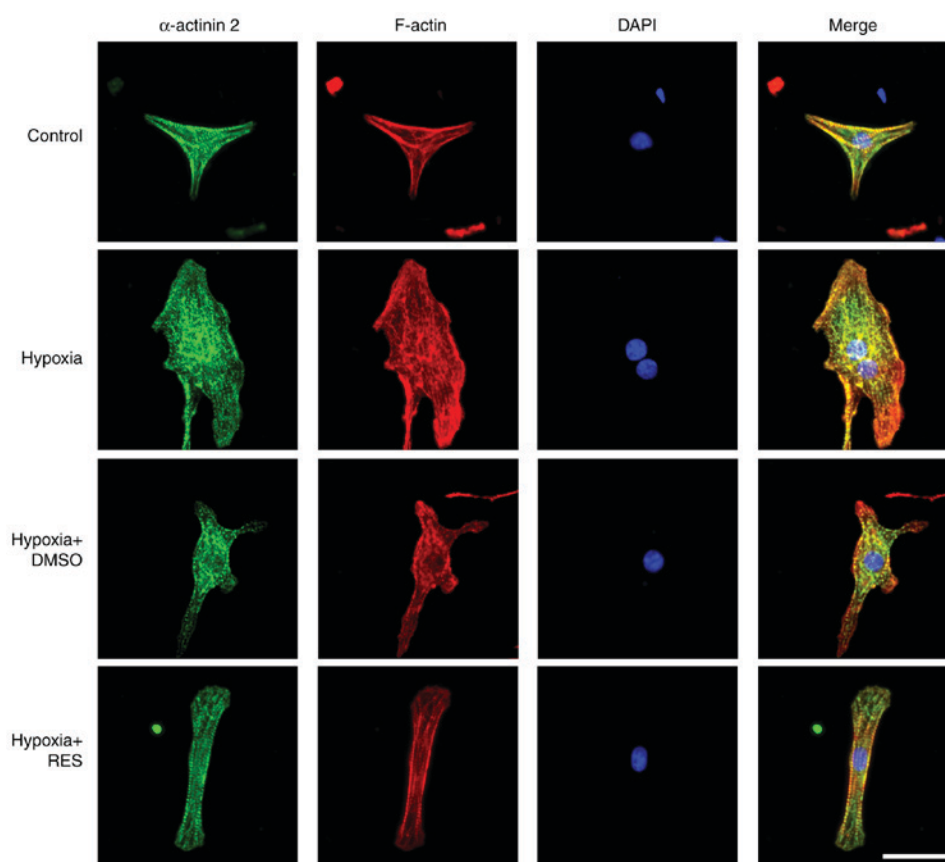


Figure 1. Images of NRCMs with immunofluorescence microscopy showing the attenuating effects of RES on H/R injury-induced structural impairment of NRCMs. α -actinin (green) and F-actin (red) indicate the T-tubule marker and cytoskeleton marker, respectively. Nuclei were tagged with DAPI (blue). Scale bar=20 μ m. NRCMs, neonatal rat cardiomyocytes; RES, resveratrol; H/R, hypoxia/reoxygenation; DMSO, dimethyl sulfoxide.

microscope (IX-81, Olympus Corporation, Tokyo, Japan). The excitation wavelengths for DAPI, DyLight[®] 488-conjugated and rhodamine phalloidin were 405, 488 and 550 nm, respectively.

Western blotting. The cells were washed with PBS and lysed with lysis buffer containing a cocktail of protease inhibitors (cat. no. 87785, Thermo Fisher Scientific, Inc.). Subsequently, 60 μ g total protein for each lane was separated with a 5% stacking gel and 10% separation gel, and then transferred onto a PVDF membrane (cat. no. IPVH00010; EMD Millipore, Billerica, MA, USA). The membrane was incubated in TBST containing 5% non-fat milk for 2 h at room temperature to block non-specific binding and was then incubated with primary antibodies (1:1,000) overnight at 4°C. The primary antibodies against Bax (cat. no. 2772; Cell Signaling Technology, Inc., Danvers, MA, USA), Bcl-2 (cat. no. ab196495; Absin, Shanghai, China), Sirt1 (cat. no. 9475; Cell Signaling Technology, Inc.) and the internal control antibody GAPDH (cat. no. sc-25778; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) were polyclonal antibodies raised in rabbit. The membrane was then incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG secondary antibody (cat. no. D110058; BBI Life Sciences, Shanghai, China; 1:1,000) for 1 h at room temperature. The membrane was incubated in chemiluminescent HRP substrate (cat. no. WBKLS0500; EMD Millipore) at room temperature for 5 min, following which images were captured with the Universal Hood II system (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Measurement of caspase 3 activity. Apoptotic cell death was determined by caspase 3 activation using a Caspase-3 activity assay kit (cat. no. G015, Nanjing Jiancheng Bioengineering Institute). Briefly, the cells were harvested using caspase lysis buffer (50 mM HEPES, pH 7.4, 0.1% Chaps, 5 mM dithiothreitol, 0.1 mM EDTA and 0.1% Triton X-100) for 5 min on ice and centrifuged at 13,000 \times g for 10 min at 4°C. The supernatant (50 μ g) was then isolated and incubated with 10 μ l caspase 3 substrate (Ac-DEVDpNA) for 1 h at 37°C. The activity of caspase 3 was detected with a microplate reader (Tecan Group, Ltd.) at 400 nm.

Statistical analysis. Data are presented as the mean \pm standard error of the mean and were analyzed by one way analysis of variance using SPSS 19.0 (IBM Corp., Armonk, NY, USA). The least significant difference test was used for further multiple group comparisons. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

RES protects against H/R injury-induced structural impairment in NRCMs. Firstly, the present study investigated the effects of RES on H/R-induced structural impairment in NRCMs using immunohistological staining techniques. As shown in Fig. 1, NRCMs without H/R injury (control group) had a clear morphology with a striated pattern (green) and normal cytoskeletal structure (red). The NRCMs with H/R

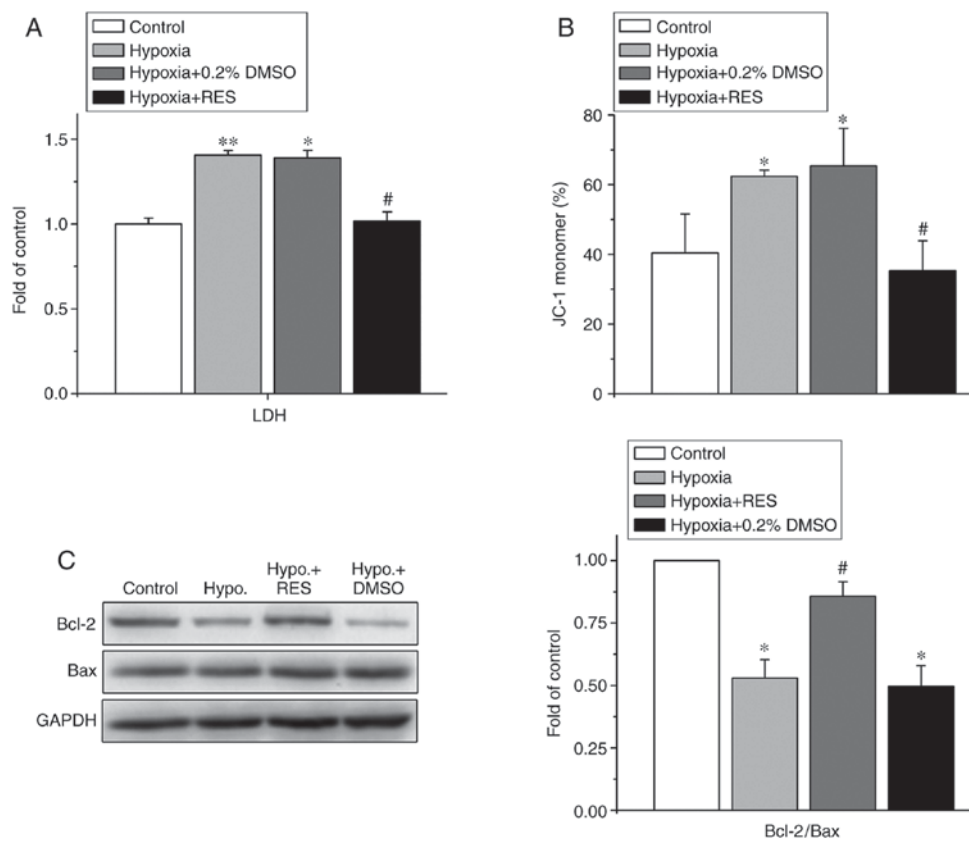


Figure 2. Effects of RES on H/R injury-induced mitochondria oxidative stress in neonatal rat cardiomyocytes. (A) Effect of RES on LDH release (n=4). (B) Effect of RES on the $\Delta\Psi_m$, as measured with the JC-1 kit. The percentage of monomer JC-1 showed the level of $\Delta\Psi_m$. The increment of monomer JC-1 represents the depolarization of $\Delta\Psi_m$, whereas the decrement of monomer JC-1 indicates the hyperpolarization of $\Delta\Psi_m$. (C) Western blotting of the protein levels of Bcl-2 and Bax (left) and the ratio of Bcl-2/Bax (right) (n=5). *P<0.05, vs. control; #P<0.05, vs. H/R treatment. RES, resveratrol; H/R, hypoxia/reoxygenation; LDH, lactate dehydrogenase; $\Delta\Psi_m$, mitochondrial membrane potential; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein; Hypo, hypoxia; DMSO, dimethyl sulfoxide.

injury exhibited significant alterations in morphology and cytoskeletal structure, characterized by disordered α -actin and F-actin. In contrast to the NRCMs in the H/R injury group, the impairment of cell structure induced by H/R injury in the NRCMs was significantly attenuated by treatment with 100 μ M RES. Treatment with 0.2% DMSO, the final concentration used for diluting RES, did not affect H/R injury-induced structural impairment.

Effects of RES on H/R injury-induced oxidative stress of mitochondria. Mitochondrial oxidative stress is a hallmark of I/R injury in cardiomyocytes. The present study further examined the effect of RES on mitochondrial oxidative stress injury and cell apoptosis, including the determination of LDH release, $\Delta\Psi_m$ and the Bcl2/Bax ratio. The Bcl2/Bax ratio in each group was normalized to the value in the control group. As shown in Fig. 2A, exposure to H/R increased LDH release by 1.41±0.03-fold compared with that of the control group (P<0.01, n=4), whereas 100 μ M RES significantly attenuated the increased release of LDH induced by H/R injury in the NRCMs from 1.41±0.03-fold (H/R) to 1.02±0.06-fold (P<0.01, n=4). Treatment with 0.2% DMSO did not affect LDH release when compared with the H/R group. Treatment with H/R induced the depolarization of $\Delta\Psi_m$ by shifting the ratio of JC-1 monomers from 40.43±11.21% (control) to 62.39±1.82% (H/R) (P<0.05, n=4), whereas treatment with 100 μ M RES alleviated

the H/R-induced depolarization of $\Delta\Psi_m$ by shifting the ratio of JC-1 monomers from 62.39±1.82% (H/R) to 35.31±8.63% (H/R +100 μ M RES) (P<0.05, n=4), as shown in Fig. 2B.

Furthermore, the ratio of Bcl2/Bax expression was examined, as detected by western blotting (Fig. 2C). Exposure to H/R decreased the Bcl2/Bax ratio to 0.53±0.08-fold of the control (P<0.05, n=5), whereas treatment with 100 μ M RES rescued the H/R-induced decrease in the Bcl2/Bax ratio to 0.86±0.06-fold (P<0.05, n=5).

Effects of RES on H/R-injury-induced apoptosis of NRCMs.

The present study also detected the effect of RES on apoptosis induced by H/R injury in NRCMs, as shown in Fig. 3. The results of the flow cytometry (Fig. 3A and B) showed that RES attenuated the H/R injury-induced cell apoptosis of NRCMs, with a decrease in the cell apoptotic rate from 84.25±7.41% (H/R) to 46.39±5.43% (H/R+RES) (P<0.05, n=4). Treatment with 0.2% DMSO alone did not significantly alter the effect of H/R on the apoptotic rate of cells (P>0.05, n=4).

In addition, caspase 3 is a common downstream signaling molecule involved in cell apoptosis induced by different factors. The present study found that RES alleviated the H/R injury-induced apoptosis of NRCMs (Fig. 3C). The activity of caspase 3 in each group was normalized to the value in the control group. Exposure to H/R increased the activity of caspase 3 to 1.32±0.06-fold compared with the control group (P<0.05,

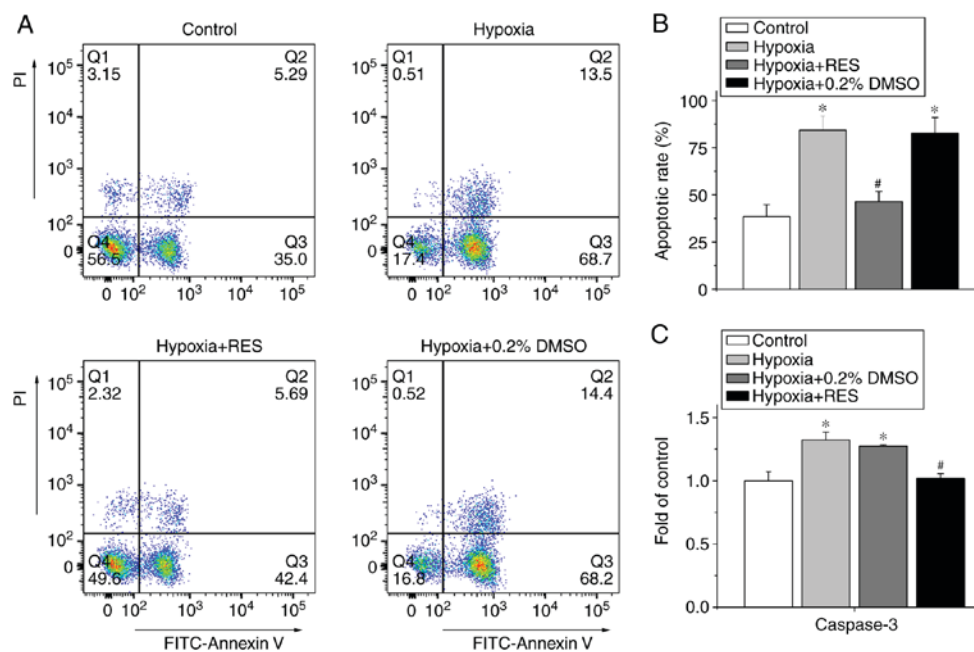


Figure 3. Effects of RES on H/R injury-induced apoptosis of NRCMs. (A) Typical original FCM plots showing the effect of RES on H/R injury-induced apoptosis of NRCMs. FITC Annexin V- and PI-negative cells were viable and did not undergo apoptosis; FITC Annexin V-positive and PI-negative cells were in early stage apoptosis; FITC Annexin V- and PI-positive cells were in the late stage of apoptosis. (B) Statistical bar graph showing the effect of RES on the apoptotic ratio (n=4). (C) Statistical bar graph showing the activity of caspase 3 in NRCMs that underwent different treatments (n=5). *P<0.05, vs. control; #P<0.05, vs. H/R treatment. NRCMs, neonatal rat cardiomyocytes; H/R, hypoxia/reoxygenation; RES, resveratrol; DMSO, dimethyl sulfoxide.

n=5), whereas treatment with 100 μ M RES alleviated the increased activity of caspase 3 to 1.02 \pm 0.04-fold (P<0.05, n=5).

Sirt1 is involved in the effect of RES on H/R injury in NRCMs. Sirt1 is reportedly an important target of RES, involved in the cardioprotective effects of several drugs indicated for cardiovascular diseases. Therefore, the present study examined whether Sirt1 was involved in the effects of RES on H/R injury in NRCMs. The data are shown in Fig. 4. The expression of Sirt1 in each group was normalized to the value in the control group. Treatment of H/R decreased the expression of Sirt1 to 0.61 \pm 0.06-fold compared with that in the control group (P<0.05, n=5), whereas treatment with RES alleviated the decrease of Sirt1 induced by H/R injury to 1.01 \pm 0.05-fold (P<0.05, n=5).

Discussion

CVDs are the major causes of morbidity and mortality in most countries. I/R injury is a leading cause of myocardial cell death following myocardial infarction. Oxidative stress is the main pathophysiological process involved in I/R injury and other CVDs, including heart failure and hypertension (23,24). Active cardiomyocytes consume energy from the aerobic metabolic pathway, and cardiomyocytes are more sensitive to hypoxia than other cell types. Cardiomyocytes are more prone to metabolic dysfunction, as they are decompensated during the acute I/R condition. Although several studies have verified the cardioprotective effects of RES through inhibiting inflammation and platelet aggregation, alleviating endothelial damage and modulating autophagy, current knowledge is limited regarding the role of oxidative stress injury on RES cardioprotection (9,25). In the present study, an *in vitro* H/R

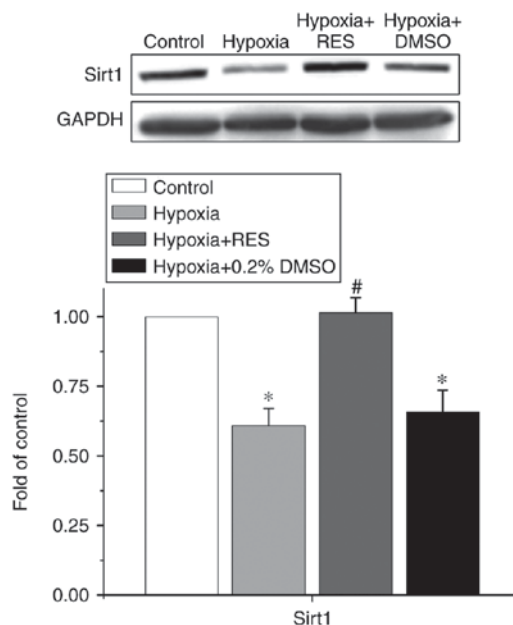


Figure 4. Sirt1 is involved in the effect of RES on H/R injury in NRCMs. Results showing the effects on Sirt1. *P<0.05, vs. control; #P<0.05, vs. H/R treatment (n=5). H/R, hypoxia/reoxygenation; RES, resveratrol; Sirt1, sirtuin 1; DMSO, dimethyl sulfoxide.

model was established using NRCMs to imitate I/R injury, and the role of RES on H/R-induced NRCM injury and underlying mechanism were examined. It was found that RES alleviated H/R-induced NRCM injury and apoptosis through attenuating the mitochondria-mediated oxidative stress pathway.

Dong *et al* (26) reported that resveratrol protected against pressure-overload-induced cardiac structure injury, and

exerted beneficial effects on cardiac hypertrophy in a rat model. In the present study, it was found that treatment with RES ameliorated H/R-induced cardiomyocyte structural impairment with F-actin and α -actinin 2, indicating the cytoskeleton and T-tubules (Fig. 1).

Mitochondria are the main organelle involved in biological oxidative reactions. Mitochondria are abundant in cardiomyocytes and the mitochondria-mediated oxidative stress pathway is involved in the cardiac I/R injury process (5,17). LDH levels, $\Delta\Psi_m$ and the ratio of Bcl-2 to Bax are important indices for reflecting the mitochondria-mediated oxidative stress status (27). The present study found that H/R treatment induced mitochondria oxidative stress, whereas treatment with RES alleviated H/R-induced mitochondrial injury through decreasing the release of LDH, inhibiting the depolarization of $\Delta\Psi_m$ and increasing the ratio of Bcl-2 to Bax (Fig. 2). These data suggest that RES attenuates H/R-induced cardiomyocyte injury through alleviating mitochondria-mediated oxidative stress; mitochondria are targets of RES involved in the cardioprotective effect to attenuate the H/R-induced injury of NRCMs.

The mitochondria-mediated route is an important apoptotic pathway in cells. Enhanced oxidative stress induces cell injury through mitochondria-mediated cell apoptosis. In the present study, it was found that treatment with RES inhibited the apoptosis induced by H/R injury through alleviating the cell apoptotic rate and activity of caspase 3, which reflects the status of cell apoptosis (Fig. 3). Therefore, these results support the hypothesis that RES alleviates H/R injury and exerts cardioprotective effects through the mitochondria-mediated signaling pathway.

In addition, Sirt1, a member of the conserved sirtuin family, is an NAD⁺-dependent histone deacetylase, which is involved in the various cardiac pathophysiological process and cardioprotective effects of certain drugs. Previous studies have suggested that Sirt1 is required for the RES-mediated cardioprotective effect (28,29). Sin *et al* (30) reported that Sirt1 was involved in the effect of RES for alleviating doxorubicin-induced cardiotoxicity in aged hearts. Recently, Li *et al* (21) reported that Sirt1 is involved in hypoxia-induced pulmonary artery smooth muscle cell apoptosis. The data obtained in the present study are consistent with those of former studies, and also provide novel evidence that Sirt1 is involved in the cardioprotective effect of RES with respect to H/R injury.

The present study found that the cardioprotective effect of RES in H/R-induced cardiomyocyte injury occurs through alleviating mitochondrial oxidative stress and restoring the expression of Sirt1. The results further elucidated the underlying mechanism of RES cardioprotection, and support the widespread clinical use of RES for cardiovascular disease.

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Availability of data and materials

The datasets and materials used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

XQT, TL and LC designed the experiments. TL, LC, YY, BY and PL performed the experiments and collected the data. XQT, TL and LC analyzed and interpreted the data. XQT and TL wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The experimental protocols were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (National Institutes of Health, Bethesda, MD, USA) and with the approval of the Animal Care and Use Committee of the Southwest Medical University, (Sichuan, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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