Journal of Physiology and Biochemistry (2019) 75:607–618
https://doi.org/10.1007/s13105-019-00704-5

ORIGINAL ARTICLE

Resveratrol pretreatment alleviates myocardial ischemia/reperfusion injury by inhibiting STIM1-mediated intracellular calcium accumulation

Han Xu[1,2] · Jing Cheng[3] · Xianbao Wang[4,5,6,7] · Haiqiong Liu[4,5,6,7] · Siyi Wang[4,5,6,7] · Jixiong Wu[1] · Banglong Xu[1] · Aihua Chen[4,5,6,7] · Fei He[1]

Received: 18 January 2019 / Accepted: 2 September 2019 / Published online: 30 November 2019

Abstract

Previous studies have shown that stromal interaction molecule1 (STIM1)-mediated store-operated Ca^{2+} entry (SOCE) contributes to intracellular Ca^{2+} accumulation in H9C2 cells subjected to hypoxia/reoxygenation(H/R) injury. The aim of the present study was to investigate the effect of resveratrol on STIM1-mediated intracellular Ca^{2+} accumulation and subsequent cell death in the context of myocardial ischemia/reperfusion (I/R) injury. C57 BL/6 mice were fed with either saline or resveratrol (50 mg/kg daily for 2 weeks) and then subjected to myocardial I/R injury. TTC/Evans Blue staining and TUNEL assay were performed to quantify the infarct size and apoptosis index. The cardiac function was evaluated by echocardiography. Neonatal rat ventricular cardiomyocytes (NRVCs) underwent hypoxia/reoxygenation (H/R) to establish the in vitro model. To achieve over-expression, NRVCs were transfected with STIM1-adenovirus vector. Apoptosis was analyzed by TUNEL assay. Cell viability was measured using MTS assay and cell necrosis was determined by LDH release assay. Intracellular Ca^{2+} concentration was detected by laser scanning confocal microscopy using a Fluo-3AM probe. Resveratrol significantly reduced apoptosis, decreased infarct size, and improved cardiac function in mice subjected to myocardial I/R injury. In NRVCs, resveratrol also downregulated STIM1 expression accompanied by decreased intracellular Ca^{2+} accumulation elicited by H/R injury. In addition, resveratrol reduced cell apoptosis, upregulated the Bcl-2, decreased Bax, and cleaved caspase-3 expression. Furthermore, the effects of resveratrol on STIM1-mediated intracellular Ca^{2+} accumulation, apoptotic proteins, and H/R-induced cell injury were exacerbated by STIM1 over-expression and were partly abolished by SOCE inhibitor SKF96365 in NRVCs in vitro. Our findings demonstrate that resveratrol exerts anti-apoptotic activity and improves cardiac functional recovery following myocardial I/R by inhibiting STIM1-induced intracellular Ca^{2+} accumulation.

Keywords Resveratrol · Ischemia reperfusion injury · STIM1 · Calcium · Apoptosis

Han Xu and Jing Cheng contributed equally to this work.

✉ Aihua Chen
13380092288@189.cn

✉ Fei He
coffeelove3344@163.com

[1] Department of Cardiology, Second Affiliated Hospital of Anhui Medical University, Hefei, Anhui, China

[2] Clinical Medical College of Anhui Medical University, Hefei, Anhui, China

[3] School of Nursing, Anhui University of Traditional Chinese Medicine, No.103 of Middle Meishan Road, Hefei, Anhui, China

[4] Department of Cardiology, Heart Center, Zhujiang Hospital, Southern Medical University, Guangzhou, Guangdong, China

[5] Guangdong Provincial Biomedical Engineering Technology Research Center for Cardiovascular Disease, Guangzhou, Guangdong, China

[6] Sino-Japanese Cooperation Platform for Translational Research in Heart Failure, Guangzhou, Guangdong, China

[7] Laboratory of Heart Center, Zhujiang Hospital, Southern Medical University, Guangzhou, Guangdong, China

Abbreviations

AAR	Area at risk
PCI	Percutaneous coronary intervention
NCX	Na^+-Ca^{2+} exchanger
ACS	Acute coronary syndrome
AMI	Acute myocardial infarction
STEMI	ST segment elevation myocardial infarction
SOCE	Store-operated calcium channels
STIM	stromal interaction molecule
RSV	Trans-3,4′,5-trihydroxystilbene
I/R	Ischemia/reperfusion
DMEM	Dulbecco's modified Eagle's medium
FBS	Fetal bovine serum

Introduction

Acute myocardial infraction (AMI) is a common disease associated with death and disability worldwide. After the onset of acute myocardial infraction, the most effective therapy for reducing myocardial ischemic injury and limiting infraction size is timely myocardial reperfusion including percutaneous coronary intervention therapy. Nevertheless, reperfusion therapy itself can cause unexpected myocardial damage and dysfunction including ventricular arrhythmia, myocardial stunning, and microvascular obstruction, which is also known as myocardial ischemia reperfusion (I/R) injury. Even though the substantial processes of myocardial I/R injury have been consistently investigated, there are still no effective therapies for preventing myocardial reperfusion injury in clinical practice [1]. Therefore, it is absolutely essential to explore underlying mechanisms and effective strategies for preventing and reducing myocardial I/R injury.

As an important second messenger, calcium (Ca^{2+}) is involved in the signaling networks that modulate pathological conditions, in addition to myocardial contraction. Experimental studies have shown that Ca^{2+} overload is a crucial modulator of mitochondrial dysfunction and cardiomyocyte apoptosis in the process of myocardial I/R injury [2–5]. Over the past decades, both the Na^+/H^+ exchanger (NHE) and the Na^+-Ca^{2+} exchanger (NCX) have been considered responsible for the sustained rise of intracellular Ca^{2+} in the ischemia stage [4, 5]. In the subsequent reperfusion stage, reactive oxygen species (ROS) is produced to mediate dysfunction of the endoplasmic reticulum (ER) and cytomembrane by inducing the opening of mitochondrial permeability transition pore (mPTP). mPTP is an unselective channel, whose opening can activate apoptosis of protein caspases, Bcl-2, and Bax, finally leading to cell death [2].

Recent studies have suggested that store-operated calcium channels (SOCE) also account for the influx of extracellular calcium in several excitable cells, including the cardiomyocytes [6]. Both stromal interaction molecule (STIM) and Orai1 protein have been identified as the core components of SOCE. Decreased Ca^{2+} concentration in ER activates SOCE that promotes Ca^{2+} influx, followed by a sustained raise in cytosolic Ca^{2+} concentration and subsequent activation of numerous signal transduction pathways [6]. STIM1 is the predominant isoform of the STIM family. In our previous study, we have found that STIM1 significantly increases in the transcript and translation level after myocardial I/R injury in vivo and H/R in vitro. We have further demonstrated that suppression of STIM1 reduced intracellular calcium concentration and attenuated hypoxia-reoxygenation-induced apoptosis in H9C2 cells, providing a new perspective on the STIM1-mediated calcium overload in the myocardial I/R injury [7].

Resveratrol (trans-3,4′,5-trihydroxystilbene (RSV)) is an anti-inflammatory antioxidant and anti-apoptotic polyphenol found in grapes, berries, and other plants [8]. Several studies have reported that resveratrol confers beneficial effects on cardiovascular disease, containing the myocardial I/R injury [9–11]. Previous studies have suggested that resveratrol can significantly attenuate the intercellular Ca^{2+} level and reduce the myocardial apoptosis and myocardial infarct size [12]. Moreover, several studies have reported opposite effects of resveratrol on STIM1 expression in different types of cells [13–15]. Thus, the effect of resveratrol on STIM1 expression in cardiomyocytes and subsequent Ca^{2+} concentration as well as their role in myocardial I/R injury remains unknown. The present study was designed to investigate the effects of resveratrol on STIM1-induced Ca^{2+} influx in the context of myocardial I/R injury.

Materials and methods

Animals

Adult male C57 BL/6 mice (10–12 weeks old; 26–30 g) were obtained from the animal center of Southern Medical University. All experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals (National Institutes of Health publication, No. 86-23, revised 1996) and were approved by the Bioethics Committee of the Anhui Medical University. The animals were purchased and housed in a temperature-controlled environment with 12-h light/dark cycles. They were given free access to food and water.

Myocardial I/R injury model

Murine myocardial I/R injury model was established as previously described [7, 16, 17]. Briefly, after an adequate anesthesia attained by intraperitoneal injection of 5% chloral hydrate (0.01 mL/g), mice were fixed in the supine position with

a thermostatic tape. The upper thorax region was shaved and the tongue was retracted. Then, a 24-gauge i.v. catheter was inserted into the trachea. The catheter was subsequently connected to a small animal ventilator (HX-101E, Techman Soft Co., Ltd., Chengdu, China) via the Y-shaped connector. Mice were ventilated with a tidal volume of 2.0 to 2.5 mL and their respiratory rate was 120 breaths per minute. To properly expose the heart, a left thoracotomy was performed by separating the fourth and fifth intercostal spaces. To establish the I/R model, the left descending artery (LAD) was occluded using an 8–0 silk suture tied transiently over PE-10 tubing for 30 min. Successful ischemia was visually confirmed by rapid myocardial blanching as well as ST segment elevation on continuous electrocardiography (ECG). After 120-min reperfusion, the knot on the PE-10 tubing was cut. Reperfusion was determined by recovery of the elevated ST segment on surface ECG. Sham-operated mice underwent the same surgical procedures with the exception of LAD occlusion.

Measurement of myocardial infarct size

2,3,5-Triphenyltetrazolium chloride (Sigma-Aldrich, St. Louis, MO, USA) and Evans blue (Sigma-Aldrich, St. Louis, MO, USA) dye were applied for the measurement of myocardial infract size (IS) as previously described [7, 16]. Briefly, at the end of reperfusion, the knot was re-tied and 2 mL 2% Evans blue was injected through right jugular vein. Next, the whole heart was immediately removed and gently washed with PBS in order to get rid of residual blood. Subsequently, the heart was cut into cross-sectional pieces and immersed into 1% TTC solution until the pieces became red which were then fixed in 10% formaldehyde for 12 min. Generally, the area stained with Evans blue represented the non-I/R myocardium, whereas the unstained area was the I/R myocardium, or area at risk (AAR). Within the AAR, I/R but viable myocardium was stained brick red by TTC, whereas dead myocardium (infarct) was white. The IS was calculated as IS/AAR (%). Images were captured using a camera and the area of the infarcted myocardium was analyzed with ImageJ software.

Echocardiography

Cardiac function was evaluated by non-invasive transthoracic echocardiography using a Visual Sonics Vevo 770 system with a RMV707B probe (Visual Sonics, France) 7 days after surgery [16]. Mice were anesthetized using 2% isoflurane, and the left chest was denuded. M-mode echocardiographic views of the mid-ventricular short axis were obtained at the level of the papillary muscle tips below the mitral valve. The left ventricular end-diastolic (Did) and systolic (Dis) diameter and end-diastolic (Vid) and systolic (Vis) dimension were measured. Left ventricular fractional shortening (LVFS) and ejection fraction (EF) were calculated as follows: LVFS% = (Did − Dis) × 100/Did EF% = (Vid − Vis) × 100/Vid

Isolation of neonatal rat ventricular cardiomyocytes

Neonatal rat ventricular cardiomyocytes (NRVCs) were isolated from the whole heart of Sprague–Dawley rats using a modified protocol as described in our previous study [18]. In brief, the heart was rinsed with D-Hank's (Hyclone, China) solution to remove blood and was immersed into 0.25% trypsin solution for 10 min at 37 °C for preliminary digestion. Next, digestion was terminated by adding Dulbecco's modified Eagle's medium (Hyclone, China) containing 10% fetal bovine serum (Hyclone, China) at 37 °C for 5 min. Then, the heart underwent subsequent digestion with 0.1% type II collagenase at 37 °C for 5 min several times until there were no visible tissues left. Finally, cells were resuspended in DMEM supplemented with 10% FBS and antibiotics. To remove myocardial fibroblasts, the differential adhesion method and bromodeoxyuridine (BrdU, 1 mM, Sigma, USA) were applied for the first 24 h.

Cardiomyocyte H/R injury model

NRVCs were randomly divided into three groups: (1) Control group NRVCs were incubated with 5%CO_2 and 95% air in regular culture medium for experimental period. (2) H/R group NRVCs were exposed to hypoxia by transferring the culture plates to a humidified incubation chamber (37 °C, 5%CO_2, and 95%N_2). After hypoxia for 3 h, NRVCs were switched to standard condition with 5%CO_2 and 95% air for reoxygenation for 3 h. (3) H/R + resveratrol group NRVCs were pretreated with resveratrol (Sigma-Aldrich, St. Louis, MO, USA) at a final concentration of 10, 30, and 50 μmol to the culture medium 24 h before hypoxia and then underwent H/R procedure same as the H/R group.

Cell transfection

STIM1 over-expression recombinant adenovirus (Ad-STIM1) and empty carrier recombinant adenovirus (Ad-control) were produced from Shanghai ALLABio. NRVCs were transfected with GFP-STIM1 by adenovirus as described by the manufacturer. After 48 h of transfection, NRVCs were washed by PBS for three times and images were collected using a MRC1024-krypton/argon laser scanning confocal equipped with a Zeiss LSM 510 Meta photomicroscope.

TUNEL staining

Apoptosis was analyzed using TUNEL (Invitrogen) assay according to the manufacturer's instructions [7, 16]. Apoptotic nucleuses were visualized with light microscopy or fluorescent microscopy. The TUNEL-stained cell (%) was calculated according to the distribution of myocardial cells under microscopy (× 100). Five random fields were chosen in each section, and 200 cells were counted in each field, after which the average percentage of the apoptotic cell was calculated as the apoptotic index.

Cell viability measurement

Cell viability was measured by the MTS assay kit (Promega, Beijing, China) according to the manufacture's instruction. NRVCs were seeded in 96-well plates at a density of 1×10^4 cells per well and were pretreated with different concentrations of resveratrol (0, 10, 30, 50 μM). After 24-h treatment, 10 μL MTS solution was added to each well of a 96-well plate and incubated for 2 h at 37 °C. The optical density value was measured at an absorption wavelength of 490 nm.

LDH detection

Cells (1×10^4/well) were seeded in 96-well plates and treated with resveratrol for 24 h. Lactate dehydrogenase (LDH) is a cytoplasmic enzyme released by dying cells. LDH was determined by a cytotoxicity assay kit (Beyotime, Shanghai, China) according to the manufacturer's protocol [18]. The optical density was spectrophotometrically measured at 490 nm on a microplate reader (ELx800; Bio-Tek, Winooski, VT). The percentage of specific lysis was calculated as [(experimental release-spontaneous release)/(maximum release − spontaneous release)] × 100.

Measurement of intracellular Ca^{2+} concentration

Fluo-3 AM (Beyotime, Shanghai, China) is a fluorescent dye that can penetrate the cell membrane. After entering the cell, it can be cut by esterase into Fluo-3 to combine with Ca2+. For measuring the cytosolic [Ca^{2+}], the cell-culture medium was removed and the cells were washed once with modified Krebs solution (135 mM NaCl, 5.9 mM KCl, 1.2 mM MgCl2, 11.6 mM HEPES, 11.5 mM glucose, and 1.5 mM CaCl2). Cells were loaded with Fluo-3AM (5 μM) for 30 min at room temperature in modified Krebs solution. The cells were then washed with the same solution and incubated for 30 min in the absence of Fluo-3AM.

Western blot

Samples were obtained from NRVCs or mouse hearts. Protein concentrations were measured via the BCA method (Thermo Fisher), and equal amounts of protein were resolved via SDS/PAGE and transferred to PVDF membranes (Millipore). After blocking in 5% non-fat milk at room temperature for 2 h, membranes were incubated with the following primary antibodies overnight at 4 °C: STIM1, β-actin, Bcl-2, Bax, and cleaved caspase-3 (all from Cell Signaling Technology). All dilutions were 1:1000. After incubation for 2 h at room temperature with secondary antibodies (Boster), the bands were visualized using enhanced chemiluminescence (Millipore). The blots were quantified by densitometry using ImageJ software (Media Cybernetic) and the relative protein expression was compared with β-actin.

Statistical analyses

All measurements are expressed as the mean ± SEM. The differences between groups were analyzed with one-way ANOVA followed by Student-Newman-Keuls post hoc analysis for pair-wise comparisons. $P < 0.05$ was considered statistically significant.

Results

Resveratrol improves cardiac function and downregulates STIM1 expression

Administration of resveratrol at a dosage of 50 mg/kg showed the most obvious apoptosis-inhibiting effect. Thus, to explore the effect of resveratrol on the cardiac function, mice were pretreated with resveratrol at this dose and then subjected to Doppler echocardiography 7 days after surgical procedure ($n = 10$ in each group). The representative echocardiographic figures are shown in Fig. 1a . We found that both LVFS and EF were significantly lower in the I/R group compared with the sham group and would dramatically improve following the resveratrol pretreatment (Fig. 1b and c).

To determine the effect of resveratrol on the extent of myocardial I/R injury, myocardial infarct size was analyzed using TTC/Evans blue dye 24 h after surgical procedure. The representative mid-ventricular cross-sections are shown in Fig. 1d ($n = 10$ in each group). IS in the I/R + RVS group was significantly reduced compared with the I/R group (Fig. 1e). There were no significant differences in AAR/LV among the groups (Fig. 1f).The results showed that I/R injury led to a dramatic increase

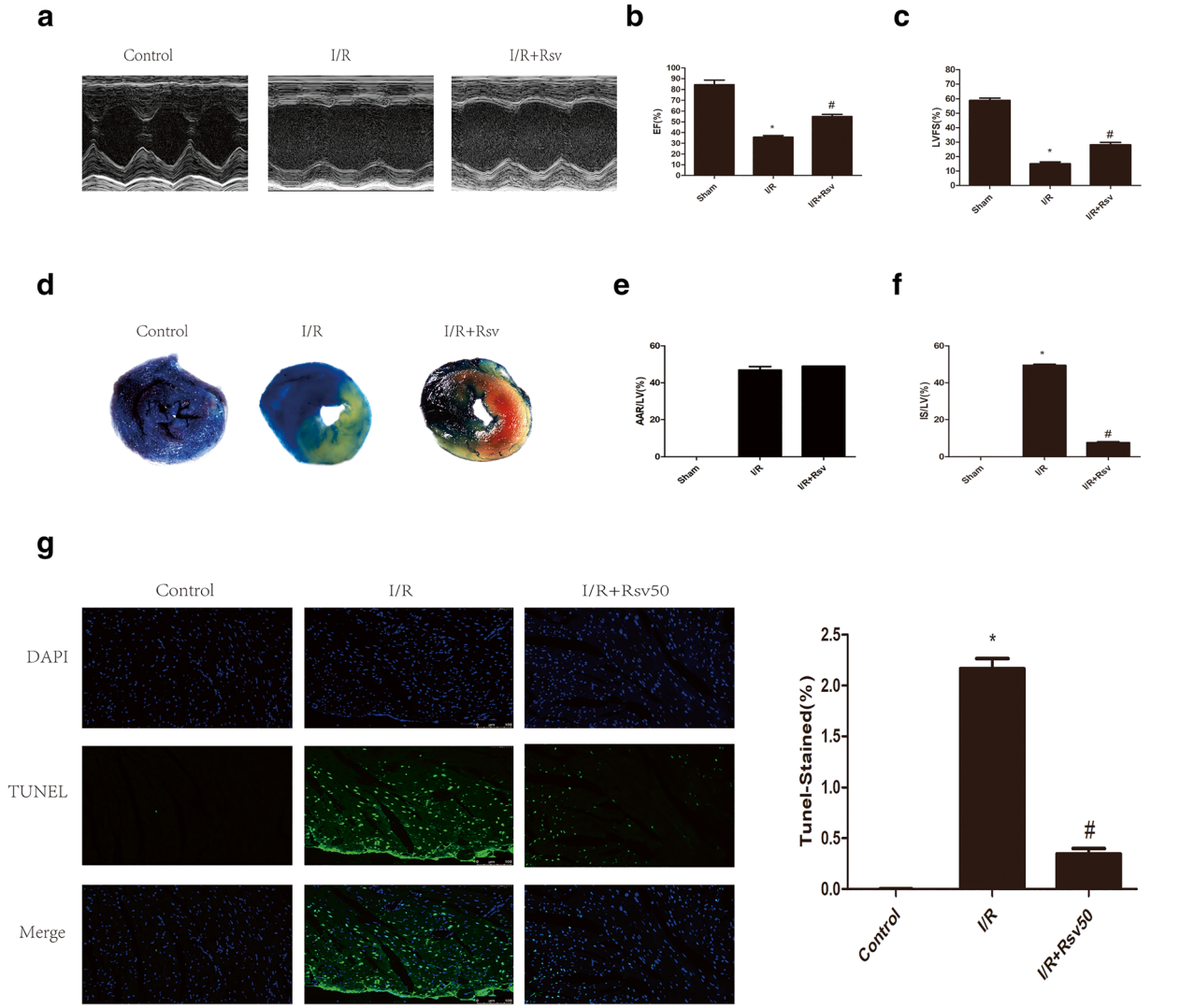

Fig. 1 Resveratrol reduced myocardial infarct size and improved cardiac function in I/R mice. Resveratrol improved cardiac function and down-regulated STIM1 expression. **a** Representative echocardiograms obtained from each group. EF (**b**) and LVFS (**c**) were measured by M-mode echocardiography ($n = 10$ in each group). $^{*}P < 0.01$ compared with the sham group, $^{\#}P < 0.05$ compared with the I/R group. **d** Representative TTC–Evans Blue stained sections of hearts from each group. Brick red–stained area represents viable myocardium, whereas the unstained (white) area represents infarcted myocardium. **e** The ratio of AAR/LV and **f** IS/AAR. **g** Effects of Rsv (50 mg/kg) on cardiomyocyte apoptosis in mice subjected to I/R were also evaluated.$^{*}P < 0.05$ compared with the control group, $^{\#}P < 0.05$ compared with the I/R group

in IS compared with the Sham group, suggesting the successful attainment of the in vivo myocardial I/R injury model.

To evaluate the effect of resveratrol on the apoptosis elicited by myocardial I/R injury, myocardial apoptosis was analyzed using TUNEL assay 24 h after the I/R procedure. The representative figure of TUNEL-positive cells in the myocardial slices from mice is displayed in Fig. 1g. The obtained results showed that the apoptotic index in the myocardial I/R injury group was obviously higher than that in the Sham operation group, suggesting the successful attainment of the in vivo myocardial I/R injury model. In addition, resveratrol pretreatment at a dose level of 50 mg/kg significantly alleviate myocardial apoptosis assessed by TUNEL (Fig. 1g, $n = 10$ in each group), suggesting that resveratrol pretreatment could significantly reduce myocardial infarct size and apoptosis in mice underwent I/R injury.

Effects of resveratrol on STIM1 and apoptosis-related protein expression

Since Bcl-2, Bax, and caspase-3 protein may have an active role in the process of apoptosis, expression of these proteins was measured by western blot. As shown in Fig. 2a , myocardial I/R injury led to a significant decrease in Bcl-2 and increase in Bax expression. Resveratrol pretreatment at a dose of 50 mg/kg significantly upregulated the Bcl-2 level and down-regulated the Bax expression. Besides, cleaved caspase-3 was obviously increased after myocardial I/R injury.

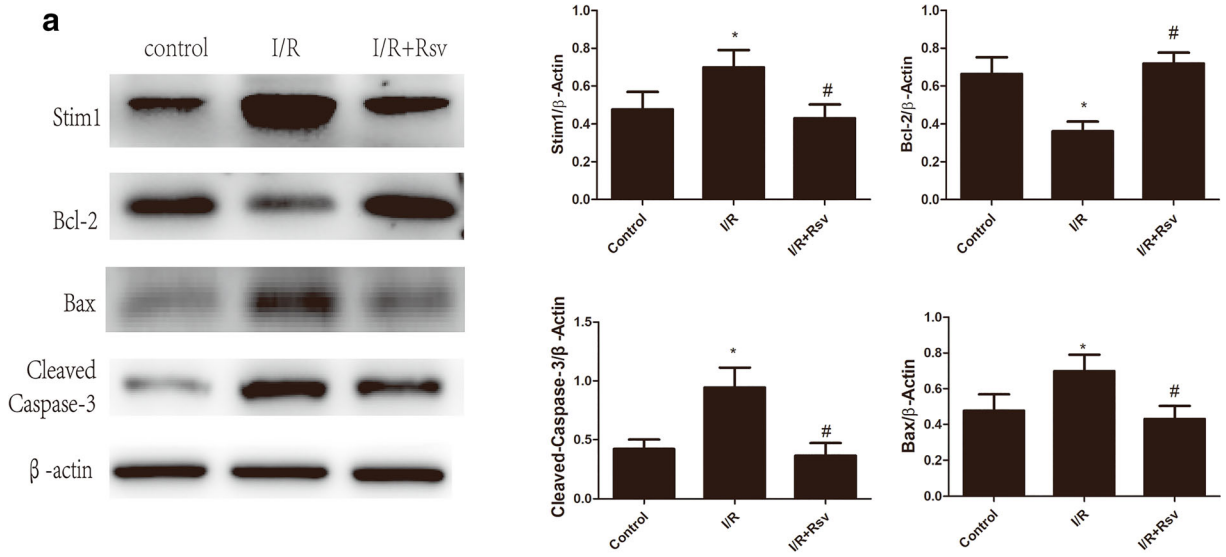

Fig. 2 Effects of resveratrol on STIM1 and apoptosis-related protein expression. **a** Western blot of STIM1, Bcl2, Bax, and cleaved caspase-3 in each group. $^{*}P < 0.05$ compared with the control, $^{\#}P < 0.05$ compared with the I/R group

Administration of resveratrol dramatically inhibited cleaved caspase-3 expression elicited by the I/R injury. The above data revealed that resveratrol significantly improves cardiac function and downregulates STIM1 and pro-apoptotic protein expression.

Resveratrol alleviates H/R-induced cell injury in NRVCs

To establish the in vitro hypoxia/reoxygenation (H/R) injury model, NRVCs underwent hypoxia for 180 min followed by reoxygenation for another 180 min. The validity of the H/R model was evaluated by cell viability and LDH release. Results showed that the cell viability measured by the MTS assay in the H/R injury group was significantly lower compared with that in the control group (Fig. 3a). Furthermore, resveratrol pretreatment rescued cell viability in a dose-dependent manner. Resveratrol pretreatment at a dose level of 50 μmol/L had the most significant effect (Fig. 3a). In contrast, cell necrosis measured by LDH release in the H/R injury group was significantly higher than that in the control group (Fig. 3b). Similarly, resveratrol pretreatment reduced LDH release in a dose-dependent manner. Resveratrol pretreatment at a dose level of 50 μmol/L had the most significant effect in reducing LDH release elicited by H/R injury (Fig. 3b).

Resveratrol downregulates STIM1 and apoptotic protein expression and reduces intracellular Ca^{2+} accumulation in NRVCs suffering from H/R injury

STIM1 levels were significantly higher in the H/R group than in the control group. Administration of resveratrol led to downregulation of STIM1 expression in a dose-dependent manner. Resveratrol pretreatment at the dosage of 50 μmol/L had the most significant effect on downregulating STIM1 expression induced by the H/R injury (Fig. 3c).

Similarly as the in vivo study, H/R injury also led to an obvious decrease in Bcl-2 and increase in Bax expression. Resveratrol upregulated Bcl-2 and downregulated Bax expression. However, only pretreatment at the dose of 50 μmol/L had the significant effect on Bcl-2 and Bax expressions (Fig. 3c).

In addition, cleaved caspase-3 was obviously elevated after cell H/R injury. Administration of resveratrol at 30 μmol/L and 50 μmol/L dramatically inhibited cleaved caspase-3 level elicited by the H/R injury. The above data revealed that resveratrol significantly alleviates cell injury and downregulates STIM1 and pro-apoptotic protein expression in NRVCs. Administration of resveratrol at the dose of 50 μmol/L had the most significant protective effect.

Since STIM1 could mediate Ca^{2+} influx, we also measured intracellular Ca^{2+} level to confirm whether resveratrol pretreatment could reduce Ca^{2+} accumulation in the setting of cell H/R injury. As shown in Fig. 3d , intracellular Ca^{2+} level significantly increased as measured by Fluo-3AM probe. As expected, resveratrol pretreatment (50 μmol/L) obviously reduced intracellular Ca^{2+} accumulation. However, it remained uncertain whether resveratrol could directly inhibit STIM1-mediated Ca^{2+} influx.

STIM1 over-expression exacerbated apoptosis and induces intracellular Ca^{2+} accumulation and apoptotic protein expression in NRVCs suffering from H/R injury

To demonstrate that resveratrol directly regulates STIM1-mediated Ca^{2+} influx, NRVCs were transfected with Ad-

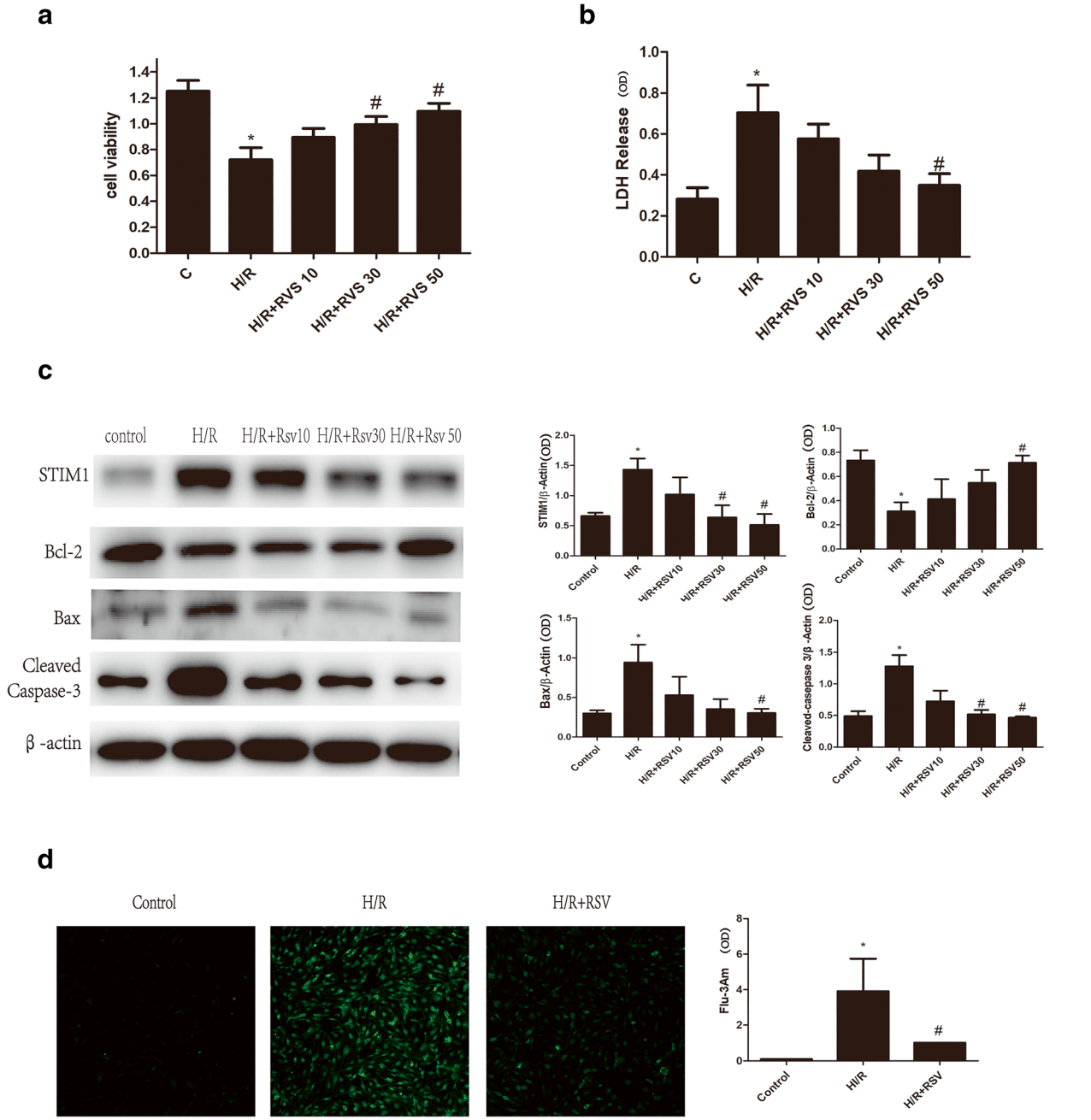

Fig. 3 Resveratrol alleviated H/R-induced cell injury in NRVCs. **a** Effects of different doses of resveratrol (10, 30, and 50 μM) on cell viability determined by MTS assay in cultured NRVCs.*$P < 0.05$ compared with the control group, $^{\#}P < 0.05$ compared with the H/R group. **b** Effects of different doses of resveratrol (10, 30, and 50 μM) cell necrosis determined by LDH release assay in cultured NRVCs. *$P < 0.05$ compared with the control group, $^{\#}P < 0.05$ compared with the H/R group. **c** Western blot of STIM1, Bcl2, Bax, and cleaved caspase-3 in the different dose group. *$P < 0.05$ compared with the control, $^{\#}P < 0.05$ compared with the H/R group. **d** Intensity of Ca^{2+} fluorescence marked by Fluo-3AM probe under fluorescence microscope significantly decreased after resveratrol treatment. *$P < 0.05$ compared with the control, $^{\#}P < 0.05$ compared with the H/R group

STIM1 to establish STIM1 over-expression which then underwent H/R injury. Transfection efficacy was determined by both fluoroscopy and Western blot (Fig. 4a). As illustrated in Fig. 4b , resveratrol pretreatment at a dosage of 50 μmol/L markedly alleviated apoptosis and necrosis in NRVCs that suffered from H/R injury. The protective effect of resveratrol pretreatment was partly eliminated by STIM1 over-expression as evidenced by augmented TUNEL-positive cell, impaired cell viability, and increased LDH release (Fig. 4b–d). In addition, the intracellular Ca^{2+} level assessed by Fluo-3AM in the H/R injury group was also significantly higher than that in the control group. Resveratrol pretreatment obviously reduced

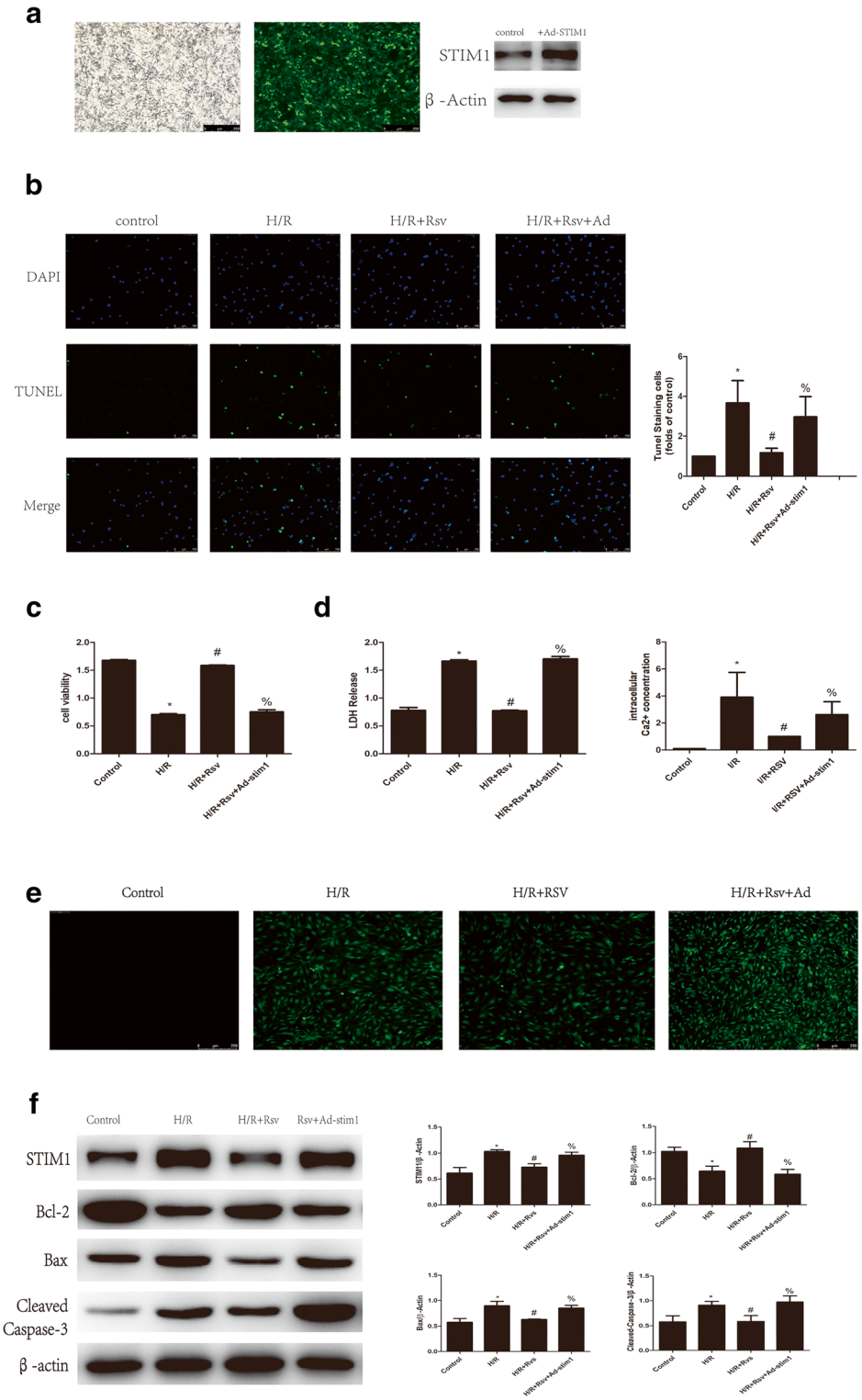

Fig. 4 STIM1 over-expression exacerbated apoptosis and induced intracellular Ca^{2+} accumulation and apoptotic protein expression in NRVCs suffering from H/R injury. **a** Infective efficiency in NRVCs after 48-h infection, over-expression of STIM1 was proven. **b** Representative TUNEL staining of NRVCs transfected with adenovirus. $^{*}P < 0.05$ compared with the control, $^{\#}P < 0.05$ compared with the I/R group, and $^{\%}P < 0.05$ compared with the resveratrol + H/R group. The protective effect of resveratrol pretreatment could be partly abolished by STIM1 over-expression as evidenced by impaired cell viability and increased LDH release (**c** and **d**). **e** Intensity of Ca^{2+} fluorescence marked by Fluo-3AM probe under fluorescence microscope was significantly enhanced after STIM1 transfection. **f** Western blot of STIM1, Bcl-2, Bax, and cleaved caspase-3 in STIM1 over-expressed NRVCs pretreated with resveratrol.$^{*}P < 0.05$ compared with the control, $^{\#}P < 0.05$ compared with the H/R group, and $^{\%}P < 0.05$ compared with the resveratrol + H/R group

intracellular Ca^{2+} accumulation. STIM1 over-expression before the H/R procedure significantly decreased intracellular Ca^{2+} concentration compared with the cells that were pretreated with resveratrol alone (Fig. 4e). Furthermore, over-expression of STIM1 by adenovirus transfection resulted in a noticeable decrease in Bcl-2 and increase in Bax compared with that of the resveratrol group. Cleaved caspase-3 significantly decreased in

NRVCs pretreated with resveratrol, and this effect was partly blocked by STIM1 over-expression.

Effects of STIM1 over-expression on apoptosis, intracellular Ca^{2+} accumulation, and apoptotic protein expression are partly abolished by the SOCE inhibitor

SKF96365 has been reported to selectively inhibit the SOCE-mediated Ca^{2+} entry. To further confirm that resveratrol directly regulates STIM1-mediated Ca^{2+} influx, NRVCs were treated with SKF96365 (5 μmol/L) to block STIM1-mediated SOCE before adenovirus transfection and the H/R procedure. As shown in Fig. 5 a, the intracellular Ca^{2+} level of NRVCs after H/R injury significantly decreased when treated with SKF96365. In addition, cell apoptosis induced by the H/R injury was distinctly reduced when treated with SKF96365. Furthermore, SOCE inhibition by SKF96365 pretreatment resulted in a visible increase in Bcl-2 and decrease in Bax compared with STIM1 over-expression plus the resveratrol pretreatment group. Cleaved caspase-3 was significantly increased in pretreated NRVCs transfected with Ad-STIM1 and treatment with resveratrol. This harmful effect of STIM1 over-expression was partly relieved by SKF96365 pretreatment.

Discussion

The present study found that resveratrol alleviated myocardial I/R injury in a murine model as evidenced by the obviously limited infarct size, decreased apoptotic index, and preserved cardiac function. Meanwhile, we found that resveratrol significantly downregulated STIM1 expression. To further explore whether resveratrol alleviated I/R injury through inhibiting STIM1-mediated Ca^{2+} influx, we performed an in vitro study in a simulated H/R injury model using NRVCs. The results showed that resveratrol attenuated H/R-induced cell injury of NRVCs by inhibiting STIM1 expression and subsequent Ca^{2+} entry, thereby altering the expression levels of apoptosis-related proteins including Bcl-2, Bax, and caspase-3. These results revealed the novel therapeutic mechanism of resveratrol for protection against the myocardial I/R injury.

Numerous studies have provided convincing evidence that resveratrol exerts beneficial effects on several cardiovascular diseases including hypertension, atherosclerosis, arrhythmia, diabetic cardiomyopathy, heart failure, and myocardial I/R injury [11]. For instance, Liao et al. have demonstrated that long-term oral resveratrol intake significantly improves left ventricular function and reduces the infarction size by down-regulating voltage-dependent anion channel 1 (VDAC1) expression and preventing mitochondrial permeability transition pore opening, which can eventually lead to myocardial apoptosis [19]. In addition, Yang et al. reported that resveratrol attenuated myocardial I/R injury via activating antioxidant signaling pathway [10]. Similarly, the present study revealed that resveratrol significantly improved left ventricular function and decreased infarct size. It has been reported by Selvaraj that resveratrol treatment decreases STIM1 expression in a time-dependent manner and reduces ER calcium storage and store-operated calcium entry (SOCE) in prostate cancer cells [15]. As far as we know, the interaction between resveratrol and STIM1-mediated SOCE in the context of myocardial I/R injury has not yet been elucidated. Thus, we performed a subsequent study to investigate whether resveratrol exerted cardioprotective effect by regulating STIM1-mediated SOCE.

Calcium overload has been widely considered as one of major mechanisms responsible for the pathogenesis of the myocardial I/R injury [20]. In general, strategies that inhibit Ca^{2+} influx or alleviate Ca^{2+} overload have been shown to partially relieve the myocardial I/R injury [2, 4, 21]. Recent studies have revealed that SOCE also accounts for Ca^{2+} entry in the cardiomyocytes [22–24]. In response to initial Ca^{2+} release from endoplasmic reticulum (ER), SOCE promotes the Ca^{2+} influx and leads to a sustained raise of intracellular Ca^{2+} concentrations [25, 26]. It has been demonstrated that STIM1 is one of the key mediators of SOCE [9, 13, 27, 28]. Our recent work has revealed that suppression of STIM1 reduces intracellular Ca^{2+} level and attenuates H/R-induced apoptosis in H9C2 cells [7]. Thus, manipulating STIM1-mediated SOCE in the setting of myocardial I/R injury might also confer cardioprotective effect. In the present study, we found that STIM1 expression was significantly upregulated and intracellular Ca^{2+} concentration was obviously elevated in NRVCs that underwent H/R injury. Furthermore, resveratrol treatment at different levels before H/R procedure dramatically alleviated cell injury in a dose-dependent manner. Additionally, we found that resveratrol pretreatment at 50 μmol/L markedly downregulated STIM1 expression and deceased intracellular Ca^{2+} accumulation. Besides, STIM1 over-expression achieved by adenovirus vector transfection significantly elevated intracellular Ca^{2+} level simultaneously with increased STIM1 expression in NVRCs in the context of H/R injury. This effect was in part canceled by the blockade of SOCE with the specific inhibitor SKF96365. Generally, the above data suggested that resveratrol downregulated STIM1 expression and SOCE-mediated Ca^{2+} accumulation in NRVCs under the condition of H/R injury.

Programmed cell death, including apoptosis, has been confirmed to remarkably contribute to the loss of cardiomyocytes in the pathogenesis of myocardial I/R injury [22]. Several apoptotic proteins, including Bcl-2, Bax, and caspase-3, are thought to have a major role in the determination of cell survival or death after apoptotic stimuli. Their expression levels in NRVCs were measured to determine the mechanism through which resveratrol decreases cell injury. Thus, we

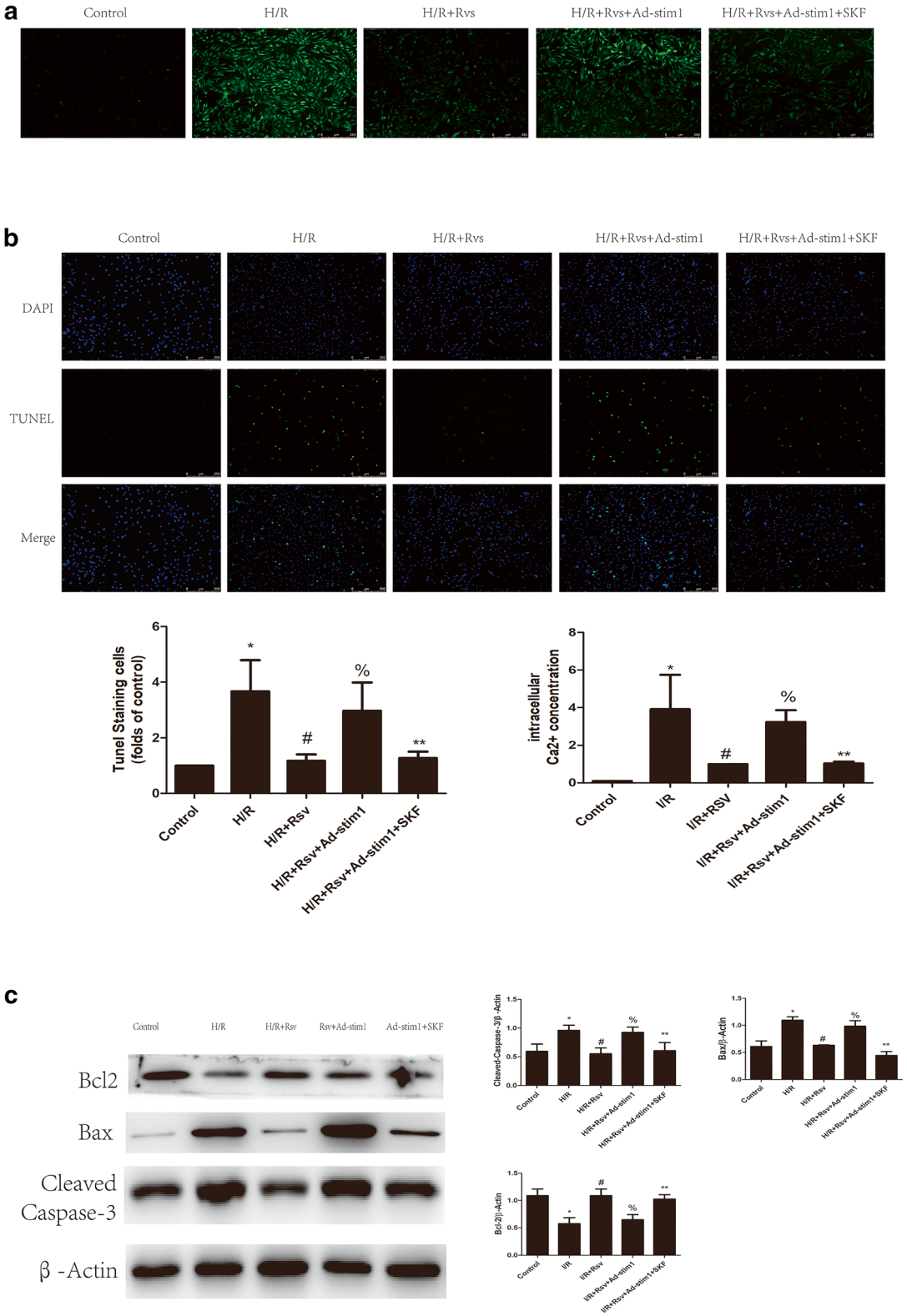

Fig. 5 Effects of STIM1 over-expression on apoptosis, intracellular Ca^{2+} accumulation, and apoptotic protein expression were partly abolished by the SOCE inhibitor. **a** Representative pictures of the intensity of Ca^{2+} fluorescence marked by Fluo-3AM probe under fluorescence microscope. **b** NRVCs were treated with SKF96365 before adenovirus transfection and the H/R procedure. SKF96265 could relieve the harmful effect of STIM1 over-expression. **c** The Western blot of Bcl-2, Bax, and cleaved caspase-3. $^{*}P < 0.05$ compared with the control, $^{\#}P < 0.05$ compared with the H/R group, $^{\%}P < 0.05$ compared with the resveratrol + H/R group, and $^{**}P < 0.05$ compared with the resveratrol + H/R + Ad-STIM1 group

measured apoptosis and apoptotic proteins in NRVCs that underwent H/R injury to further elucidate the protective effect of resveratrol. The results showed that resveratrol obviously reduced apoptosis in NRVCs caused by H/R injury as assessed by TUNEL. Meanwhile, we found that resveratrol significantly upregulated Bcl-2 and downregulated Bax and cleaved caspase-3 expression in NRVCs. Additionally, NRVCs transfected with STIM1-specific adenovirus vector exhibited increased apoptosis despite resveratrol pretreatment. Furthermore, STIM1 over-expression led to decreased Bcl-2 and increased Bax and cleaved caspase-3 in NRVCs after resveratrol treatment and H/R injury. As expected, the augmented apoptosis of NRVCs resulting from STIM1 overexpression was markedly reversed by co-administration with SOCE inhibitor SKF96365. Finally, Bcl-2 level was lower, Bax and cleaved caspase-3 were higher in NRVCs compared with those treated without SOCE inhibitor. Therefore, the above data suggested that inhibiting STIM1-mediated Ca^{2+} accumulation and subsequent apoptosis of NRVCs might be the possible mechanism through which resveratrol exerted cardioprotective effects during H/R injury.

There are some limitations in the present study. First, the interaction between resveratrol and STIM1 was investigated in cultured NRVCs using an in vitro cellular model, which may not exactly behave as cardiomyocytes of the adult heart in vivo. For another, cell injury was measured only after reoxygenation. Thus, hypoxia-induced cell injury might partly account for cell viability and LDH release assay. Therefore, further studies on the effect of resveratrol on STIM1-mediated SOCE and Ca^{2+} influx in the case of myocardial I/R injury in an in vivo animal model are warranted.

Funding information This work was supported by the Anhui Provincial Natural Science Foundation (grant no. 1808085QH235 to He Fei), Youth Elites Support Plan in universities of Anhui Province (grant no. gxyq2019013 to He Fei), Science and Technology New Star Program of Second affiliated Hospital of Anhui Medical University (grant no. 2018KA06 to He Fei), Key Natural Science Project of Anhui Colleges and Universities (grant no. KJ2018A0284 to Cheng Jing), Natural Science Foundation of China (grant no. 81903833 to Cheng Jing), and Natural Science Foundation of China (grant no. 81873460 to Chen Aihua).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Heusch G, Gersh BJ (2017) The pathophysiology of acute myocardial infarction and strategies of protection beyond reperfusion: a continual challenge. Eur Heart J 38:774–784. https://doi.org/10.1093/eurheartj/ehw224
2. Correll RN, Goonasekera SA, van Berlo JH, Burr AR, Accornero F, Zhang H, Makarewich CA, York AJ, Sargent MA, Chen X, Houser SR, Molkentin JD (2015) STIM1 elevation in the heart results in aberrant Ca2 + handling and cardiomyopathy. J Mol Cell Cardiol 87:38–47. https://doi.org/10.1016/j.yjmcc.2015.07.032
3. Garcia-Dorado D, Ruiz-Meana M, Inserte J, Rodriguez-Sinovas A, Piper HM (2012) Calcium-mediated cell death during myocardial reperfusion. Cardiovasc Res 94:168–180. https://doi.org/10.1093/cvr/cvs116
4. Ibanez B, Heusch G, Ovize M, Van de Werf F (2015) Evolving therapies for myocardial ischemia/reperfusion injury. J Am Coll Cardiol 65:1455–1471
5. Qian CP, Ma JH, Zhang PH, Luo AT, Wang C, Ren ZQ, Kong LH, Zhang S, Wang XJ, Wu Y (2012) Resveratrol attenuates the Na+ −dependent intracellular Ca2+ overload by inhibiting H_2O_2-induced increase in late sodium current in ventricular myocytes. PLoS One 7:e51358. https://doi.org/10.1371/journal.pone.0051358
6. Ambudkar IS, de Souza LB, Ong HL (2017) TRPC1, Orai1, and STIM1 in SOCE: friends in tight spaces. Cell Calcium 63:33–39. https://doi.org/10.1016/j.ceca.2016.12.009
7. Hausenloy DJ, Yellon DM (2016) Ischaemic conditioning and reperfusion injury. Nat Rev Cardiol 13:193–209. https://doi.org/10.1038/nrcardio.2016.5
8. Voelkers M, Salz M, Herzog N, Frank D, Dolatabadi N, Frey N, Gude N, Friedrich O, Koch WJ, Katus HA, Sussman MA, Most P (2010) Orai1 and Stim1 regulate normal and hypertrophic growth in cardiomyocytes. J Mol Cell Cardiol 48:1329–1334. https://doi.org/10.1016/j.yjmcc.2010.01.020
9. Ayub A, Poulose N, Raju R (2015) Resveratrol improves survival and prolongs life following hemorrhagic shock. Mol Med 21(1):305–312. https://doi.org/10.2119/molmed.2015.00013
10. Wang B, Yang Q, Y-y S, Y-f X, Y-b W, X-t L, W-w B, X-q L, Y-x Z (2014) Resveratrol-enhanced autophagic flux ameliorates myocardial oxidative stress injury in diabetic mice. J Cell Mol Med 18:1599–1611. https://doi.org/10.1111/jcmm.12312
11. Yang L, Zhang Y, Zhu MM, Zhang Q, Wang XL, Wang YJ, Zhang JC, Li J, Yang L, Liu J, Liu F, Yang YA, Kang LC, Shen YN, Qi Z (2016) Resveratrol attenuates myocardial ischemia/reperfusion injury through up-regulation of vascular endothelial growth factor B. Free Radical Bio Med 101:1–9. https://doi.org/10.1016/j.freeradbiomed.2016.09.016
12. Madsen CP, Klausen TK, Fabian A, Hansen BJ, Pedersen SF, Hoffmann EK (2012) On the role of TRPC1 in control of Ca2+ influx, cell volume, and cell cycle. Am J Phys Cell Phys 303:C625–C634. https://doi.org/10.1152/ajpcell.00287.2011
13. Benard L, Oh JG, Cacheux M, Lee A, Nonnenmacher M, Matasic DS, Kohlbrenner E, Kho C, Pavoine C, Hajjar RJ, Hulot JS (2016) Cardiac Stim1 silencing impairs adaptive hypertrophy and promotes heart failure through inactivation of mTORC2/Akt signaling. Circulation 133:1458–1471. https://doi.org/10.1161/circulationaha.115.020678
14. Lopez JJ, Albarran L, Gomez LJ, Smani T, Salido GM, Rosado JA (2016) Molecular modulators of store-operated calcium entry. Biochim Biophys Acta-Mol Cell Res 1863:2037–2043. https://doi.org/10.1016/j.bbamcr.2016.04.024
15. Sasamori J, Hasegawa T, Takaya A, Watanabe Y, Tanaka M, Ogino Y, Chiba T, Aihara K (2014) The cardioprotective effects of novel Na+/H+ exchanger inhibitor TY-51924 on ischemia/reperfusion injury. J Cardiovasc Pharmacol 63:351–359. https://doi.org/10.1097/FJC.0000000000000055
16. He F, Wu Q, Xu B, Wang X, Wu J, Huang L, Cheng J (2017) Suppression of Stim1 reduced intracellular calcium concentration and attenuated hypoxia/reoxygenation induced apoptosis in H9C2 cells. Biosci Rep 37. https://doi.org/10.1042/bsr20171249
17. Liao Z, Liu D, Tang L, Yin D, Yin S, Lai S, Yao J, He M (2015) Long-term oral resveratrol intake provides nutritional

preconditioning against myocardial ischemia/reperfusion injury: involvement of VDAC1 downregulation. Mol Nutr Food Res 59: 454–464. https://doi.org/10.1002/mnfr.201400730
18. Casas-Rua V, Alvarez IS, Pozo-Guisado E, Martin-Romero FJ (2013) Inhibition of STIM1 phosphorylation underlies resveratrol-induced inhibition of store-operated calcium entry. Biochem Pharmacol 86:1555–1563. https://doi.org/10.1016/j.bcp.2013.09.018
19. Kojima A, Kitagawa H, Omatsu-Kanbe M, Matsuura H, Nosaka S (2012) Presence of store-operated Ca-2 entry in C57BL/6J mouse ventricular myocytes and its suppression by sevoflurane. Br J Anaesth 109:352–360. https://doi.org/10.1093/bja/aes212
20. Halestrap AP, Clarke SJ, Javadov SA (2004) Mitochondrial permeability transition pore opening during myocardial reperfusion - a target for cardioprotection. Cardiovasc Res 61:372–385. https://doi.org/10.1016/s0008-6363(03)00533-9
21. Imahashi K, Pott C, Goldhaber JI, Steenbergen C, Philipson KD, Murphy E (2005) Cardiac-specific ablation of the Na+-Ca2+ exchanger confers protection against ischemia/reperfusion injury. Circ Res 97:916–921. https://doi.org/10.1161/01.RES.0000187456.06162.cb
22. He F, Xu BL, Chen C, Jia HJ, Wu JX, Wang XC, Sheng JL, Huang L, Cheng J (2016) Methylophiopogonanone A suppresses ischemia/reperfusion-induced myocardial apoptosis in mice via activating PI3K/Akt/eNOS signaling pathway. Acta Pharmacol Sin 37:763–771. https://doi.org/10.1038/aps.2016.14
23. Javadov S, Choi A, Rajapurohitam V, Zeidan A, Basnakian AG, Karmazyn M (2008) NHE-1 inhibition-induced cardioprotection against ischaemia/reperfusion is associated with attenuation of the mitochondrial permeability transition. Cardiovasc Res 77:416–424. https://doi.org/10.1093/cvr/cvm039
24. Selvaraj S, Sun Y, Sukumaran P, Singh BB (2016) Resveratrol activates autophagic cell death in prostate cancer cells via downregulation of STIM1 and the mTOR pathway. Mol Carcinog 55:818–831. https://doi.org/10.1002/mc.22324
25. Cheng J, Wu Q, Lv R, Huang L, Xu B, Wang X, Chen A, He F (2018) MicroRNA-449a inhibition protects H9C2 cells against hypoxia/reoxygenation-induced injury by targeting the notch-1 signaling pathway. Cell Physiol Biochem 46:2587–2600. https://doi.org/10.1159/000489686
26. Ling YN, Chen GM, Deng Y, Tang HX, Ling L, Zhou XM, Song XD, Yang PZ, Liu YF, Li ZL, Zhao C, Yang YF, Wang XB, Kitakaze M, Liao YL, Chen AH (2016) Polydatin post-treatment alleviates myocardial ischaemia/reperfusion injury by promoting autophagic flux. Clin Sci 130:1641–1653. https://doi.org/10.1042/cs20160082
27. Collins HE, Zhu-Mauldin X, Marchase RB, Chatham JC (2013) STIM1/orai1-mediated SOCE: current perspectives and potential roles in cardiac function and pathology. Am J Phys Heart Circ Phys 305:H446–H458. https://doi.org/10.1152/ajpheart.00104.2013
28. Lu T, Zhou D, Gao P, Si L, Xu Q (2017) Resveratrol attenuates high glucose-induced endothelial cell apoptosis via mediation of store-operated calcium entry. Mol Cell Biochem https://doi.org/10.1007/s11010-017-3194-7. doi:https://doi.org/10.1007/s11010-017-3194-7
29. Zordoky BN, Robertson IM, Dyck JR (2015) Preclinical and clinical evidence for the role of resveratrol in the treatment of cardiovascular diseases. Biochim Biophys Acta 1852:1155–1177. https://doi.org/10.1016/j.bbadis.2014.10.016