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Cardioprotective effect of breviscapine: inhibition of apoptosis in H9c2 cardiomyocytes *via* the PI3K/Akt/eNOS pathway following simulated ischemia/reperfusion injury

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Breviscapine (BE) is a standardized Chinese herbal medicine extracted from *Erigeron breviscapus* (Vant.) Hand.-Mazz. It has been widely used to treat cardiovascular and cerebrovascular diseases. However, there are no reports on the protective effects and underlying molecular mechanisms of BE action on myocardial ischemia/reperfusion (MI/R)-induced cardiomyocyte apoptosis. In the present study, we aimed to confirm the cardioprotective effect of BE from MI/R injury *in vivo*, and investigate the potential molecular mechanisms against simulated ischemia/reperfusion (SI/R)-induced cardiomyocyte apoptosis *in vitro*. The rat model of MI/R injury was induced by 30 min of transient vessel occlusion followed by 3 h of reperfusion. BE significantly reduced the myocardium infarct size and production of cardiac troponin (cTnI) in serum. In an *in vitro* experiment, H9c2 cardiomyocytes were incubated with vehicle or ischemic buffer during hypoxia; then, they were reoxygenated with or without BE. BE markedly improved the cell viability and decreased lactate dehydrogenase (LDH) release. We confirmed the anti-apoptotic effect of BE with the Hoechst 33258 staining assay, and this effect was associated with an increase in Bcl-2 and a decrease in active caspase-3 expression. Western blot analysis also showed that BE increased the phosphorylation of Akt and eNOS in H9c2 cells, and the protective effects of BE were partially inhibited by the phosphatidylinositol 3'-kinase (PI3K) specific inhibitor LY294002. Our results suggested that BE could provide significant cardioprotection against MI/R injury, and the potential mechanisms might involve suppression of cardiomyocyte apoptosis through activating the PI3K/Akt/eNOS signaling pathway.

1. Introduction

In developed countries, cardiovascular diseases are the major cause of disability and mortality. Myocardial ischemia/reperfusion (MI/R) is a common clinical entity that occurs in many situations, including cardiac transplantation, coronary bypass surgery and thrombolytic therapy (Yellon et al. 2007). The effects of reperfusion for ischemia heart disease are favorable in most cases, but reperfusion can also have a harmful effect on cardiomyocytes (Hausenloy et al. 2013). This form of reperfusion injury can induce extensive cardiomyocyte death and increase the infarct size (Cannon. 2005). It is well known that apoptosis is a significant cellular mechanism responsible for MI/R injury in the myocardium, and exploring anti-apoptotic agents is a novel therapeutic option for related heart disease, especially for MI/R injury (Liu et al. 2014; Crow et al. 2004; Zhao et al. 2000).

In the past decades, there has been an increase in the use of complementary treatments such as traditional Chinese medicine (TCM), which has been used for thousands of years in China (Guo et al. 2013; Fan et al. 2014; Jun. 1992) to treat heart disease. TCM offers many advantages in this respect (Uzuner et al. 2012). Therefore, searching for TCM agents with minimal side effects probably represents an ideal strategy for developing safe and effective drugs for MI/R injury treatment. Breviscapine (BE), produced by Shineway Pharmaceutical Co., Ltd of China, is a standardized TCM product extracted from *Erigeron breviscapus* (Vant.) Hand.-Mazz (Guo et al. 2014). It has been used to treat disorders in the blood supply to the heart and brain (Lin et al. 2007). Previous studies have demonstrated that BE can induce neuroprotective effects by decreasing the infarct size and improving neurological function (Guo et al. 2014). However, there are no reports on the protective effects and underlying molecular mechanisms of BE on MI/R-induced cardiomyocytes apoptosis.

In the current study, BE was tested *in vivo* in an MI/R injury rat model and *in vitro* in the H9c2 cardiomyocyte cell line sub-

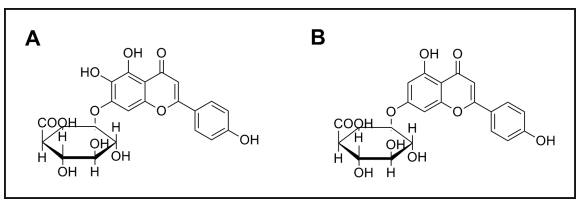

Fig. 1: Chemical constituents of breviscapine. (A) Scutellarin-7-O-glucuronide. (B) Apigenin-7-O-glucuronide.

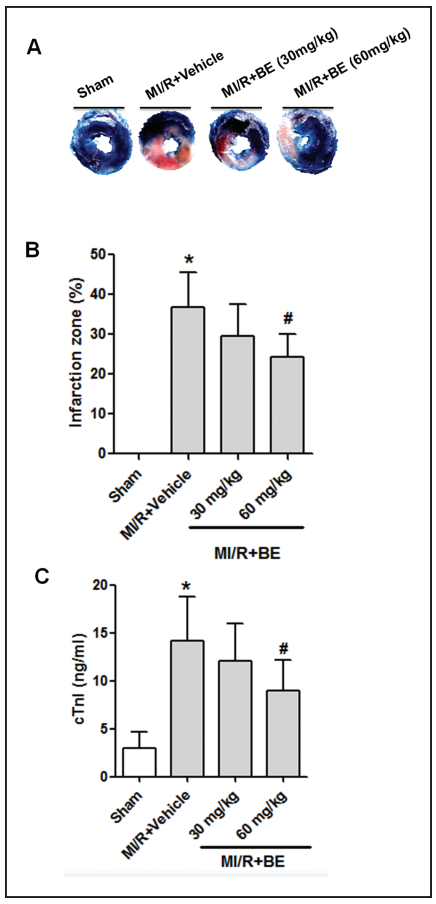

Fig. 2: BE protected the rats from MI/R injury. (A) Effect of MLB on myocardial infarct size. Myocardial risk and infarct area were determined by Evans blue and TTC method. (B) The effect of BE administration on infarct zone in rat after MI/R. (C) The effect of BE on the level of cTnI in serum. IZ, infarct zone; MI/R, myocardial ischemia/reperfusion; BE, breviscapine. n = 8; values are expressed as the mean ± S.D. Significance was determined by ANOVA followed by Tukey's test. $^{*}p < 0.05$ *versus* Sham group. $^{\#}p < 0.05$ *versus* MI/R + vehicle group.

jected to simulated ischemia/reperfusion (SI/R) to investigate the therapeutic effects of BE against cardiomyocyte apoptosis.

2. Investigations and results

2.1. BE protected the rats from MI/R injury

As reliable indicators of MI/R injury, the infarct size and activities of cTnI in serum were measured. As illustrated in Fig. 2A and B, 30 min of ischemia followed by 3 h of reperfusion resulted in the development of substantial myocardial infarcts, which were significantly attenuated by BE (60 mg/kg) treatment (24.4 ± 5.6 *vs*. $36.6 \pm 8.9\%$, $p < 0.05$, Fig. 2A and B). In addition, as shown in Fig. 2C, the elevated activities of cTnI in serum that resulted from MI/R were also markedly decreased by the administration of BE (60 mg/kg) (9.0 ± 3.1 *vs*. $14.2 \pm 4.5\%$, $p < 0.05$, Fig. 2C).

2.2. BE protected H9c2 cardiomyocytes against SI/R-induced injury

Cell viability was determined using the MTT assay. The result is shown in Fig. 3A. When exposed to SI/R, there were only $60.0 \pm 10.5\%$ viable cells. However, BE (1 and 10 μmol), to a great extent, protected the neonatal rat cardiomyocytes against SI/R-induced cell viability loss and restored cell survival to

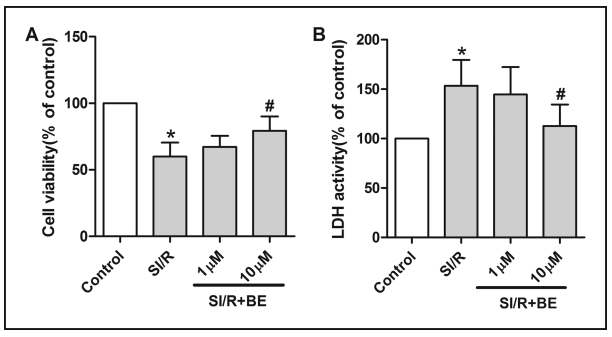

Fig. 3: BE protected H9c2 cardiomyocytes against injury induced by SI/R. (A) Cardiomyocytes were treated with BE followed by SI/R and cell viability was determined by MTT assay. (B) The release of LDH in culture medium at the end of reperfusion was determined. SI/R: simulated ischemia/reperfusion; BE, Breviscapine. n = 8; values are expressed as the mean ± S.D. Significance was determined by ANOVA followed by Tukey's test. $^{*}p < 0.05$ *versus* Control group. $^{\#}p < 0.05$ *versus* SI/R group.

$67.2 \pm 8.4\%$ ($p < 0.05$, Fig. 3A) and $79.3 \pm 10.7\%$ ($p < 0.05$, Fig. 3A).

As marker of cardiomyocyte injury, the LDH activity in the culture medium was markedly elevated in the SI/R group ($153.3 \pm 26.3\%$, $p < 0.05$, Fig. 3B) compared to the control group, but it was significantly decreased by BE (10 μmol) (112.7 ± 21.7 *vs*. $153.3 \pm 26.3\%$, $p < 0.05$, Fig. 3B).

2.3. BE inhibited H9c2 cardiomyocytes from SI/R-induced apoptosis

The anti-apoptotic effect of BE was assessed by Hoechst 33258 staining and flow cytometry in the present study. Compared with the control group, SI/R significantly increased the number of apoptotic cells with distinctive morphological changes, including chromatin and/or cell nuclear fragmentation. However, the morphological changes were markedly attenuated by pretreatment with different concentrations of BE (Fig. 4).

To further characterize the inhibitory effects of BE on myocardial cell apoptosis, we examined whether BE could inhibit caspase-3 activity. As illustrated in Fig. 5, SI/R resulted in a noticeable increase in caspase-3 activity compared with the control group ($164.5 \pm 20.9\%$, $p < 0.05$, Fig. 5), while 10 μmol of BE significantly reduced the level of caspase-3 compared with the SI/R group ($125.7 \pm 10.0\%$, $p < 0.05$, Fig. 5). However, compared with the BE-treated group, treatment with BE plus LY-294002 increased the production of active caspase-3 ($156.0 \pm 11.6\%$, $p < 0.05$, Fig. 5).

2.4. Intracellular mechanisms of anti-apoptosis by BE

The expression levels of phosphorylated Akt and eNOS (p-Akt, p-eNOS) were determined by western blot analysis (Fig. 6A). BE (10 μmol) pretreatment for 24 h before OGD significantly increased the p-Akt and p-eNOS levels compared with the SI/R group (Fig. 6A). Western blot analysis showed that treatment with the PI3K inhibitor LY-294002 (10 μmol) 1 h before OGD inhibited the increase in the p-Akt level induced by BE pretreatment (Fig. 6A). It was demonstrated that the PI3K/Akt/eNOS signaling pathway was involved in the anti-apoptotic effect of BE. Furthermore, SI/R slightly increased Bcl-2 expression (Fig. 6A) in SI/R-injured H9c2 cardiomyocytes compared with the control group; 24-h pretreatment with BE (10 μmol) before OGD remarkably increased Bcl-2 expression (Fig. 6A). Treatment with LY-294002 (10 μmol) 1 h before OGD significantly reversed the expression of Bcl-2 (Fig. 6A), confirming the anti-apoptotic effect of MLB on SI/R-induced apoptosis.

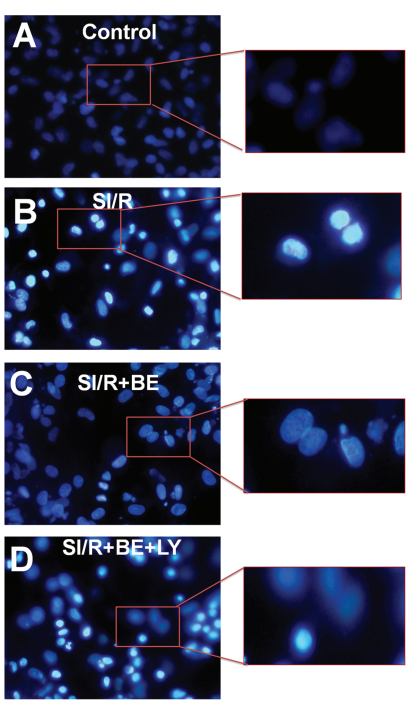

Fig. 4: BE inhibited H9c2 cardiomyocytes from apoptosis induced by SI/R. Effect of BE on the histochemical characterization of cardiomyocytes after SI/R injury (× 200). SI/R, simulated ischemia/reperfusion; BE, breviscapine.

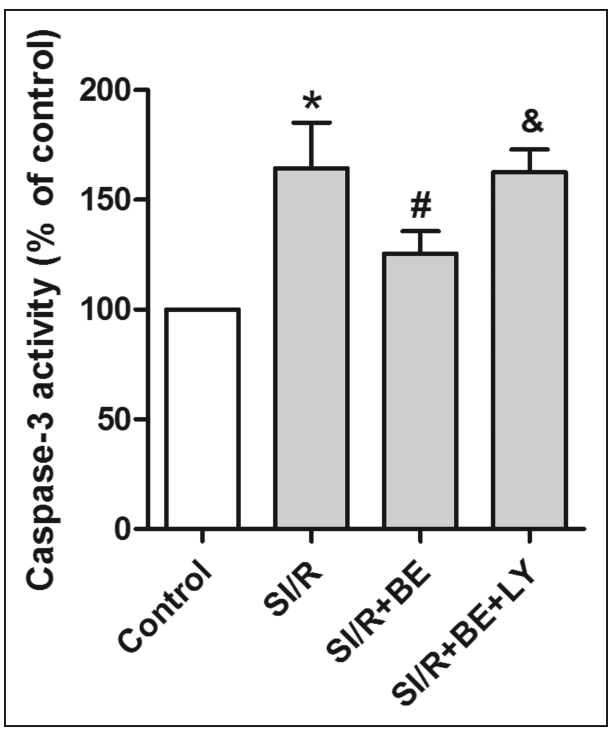

Fig. 5: BE decreased caspase-3 activity of cardiomyocytes after SI/R injury. SI/R, simulated ischemia/reperfusion; BE, Breviscapine.Valuesare expressed as the mean ± S.D. of three independent experiments. Significance was determined by ANOVA followed by Tukey's test. $^{\#}p < 0.05$ *versus* Control group. $^{*}p < 0.05$ *versus* SI/R group. $^{\&}p < 0.05$ *versus* SI/R + BE group.

3. Discussion

It is widely accepted that cardiomyocyte loss from apoptosis plays an important role in various heart diseases, including MI/R injury (Zhao et al. 2014; Freude et al. 2000). Blocking

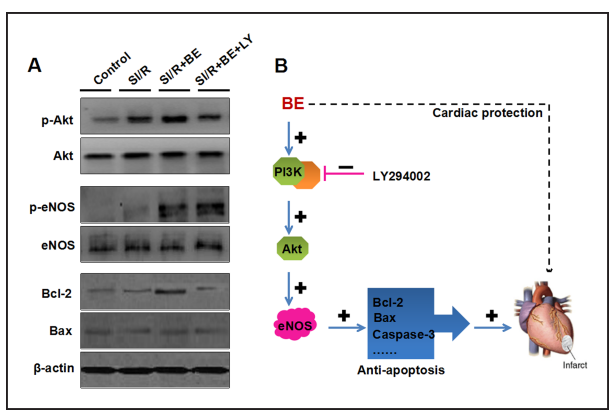

Fig. 6: Intracellar mechanisms of the anti-apoptosis by BE. (A) Regulation of proteins p-Akt, p-eNOS, Bcl-2 and Bax associated with apoptosis by BE. (B) Proposed cardioprotective effects and potential mechanisms of BE. BE, breviscapine.

the MI/R-induced apoptosis process may help to slow down or even prevent the occurrence and progression of heart failure.

In the past decades, the use of many traditional plants has been reported for the control of problems due to ischemia and associated pathologies (Zhang et al. 2013; Nishizawa et al. 1994). *Erigeron breviscapus* (Vant.) Hand.-Mazz is an endemic medicinal plant resource in Yunnan Province of China. According to records of the Chinese ancient book "Dian Nan Ben Cao", it can promote blood circulation, prevent blood stasis and dredge the meridian passage. Recent studies have reported that breviscapine (BE) is the active ingredient of *Erigeron breviscapus* for treating cerebrovascular and cardiovascular disease (Wei et al. 2012). BE has been prepared in some Chinese patent medicines, including parental preparations, and is generally used in clinical stroke treatment. However, it is poorly understood whether BE has protective effects against cardiomyocyte apoptosis *in vitro*. The particular pathway and underlying mechanisms have yet to be elucidated.

Our present study showed that MI/R (30 min/3 h) *in vivo* and SI/R (2 h/24 h) *in vitro* significantly aggravated myocardial injury, decreased cell viability, and increased cardiomyocyte apoptosis, which were evidenced by the increased infarct size, cTnI release in the serum and LDH activity in the culture supernatant that enhanced the percentage of apoptotic cells. However, treatment with EB greatly reduced infarct size, cell ability loss, cTnI, and LDH activities. The anti-apoptotic effects were also confirmed by the results of Hoechst 33258. MI/R led to a significant increase in Hoechst 33258-positive H9c2 cardiomyocytes, and incubation with BE for 24 h before SI/R injury significantly decreased SI/R-induced apoptosis. The results strongly suggested that BE might have a protect effect against MI/R injury and inhibit cardiomyocyte apoptosis in response to SI/R.

Previous studies have indicated that the caspase cascade plays a key role in apoptosis. Caspase-3 is thought to be activated during the final step of the proapoptotic signaling pathway in many cell lines. Inhibition of caspase activities has been shown to attenuate both MI/R injury and apoptosis in cardiomyocytes (Gustafsson et al. 2002; Borutaite et al. 2003). In the current study, we found that BE can up-regulate the caspase-3 level, inhibiting H9c2 cardiomyocyte apoptosis. The anti-apoptotic effects of BE may be mediated by caspase-3.

There are complex mechanisms that cause progressive heart damage during MI/R (Mozaffari et al. 2013). The PI3K/Akt/eNOS pathway has been reported to play a protective role in MI/R (Wu et al. 2011). Activated PI3K and Akt are each sufficient to protect cardiomyocytes against apoptosis (Chen et al. 2011; Lu et al. 2008). Up-regulation of eNOS has been reported to protect against MI/R injury through sup-

pressing vascular cell adhesion molecule expression, preventing excessive leukocyte tissue infiltration (Han et al. 2012). Therefore, the phosphorylated Akt and eNOS levels in cardiomyocytes treated with BE were further elevated, suggesting that the anti-apoptotic effects of BE depend on PI3K/Akt/eNOS. Meanwhile, studies have also indicated that an increase in the proapoptotic Bax family proteins and a decrease in anti-apoptotic Bcl-2 family proteins are involved in the process of apoptosis (Produit-Zengaffinen et al. 2009). Our results indicated that the induction of apoptosis by SI/R was associated with a increase in Bcl-2, and incubation with BE resulted in up-regulation of the Bcl-2 protein level. However, Bax was not affected, suggesting that Bax does not play an important role here. We aimed to dissect the role of BE in protecting cardiomyocytes against SI/R-induced injury. LY294002 (specific inhibitor of P13K) significantly reversed the anti-apoptotic effects of BE, suggesting the crucial participation of the PI3K/Akt/eNOS pathway in BE-induced survival signaling in H9c2 cardiomyocytes.

In conclusion, we revealed that BE protects the myocardium from MI/R injury *in vivo* and inhibits SI/R-induced cardiomyocyte apoptosis. Our data show that increased expression of p-Akt, p-eNOS, and Bcl-2 and decreased expression of caspase-3 play an essential role in the cardioprotective effects of BE. These findings might be meaningful for further understanding the molecular mechanisms of BE-mediated cardioprotection.

4. Experimental

4.1. Preparation and quality control of breviscapine injection

Approximately 4 g pure breviscapine powder was dissolved into 700 ml of 0.1% edetate disodium. Then, a small amount of sodium bicarbonate was added to obtain a physiological solution with a pH of 7.0-7.5. Subsequently, water was added to the mixed solution for injection up to 1000 ml; then, the pyrogens were removed by treatment with activated charcoal. Finally, the solution was filtered, sterilized and encapsulated into ampoules (Ministry of Public Health, 1998).

The main active components of breviscapine are two flavonoids: the main scutellarin-7-O-glucuronide and a small level of apigenin-7-O-glucuronide (Fig. 1. In accordance with the corresponding quality control standard, breviscapine injection should contain no less than 95.0% and no more than 105.0% of the labeled level of scutellarin-7-O-glucuronide (molecular formula: $C_{21}H_{18}O_{12}$) when determined by ultraviolet spectrophotometry under 335 nm (Ministry of Public Health, 1998).

4.2. Animals

Adult male Sprague-Dawley rats (250 ± 20 g) were supplied by the animal research center at Fourth Military Medical University, Xi'an, China [Animal Certificate of Conformity: SCXK-(Army) 2007-007]. Rats were housed individually under constant temperature ($23 \pm 3\,^{\circ}C$) and humidity conditions with a 12-h light/dark cycle, and they had free access to chow and water. All animal protocols were approved by the Fourth Military Medical University Committee on Animal Care.

4.3. MI/R injury model and experimental protocols

The MI/R surgical procedure was used as previously described (Zhu et al. 2008). Briefly, Sprague-Dawley rats were intubated and artificially ventilated with a rodent ventilator (HX-100E, Taimeng, China) under anesthesia with 3% pentobarbital sodium (40 mg/kg, i.p.). The normal electrocardiogram was recorded after electrodes were placed subcutaneously and connected to an electrocardiograph (BL-420S, Taimeng, China). Coronary artery ligation was achieved with a gab occluder fixed onto the LAD coronary artery. A 7-0 silk suture was passed underneath the LAD (2-3 mm inferior to the left auricle) and tied. MI/R was induced by 30 min of ischemia followed by 3 h of reperfusion.

Rats were divided into the following four groups: (I) Sham group, the silk suture crossed without ligation; (II) MI/R + vehicle group (model group), rats received saline alone; (III) MI/R + BE (30 mg/kg) group, rats received 30 mg/kg BE; and (IV) MI/R + BE (30 mg/kg) group, rats received 30 mg/kg BE. BE was dissolved in saline to reach the final concentration. The BE groups received intravenous (i.v.) BE injection at the onset of reperfusion.

4.4. Measurement of myocardial infarct size

At 3 h after reperfusion, 2 ml of 3% Evans blue was perfused into the aorta and coronary arteries after coronary artery ligation. The entire ventricular tissue was sliced into five sections through the transverse axis from the apex to the atrioventricular groove. Tissues were incubated in 3 ml of 2% TTC at $37\,^{\circ}C$ for 15 min as previously described (Gao et al. 2004). TTC stains viable tissue dark red, while the infarct portion remains grayish-white. The area of infarction was determined by computerized planimetry (Image-ProPlus, Media Cybernetics, Bethesda, MD). The area as a percentage of the risk zone was used to estimate the size of the infarct zone.

4.5. Measurement of cTnI release in serum

At 3 h after reperfusion, carotid artery blood was collected and stored at $20\,^{\circ}C$ after centrifugation at 4000 g for 15 min (TDZ4A-WS, Xiangyi, China) as previously described (Li et al. 2011). The levels of cTnI in serum were measured using ELISA kits according to the manufacturer's instructions.

4.6. Cell culture

The rat H9c2 cardiomyocyte cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The H9c2 cells were maintained in DMEM supplemented with 10% fetal calf serum at $37\,^{\circ}C$ in 5% CO_2. The medium was replaced every 2-3 days, and cells were subcultured or subjected to experimental procedures at 80-90% confluence (Yin et al. 2013).

4.7. SI/R injury model in vitro and experimental protocols

The oxygen and glucose deprivation (OGD) technique was based on a previously described protocol (Yin et al. 2013). In our study, the OGD injury was produced by incubation with blank solution and exposure to a hypoxic environment of 95% N_2 and 5% CO_2 in airtight gas chambers at $37\,^{\circ}C$ for 2 h (Billups-Rothenberg,USA). After OGD treatment, cells were removed from the gas chamber, and the OGD solution was replaced with warmed culture medium for 24 h (recovery period) in a CO_2 incubator at $37\,^{\circ}C$. After 24 h in culture, H9c2 cardiomyocytes were used in subsequent experiments. H9c2 cardiomyocytes were randomly divided into the following five groups: (1) Control group without any treatment; (2) SI/R group (model group), which was cultured under OGD for 2 h and then under recovery conditions for 24 h; (3-4) SI/R + BE groups, which were pretreated with BE at concentrations of 1 and 10 μmol for 24 h before OGD; and (5) SI/R + BE + LY group, pre-treated with BE (10 μmol) for 24 h before OGD, after which the cardiomyocytes were pretreated with LY-294002 (PI3K inhibitor, 10 μmol) for 1 h before OGD.

4.8. Cell viability

Cell viability was determined colorimetrically using the MTT assay. Cells at the exponential phase were seeded with 1×10^4 cells/well in 96-well plates. After different treatments, 20 μl of 5 mg/ml MTT solution was added to each well (0.5 mg/ml final concentration in medium), and wells were incubated for 4 h at $37\,^{\circ}C$. The supernatants were aspirated, the formazan crystals in each well were dissolved in 150 μl DMSO, and the optical density at 490 nm was read on a microplate reader. The reduction in optical density was considered to be due to the decrease in cell viability.

4.9. Measurement of LDH release in the culture supernatant

The activities of LDH in the cultured supernatant were measured with a microplate reader (Model 550, USA) using diagnostic kits according to the manufacturer's instructions.

4.10. Hoechst 33258 staining assay

Briefly, H9c2 cardiomyocytes were washed in ice-cold PBS, fixed in 10% neutral buffered formalin for 10 min at room temperature, and washed again in ice-cold PBS. Then, the H9c2 cardiomyocytes were exposed to Hoechst 33258 (2 μg/ml in PBS) and incubated for 20 min at room temperature. The H9c2 cardiomyocytes were then washed three times in PBS and examined under a fluorescence microscope with an appropriate filter (Quan et al. 2014).

4.11. Measurement of caspase-3 activity

Caspase-3 activity was measured using a colorimetric activity assay kit (Beyotime Institute of Biotechnology, Jiangsu, China) according to the manufacturer's protocol. In brief, control or SI/R-treated H9c2 cardiomyocytes were lysed in ice-cold lysis buffer, placed on ice for 30 min, and then centrifuged at $4\,^{\circ}C$ for 15 min at 16,000 rpm. After determining the protein concentration, the supernatant was incubated with the caspase-3

substrate (Ac-DEVD-pNA) on a 96-well-plate. The activity of caspase-3 was determined using a microplate reader at 405 nm.

4.12. Protein extraction and western blot analysis

Cells cultured on 6-well plates were lysed in lysis buffer (Beyotime Institute of Biotechnology, Jiangsu, China) on ice for 30 min, and the lysates were centrifuged at 4 °C for 20 min at 10,000 rpm. After quantitation of the protein concentration, 50 μg of total protein was resolved on a 15% SDS-poly-acrylamide gel. The fractionated proteins were electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes and probed with rabbit anti-phospho-Akt, anti-Akt, ant-phospho-eNOS, anti-eNOS, anti-Bcl and anti-Bax overnight at 4 °C, which was followed by incubation with the corresponding secondary antibodies for 2 h at room temperature. The blots were visualized with ECL-Plus reagent (GE Healthcare, Pisca-taway,NJ). In some immunoblotting experiments, the blots were reprobed with an anti-β-actin antibody to control for protein loading.

4.13. Statistical analysis

Because all data were normally distributed, we conducted parametric analysis. Continuous variables that approximated the normal distribution are expressed as the mean ± standard deviation (S.D.). Comparison between groups was subjected to ANOVA followed by Turkey's multiple comparison tests. A p-value < 0.05 was considered statistically significant. Statistical analyses were performed using GraphPad Prism software Version 5.01 (La Jolla, CA).

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