International Immunopharmacology 73 (2019) 98–107

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Nobiletin ameliorates myocardial ischemia and reperfusion injury by attenuating endoplasmic reticulum stress-associated apoptosis through regulation of the PI3K/AKT signal pathway

Bo-fang Zhang[a,c,d,1], Hong Jiang[a,c,d,*], Jing Chen[a,c,d,*], Xin Guo[b,1], Yue Li[a,c,d], Qi Hu[a,c,d], Shuo Yang[a,c,d]

[a] Department of Cardiology, Renmin Hospital of Wuhan University, Wuhan 430060, China
[b] Department of Cardiology, The Central Hospital of Wuhan, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 43000, Hubei Province, China
[c] Cardiovascular Research Institute, Wuhan University, Wuhan 430060, China
[d] Hubei Key Laboratory of Cardiology, Wuhan 430060, China

ARTICLE INFO

Keywords:
Nobiletin
Myocardial ischemia and reperfusion injury
Endoplasmic reticulum stress
Apoptosis
PI3K/AKT

ABSTRACT

Background: Nobiletin is a natural polymethoxylated flavone that confers antioxidative, anti-inflammatory and anti-apoptotic efficacies. However, the potential benefits of nobiletin preconditioning on myocardial ischemia and reperfusion injury (MIRI) remains largely unknown.

Methods: MIRI was induced by ligation of the left anterior descending coronary artery and reperfusion. Pre-treatment with nobiletin, with or without PI3K/AKT inhibitor LY294002, was performed at the onset of reperfusion. Histological analyses, apoptotic evaluation, plasma biomarkers of myocardial injury, echocardiographic evaluation of cardiac function and myocardial levels of endoplasmic reticulum stress (ERS)-related molecules were observed.

Results: Nobiletin pre-treatment significantly deceased the infract size and number of apoptotic cells in the myocardium of MIRI rats, as determined by Terminal deoxynucleotidyl transferase dUTP nick end labeling staining. Moreover, the plasma levels of lactate dehydrogenase (LDH) and creatine kinase-MB (CK-MB) also markedly decreased. In addition, pre-treatment with nobiletin restored the impaired cardiac systolic function, as evidenced by echocardiographic evaluation results. Importantly, pre-treatment with nobiletin significantly downregulated the myocardial mRNA and protein levels of ERS-related signal molecules, including GRP78, CHOP and caspase-12, but upregulated the levels of p-PI3K and p-AKT. Interestingly, co-treatment with LY294002 significantly abolished the benefits of nobiletin pre-treatment on cardiac function, myocardial apoptosis, cardiomyocyte injuries, and changes in myocardial levels of ERS-related signaling molecules.

Conclusion: Nobiletin pre-treatment may alleviate MIRI probably *via* the attenuation of PI3K/AKT-mediated ERS-related myocardial apoptosis.

1. Introduction

Acute myocardial infarction (AMI) is a clinical disease characterized by the abrupt obstruction of the coronary artery, which results in substantial myocardial ischemia, cardiomyocyte necrosis, and impaired cardiac function [1]. Despite the significant advances in its diagnosis and treatment in recent decades, AMI remains one of the leading causes of morbidity and mortality worldwide [2]. Revascularization *via* thrombolysis, percutaneous coronary intervention and coronary artery bypass grafting are the most effective treatments to relieve symptoms and preserve the myocardium in patients with AMI [3,4]. However, it has been proposed that myocardial reperfusion after ischemia may paradoxically lead to additional damage to the myocardium, which is defined as myocardial ischemia and reperfusion injury (MIRI) [5,6]. Indeed, it has been estimated that MIRI may account for more than half of the infarcted myocardium, which further deteriorates cardiac

* Corresponding authors at: Department of Cardiology, Renmin Hospital of Wuhan University, Cardiovascular Research Institute of Wuhan University, Wuhan 430060, Hubei Province, China.
E-mail addresses: hong-jiang@whu.edu.cn (H. Jiang), 364580342@qq.com (J. Chen).
[1] Equally contributed to this work.

https://doi.org/10.1016/j.intimp.2019.04.060
Received 31 January 2019; Received in revised form 29 March 2019; Accepted 29 April 2019
Available online 10 May 2019
1567-5769/

function and prognosis in patients with AMI [5]. Although many potential pathophysiological mechanisms have been proposed underlying the pathogenesis of MIRI, including myocardial apoptosis [7,8], inflammation [9], calcium overloading [10] and reactive oxygen species (ROS)-related injury [11], no evidence-based strategies for the prevention of MIRI have been confirmed, at present. Therefore, the development of novel treatment strategies to attenuate MIRI may be important for improving the prognosis for patients with AMI.

Conventionally, the endoplasmic reticulum (ER) has been recognized as an organelle responsible for protein biosynthesis, folding, processing, secretion and transportation [12]. Recent findings have indicated other important functions of ER, such as the regulation of cellular apoptosis. Furthermore, the disturbance of ER homeostasis caused by nutrient/glucose deprivation or ischemia/hypoxia can lead to the inaccurate synthesis and assembly of proteins [13], thereby causing these misfolded proteins to accumulate within the ER, which is collectively defined as ER stress (ERS) [14]. Initially, an endogenous self-protective response to ERS will be activated, attempting to inhibit protein secretion, assist protein refolding and obliterate the misfolded proteins [15], namely, the unfolded protein response (UPR). UPR is initiated through the activation of the protein kinase RNA (PKR)-like ER kinase (PERK), inositol requiring 1a (IRE1a), and activating transcription factor-6 (ATF6) pathways [14,16,17] after the detachment of ER-resident chaperone glucose-regulated protein-78 (GRP78) [18–20]. However, if ERS is sustained and over-activated, the above transducers will subsequently provoke ERS-associated apoptosis signaling pathways, such as C/EBP homologous protein (CHOP), caspase-12 and c-Jun NH2 terminal kinase (JNK), to initiate the cell apoptotic procedure, and protect organisms by eliminating injured cells [14,15,21]. Increasing evidence from experimental studies in the cardiovascular system suggests that persistent activated ERS and subsequent apoptotic pathways may be the crucial underlying mechanisms for MIRI [22,23]. Therefore, therapeutic approaches that target the alleviation of ERS-related myocardial apoptosis may be of preventative significance for MIRI.

Nobiletin is a ubiquitous bioflavonoid and polyphenolic compound predominantly isolated from the peels of citrus, which has been confirmed to confer anti-oxidative, anti-inflammatory and anti-apoptotic properties [24] involved in the pathogenesis of ischemia and reperfusion (I/R) injury. Moreover, the potential benefits of flavonoids compounds against I/R injury have been proposed in early studies [25]. Interestingly, recent studies have shown that nobiletin could exert a neuroprotective and anti-apoptosis effect during the process of cerebral I/R injury [26,27]. In addition, nobiletin could also ameliorate I/R injury after liver transplantation [28]. In studies of the cardiovascular system, nobiletin has been proven to be effective for the attenuation of myocardial dysfunction in a rat model of diabetes [29]. Taken together, it could be hypothesized that nobiletin may attenuate MIRI. Therefore, using a rat model of MIRI, the present study aimed to evaluate whether pre-treatment with nobiletin could relieve MIRI. Moreover, it was also evaluated whether the regulation of ERS-related apoptosis played an important role in this pathophysiological process.

2. Materials and methods

2.1. Establishment of the rat MIRI model

Male Sprague Dawley (SD) wild-type rats (SPF grade, 200–250 g) were obtained from Wuhan University Experiment Animal Center. These rats were allowed free access to standard rat chow and water, and were kept in an environment with controlled temperature and lighting (24 °C, 12/12 hour-light/dark cycle). All experimental procedures were approved by the Animal Care and Use Committee of Wuhan University, and performed in accordance with the Institutional Guidelines and Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996).

The animals were subjected to myocardial ischemia for 30 min and reperfusion for 2 h to establish the I/R injury model. At 12 h before surgery, food, but not water, was taken away from the cages. After anesthetization by intraperitoneal injection with pentobarbital sodium (60 mg/kg), the rats were fixed in the supine position. With the temperature maintained within a normal range of 35–37 °C, the rats were artificially ventilated at 70 strokes per minute using a volume-controlled small animal respirator. Four stainless steel electrodes were subcutaneously inserted into the limb of these rats for continuous recording of standard body part II-lead ECG. Then, a left thoracotomy was performed, and a small curved needle with a 6-0 silk suture was passed around the myocardium beneath the left arterial descending (LAD) branch of the coronary artery. With a small piece of polyethylene tubing placed to the superior surface of the LAD, a ligation of approximately 2–3 mm from the tip of the left auricle was placed to induce the ischemia of the myocardium. The model of MIRI was considered to be successful when the elevation of the ST segment in leads II could be observed with the appearance of cyanosis in the regional myocardial surface. After 30 min of ischemia, the LAD ligature was released for the two-hour reperfusion of the myocardium. Then, the rats were sacrificed, and the myocardium around the cardiac apex was obtained for subsequent analyses.

2.2. Experimental design

Nobiletin was purchased from Shanghai Winherb Medical Science Co. Ltd. (Shanghai, China), and its purity was > 98% as determined by high-performance liquid chromatography (HPLC) analysis. Nobiletin was dissolved in normal saline containing 0.05% Tween-80(Sigma, USA), and applied *via* the tail vein at the start of the myocardial reperfusion. First, in order to determine the optimal dose of nobiletin to attenuate MIRI, a concentration gradient study was designed, in which 30 rats were randomized into five groups: (1) sham-operated group (SO group); (2) ischemia and reperfusion group (I/R); (3) nobiletin (15 mg/kg) + I/R (NOB15 + IR group); (4) nobiletin (30 mg/kg) + I/R (NOB30 + IR group); (5) nobiletin (45 mg/kg) + I/R (NOB45 + IR group). For rats in the SO group, surgical manipulation without ligature of the LAD was performed. However, rats in the other groups were subjected to the occlusion of LAD and reperfusion for 2 h.

In order to further explore the specific protective mechanism of nobiletin against MIRI, another experiment was implemented, in which rats were randomized into five equal groups with the following treatments: (1) Sham-operated control group (SO group, $n = 10$), rats were subjected to a similar surgical process without LAD ligation; (2) Ischemia and reperfusion control group (I/R group, $n = 10$), rats were subjected to LAD occlusion for 30 min, followed by reperfusion for 2 h; (3) Nobiletin + ischemia and reperfusion group (NOB-30 + I/R group, $n = 10$), rats were subjected to LAD occlusion for 30 min, followed by reperfusion for 2 h, with the administration of nobiletin (30 mg/kg) at the beginning of the reperfusion; (4) Nobiletin + ischemia and reperfusion + LY294002 group (NOB-30 + IR + LY group, $n = 10$), rats were subjected to LAD occlusion for 30 min, followed by reperfusion for 2 h, and co-treatments with nobiletin (30 mg/kg) and LY394002 (0.3 mg/kg) were applied at the beginning of the reperfusion; (5) LY294002 + ischemia and reperfusion group (LY + I/R group, $n = 10$), rats were subjected to LAD occlusion for 30 min, followed by reperfusion for 2 h, and LY394002 (0.3 mg/kg) was injected ahead of the reperfusion. Rats in the NOB + I/R group and NOB + IR + LY group were pre-administered with nobiletin at a dose of 30 mg/kg *via* the tail vein at the beginning of the reperfusion. An equal volume of normal saline, including 0.05% Tween-80, was similarly applied in rats in the SO, I/R and LY + IR groups. As for LY294002 (Sigma-Aldrich, St. Louis, USA), a specific inhibitor of the IP3K/AKT signaling pathway, this was dissolved in DMSO at a concentration of 0.3 mg/ml, and intraperitoneally injected at the beginning of the reperfusion.

2.3. Histological examination

Myocardial tissue was harvested at the end of the experiment and fixed in 4% paraformaldehyde solution for 24 h. After these were embedded in paraffin, the samples were cut into 5-μmin thick sections, stained with hematoxylin and eosin, (H&E) and observed under an optical microscope. In order to evaluate the severity of the myocardial injury, five fields were randomly selected from each group, and these were scored by two individuals who are blinded to the experiment, according to the following criteria: 0, no damage; 1 (mild injury), appearance with interstitial edema and focal necrosis; 2 (moderate injury), appearance with cardiomyocyte swelling and necrosis; 3 (severe injury), appearance of the formation of necrotic contraction bands and inflammatory cell enrichments; 4 (highly severe injury), appearance of the expanded necrosis of contraction bands, and inflammation cells infiltrate and hemorrhage.

2.4. Evaluation of infarct size

After reperfusion for 2 h, the LAD was retied at the original ligation site, and 1 ml of Evans blue (2.0%) (Sigma-Aldrich, St. Louis, USA) was injected into the aorta to demarcate the ischemic area at risk (AAR). Then, the heart was quickly isolated and frozen at −80 °C. The atria and right ventricular wall were resected, and the left ventricular wall was sliced into 2-mm thick transverse sections from the cardiac apex to the base. All sections were incubated with 1% 2,3,5-triphenyltetrazolium chloride (TTC, in 0.2 M of Tris buffer, pH 7.4) (Sigma-Aldrich, St. Louis, USA) solution for staining for 30 min at 37 °C. Areas that were stained blue indicated normal myocardium tissue, while areas that were stained red were on behalf of the ischemic area, but were viable myocardial tissues, namely, AAR. Furthermore, negatively stained areas (white areas) represented the infarcted myocardium. Areas of the infarcted myocardium (white areas) and myocardium at risk (red areas) were quantitatively evaluated using ImageJ software. The infarct rate was calculated as the ratio of the infarcted area over the total myocardium area in the papillary muscle plane.

2.5. Measurement of markers of myocardial injury

The plasma levels of lactate dehydrogenase (LDH) and creatine kinase-MB (CK-MB) were used as indicators of the severity of injuries of the myocardial tissue. These myocardium enzymes leaked from the damaged cells and provided information about the severity of the cytomembrane integrity destruction. Before sacrifice, blood samples were collected though the jugular vein using vacuum blood collection tubes, and the samples were centrifuged (3000 rpm, 10 min) to obtain the plasma. LDH and CK-MB levels were measured using commercially available assay kits, according to manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, China).

2.6. Detection of myocardial apoptosis

Myocardial apoptosis associated with MIRI was quantitatively analyzed by terminal deoxyribonucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) staining. Briefly, the myocardial samples were fixed in paraformaldehyde (4%) solution for 24 h, and graded dehydration in a series of ethanol dilutions was performed. After the samples were embedded in paraffin and horizontally cut into 5-μm sections, the sections were deparaffinized and rehydrated, and TUNEL staining was performed using a TUNEL assay kit (Roche Applied Science, Basle, Switzerland), according to manufacturer's instructions. Five randomly selected high-power fields (×400) of each slice were used to count the TUNEL-positive cardiomyocytes. The apoptosis index (AI) was calculated as the ratio of TUNEL-positive cells *vs.* total cardiomyocytes.

2.7. Assessment of cardiac function

Transthoracic echocardiography was performed to determine left ventricular function and cardiac remodeling before the animals were sacrificed using the MyLab 30CV ultrasound system (Biosound Esaote, Inc.). After the induction of general anesthesia with isoflurane, the images were observed from the parasternal short axis at the level of the mid-papillary muscle from at least three separate cardiac cycles. The parameters of left ventricular end-diastolic volume (LVEDV), left ventricular end-systolic volume (LVESV), left ventricular internal diameter at end-diastole (LVIDd) and left ventricular internal diameter at end-systole (LVIDs) were measured. Left ventricular ejection fraction (LVEF) and left ventricular fractional shortening (LVFS) were subsequently calculated using equations based on the M-mode echocardiographic results (EF = [LVEDV − LVESV] × 100%/LVEDV; FS = [LVIDd − LVIDs] × 100%/LVIDd).

2.8. Quantitative real-time polymerase chain reaction assay

The animals were sacrificed at 2 h after reperfusion, and myocardial tissues were collected for mRNA analysis. After the extraction and purification of total RNA from the myocardial samples using a Trizol reagent kit (Invitrogen, USA), reverse transcription was performed using 1 μg of isolated RNA to synthesize the complementary DNA (cDNA) using a commercially available cDNA synthesis kit (TaKaRa, Japan), according to manufacturer's instructions. With the presence of primer sequences and the SYBR Green Supermix (Bio-Rad) and cDNA in proper proportions, the ABI Prism 7500 Sequence Detection System (Applied Bio-systems) was used to perform the real-time RT-PCR. The polymerase chain reaction cycling condition consisted of pre-denaturing at 50 °C for 120 s, followed by 40 cycles of denaturation at 95 °C for 15 s, and subsequently, annealing at 60 °C for 30 s and extension at 60 °C for 30 s. Using the housekeeping gene GAPDH as the internal control, the expression of the target genes were normalized using the comparative quantification method ($2^{-\Delta\Delta CT}$). The primer sequences for real-time RT-PCR were as follows: GRP78 forward: 5′-TCAGCCCACCG TAACAATCAAG-3′, GRP78 reverse: 5′-GAGCAGGAGGGATTCCAGT CAG-3′, CHOP forward: 5′-CCTTCACTACTCTTGACCCTG-3′, and CHOP reverse: 5′-GACCACTCTGTTTCCGTTTC-3′, caspase-12 forward: 5′-CATTCCTGGTCTTTATGTCCC-3′, caspase-12 reverse: 5′-GTATCAGC AGTGGCTATCCCT-3′. GAPDH forward: 5′-ACAGCAACAGGGTGGTG GAC-3′. GAPDH reverse: 5′-TTTGAGGGTGCAGCGAACTT-3′.

2.9. Western blotting

Total protein extraction from myocardial tissues was performed, according to a commercially available kit (Beyotime, China). After centrifugation, the supernatant was collected, and the concentration of protein samples was quantitatively determined *via* bicinchoninic acid protein assay (BCA, Beyotime, China). Then, using 10% SDS-polyacrylamide gel electrophoresis, the protein samples from each group were separated and transferred onto nitrocellulose membranes (Millipore). After blocking the nonspecific binding sites of the membranes with 5% nonfat dried milk for 1 h, primary antibodies specific for GRP78 (Abcam, ab21685), CHOP (Cell signaling, #2895), caspase-12 (Abcam, ab62484), AKT (Cell signaling, #2938), p-AKT (Abcam, ab32509), PI3K (Abcam, ab191606), p-PI3K (Abcam, ab182651) , cytochrome C (Abcam, ab133504), cleaved-caspase-8 (Abcam, ab25901) and GAPDH (Abcam, ab181602) were used to seal the membrane overnight at 4 °C. After incubation with the secondary antibodies (room temperature for 1 h), an enhanced chemiluminescence (ECL) system was used to display the protein bands, and GAPDH was used as the internal loading control.

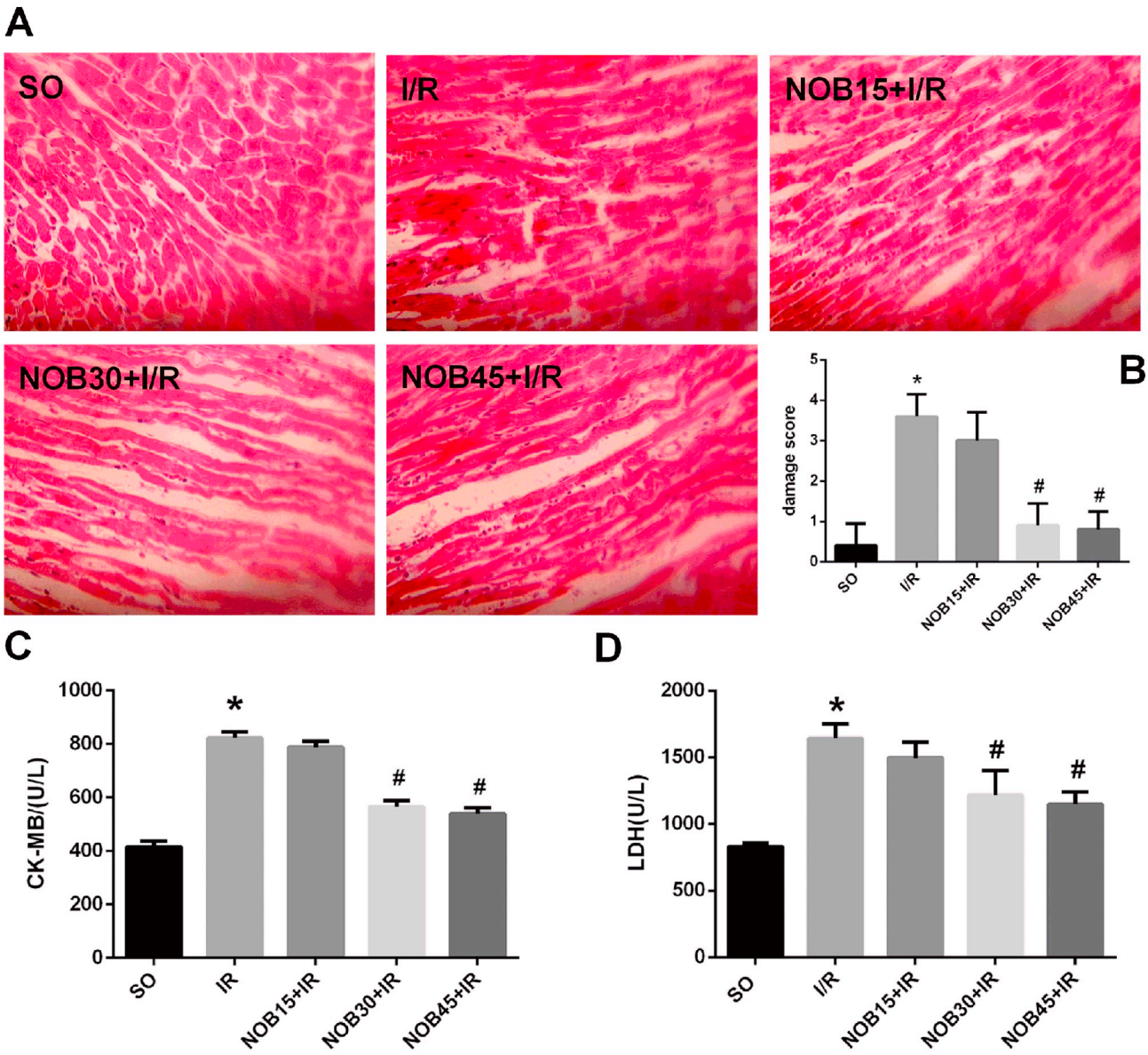

Fig. 1. Pre-treatment with nobiletin alleviates the pathological damage of myocardial tissues and mitigates myocardial injury caused by reperfusion. (A) Representative photographs of hematoxylin and eosin (H&E) staining after treatment with different doses of nobiletin at the beginning of the reperfusion (×200). (B) Bar graphs of the damage scores ($n = 5$). (c) Serum levels of CK-MB in each group ($n = 5$). (D) Serum levels of LDH in each groups ($n = 5$). The values were expressed as mean ± standard deviation (SD). $^{*}P < 0.05$ *vs.* the SO group; $^{\#}P < 0.05$ *vs.* the I/R group.

2.10. Statistical analysis

Continuous values were presented as mean ± standard deviation (SD). Student's *t*-test was used for comparisons between two groups. One-way analysis of variance (ANOVA) was used for comparisons among multiple groups. Student–Newman–Keuls (SNK)-q tests were used for subsequent analyses between multiple comparisons. A *P*-value < 0.05 was considered statistically significant. Statistical analyses were performed using SPSS 19.0 software.

3. Results

3.1. Nobiletin at doses of 30 and 45 mg/kg attenuated histological changes and reduced I/R injury in the myocardium

In order to evaluate the efficacy of different doses of nobiletin on myocardial injury caused by I/R, treatments with multiple doses of nobiletin were performed. The morphological changes of cardiomyocytes were observed under an optical microscope using H&E staining. Moreover, the biomarkers of myocardial injury (LDH and CK-MB) were also detected. As shown in the Fig. 1A, in the SO group, myocardial fibers were intact and regularly arranged, the cardiomyocytes were integral, and the cell structures were clear, with no apomorphosis, necrosis, or inflammatory cell infiltration. In the I/R group, myocardial fibers were partially ruptured and disorganized, cells fell apart and lysed, and karyopyknosis, karyolysis, hypochromatosis and interstitial edema could be observed. However, in all NOB + I/R groups at different doses, the cell structure and cell sharp were kept grossly normal, although the myocardial fibers appeared to be swelled, atrophied and partially ruptured. The damage scores in all nobiletin treated groups were significantly lower, when compared with the I/R group. In addition, the serum levels of LDH and CK-MB in all nobiletin treated groups were lower, when compared with the I/R group (Fig. 1C and D; $P < 0.05$). These data indicate that nobiletin could relieve MIRI. However, significantly improved morphological features of the myocardial tissue could only be observed in groups administered with

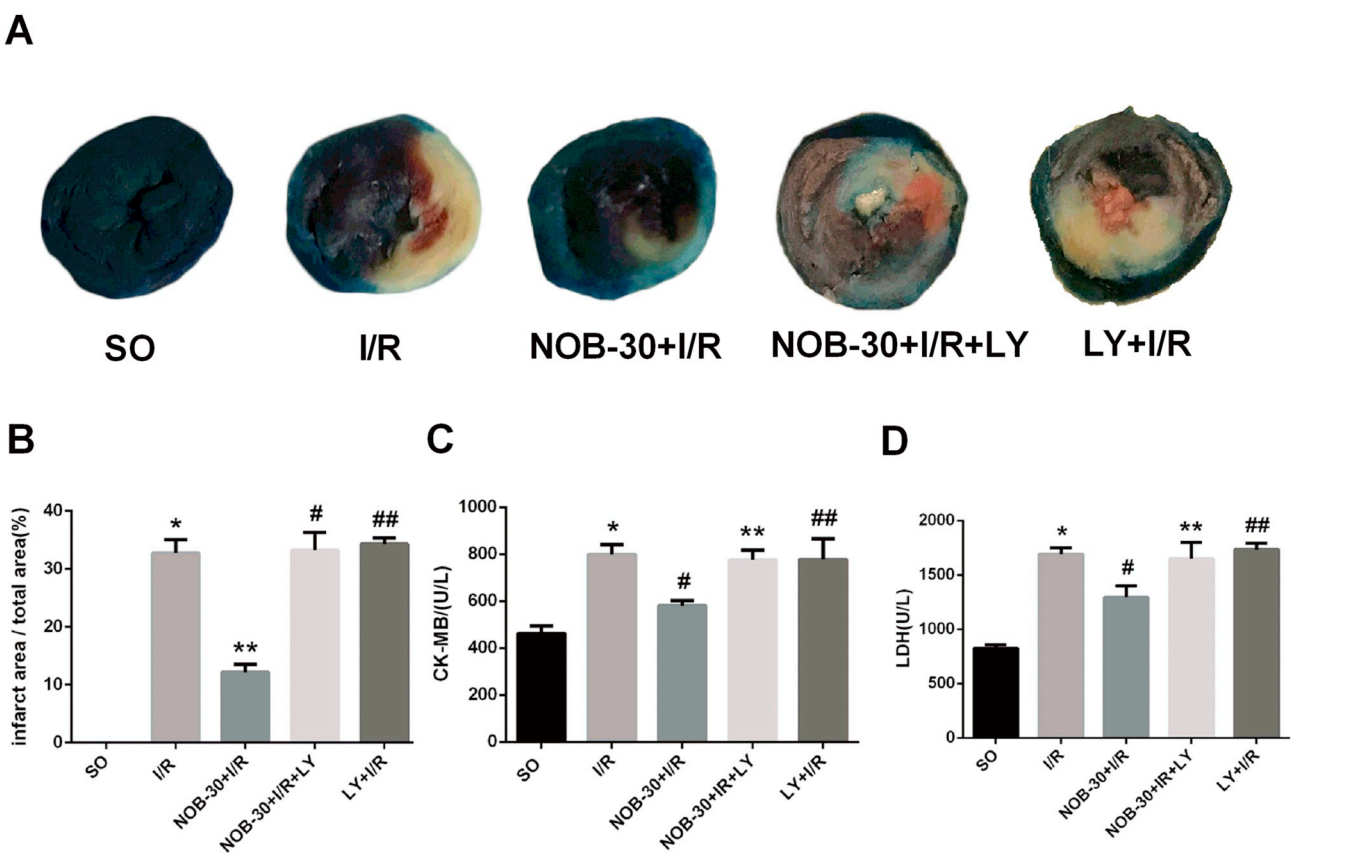

Fig. 2. Pre-treatment with nobiletin reduced the infract size and myocardial damage after MIRI. However, co-treatments with LY294002 attenuated the beneficial effect of nobiletin. (A) Representative images of Evans and TTC staining for the areas of myocardial infarction. White area, infarcted area; red-stained area, area at risk ($n = 6$). (B) Bar graphs of the percentages of myocardial infarction size ($n = 6$). (C) Serum levels of CK-MB in the different groups ($n = 6$). (D) Serum levels of LDH in the different groups ($n = 6$). Values were expressed as mean ± standard deviation (SD). $^{*}P < 0.05$ *vs.* the SO group; $^{\#}P < 0.05$ *vs.* the I/R group; $^{**}P < 0.05$ *vs.* the NOB-30 + I/R group; $^{\#\#}P > 0.05$ *vs.* the I/R group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

nobiletin at doses of 30 and 45 mg/kg. In addition, no significant differences were detected for the damage score or biomarkers of myocardial injury between groups treated with 30 or 45 mg/kg of nobiletin (Fig. 1B). Therefore, 30 mg/kg was determined as the optimal treatment dose for the subsequent experiment.

3.2. Nobiletin reduced myocardial infarction size and attenuated myocardial injury caused by I/R, while these beneficial effects could be blocked by LY294002

Evans blue and TTC double-staining were performed to measure the infraction size of the myocardium in each group. After 2 h of reperfusion, the hearts were harvested to measure the infarct size. As shown in Fig. 2A, infarct size markedly decreased in the NOB-30 + I/R group, when compared with the I/R group ($P < 0.05$). When co-treated with nobiletin and LY294002 at the beginning of the reperfusion, the infarct size of the myocardium significantly increased, when compared with rats treated with nobiletin only ($P < 0.05$). However, LY294002 alone did not affect the infract size, when compared to rats in the I/R group ($P > 0.05$).

The extent of myocardial injury was also evaluated by determining the plasma levels of LDH and CK-MB. I/R injury resulted in significant myocardial tissue injuries, and plasma LDH and CK-MB levels were remarkably upregulated ($P < 0.05$). However, pre-treatment with nobiletin alone decreased LDH and CK-MB levels by 23.3% and 37.4%, respectively, when compared with those in the I/R group. Interestingly, the co-treatments of nobiletin and LY294002 did not significantly affect the plasma levels of LDH and CK-MB, when compared to those in the I/R group ($P > 0.05$). Moreover, treatment with LY294002 alone had no significant influence on the levels of LDH or CK-MB, when compared to those in the I/R group ($P > 0.05$).

3.3. Nobiletin reduced I/R injury-induced myocardial apoptosis, while LY294002 obliterates its beneficial effect

TUNEL staining was performed to detect the cardiomyocyte apoptosis in each group. As shown in Fig. 3A, minimal TUNEL-positive cells were observed in the SO group, while I/R injury dramatically enhanced the cellular apoptosis of the myocardium ($P < 0.05$). As expected, the number of TUNEL-positive cells were significantly lower in the myocardial tissues of rats treated with nobiletin at the beginning of the reperfusion, when compared to rats in the I/R group ($P < 0.05$). However, apoptotic cardiomyocytes significantly increased in the NOB + LY + I/R group, when compared with the NOB + I/R group ($P < 0.05$), while treatment with LY294002 alone did not significantly affect the apoptosis of cardiomyocytes, when compared with the I/R group ($P > 0.05$).

3.4. Nobiletin attenuates cardiac dysfunction caused by I/R injury, while PI3K inhibition blunts its I/R-protective effects

As shown in Fig. 4A, I/R injury resulted in significantly deteriorated cardiac systolic function. However, EF and FS were improved by 18.2% and 20.9%, respectively, after the administration of nobiletin. Nevertheless, treatment with LY294002 alone significantly blocked the beneficial effects of nobiletin on cardiac function ($P < 0.05$). Furthermore,

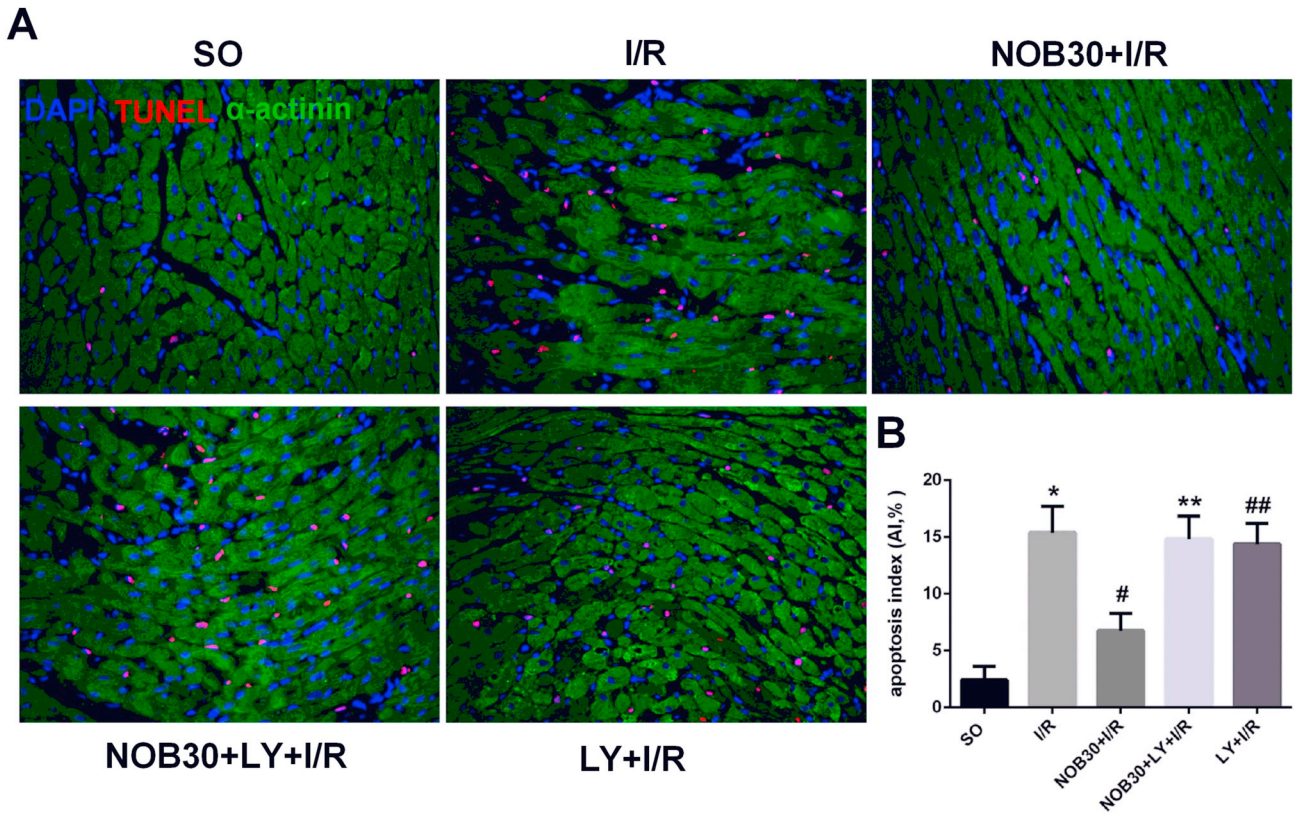

Fig. 3. Pre-treatment with nobiletin significantly relieved MIRI–induced myocardial apoptosis, while co-treatments with LY294002 partially abolished its anti-apoptosis effects. (A) Representative pictures of TUNEL staining from the five groups (×400): DAPI-labeled nuclei of cardiomyocytes (blue); α-actinin-labeled cardiomyocytes (green); TUNEL-labeled nuclei of apoptotic cardiomyocytes (red). (B) Bar graphs of the apoptosis index of each group ($n = 6$). Values were expressed as mean ± standard deviation (SD). $^{*}P < 0.05$ *vs.* the SO group; $^{\#}P < 0.05$ *vs.* the I/R group; $^{**}P < 0.05$ *vs.* the NOB-30 + I/R group; $^{\#\#}P > 0.05$ *vs.* the I/R group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

no significant differences among these parameters were demonstrated between the I/R group and LY + I/R group ($P > 0.05$).

3.5. Nobiletin ameliorates I/R injury-induced myocardial ERS, while the inhibition of the PI3K/AKT signaling pathway counteracts this ameliorative effect

GRP78 is a special biomarker of ERS. In order to clarify the effect of nobiletin on ERS, the expression of GRP78 in myocardial tissues in each group was evaluated at both the mRNA and protein level. As shown in Fig. 5A and D, the expression of GRP78 significantly decreased after pre-treatment with 30 mg/kg of nobiletin further during the reperfusion ($P < 0.05$), indicating that nobiletin attenuated the myocardial ERS induced by I/R injury. However, when pre-treated with both nobiletin and LY294002, GRP78 levels obviously increased ($P < 0.05$). Treatment with LY294002 alone did not significantly affect the expression of GRP78, when compared with the I/R group ($P > 0.05$). Thus, it was speculated that nobiletin ameliorated MIRI-induced ERS possibly through the PI3K/AKT signaling pathway.

3.6. Nobiletin inactivates the ERS-associated apoptosis signaling pathway, while the inhibition of the PI3K/AKT signaling pathway with LY294002 eliminates this anti-apoptotic effect

In order to determine whether nobiletin could mitigate ERS-induced myocardial apoptosis and explore the potential underlying mechanisms, the expression of CHOP and caspase-12 were analyzed using RT-PCR and Western blotting. As shown in Fig. 5, I/R injury significantly induced the expression of CHOP and caspase-12 at both the mRNA and protein level ($P < 0.05$). However, the administration of nobiletin at the onset of reperfusion notably decreased the mRNA and protein expression levels of CHOP by 27.1% and 32.4%, respectively. Moreover, the mRNA and protein expression levels of caspase-12 were also markedly reduced by 37.0% and 26.6%, respectively.

Since the PI3K/AKT signaling pathway has been reported to play a vital role in ERS-associated apoptosis, it was further determined whether the anti-apoptotic effect of nobiletin was mediated through the PI3K/AKT signaling pathway. As shown in Fig. 5F and G, pre-treatment with nobiletin remarkably facilitated the phosphorylation of AKT and PI3K. However, co-treatments with nobiletin and LY294002 significantly decreased the levels of p-PI3K and p-AKT ($P < 0.05$). In addition, the inhibition of the PI3K/AKT signaling pathway could abrogate the inhibitory effect of nobiletin on CHOP and caspase-12 at both the mRNA and protein level ($P < 0.05$). However, treatment with LY294002 alone did not significantly affect the levels of p-PI3K, p-AKT, CHOP and caspase-12, when compared to those in the I/R group ($P > 0.05$). Meanwhile, as shown in the Fig. 5H and I, compared with I/R group, nobiletin pretreatment could not alter cytochrome *c* and cleaved caspase-8 protein levels ($P < 0.05$).

4. Discussion

Increasing evidence suggests that nobiletin may be beneficial for various chronic diseases, including ischemic stroke, Alzheimer's disease, gastric cancer, atherosclerosis, and debate-associated vascular and myocardium damage [26,29–31], which is probably due to its multiple health-promoting biological functions. Numerous studies have demonstrated that nobiletin exerts its protective effect in the pathological process of I/R injury in different organs. Yasuda and Zheng revealed that both short-term and long-term nobiletin pre-treatment could

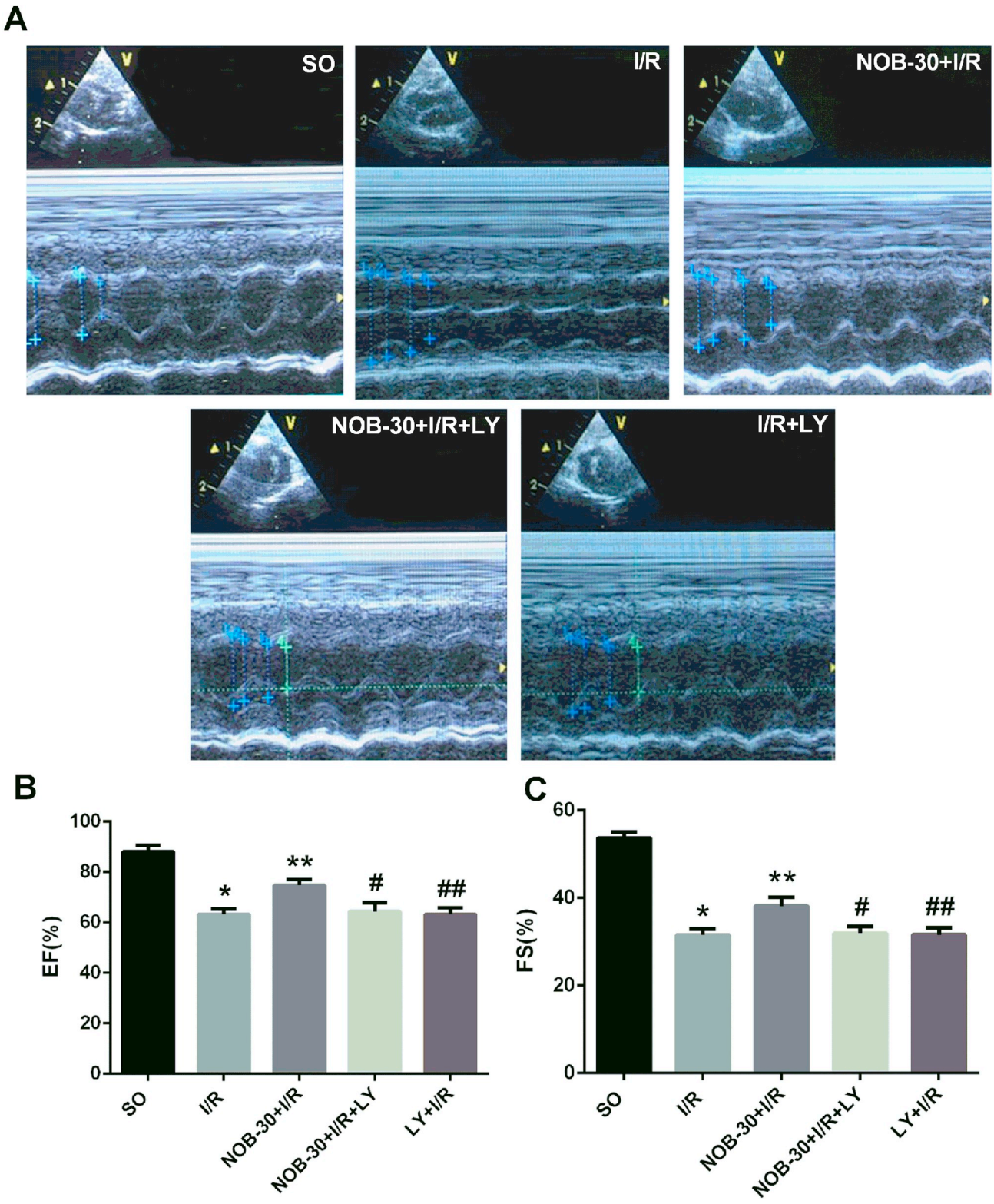

Fig. 4. Pre-treatment with nobiletin improved cardiac function after MIRI. However, co-treatment with LY294002 partially abrogated this effect. (A) Representative ultrasound images of the heart for the evaluation of cardiac function in each group. (B) Bar graphs for ejection fraction (EF) ($n = 6$). (C) Bar graphs for fractional shortening (FS) ($n = 6$). Values were expressed as mean ± standard deviation (SD). $^{*}P < 0.05$ *vs.* the SO group; $^{\#}P < 0.05$ *vs.* the I/R group; $^{**}P < 0.05$ *vs.* the NOB-30 + I/R group; $^{\#\#}P > 0.05$ *vs.* the I/R group.

significantly mitigate neutrophil invasion and decrease neurocyte apoptosis in the ischemic brain hemisphere, respectively [26,27]. In addition, Wu et al. indicated that nobiletin pre-treatment once a day for one week before liver transplantation could notably suppress the expression of inflammatory cytokines, reduce hepatocyte apoptosis and alleviate the histopathology changes induced by IR injury [28]. Moreover, Tobias and his colleges uncovered that nobiletin could reverse the deleterious effects of Midazolam on MIRI by upregulating the

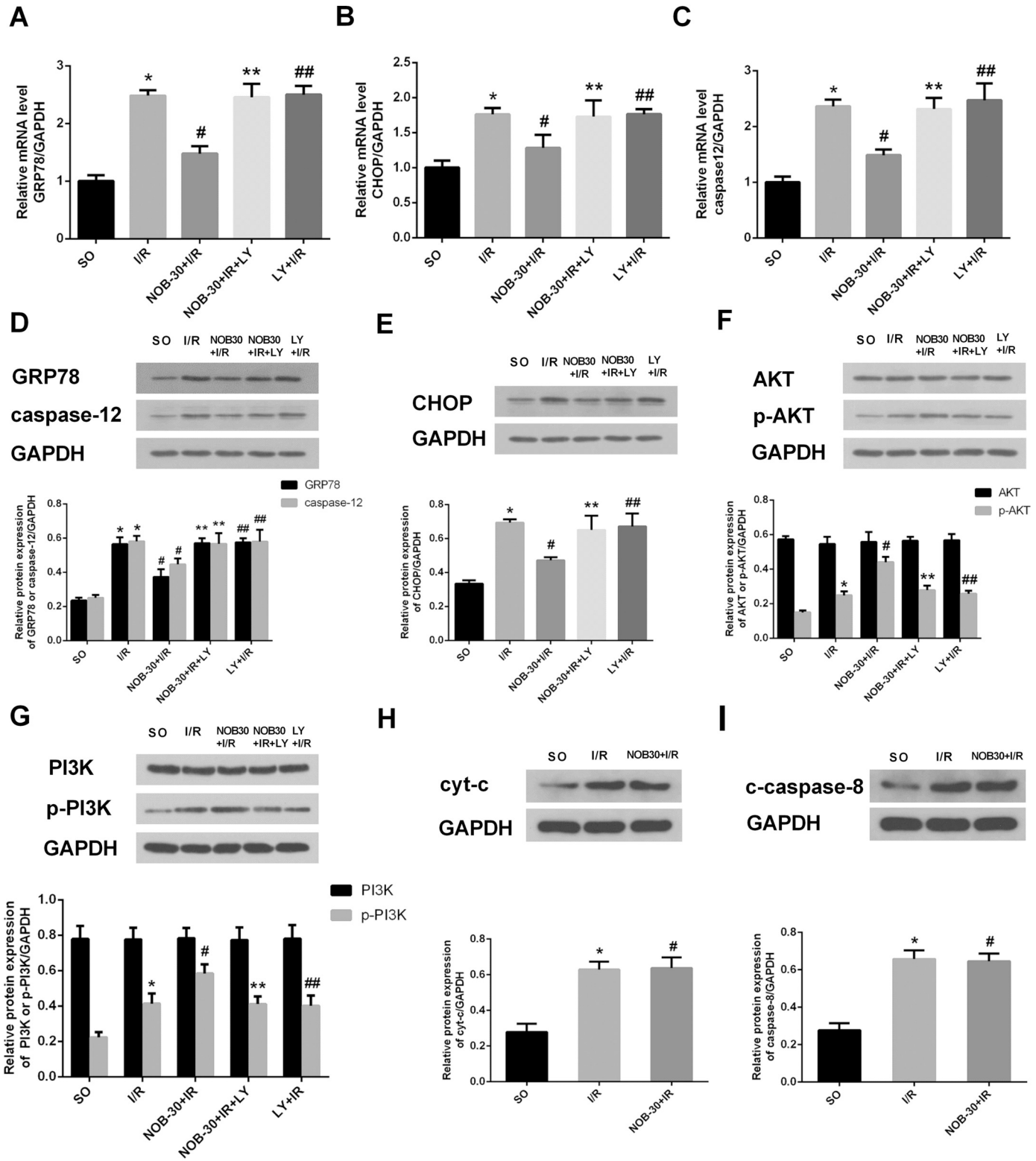

Fig. 5. Pre-treatment with nobiletin remarkably downregulated the regulatory factors involved in ERS and upregulated the expression of p-AKT, while treatment with LY294002 significantly reversed these changes. (A) The mRNA expression levels of GRP78 in each group. (B) The mRNA expression levels of CHOP in each group. (C) The mRNA expression levels of caspase-12 in each group. (D) Upper panel: Representative immunoblots of GRP78, caspase-12 and GAPDH. Lower panel: Bar graphs of the corresponding densitometry analyses. (E) Upper panel: Representative immunoblots of CHOP and GAPDH. Lower panel: Bar graphs of the corresponding densitometry analyses. (F) Upper panel: Representative immunoblots of AKT, p-AKT and GAPDH. Lower panel: Bar graphs of the corresponding densitometry analyses. (G) Upper panel: Representative immunoblots of PI3K, p-PI3K and GAPDH. Lower panel: Bar graphs for the corresponding densitometry analyses. (H) Upper panel: Representative immunoblots of cytochrome *c* and GAPDH. Lower panel: Bar graphs of the corresponding densitometry analyses. (I) Upper panel: Representative immunoblots of cleaved caspase-8 and GAPDH. Lower panel: Bar graphs of the corresponding densitometry analyses. Values were expressed as mean ± standard deviation (SD). $n = 3$; $P < 0.05$ *vs.* the SO group; $^{\#}P < 0.05$ *vs.* the I/R group; $^{**}P < 0.05$ *vs.* the NOB-30 + I/R group; $^{\#\#}P > 0.05$ *vs.* the I/R group.

expression of PER2 in cardiac endothelial cells and fibroblasts [32]. However, the effect of nobiletin on cardiomyocytes during the pathologic process of MIRI and the underlying mechanism is still unknown. In the present study, using a model of MIRI in rats, it was found that pre-treatment with 30 mg/kg of nobiletin at the onset of reperfusion could significantly minimize infract size, reduce myocardial apoptosis and improve cardiac function. These detrimental outcomes were appealing, and comprised of the pronounced alleviation of ERS and the inactivation of ERS-associated apoptosis signaling pathways. Further investigations regarding these potential molecular pathways indicated that the activation of the PI3K/AKT signaling pathway and the amelioration of ERS secondary to MIRI may be involved in the benefits of nobiletin on MIRI-induced myocardial apoptosis. Taken together, our experiment revealed that nobiletin might be a promising therapeutic option to mitigate MIRI.

It has been accepted that the most rapid and efficient treatment strategy for AMI is the early and successful reperfusion of the ischemic myocardium [3]. However, subsequent I/R injury could counteract its benefit to a great extent. Despite the considerable advances made in understanding the pathophysiologic mechanisms of MIRI, an ideal method to eliminate the adverse impact of reperfusion remains to be determined. Apoptosis has been considered as one of the most important underlying mechanisms of MIRI. As for signaling pathways that mediate cellular apoptosis, the ERS-associated apoptotic signaling pathway has been recognized as an important mechanism alternative to mitochondria-dependent and death receptor-dependent apoptotic signaling pathways [33]. Previous *in vivo* and *in vitro* studies have both indicated that MIRI could lead to severe ERS, since the level of GRP78 is significantly upregulated following MIRI [34,35]. Accumulated unfolded and misfolded proteins competitively bond with the interacting sites of GRP78, which lead to the depolymerization between GRP78 and ER-transmembrane transducers, resulting in the initiation of apoptosis cascades. CHOP is a specific transcription molecule in ERS-induced apoptosis, and belongs to the C/EBP family [36]. The expression of CHOP was maintained at a relative low level in physiological conditions, while ERS was prolonged, and all three signaling pathways activated by the UPR could promote the expression of CHOP. Subsequently, the homeostasis between its downstream molecule pro-apoptotic protein Bax and anti-apoptotic protein Bcl-2 were disturbed, leading to poor cell survival [36,37]. As for the potential role of CHOP in MIRI, Yuji Miyazaki et al. revealed that the knockout of CHOP significantly decreased ERS-induced cardiomyocyte apoptosis following MIRI [38]. Caspase-12 is another key factor involved in the ERS-dependent apoptosis signaling pathway [39]. Investigators have demonstrated that the suppression of Caspase-12 significantly alleviated ERS and protected against MIRI [40]. In line with the results of previous studies, the present data revealed that MIRI led to remarkable myocardial injury and sustained ERS, as reflected by the increased expression of GRP78 at both mRNA and protein level. More importantly, the expression of CHOP and caspase-12 were concomitantly upregulated. However, the administration of nobiletin at the beginning of the reperfusion significantly reduced the expression of GRP78, CHOP and caspase-12 at both the gene and protein level, with improvements in other parameters related to the severity of MIRI, including infract size, TUNEL positive cell number and myocardial enzymes. It is worth pointing out that except for ERS-associated apoptotic signaling pathway, mitochondria-dependent and death receptor-dependent apoptotic signaling pathways also play important roles in MIRI [41,42]. We assess the expression of cytochrome *c* (markers of mitochondrial stress) and caspase-8 (the extrinsic apoptotic marker) as well. However, nobiletin pretreatment could not alter cytochrome *c* and cleaved caspase-8 protein levels in the pathological process of MIRI. As a result, it was considered that the myocardial salvaging virtue of nobiletin is primarily mediated through the alleviation of ERS and attenuation of subsequent apoptotic signaling pathways.

Phosphoinositide 3-kinases (PI3K)/serine/threonine kinase (AKT) is a widespread signaling pathway, which has been recognized to participate in multiple cellular biological processes [43]. This signaling pathway would be activated in response to various pathological or stress stimuli, such as inflammation, ischemia, hypoxia, inflammation and oxidative stress [44]. AKT is one of the key downstream mediators of PI3K, activated PI3K converts membrane-bound phosphatidylinositol 4,5-biphosphate (PIP2) into phosphatidylinositol 3,4,5-triphosphate (PIP3), and PIP3 works as an important second messenger and recruits phosphoinositide-dependent kinase 1 (PDK1) to phosphorylate AKT at threonine 308 (Thr308) [45]. Therefore, the activation form of AKT (phosphorylated AKT) could further interact with a variety of apoptosis- and survival-related regulatory factors, leading to the promotion of cell survival and the inhibition of cell apoptosis [31]. It has been well-accepted that the activation of the PI3K/AKT signaling pathway could attenuate I/R induced cardiomyocyte apoptosis [46,47]. Furthermore, PI3K/AKT not only directly interacts with intrinsic and extrinsic cell death pathways, such as the Bcl-2 family and caspase family, but also regulates numerous key molecules associated with apoptosis, including HMGB1, HIF-1α, TNF-α, Nrf2 and so on [48]. In addition, recent studies have shown that PI3K/AKT has an intimate relationship with ERS. Kanae H et al. revealed that the inhibition of the PI3K/AKT signaling pathway significantly upregulated the expression of CHOP and exacerbated ERS in mouse fibroblast L929 cells [49]. Moreover, Xu et al. and Wu et al. further demonstrated that activation of the PI3K/AKT signaling pathway ameliorated MIRI by alleviating ERS-induced apoptosis [22,50]. Interestingly, previous studies have suggested that there might be an intimate association between nobiletin and the PI3K/AKT signaling pathway. Indeed, nobiletin has been found to postpone the development of ovarian cancer *via* the AKT signaling pathway [51]. Moreover, it has been recently shown that nobiletin could protect against cerebral ischemia through the activation of the PI3K/AKT signaling pathway [31]. In addition, researchers have also found that nobiletin plays an important protective role in ER-stress mediated apoptosis in human gastric cancer SNU-16 cells [52]. On the basis of the above investigations, it could be considered that nobiletin may ameliorate MIRI by attenuating cell apoptosis secondary to ERS through the activation of the PI3K/AKT signaling pathway.

In the present study, it was found that pre-treatment with nobiletin at the beginning of the reperfusion significantly enhanced the levels of p-PI3K and p-AKT, when compared with the I/R group. In order to explore the role of the PI3K/AKT signaling pathway in this process, both nobiletin and the specific inhibitor of PI3K were applied at the onset of reperfusion. As expected, the inhibition of the PI3K/AKT signaling pathway with LY294002 significantly blocked the phosphorylation of PI3K and its downstream mediator AKT, and simultaneously abolished the beneficial effect of nobiletin on the cardiac functional and morphological characteristics of MIRI. Similarly, co-treatments with nobiletin and the specific inhibitor of PI3K significantly abolished the inhibitory efficacy of nobiletin on the mRNA and protein expression levels of GRP78, CHOP and caspase-12. Taken together, these findings indicate that the activation of the PI3K/AKT signaling pathway and the attenuation of ERS-associated apoptosis may be the key regulatory mechanisms underlying the potential preventative effects of nobiletin on MIRI.

In conclusion, the present study reveals the protective effect of nobiletin for myocardial injury induced by I/R. More importantly, this compelling evidence highlights that nobiletin could attenuate ERS-induced cardiomyocyte apoptosis in a PI3K/AKT-dependent manner. It is noteworthy that other than anti-apoptosis, nobiletin also possess antioxidant and anti-inflammation properties. This pharmacological activity might also be the potential underlying mechanism for the preventative efficacy of nobiletin for MIRI. However, these assumption needs to be further confirmed. Taken together, the present study comprehensively elaborates the theoretical basis of nobiletin resistance to ERS induced by I/R, indicating that nobiletin might be a promising therapeutic agent to prevent MIRI.

Disclosure statement

The authors declare no conflict of interest.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (81200156 and 81570331), and the Fundamental Research Funds for the Central Universities (2042017kf0056).

References

[1] P.J. McCartney, H. Eteiba, A.M. Maznyczka, M. McEntegart, J.P. Greenwood, D.F. Muir, et al., Effect of low-dose intracoronary alteplase during primary percutaneous coronary intervention on microvascular obstruction in patients with acute myocardial infarction: a randomized clinical trial, JAMA 321 (2019) 56–68.
[2] A.C. Kentner, S.L. Grace, Between mind and heart: sex-based cognitive bias in cardiovascular disease treatment, Front. Neuroendocrinol. 45 (2017) 18–24.
[3] B. Vogel, S.R. Mehta, R. Mehran, Reperfusion strategies in acute myocardial infarction and multivessel disease, Nat. Rev. Cardiol. 14 (2017) 665–678.
[4] K.H. Humphries, M. Izadnegandar, T. Sedlak, J. Saw, N. Johnston, K. Schenck-Gustafsson, et al., Sex differences in cardiovascular disease - impact on care and outcomes, Front. Neuroendocrinol. 46 (2017) 46–70.
[5] N. Otaka, R. Shibata, K. Ohashi, Y. Uemura, T. Kambara, T. Enomoto, et al., Myonectin is an exercise-induced myokine that protects the heart from ischemia-reperfusion injury, Circ. Res. 123 (2018) 1326–1338.
[6] L.M. Yu, W.C. Di, X. Dong, Z. Li, Y. Zhang, X.D. Xue, et al., Melatonin protects diabetic heart against ischemia-reperfusion injury, role of membrane receptor-dependent cGMP-PKG activation, Biochim. Biophys. Acta Mol. basis Dis. 1864 (2018) 563–578.
[7] X. Guo, H. Jiang, J. Chen, RP105-PI3K-Akt axis: a potential therapeutic approach for ameliorating myocardial ischemia/reperfusion injury, Int. J. Cardiol. 206 (2016) 95–96.
[8] M. Zhai, B. Li, W. Duan, L. Jing, B. Zhang, M. Zhang, et al., Melatonin ameliorates myocardial ischemia reperfusion injury through SIRT3-dependent regulation of oxidative stress and apoptosis, J. Pineal Res. 63 (2017).
[9] Q. Fan, R. Tao, H. Zhang, H. Xie, L. Lu, T. Wang, et al., Dectin-1 contributes to myocardial ischemia/reperfusion injury by regulating macrophage polarization and neutrophil infiltration, Circulation 139 (2019) 663–678.
[10] H.A. Cooper, S. Eguchi, Inhibition of mitochondrial fission as a novel therapeutic strategy to reduce mortality upon myocardial infarction, Clin. Sci. (Lond.) 132 (2018) 2163–2167.
[11] T. Tanimoto, M.H. Parseghian, T. Nakahara, H. Kawai, N. Narula, D. Kim, et al., Cardioprotective effects of HSP72 administration on ischemia-reperfusion injury, J. Am. Coll. Cardiol. 70 (2017) 1479–1492.
[12] A. Gallo, C. Vannier, T. Galli, Endoplasmic reticulum-plasma membrane associations:structures and functions, Annu. Rev. Cell Dev. Biol. 32 (2016) 279–301.
[13] F.N. Penaranda, C. Meijer, F.A. Kruyt, The endoplasmic reticulum stress/unfolded protein response in gliomagenesis, tumor progression and as a therapeutic target in glioblastoma, Biochem. Pharmacol. 118 (2016) 1–8.
[14] H.Y. Fu, S. Sanada, T. Matsuzaki, Y. Liao, K. Okuda, M. Yamato, et al., Chemical endoplasmic reticulum chaperone alleviates doxorubicin-induced cardiac dysfunction, Circ. Res. 118 (2016) 798–809.
[15] X. Li, Y. Wang, H. Wang, C. Huang, Y. Huang, J. Li, Endoplasmic reticulum stress is the crossroads of autophagy, inflammation, and apoptosis signaling pathways and participates in liver fibrosis, Inflamm. Res. 64 (2015) 1–7.
[16] P. Walter, D. Ron, The unfolded protein response: from stress pathway to homeostatic regulation, Science 334 (2011) 1081–1086.
[17] J. Flores-Martin, L. Reyna, M.E. Ridano, G.M. Panzetta-Dutari, S. Genti-Raimondi, Suppression of StarD7 promotes endoplasmic reticulum stress and induces ROS production, Free Radic. Biol. Med. 99 (2016) 286–295.
[18] H. Wu, Q. Tang, J. Yang, J. Ding, M. Ye, W. Dong, Atorvastatin ameliorates myocardial ischemia/reperfusion injury through attenuation of endoplasmic reticulum stress-induced apoptosis, Int. J. Clin. Exp. Med. 7 (2014) 4915–4923.
[19] H. Wu, M. Ye, J. Yang, J. Ding, Modulating endoplasmic reticulum stress to alleviate myocardial ischemia and reperfusion injury from basic research to clinical practice: a long way to go, Int. J. Cardiol. 223 (2016) 630–631.
[20] S. Jiang, T. Li, Z. Yang, W. Yi, S. Di, Y. Sun, et al., AMPK orchestrates an elaborate cascade protecting tissue from fibrosis and aging, Ageing Res. Rev. 38 (2017) 18–27.
[21] M. Boyce, J. Yuan, Cellular response to endoplasmic reticulum stress: a matter of life or death, Cell Death Differ. 13 (2006) 363–373.
[22] X. Teng, J. Song, G. Zhang, Y. Cai, F. Yuan, J. Du, et al., Inhibition of endoplasmic reticulum stress by intermedin(1-53) protects against myocardial injury through a PI3 kinase-Akt signaling pathway, J. Mol. Med. (Berl.) 89 (2011) 1195–1205.
[23] J. Xu, H. Hu, B. Chen, R. Yue, Z. Zhou, Y. Liu, et al., Lycopene protects against hypoxia/reoxygenation injury by alleviating ER stress induced apoptosis in neonatal mouse cardiomyocytes, PLoS One 10 (2015) e136443.
[24] S. Malik, J. Bhatia, K. Suchal, N. Gamad, A.K. Dinda, Y.K. Gupta, et al., Nobiletin ameliorates cisplatin-induced acute kidney injury due to its anti-oxidant, anti-inflammatory and anti-apoptotic effects, Exp. Toxicol. Pathol. 67 (2015) 427–433.
[25] N. Rani, S. Bharti, M. Manchanda, T.C. Nag, R. Ray, S.S. Chauhan, et al., Regulation of heat shock proteins 27 and 70, p-Akt/p-eNOS and MAPKs by Naringin Dampens myocardial injury and dysfunction in vivo after ischemia/reperfusion, PLoS One 8 (2013) e82577.
[26] N. Yasuda, T. Ishii, D. Oyama, T. Fukuta, Y. Agato, A. Sato, et al., Neuroprotective effect of nobiletin on cerebral ischemia-reperfusion injury in transient middle cerebral artery-occluded rats, Brain Res. 1559 (2014) 46–54.
[27] Y. Zheng, J. Bu, L. Yu, J. Chen, H. Liu, Nobiletin improves propofol-induced neuroprotection via regulating Akt/mTOR and TLR 4/NF-kappaB signaling in ischemic brain injury in rats, Biomed. Pharmacother. 91 (2017) 494–503.
[28] Y. Wu, W. Zhang, M. Li, D. Cao, X. Yang, J. Gong, Nobiletin ameliorates ischemia-reperfusion injury by suppressing the function of Kupffer cells after liver transplantation in rats, Biomed. Pharmacother. 89 (2017) 732–741.
[29] N.A. Parkar, L.K. Bhatt, V. Addepalli, Efficacy of nobiletin, a citrus flavonoid, in the treatment of the cardiovascular dysfunction of diabetes in rats, Food Funct. 7 (2016) 3121–3129.
[30] Z.R. Li, L. Yang, J. Zhen, Y. Zhao, Z.N. Lu, Nobiletin protects PC12 cells from ERS-induced apoptosis in OGD/R injury via activation of the PI3K/AKT pathway, Exp. Ther. Med. 16 (2018) 1470–1476.
[31] L. Zhang, H. Zhao, X. Zhang, L. Chen, X. Zhao, X. Bai, et al., Nobiletin protects against cerebral ischemia via activating the p-Akt, p-CREB, BDNF and Bcl-2 pathway and ameliorating BBB permeability in rat, Brain Res. Bull. 96 (2013) 45–53.
[32] Y. Oyama, C.M. Bartman, J. Gile, D. Sehrt, T. Eckle, The circadian PER2 enhancer nobiletin reverses the deleterious effects of midazolam in myocardial ischemia and reperfusion injury, Curr. Pharm. Des. 24 (2018) 3376–3383.
[33] X.B. Wang, X.M. Huang, T. Ochs, X.Y. Li, H.F. Jin, C.S. Tang, et al., Effect of sulfur dioxide preconditioning on rat myocardial ischemia/reperfusion injury by inducing endoplasmic reticulum stress, Basic Res. Cardiol. 106 (2011) 865–878.
[34] Y. Gao, P. Jia, W. Shu, D. Jia, The protective effect of lycopene on hypoxia/reoxygenation-induced endoplasmic reticulum stress in H9C2 cardiomyocytes, Eur. J. Pharmacol. 774 (2016) 71–79.
[35] R. Wang, M. Yang, M. Wang, X. Liu, H. Xu, X. Xu, et al., Total saponins of aralia elata (Miq) seem alleviate calcium homeostasis imbalance and endoplasmic reticulum stress-related apoptosis induced by myocardial ischemia/reperfusion injury, Cell. Physiol. Biochem. 50 (1) (2018) 28–40.
[36] S. Nashine, Y. Liu, B.J. Kim, A.F. Clark, I.H. Pang, Role of C/EBP homologous protein in retinal ganglion cell death after ischemia/reperfusion injury, Invest. Ophthalmol. Vis. Sci. 56 (2014) 221–231.
[37] K. Jaikumkao, A. Pongchaidecha, L.O. Thongnak, K. Wanchai, P. Arjinajarn, V. Chatsudthipong, et al., Amelioration of renal inflammation, endoplasmic reticulum stress and apoptosis underlies the protective effect of low dosage of atorvastatin in gentamicin-induced nephrotoxicity, PLoS One 11 (2016) e164528.
[38] Y. Miyazaki, K. Kaikita, M. Endo, E. Horio, M. Miura, K. Tsujita, et al., C/EBP homologous protein deficiency attenuates myocardial reperfusion injury by inhibiting myocardial apoptosis and inflammation, Arterioscler. Thromb. Vasc. Biol. 31 (2011) 1124–1132.
[39] H. Li, H. Chen, R. Li, J. Xin, S. Wu, J. Lan, et al., Cucurbitacin I induces cancer cell death through the endoplasmic reticulum stress pathway, J. Cell. Biochem. (2018).
[40] J.G. Xia, F.F. Xu, Y. Qu, D.G. Song, H. Shen, X.H. Liu, Atorvastatin post-conditioning attenuates myocardial ischemia reperfusion injury via inhibiting endoplasmic reticulum stress-related apoptosis, Shock 42 (2014) 365–371.
[41] Q. He, J. Pu, A. Yuan, W.B. Lau, E. Gao, W.J. Koch, et al., Activation of liver-X-receptor alpha but not liver-X-receptor beta protects against myocardial ischemia/reperfusion injury, Circ. Heart Fail. 7 (2014) 1032–1041.
[42] J. Pu, A. Yuan, P. Shan, E. Gao, X. Wang, Y. Wang, et al., Cardiomyocyte-expressed farnesoid-X-receptor is a novel apoptosis mediator and contributes to myocardial ischaemia/reperfusion injury, Eur. Heart J. 34 (2013) 1834–1845.
[43] J. Guo, X. Dai, B. Laurent, N. Zheng, W. Gan, J. Zhang, et al., AKT methylation by SETDB1 promotes AKT kinase activity and oncogenic functions, Nat. Cell Biol. 21 (2019) 226–237.
[44] X. Li, X. Hu, J. Wang, W. Xu, C. Yi, R. Ma, et al., Short-term hesperidin pretreatment attenuates rat myocardial ischemia/reperfusion injury by inhibiting high mobility group box 1 protein expression via the PI3K/Akt pathway, Cell. Physiol. Biochem. 39 (2016) 1850–1862.
[45] H. Zhu, P. Luo, Y. Fu, J. Wang, J. Dai, J. Shao, et al., Dihydromyricetin prevents cardiotoxicity and enhances anticancer activity induced by adriamycin, Oncotarget 6 (2015) 3254–3267.
[46] C. Lu, T. Ha, X. Wang, L. Liu, X. Zhang, E.O. Kimbrough, et al., The TLR9 ligand, CpG-ODN, induces protection against cerebral ischemia/reperfusion injury via activation of PI3K/Akt signaling, J. Am. Heart Assoc. 3 (2014) e629.
[47] T. Ha, F. Hua, X. Liu, J. Ma, J.R. McMullen, T. Shioi, et al., Lipopolysaccharide-induced myocardial protection against ischaemia/reperfusion injury is mediated through a PI3K/Akt-dependent mechanism, Cardiovasc. Res. 78 (2008) 546–553.
[48] C. Zeng, W. Jiang, R. Zheng, C. He, J. Li, J. Xing, Cardioprotection of tilianin ameliorates myocardial ischemia-reperfusion injury: role of the apoptotic signaling pathway, PLoS One 13 (2018) e193845.
[49] K. Hyoda, T. Hosoi, N. Horie, Y. Okuma, K. Ozawa, Y. Nomura, PI3K-Akt inactivation induced CHOP expression in endoplasmic reticulum-stressed cells, Biochem. Biophys. Res. Commun. 340 (2006) 286–290.
[50] H. Wu, M. Ye, J. Yang, J. Ding, J. Yang, W. Dong, et al., Nicorandil protects the heart from ischemia/reperfusion injury by attenuating endoplasmic reticulum response-induced apoptosis through PI3K/Akt signaling pathway, Cell. Physiol. Biochem. 35 (2015) 2320–2332.
[51] J. Chen, A.Y. Chen, H. Huang, X. Ye, W.D. Rollyson, H.E. Perry, et al., The flavonoid nobiletin inhibits tumor growth and angiogenesis of ovarian cancers via the Akt pathway, Int. J. Oncol. 46 (2015) 2629–2638.
[52] J.Y. Moon, S.K. Cho, Nobiletin induces protective autophagy accompanied by ER-stress mediated apoptosis in human gastric cancer SNU-16 cells, Molecules 21 (2016).