



Brazilin prevents against myocardial ischemia-reperfusion injury through the modulation of Nrf2 via the PKC signaling pathway

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Background: Brazilin, a major ingredient of *Caesalpinia sappan L.*, possesses multiple pharmaceutical activities, although whether or not brazilin exerts any protective effect on myocardial ischemia-reperfusion injury (MIRI) has not yet been reported. The present study determined the cardioprotective effects of brazilin, and elucidated the role of nuclear factor E2-associated factor 2 (Nrf2) in this process.

Methods: Following treatment with brazilin, H9c2 cells were subjected to 6 h of hypoxia/3 h of reoxygenation. CCK-8 assay and flow cytometry were employed to detect cell viability and apoptosis, respectively. Furthermore, after brazilin treatment, isolated rat hearts underwent 30 min of ischemia, followed by 90 min of reperfusion. Triphenyltetrazolium chloride (TTC) and terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) staining were performed to measure myocardial infarct size and apoptosis, respectively. The changes in the levels of proteins were detected by western blotting.

Results: Brazilin treatment dose-dependently led to a significant enhancement in cell viability, a reduction in myocardial infarct size, and a decrease in release of creatine kinase-MB (CK-MB) and lactate dehydrogenase (LDH). Moreover, brazilin also remarkably inhibited apoptosis and led to various improvements in cardiac function. Additionally, brazilin treatment caused a marked alleviation of oxidative stress, as evidenced by the fact that brazilin reduced the accumulation of reactive oxygen species (ROS) and malondialdehyde (MDA), while enhancing the activities of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px). Mechanistically, it was found that brazilin induced Nrf2 nuclear translocation, with a concomitant upregulation of both heme oxygenase-1 (HO-1) and NAD(P)H quinone oxidoreductase (NQO1) expression. Furthermore, the phosphorylation level and transcriptional activity of Nrf2 were enhanced by brazilin, although these enhancements were abrogated by treatment with a protein kinase C (PKC) inhibitor. Finally, it was observed that the protective effects of brazilin could be negated through inhibition of Nrf2, which suggested that the cardioprotection afforded by brazilin was Nrf2-dependent.

Conclusions: Taken together, our results have demonstrated that brazilin may afford protection against MIRI through the activation of Nrf2 via the PKC signaling pathway. These results may lay the foundation for the further use of brazilin in the prevention of MIRI in clinical practice.

Keywords: Brazilin; myocardial ischemia-reperfusion injury (MIRI); oxidative stress; nuclear factor E2-associated factor 2 (Nrf2); protein kinase C (PKC)

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Introduction

Acute myocardial infarction (AMI), a major deadly disease worldwide, is commonly caused by acute and persistent coronary artery occlusion (1). Therefore, opening the occlusion of the coronary artery to rapidly restore the blood supply to myocardium is considered to be essential in the treatment of AMI (2). However, certain patients with AMI often experience a deterioration in cardiac function, fatal arrhythmia, and even extended infarct size when the blood supply to myocardium is allowed to recover through implementing percutaneous coronary intervention (PCI) (3,4). This paradoxical phenomenon is described as myocardial ischemia-reperfusion injury (MIRI) (5).

Even though MIRI was discovered more than half a century ago, the specific mechanism has yet to be fully elucidated. Oxidative stress, however, has long been regarded as a pivotal mechanism for MIRI (6). A large amount of reactive oxygen species (ROS) were found to be produced during the process of MIRI (7), and the excessive ROS were shown not only to trigger oxidative stress, but also to destroy cell biological macromolecules, including proteins, lipids and DNA, subsequently leading to myocardial cell death and cardiac function disorder (8). Nuclear factor E2-associated factor 2 (Nrf2) is an important transcription factor that regulates cellular oxidative stress responses, and serves as a key regulator in maintaining intracellular redox homeostasis (9). Nrf2 activity depends upon its dissociation from Kelch-like—ECH-associated protein 1 (Keap1), which is regulated by the phosphorylation of Nrf2. For example, Nrf2 phosphorylation via the protein kinase C (PKC) pathway facilitates the release of Nrf2 from Keap1 (10). Interestingly, recent studies have demonstrated that Nrf2 activation is able to render cardioprotection through coordinating upregulation of both phase II detoxifying enzyme and antioxidant enzyme genes (11,12). Therefore, alleviating oxidative stress injury through modulating Nrf2 activity is potentially a novel strategy for protection against MIRI.

Brazilin, a homoisoflavonoid (C₁₆H₁₄O₅; chemical structure shown in *Figure 1A*), is a natural product of *Caesalpinia sappan* L. and it has been widely used as a traditional medicine for a long time in history (13). Brazilin has been reported to possess multiple pharmaceutical effects, including its efficacy in treating depressive and anxiety disorders (14), ameliorating oxidative stress-induced photoaging of skin (15), protecting auditory cells against t-butyl hydroperoxide (t-BHP)-induced cell death (16,17),

suppressing high glucose-induced vascular inflammatory processes (18), extending lifespan in *C. elegans* (19) and so on. These effects are predominantly based on its powerful antioxidant activity, as evidenced by the fact that brazilin has been shown to inhibit antioxidant enzyme gene expression (16,17), lower the accumulation of intracellular ROS (18,19), and increase superoxide dismutase (SOD) activity (19). Interestingly, a recent study reported that brazilin exerted protective effects against renal ischemia-reperfusion injury in rats (20). Taken together, these findings enabled us to hypothesize that brazilin could be used therapeutically to afford protection against MIRI.

In this study, the cardioprotective effects of brazilin on MIRI, and its ability to influence Nrf2 activity, were examined. The results obtained demonstrated that brazilin is able to protect against MIRI both *in vitro* and *ex vivo* through the activation of Nrf2 via the PKC signaling pathway. The following article is presented in accordance with the ARRIVE reporting checklist (available at <http://dx.doi.org/10.21037/atm-20-4414>).

Methods

Experiments were performed under a project license (No. KT2018009) granted by the institutional/regional/national ethics/committee/ethics board of China Medical University and the Guide for the Care and Use of Laboratory Animals (NIH, USA) were followed closely.

Cell culture and hypoxia/reoxygenation (HR) model

Rat myocardial cells (the H9c2 cell line) were obtained from the Shanghai Institutes for Biological Sciences (Shanghai, China), and were routinely cultivated in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) under the conditions of 37 °C and 5% CO₂.

The HR model was established as described previously (21). Specifically, cells were relocated to an atmosphere of 94% N₂, 5% CO₂ and 1% O₂, and subsequently cultured with Earle's medium without glucose and FBS for 6 h to establish hypoxia. At the end of the incubation, cells were subjected to reoxygenation for 3 h through cultivating them with normal medium in an atmosphere of 5% CO₂.

Drug treatment in vitro

Brazilin (CAS no. 474-07-7; purity ≥98%) and

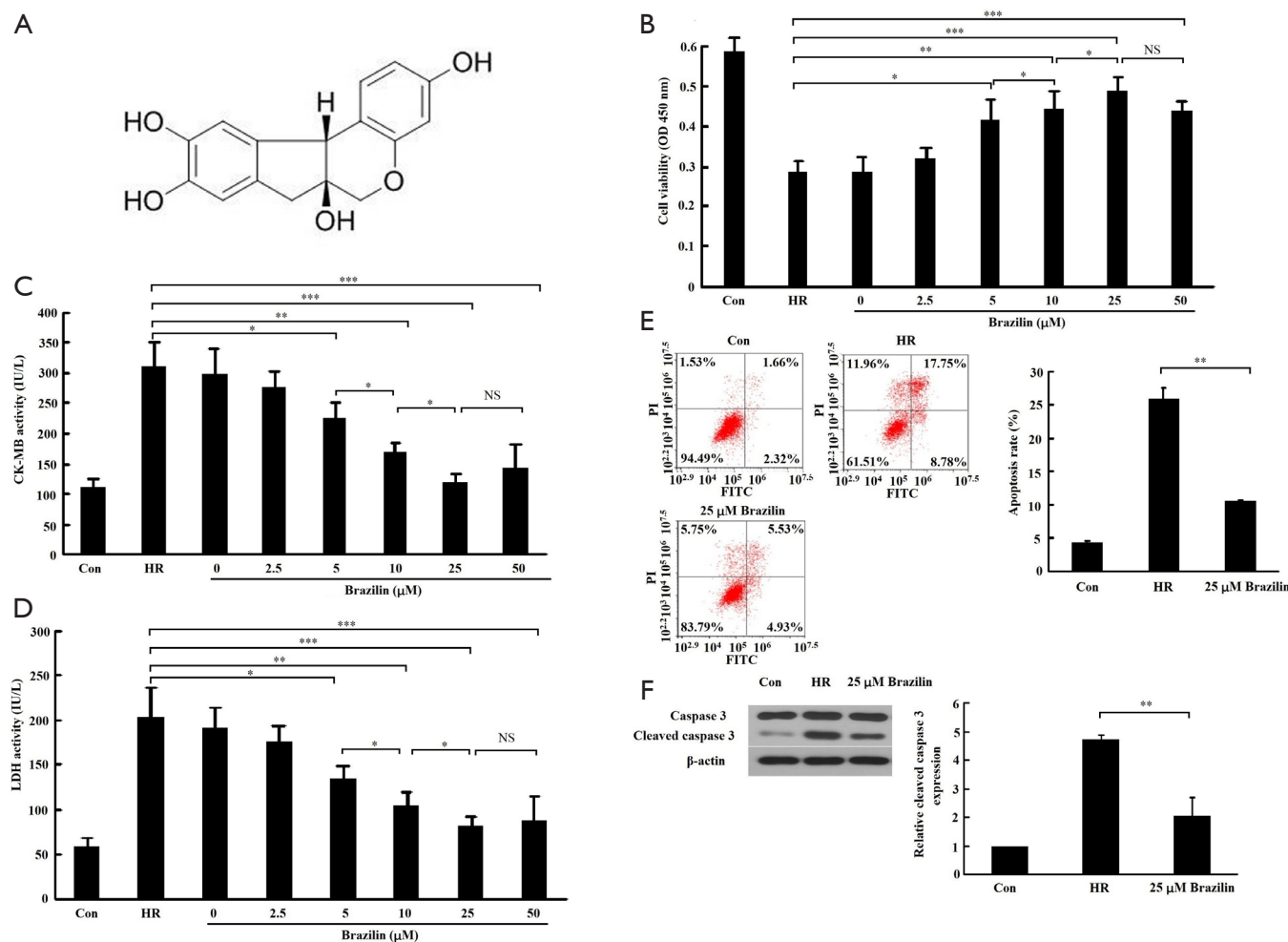


Figure 1 Brazilin protects against myocardial ischemia-reperfusion injury (MIRI) *in vitro*. Following being treated with various doses of brazilin for 1 h, myocardial cells underwent 6 h of hypoxia, followed by 3 h of reoxygenation (HR). (A) Chemical structure of sappanone A. (B) CCK-8 assay. (C) The creatine kinase-MB (CK-MB) activity. (D) Lactate dehydrogenase (LDH) activity. (E) Flow cytometry analysis of cell apoptosis. (F) Western blotting analysis of the protein expression level of caspase-3. All values are expressed as the mean \pm standard deviation (SD) from triplicate independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. NS, not significant.

chelerythrine (CAS no. 34316-15-9; purity $\geq 98\%$) were purchased from Wuhan ChemFaces Biochemical Co., Ltd. (Wuhan, China). Brazilin and chelerythrine were dissolved in dimethyl sulphoxide (DMSO) (Sigma-Aldrich; Merck KGaA), and further diluted with culture medium until DMSO concentration was $< 0.1\%$. Five different drug concentrations of brazilin (2.5, 5, 10, 25 and 50 μM) were given for 1 h prior to hypoxia stimulation. Cells were also pretreated with 1 μM chelerythrine for 1 h before brazilin treatment in order to repress PKC activation, as described previously (22).

Cell transfection

Small interfering RNA targeting Nrf2 (si-Nrf2, 5'-GG GUAAGUCGAGAAGUGUUTTAACACUUCUCGACUUACCCTT-3') and its negative control (scrambled siRNA, 5'-UUCUCCGAACGUGUCACGUTTACGUGACACGUUCGGAGAATT-3') were designed and synthesized by GenePharma Co., Ltd. (Shanghai, China). The siRNAs were transfected into cells using Invitrogen[®] Lipofectamine[®] 2000 (Thermo Fisher Scientific, Inc., Waltham, MA, USA) in accordance with the operation

guideline. Following transfection for 24 h, cells were further processed for drug treatment, followed by HR.

Cell viability assay

Cells were cultured in 96-well plates at a concentration of 5,000 cells/well. Subsequently, the cells received treatment with the brazilin and chelerythrine as described above, and underwent HR stimulation. CCK-8 assay (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was performed to evaluate cell viability following the operation guideline. The absorbance was read at 450 nm on a microplate reader.

Myocardial enzyme tests

After the cardiomyocytes had been subjected to the HR treatment, the levels of myocardial enzymes, including lactate dehydrogenase (LDH) and creatine kinase-MB (CK-MB), were notably increased. The levels of LDH and CK-MB in the culture medium and coronary effluent were measured using LDH and CK-MB assay kits, respectively (provided by Jiancheng Bioengineering Institute, Nanjing, China), following the operation guideline.

Flow cytometric analysis

To measure apoptosis in cardiomyocytes, flow cytometry was performed using an annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) kit (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) as previously described (23).

Measurement of ROS content

The cells were cultured on a tiny glass sheet and fixed with 4% paraformaldehyde for 15 min. After being washed three times with phosphate buffer saline (PBS), cells were incubated with 2,7-dichlorofluorescein diacetate (DCFH-DA), which acts as a sensitive ROS probe (Jiancheng Bioengineering Institute, Nanjing, China), at 37 °C for 1 h. DCFH-DA-stained cardiomyocytes were observed and photographed under a fluorescence microscope.

Determination of malondialdehyde (MDA), SOD and glutathione peroxidase (GSH-Px) enzymes

After being digested by trypsin, cells were collected, and subsequently resuspended in 0.5 mL PBS, prior

to being comminuted with an ultrasonic disintegrator. Cell homogenates were centrifugated at 12,000 g for 10 min, followed by collection of the supernatant. The protein concentration of the supernatant was determined using an enhanced BCA Protein Assay kit (Beyotime Institute of Biotechnology, Haimen, China) following the manufacturer's guidelines. The MDA content, and the activities of SOD and GSH-Px were measured using MDA, SOD and GSH-Px detection kits (all from Jiancheng Bioengineering Institute, Nanjing, China), respectively, following the operation guideline.

Animal experiments

The animals used in the present study were healthy male Wistar rats (8 weeks, 240–260 g), which were purchased from the Animal Laboratory Center of China Medical University (Shenyang, China). The rats were kept at 22–24 °C under 12 h daylight: 12 h dark conditions, and had free access to clean water. The body weight of the rats was monitored twice a week. The use of animals in the present study was authorized by the Institutional Animal Care and Use Committee of China Medical University, and the Guide for the Care and Use of Laboratory Animals (NIH, USA) were followed closely.

Establishment of the isolated rat heart ischemia-reperfusion model

Animal anesthesia was implemented through an intraperitoneal injection of sodium pentobarbital (30 mg/kg) (23). The rat heart was isolated from the thoracic cavity, hung on a Langendorff perfusion device, and perfused with O₂-saturated Krebs-Henseleit (K-H) solution at a constant pressure, as described previously (21). Myocardial ischemia-reperfusion was induced by stopping K-H solution perfusion for a period of 30 min, followed by reperfusion with K-H solution for 90 min. The fluid-filled latex balloon which was connected to a pressure sensor was inserted into the left ventricle to dynamically monitor the alteration of cardiac function using a homodynamic system (MP150; BIOPAC Systems, Inc., Goleta, CA, USA).

Experimental groups

The animal experiments that were performed consisted of two phases. The concentration gradient of brazilin (12.5, 25 and 50 mg/kg) was administrated to determine the most

effective dose of brazilin against MIRI based on a previous study (24). Forty-eight rats were equally separated into six groups (n=8) for the first phase, as follows: (I) the control group: the isolated heart underwent 120 min of perfusion without interruption; (II) the ischemia-reperfusion (IR) group: the isolated heart was subjected to interruption of perfusion for 30 min, followed by reperfusion for 90 min; (III) the vehicle group: the rats received an intraperitoneal injection of 1 mL saline (containing 0.1% DMSO) at 1 h ahead of heart operation, followed by IR, as described for the IR group; (IV) the 12.5 mg/kg brazilin treatment group: the rats received an intraperitoneal injection of brazilin (12.5 mg/kg) 1 h before heart isolation, followed by IR, as described for the IR group; (V) the 25 mg/kg brazilin treatment group: the rats received 25 mg/kg brazilin treatment and underwent IR, as described for the 12.5 mg/kg brazilin group; and (VI) the 50 mg/kg brazilin treatment group: the rats received 50 mg/kg brazilin treatment, and underwent IR as described for the 12.5 mg/kg brazilin group.

To explore the role of Nrf2 in the cardioprotection of brazilin, 40 rats were equally separated into five groups (n=8) for the second phase, as follows: (I) the control group: the same as described above for the first phase; (II) the IR group: also the same as the first phase; (III) the most effective dose of brazilin treatment group: the rats were administered the most effective dose of brazilin that had been confirmed in the first phase, and then underwent IR as described above for the first phase; (IV) the brazilin + ML385 group: the rats received an intraperitoneal injection of an Nrf2 inhibitor, ML385 (30 mg/kg), 30 min before brazilin administration, based on a previous study (25); and (V) the ML385 group: the rats only received an intraperitoneal injection of ML385 (30 mg/kg), followed by IR, as described above.

Measurement of infarct size

After the reperfusion finished, the hearts were firstly detached from the device and refrigerated at -20°C for 1 h, then cut into 1 mm-thick sections, finally stained with triphenyltetrazolium chloride (TTC) solution, as described previously (21).

Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay

The heart samples were subjected to formaldehyde fixation and gradient ethanol hydration, before being embedded

in wax and made into paraffin sections. TUNEL assay was employed to measure apoptosis in the myocardium using an *in situ* Cell Death detection kit (Roche Diagnostics, Indianapolis, IN, USA), in accordance with the manufacturer's instructions. The results of TUNEL staining were observed and photographed under a light microscope (Olympus BX51, Japan).

Immunofluorescence staining

Cells were placed on glass slides, fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.1% Triton X-100 for 30 min, and then blocked with goat serum for 15 min at room temperature. Subsequently, they were incubated with diluted primary antibody against Nrf2 (Abcam, Hong Kong) at 4°C overnight, followed by incubation with Cy3-labelled goat anti-mouse IgG (1:2,000; cat. no. 33208ES60; Yeason, Shanghai, China) for 60 min at room temperature. Nuclei were re-stained with DAPI, and the results of the staining process were observed under a fluorescence microscope.

Enzyme-linked immunosorbent assay (ELISA)

The nuclear lysates were extracted from cardiomyocytes using a Nuclear Extraction kit (cat. no. ab113474; Abcam, Cambridge, UK), in accordance with the operation guideline. Nrf2 transcriptional activity was then measured by ELISA using an Nrf2 Transcription Factor Assay kit (cat. no. ab207223; Abcam) in accordance with the operation guideline.

Western blotting

Total proteins were obtained from the collected cells with RIPA. Nuclear and cytoplasmic proteins were extracted using a Nuclear and Cytoplasmic Protein Extraction kit (Beyotime Institute of Biotechnology, Shanghai, China), and the protein concentration was determined using an enhanced BCA Protein Assay kit (Beyotime Institute of Biotechnology), in accordance with the manufacturer's instructions. Protein samples were thermally denatured, separated by 8% or 10% SDS-PAGE electrophoresis, transferred to PVDF membranes and blocked with 1% bovine serum albumin for 1 h at room temperature, followed by incubation with diluted primary antibodies, including anti-Nrf2 (cat. no. ab89443), anti-phospho-Nrf2 (cat. no. ab76026), anti-caspase 3 (cat. no. ab184787), anti-PKC ϵ (cat. no. ab182126), anti-phospho-PKC ϵ (cat.

no. ab108972), anti-heme oxygenase-1 (anti-HO-1) (cat. no. ab68477) and anti-NAD(P)H quinone oxidoreductase (anti-NQO1) (cat. no. ab28947) (all antibodies diluted 1:1,000 and purchased from Abcam) at 4 °C overnight. After being washed three times with PBS, the membranes were incubated with horseradish peroxidase (HRP)-labelled IgG (1:5,000; Zhongshan Jinqiao Biotechnology, Beijing, China) at room temperature for 30 min. The membranes were then developed using a BeyoECL Plus kit (Beyotime Institute of Biotechnology) following the operation guideline. Image J2x analysis software (NIH, Bethesda, MD, USA) was employed to analyze relative densitometry values.

Statistical analysis

All values are presented as the mean \pm standard deviation (SD). The statistical significance between groups were determined using one-way analysis of variance, followed by the least significant difference post-hoc test. SPSS version 17.0 software (SPSS Inc., Chicago, IL, USA) was employed to conduct all statistical analyses, and $P < 0.05$ was considered statistically significant.

Results

Brazilin protected against MIRI *in vitro*

First, the effects of various concentrations of brazilin (0–50 μ M) on cell viability and the release of cardiac enzymes in the HR-induced cardiomyocytes were examined. The results showed that concentrations of brazilin ranging from 5 to 25 μ M dose-dependently led to a notable enhancement in cell viability and a reduction in the release of CK-MB and LDH. However, no further enhancements in the effectiveness of brazilin were observed in the higher concentration of brazilin (50 μ M) treatment group (Figure 1B,C,D). Accordingly, 25 μ M brazilin was chosen as the concentration for further investigations *in vitro*. Moreover, the cell apoptotic rate (Figure 1E) and expression level of cleaved caspase 3 (Figure 1F) were shown to be repressed by brazilin treatment. Altogether, these findings suggested that brazilin could protect against MIRI *in vitro*.

Brazilin ameliorated MIRI *ex vivo*

Subsequently, the cardioprotective effects of brazilin in an isolated rat heart model were examined. As shown in Figure 2A, the myocardial infarct size was dose-dependently

reduced by brazilin, ranging from 12.5 to 25 mg/kg. Furthermore, similar results were also obtained with respect to the CK-MB and LDH (Figure 2B,C). However, no additional enhancement of the effects were shown in the higher dose of brazilin (50 mg/kg) treatment group. Therefore, 25 mg/kg brazilin was selected as the dose for further experiments *ex vivo*. Moreover, the percentage of apoptotic cells was decreased by brazilin treatment (Figure 2D). In addition, even though no significant changes were observed in terms of heart rate (Figure 2E), the higher values of LVDP and $\pm dp/dt$ were preserved in the brazilin treatment group compared with the IR group (Figure 2F,G,H). Altogether, these findings revealed that brazilin could ameliorate MIRI *ex vivo*.

Brazilin alleviated HR-induced oxidative stress

To investigate the effects of brazilin on HR-induced oxidative stress, the levels of ROS and MDA and the activities of the anti-oxidative enzymes SOD and GXH-Px following brazilin treatment were both examined. The results showed that brazilin decreased the accumulation of ROS and MDA in HR-induced cardiomyocytes (Figure 3A,B). Brazilin was also found to enhance the activities of SOD and GXH-Px (Figure 3C,D). Altogether, these results suggested that brazilin could alleviate HR-induced oxidative stress.

Brazilin modulated the activity of Nrf2 via the PKC pathway

To elucidate how the levels of oxidative stress may be regulated by brazilin, the effect of brazilin on the regulation of Nrf2, a key transcription factor to oxidative stress, was subsequently assessed. The results indicated that brazilin could induce Nrf2 nuclear translocation, as evidenced by a notable increase in the expression level of Nrf2 in the nuclei, accompanied by a marked decrease in the expression of Nrf2 in the cytoplasm (Figure 4A,B). Additionally, HO-1 and NQO1, two main downstream target genes of Nrf2, were downregulated following brazilin treatment (Figure 4C,D). Altogether, these findings revealed that brazilin could stimulate the transcriptional activity of Nrf2.

Since Nrf2 nuclear translocation may be regulated by PKC-mediated phosphorylation (26), the influence of brazilin on the phosphorylation of PKC ϵ and Nrf2 was subsequently examined. The results revealed that the phosphorylation level of PKC ϵ (Figure 5A) and Nrf2 (Figure

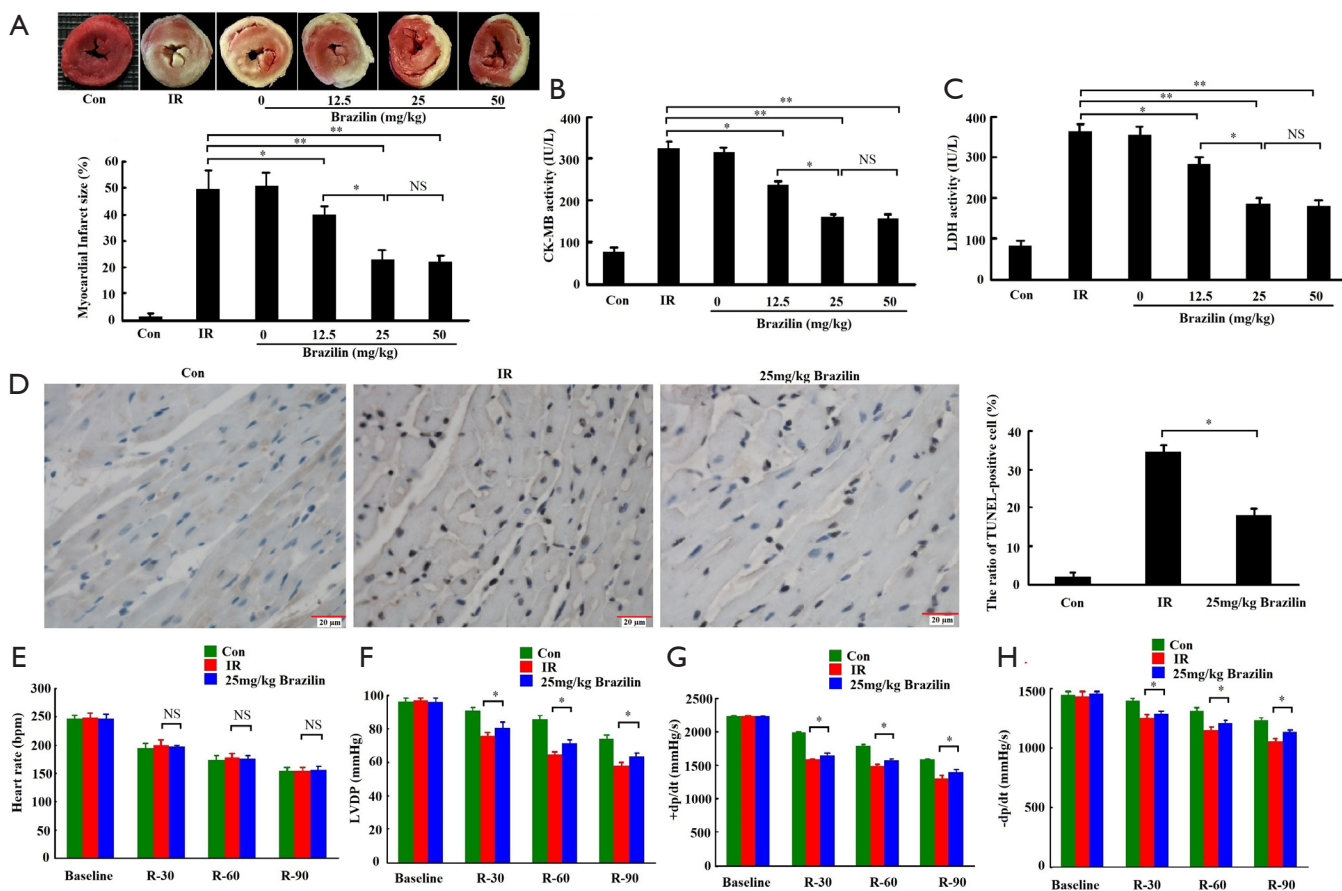


Figure 2 Brazilin ameliorates myocardial ischemia-reperfusion injury (MIRI) *ex vivo*. Wistar rats received an intraperitoneal injection of different doses of brazilin 1 h before heart isolation, and then the isolated hearts were subjected to 30 min of ischemia, followed by 90 min of reperfusion. (A) Triphenyltetrazolium chloride (TTC) staining. (B) The creatine kinase-MB (CK-MB) activity in coronary effluent. (C) Lactate dehydrogenase (LDH) activity in coronary effluent. (D) Myocardial apoptosis was measured by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) staining (magnification, $\times 400$) ($n=6$). The nuclei in apoptotic cells presented brown, and normal nuclei presented blue. Scale bar =20 μm . Changes in cardiac function parameters were collected 1 min prior to ischemia (baseline), after 30 (R-30), 60 (R-60) and 90 (R-90) min of reperfusion. (E) Heart rate. (F) Left ventricular developed pressure (LVDP). (G) Positive first-order derivative of ventricular pressure (+dp/dt). (H) Negative first-order derivative of ventricular pressure (-dp/dt). All values are expressed as the mean \pm standard deviation (SD) ($n=8$). *, $P<0.05$; **, $P<0.01$. NS, not significant.

5B), as well as the transcriptional activity of Nrf2 (Figure 5C), were increased by brazilin. However, the reinforced phosphorylation level and transcriptional activity of Nrf2 by brazilin were found to be abrogated by a PKC inhibitor (Figure 5B,C). Therefore, these results further suggested that brazilin was activating Nrf2 through the PKC pathway.

The cardioprotection of brazilin might be rescued via inhibition of Nrf2

To confirm the role of Nrf2 in the cardioprotection of

brazilin, Nrf2 was inhibited by si-Nrf2 *in vitro* or via an Nrf2 inhibitor (ML385) *in vivo*, followed by brazilin treatment. The enhancement in cell viability and reduction in the rate of release of CK-MB and LDH mediated by brazilin were effectively rescued by treatment with si-Nrf2 *in vitro* (Figure 6A,B,C). Similarly, the beneficial effects of brazilin on myocardial necrosis were also abrogated by treatment with ML385 (Figure 6D,E,F). These results suggested that the protective effects of brazilin on MIRI were abolished via the inhibition of Nrf2, and confirmed that brazilin exerted its protection against MIRI via a

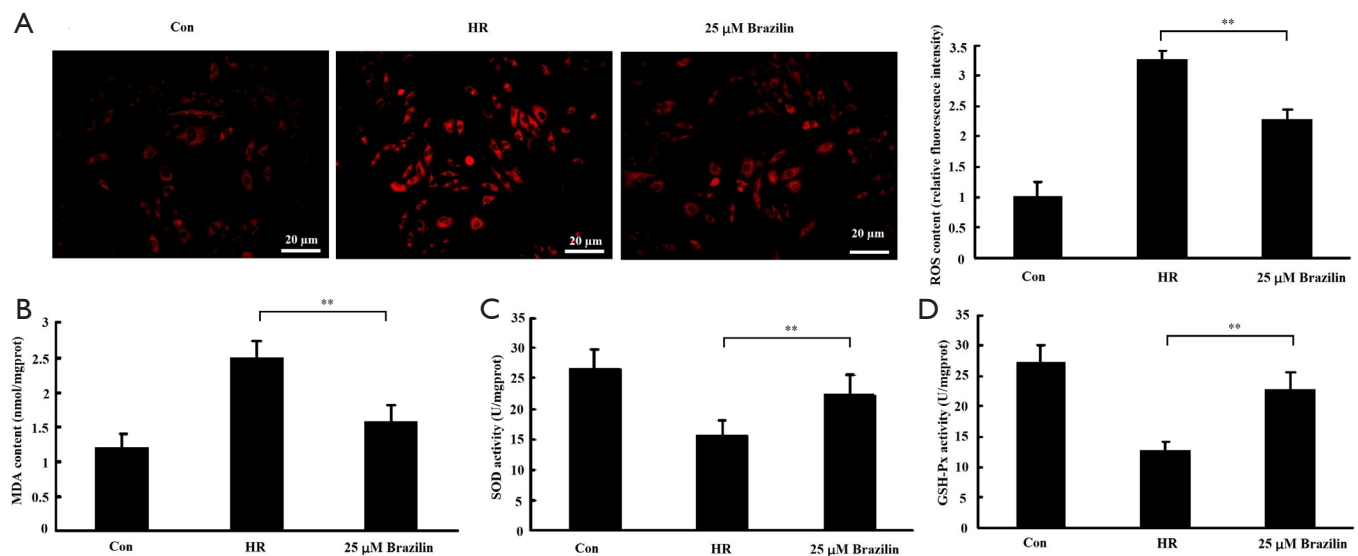


Figure 3 Brazilin alleviates hypoxia/reoxygenation (HR)-induced oxidative stress. Following being treated with 25 μ M brazilin for 1 h, myocardial cells underwent 6 h of hypoxia, followed by 3 h of reoxygenation (HR). (A) Analysis of reactive oxygen species (ROS) levels in cells using a DCFH-DA probe (immunofluorescence staining). Scale bar =20 μ m. The ROS level was calculated by the mean fluorescence intensity. (B) Malondialdehyde (MDA) content. (C) Superoxide dismutase (SOD) activity. (D) Glutathione peroxidase (GSH-Px) activity. All values are presented as the mean \pm standard deviation (SD) (n=6). **, P<0.01.

mechanism involving Nrf2.

Discussion

Caesalpinia sappan L., a type of flowering tree belonging to the legume family, is mainly distributed in certain Asian countries, including India, Myanmar and Vietnam. Its dried heartwood, usually used as a folk medicine, is used to treat ulcers, diarrhea, epilepsy, traumatic disease and menstrual disorders in those countries (27). A body of experimental evidence has demonstrated that the extract of *Caesalpinia sappan L.* possesses anti-oxidative (16), anti-inflammatory (28) and antimicrobial activities (29). In addition, sappanone A, another active component of *Caesalpinia sappan L.* that is similar to brazilin, has been demonstrated to protect against MIRI both *in vitro* and *ex vivo* (30,31). However, whether brazilin is able to exert any protective effects on MIRI has not yet been reported. In this study, it was shown that brazilin could alleviate MIRI in a dose-dependent manner *in vitro* and *ex vivo*. To the best of the authors' knowledge, this is the first study to have been published on the role of cardioprotection afforded by brazilin, and these findings will lay the foundation for the further use of brazilin in terms of the prevention of MIRI in clinical practice.

Previous studies have shown that the majority of the beneficial pharmaceutical effects exerted by brazilin are concerned with its regulation on oxidative stress (14-19). For example, Wang *et al.* (14) suggested that brazilin exerted antidepressant- and anxiolytic-like effects in mice with chronically mild stress (CMS)-induced depression, probably through inhibiting oxidative stress. Moreover, Jayakumar *et al.* (18) suggested that brazilin could suppress high glucose-induced vascular inflammatory processes partly via inhibiting ROS production. In agreement with previous findings, it was also shown in the present study that brazilin could inhibit the accumulation of ROS and MDA in HR-induced cardiomyocytes, while the activities of antioxidant enzymes in the cardiomyocytes were stimulated. These results suggested that the anti-MIRI effect of brazilin is based, at least in part, on its coordination of the cellular antioxidant defenses and maintenance of the redox balance under conditions of MIRI.

Mounting evidence has shown that Nrf2 exerts a critical role in MIRI (11,12). A significant increase in myocardial infarct size was demonstrated in Nrf2-knockout mice that were subjected to IR (32). On the other hand, Nrf2 could be activated by ischemic preconditioning and ischemic postconditioning, two well-established endogenous cardioprotective mechanisms, subsequently

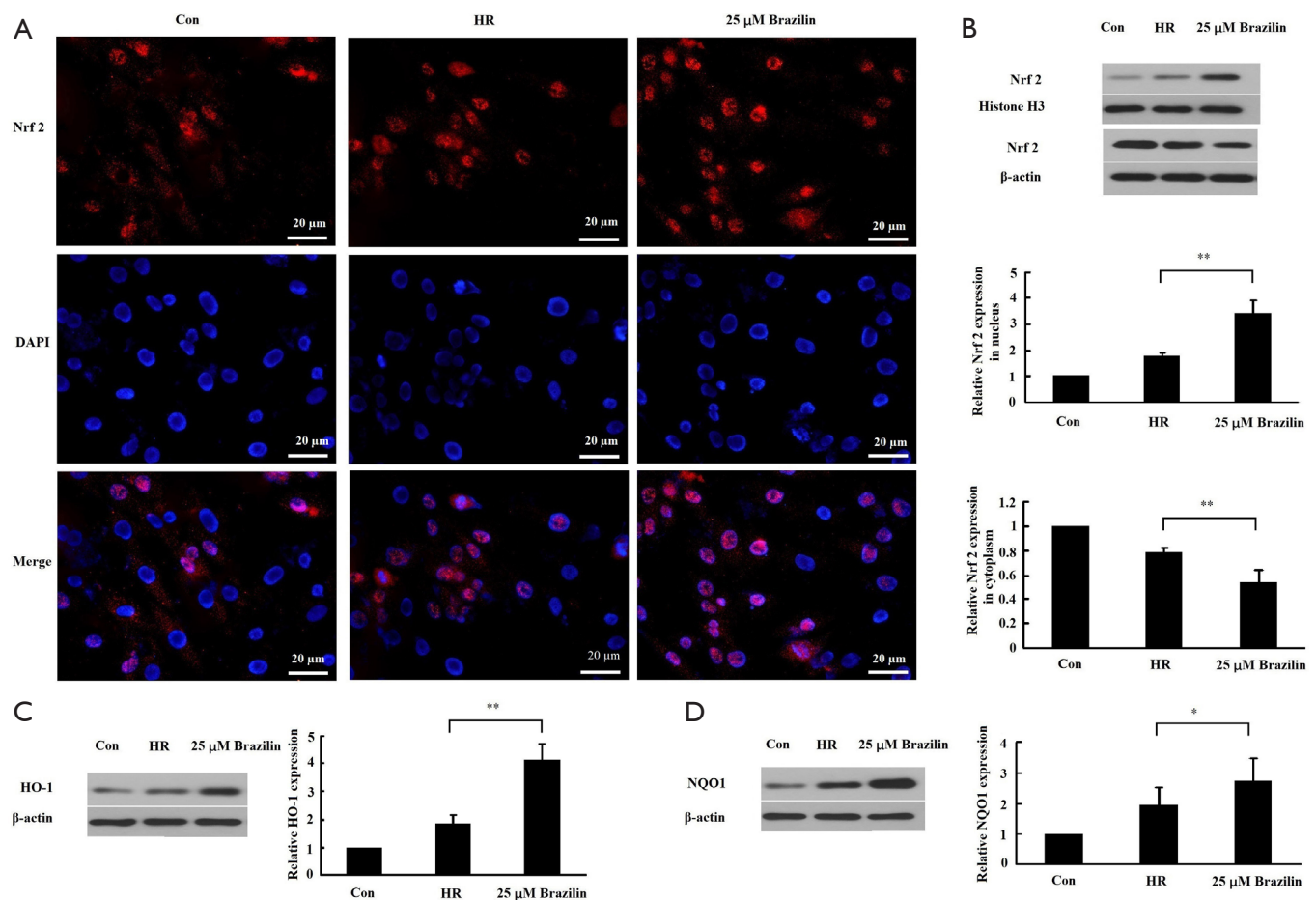


Figure 4 Brazilin promotes Nrf2 nuclear translocation and upregulates the expression of its target genes. Following being treated with various doses of brazilin for 1 h, myocardial cells underwent 6 h of hypoxia, followed by 3 h of reoxygenation (HR). (A) Nrf2 nuclear translocation was detected via immunofluorescence staining. Scale bar =20 μm. (B) Western blotting analysis of the expression level of Nrf2 in the cytoplasm and nuclei. (C) Western blotting analysis of heme oxygenase-1 (HO-1) expression. (D) Western blotting analysis of NAD(P)H quinone dehydrogenase 1 (NQO1) expression. All values are expressed as the mean ± standard deviation (SD) (n=3). *, P<0.05; **, P<0.01.

inducing antioxidant enzymes to protect the heart against MIRI (33,34). In this study, it was shown that Nrf2 was translocated into the nucleus, subsequently leading to an increase in HO-1 and NQO1 expression following brazilin treatment, findings that were consistent with those of Choi *et al.* (17), who were working with t-BHP-induced House Ear Institute-Organ of Corti 1 (HEI-OC1) cells. Additionally, the present study revealed that the protective effect of brazilin was abrogated by inhibition of Nrf2 with si-Nrf2 and an Nrf2 inhibitor, which further suggested that the cardioprotective effects of brazilin were Nrf2-dependent. Taken together, it was possible to conclude that brazilin protected against MIRI via activation of Nrf2.

It has been reported that the activation of Nrf2 induced by oxidative stress is involved with PKC and the phosphoinositide 3-kinase (PI3K)/Akt and extracellular-signal-regulated kinase (ERK) pathways (12). Choi *et al.* (17) reported that an increase in the expression of HO-1 induced by brazilin was dependent on activation of the PI3K/Akt and ERK pathways. However, whether PKC is involved with the activation of Nrf2 induced by brazilin has yet to be determined. Our present study showed that brazilin could induce PKC activation by enhancing its phosphorylation. An accumulating body of evidence has shown that PKC activation regulates Nrf2 transcription. For example, direct phosphorylation of serine-40 of Nrf2 by PKC led to the

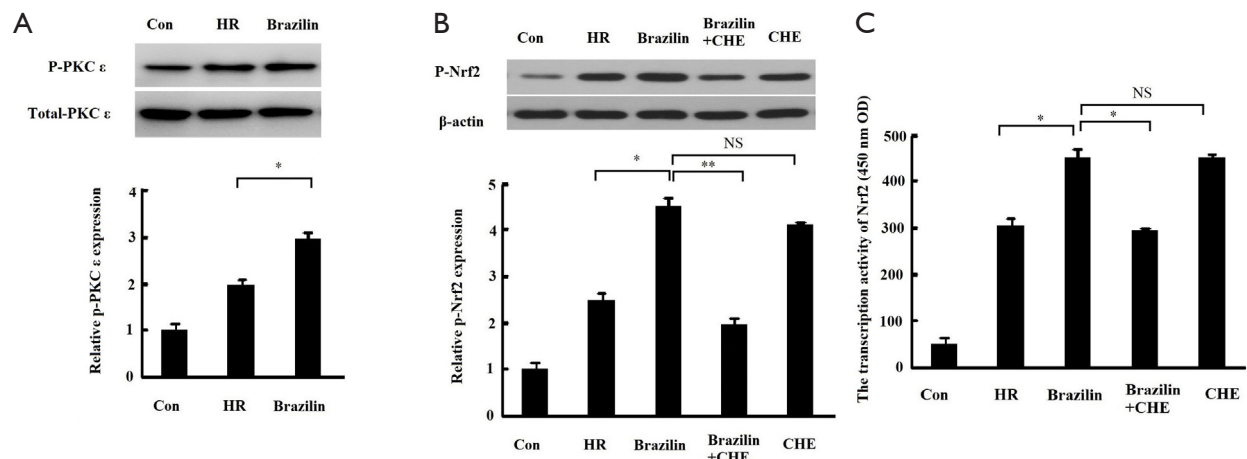


Figure 5 Brazilin activates Nrf2 through the PKC pathway. Following being treated with various doses of brazilin for 1 h, myocardial cells underwent 6 h of hypoxia, followed by 3 h of reoxygenation (HR). Cells were treated with 1 μ M chelerythrine (CHE), a PKC inhibitor, 1 h before brazilin treatment. (A) Western blotting analysis of the phosphorylation of PKC ϵ . (B) Western blotting analysis of the phosphorylation of Nrf2 (P-Nrf2). (C) The transcriptional activity of Nrf2 was measured by enzyme-linked immunosorbent assay (ELISA). All values are expressed as the mean \pm standard deviation (SD), (n=3). *, P<0.05; **, P<0.01. NS, not significant.

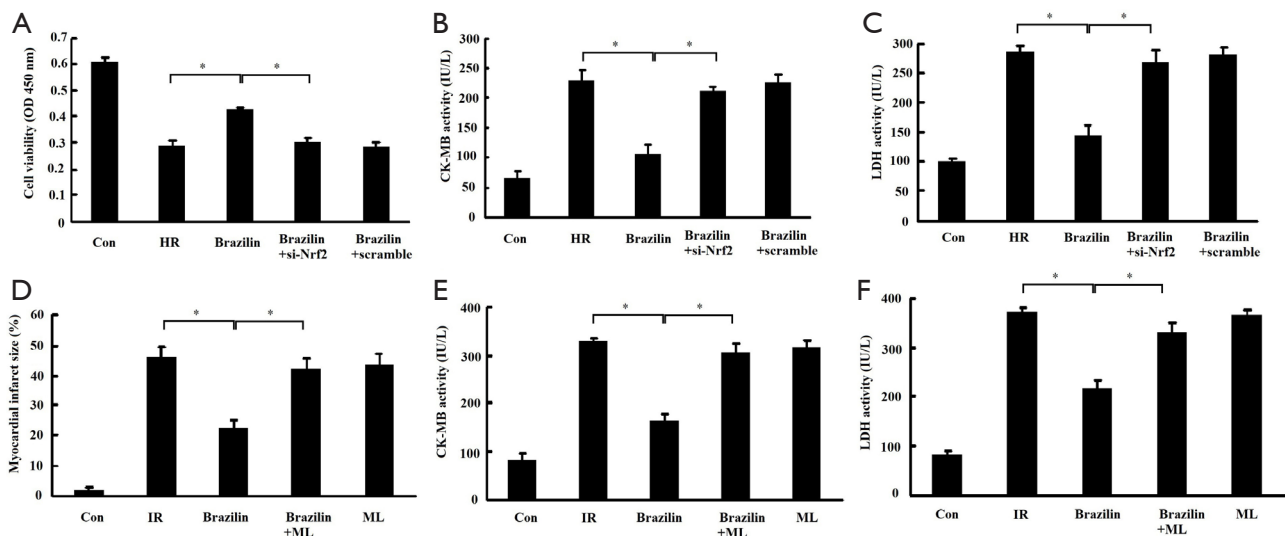


Figure 6 The cardioprotection of brazilin was rescued by inhibition of Nrf2. H9c2 cells were transfected with small interfering RNA targeting Nrf2 (si-Nrf2) and its negative control (scrambled siRNA). Following transfection for 24 h, cells were further processed for brazilin treatment, then subjected to 6 h of hypoxia, followed by 3 h of reoxygenation (HR). (A) CCK-8 assay. (B) The creatine kinase-MB (CK-MB) activity in the culture medium. (C) Lactate dehydrogenase (LDH) activity in the culture medium. All values are expressed as the mean \pm standard deviation (SD) from triplicate independent experiments. *, P<0.05. Following being intraperitoneally administered with brazilin (25 mg/kg) for 1 h, the hearts were isolated and subjected to 30-min ischemia, followed by 90-min reperfusion. ML385 (30 mg/kg), an Nrf2 inhibitor, was intraperitoneally given for 30 min before brazilin treatment. (D) Triphenyltetrazolium chloride (TTC) staining. (E) Creatine kinase-MB (CK-MB) activity in the coronary effluent. (F) Lactate dehydrogenase (LDH) activity in the coronary effluent. All values are presented as the mean \pm SD (n=8). *, P<0.05.

release of Nrf2 from Keap1, a physiological suppressor of Nrf2, thereby promoting translocation of Nrf2 to the nucleus (35,36). The results of the present study have corroborated that both the phosphorylation and the transcriptional activity of Nrf2 were reinforced by brazilin treatment, but abolished by PKC inhibitors, suggesting that the activation of Nrf2 by brazilin is mediated via the PKC pathway. These findings provide novel information in terms of understanding how the induction of Nrf2 by brazilin occurs.

There were certain limitations associated with the present study. Since the isolated heart lacks its usual neural and humoral regulation, the MIRI model established by the Langendorff method is not entirely reflective of the pathophysiological alterations during MIRI. The cardioprotective mechanism of brazilin should also be examined further *in vivo*.

In conclusion, the present results have demonstrated that brazilin may protect against MIRI via activation of Nrf2 through the PKC pathway. These results may lay the foundation for the further use of brazilin in the prevention of MIRI in clinical practice.

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Footnote

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granted by the institutional/regional/national ethics/committee/ethics board of China Medical University and the Guide for the Care and Use of Laboratory Animals (NIH, USA) were followed closely.

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