Saprirearine protects H9c2 cardiomyocytes against hypoxia/reoxygenation-induced apoptosis by activating Nrf2

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Myocardial infarction is a major cause of mortality and disability worldwide. Ischemia/reperfusion injury is the key factor that results in the increase in infarct size in pathogenesis. To find a novel therapy for myocardial infarction, we have evaluated saprirearine, a natural diterpenoid, using H9c2 cardiomyocytes injured by hypoxia/reoxygenation and explored the possible mechanisms. The results showed that saprirearine improved cell survival by increasing cell viability and blocking the release of lactate dehydrogenase. Meanwhile, saprirearine was found to attenuate mitochondrial dysfunction by inhibiting calcium overload, collapse of the mitochondrial membrane potential, and opening of the mitochondrial permeability transition pore. And oxidative stress resulting from hypoxia/reoxygenation was ameliorated by saprirearine through the reduction of reactive oxygen species and malondialdehyde as well as activation of superoxide dismutase and catalasase. Additionally, saprirearine inactivated cysteinylation-specific proteinase-3, the up-regulated B-cell lymphoma-2 and down-regulated Bcl-2-associated X protein, to inhibit hypoxia/reoxygenation-induced apoptosis. Further research revealed saprirearine-activated nuclear factor E2-related factor-2 in H9c2 cardiomyocytes, which is closely associated with its protective effects. These findings can provide evidence for the discovery of new therapies targeting myocardial infarction and the application of saprirearine in clinical practice.

Keywords: saprirearine, myocardial infarction, H9c2 cardiomyocytes, apoptosis, Nrf2

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INTRODUCTION

Myocardial infarction resulting from ischemia is one of the leading causes of death and disability worldwide (Thygesen et al., 2012). Although timely myocardial reperfusion with thrombolytic therapy or primary myocardial intervention can reduce the size of the infarct and improve the clinical outcome, restoration of blood flow to the ischemic myocardium will induce the injury and result in cardiomyocyte apoptosis, as well as increase the size of the infarct (Yellon & Hausenloy, 2007). In the pathogenesis of myocardial reperfusion injury, oxidative stress plays a central role, since overproduction of reactive oxygen species (ROS) causes calcium overload and leads to apoptosis of cardiomyocytes (Zhao, 2004). Therefore, targeting oxidative stress will provide a potential approach to attenuate myocardial reperfusion injury (Marczin et al., 2003).

Nuclear factor E2-related factor-2 (Nrf2) is an important transcription factor that regulates the transcription of target genes encoding antioxidant enzymes by binding to the promoter region of these genes (Kaspar et al., 2009). Under basal conditions, Nrf2 is sequestered in the cytosol by Kelch-like ECH associated protein 1 (Keap1) to facilitate ubiquitination and degradation by the 26S proteasome. However, under the induced condition, oxidants or electrophiles can interact with Keap1 and result in activation of Nrf2 and translocation into the nucleus of the cytosol to implement transcriptional regulation (Yamamoto et al., 2018). As in a previous report, many antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) are regulated by Nrf2 (Shaw & Chattopadhyay, 2020). Therefore, activation of Nrf2 can provide a potential therapeutic approach for the treatment of diseases related to oxidative stress, including myocardial ischemia and reperfusion injury (Shen et al., 2019).

In the discovery of a new therapy for myocardial infarction, phytochemicals have played a crucial role (Kumar & Nayak, 2017). Salvia is a genus that belongs to the Lamiaceae family, and most plants in this genus are used for the treatment of various diseases, including cardiovascular disease. Especially Salvia miltiorrhiza, a member of this genus, has been used for the treatment of cardiovascular diseases for thousands of years in traditional Chinese medicine (Xu et al., 2018). Salvia przewalskii is another plant of the genus Salvia, which is used in Chinese folk medicine as an antiphlogistic, antibacterial, and antitubercular drug (Chen et al., 2002). Phytochemical investigations have revealed there are diterpenoids, polyphenols, and alkaloids in this plant (Zhao et al., 1996; Li et al., 2000; Chang et al., 2005; Xu et al., 2006). As the main phytochemicals, diterpenoids have shown antimicrobial, antioxidant, anti-inflammatory, cytotoxic, neuroprotective, and cardioprotective effects (Li et al., 2018). Saprirearine (Fig. 1A) is one of the diterpenoids found in Salvia przewalskii (Chen et al., 2002). However, to the best of our knowledge, there are no reports on its activity. In our interest to find bioactive phytochemicals for the treatment of myocardial infarction, we have investigated the protective effects of saprirearine using H9c2 cardiomyocytes induced by hypoxia/reoxygenation.
MATERIALS AND METHODS

Chemicals and reagents

Saprirearine was provided by Qincheng Biotechnology (Shanghai, China) with purity of more than 98% analyzed by HPLC. Dulbecco’s modified Eagle’s medium (DMEM) and fetal bovine serum were obtained from Invitrogen Gibco Co. (Grand Island, NY). 3-[4,5-di-methylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO). Fluo-3 AM, ROS assay kit, lactate dehydrogenase (LDH) activity assay kit, bicinchoninic acid (BCA) protein assay kit, SOD activity assay kit, CAT activity assay kit, malondialdehyde (MDA) assay kit, mitochondrial membrane potential assay kit with JC-1 and horseradish peroxidase conjugated secondary antibody together with enhanced chemiluminescence (ECL) assay kit were provided by Beyotime Biotechnology Institute (Shanghai, China). The primary antibody for cysteinyl aspartate specific proteinase-3 cleaved (caspase-3) (#9661) was supplied by Cell Signaling Technology (Danvers, MA), and others, including B-cell lymphoma-2 (Bcl-2) (ab196495), Bcl-2-associated X protein (Bax) (ab32503) Nrf2 (ab196495), β-actin (ab8226) and lamin B1 (ab16048) were obtained from Abcam (Cambridge, UK). Calcein-AM was purchased from Dojindo Laboratories (Kumamoto, Japan). The ARE-luciferase reporter plasmid (pGL4.37[luc2P/ARE/Hygro]) (E3641), the renilla luciferase reporter plasmid (pRL-TK) (E2241) and the dual luciferase reporter assay system (E1910) were supplied by Promega (Madison, WI). Lipofectamine 2000 and sulforaphane were supplied by Thermo Fisher Scientific (Waltham, MA). Negative control siRNA (NC-siRNA) (sc-37007) and Nrf2-siRNA (sc-156128) were obtained from Santa Cruz Biotechnology (Dallas, TX).

Cell culture and treatment

Rat H9c2 cardiomyocytes were supplied by the American Type Culture Collection (ATCC, Manassas, VA) and kept in DMEM, including 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin in humid conditions with 5% CO₂ and 95% air at 37°C. The cells were divided into control group, hypoxia/reoxygenation (HR) group, and experimental groups. Cells in experimental groups were pretreated with certain saprirearine or sulforaphane for 24 h and then exposed to a humid atmosphere with 95% N₂ and 5% CO₂ at 37°C for 3 h followed by reoxygenation at 37°C in 5% CO₂ and 95% air for another 3 h. Cells in the HR group were just subjected to hypoxia/reoxygenation without treatment with saprirearine or sulforaphane while normoxic control cells were incubated at 37°C in 5% CO₂ and 95% air.

Cell viability

Cell viability was detected using the MTT assay. Briefly, cells were seeded in 96-well microplates and treated as previously, then 20 μL MTT (5 mg/mL) was added to each well and incubation was carried out at 37°C for 4 h. Subsequently, 100 μL DMSO was added to dissolve the formazan crystals. The absorbance was recorded on a microplate reader (Bio-Rad, Hercules, CA) at 570 nm.

LDH release

To further assess the damage to H9c2 cardiomyocytes, LDH release was evaluated here using the commercially available kit. The treated cells were centrifuged at 400×g for 5 min and then the supernatant was collected and
handled according to the manufacturer’s instructions. Finally, the absorbance was measured on a microplate reader at 450 nm.

**Intracellular calcium level**

Intracellular calcium in H9c2 cardiomyocytes was detected using the fluorescence dye Fluo-3 AM. Following the supplier’s protocol, treated cells were loaded in the dark with 5 μM Fluo-3 AM at 37°C for 30 min, then washed with PBS three times to remove excessive dye. Fluo-3 AM can react with calcium to produce calcium-chelated Fluo-3, which can emit fluorescence at 525 nm after excited at 488 nm. The fluorescence intensity was read on a fluorescence microplate reader (Molecular Devices, Sunnyvale, CA).

**Mitochondrial membrane potential**

The mitochondrial membrane potential of H9c2 cardiomyocytes was monitored using the JC-1 assay kit. Following the manufacturer’s instructions, the treated H9c2 cardiomyocytes were incubated with JC-1 at 37°C for 20 min in the dark. Then the fluorescence intensity was read on a fluorescence microplate reader at 488 nm/530 nm as an excitation/emission wavelength.

**Mitochondrial permeability transition pore**

To explore the mitochondrial permeability transition pore, calcine-AM was used. Briefly, treated H9c2 cardiomyocytes were incubated with 2 μM calcine-AM and 1 mM CoCl₂ at room temperature for 30 min. After removing free calcine-AM and CoCl₂, cells were incubated with CoCl₂ for another 20 min at 37°C to quench the fluorescence of cytosolic calcine. The fluorescence intensity of mitochondrial calcine in cardiomyocytes was recorded on a fluorescence microplate reader at 490 nm for excitation and 515 nm for emission. The loss of calcine fluorescence in cardiomyocytes indicated the opening of the mitochondrial permeability transition pore.

**Generation of intracellular ROS**

The generation of intracellular ROS was monitored by the fluorescence method using a ROS assay kit. After treatment, the medium was replaced and the cells were rinsed with PBS. Then 10 μM DCFH-DA in DMEM was loaded and incubation was carried out at 37°C for 30 min. After being washed with PBS to remove excess dye, the fluorescence intensity was recorded on a fluorescence microplate reader at ex/em wavelength of 485/520 nm.

**MDA content**

The content of MDA in H9c2 cardiomyocytes was detected using the thiobarbituric acid-based assay kit. The treated cells were lysed on ice and centrifuged at 16000×g for 10 min. The supernatant was collected and the absorbance was measured on a microplate reader at 532 nm.

**Activity of SOD and CAT**

The activity of SOD and CAT in H9c2 cardiomyocytes was assessed using the assay kits. For CAT activity, the treated cells were centrifuged and the supernatant was collected. After being treated according to the supplier’s instructions, the absorbance was recorded at 520 nm. For SOD activity, the treated cells were lysed on ice and the supernatant was collected after centrifugation. After treatment following the supplier’s instructions, the absorbance was read at 560 nm.

**Immunofluorescence staining**

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**Immunofluorescence staining**

To localize Nrf2 in H9c2 cardiomyocytes, immunofluorescence staining was employed. Cells were seeded in 12-well microplates with a coverlip and treated as above. The cells on the coverslips were then washed and fixed using a fixation mixture (acetonemethanol = 1:1) for 10 min. Then the primary Nrf2 antibody (diluted in 10% FBS-PBS) was added to the coverlip and the incubation was carried out at 4°C overnight. After washing, cells were exposed to Alex-594 conjugated secondary antibody (diluted in 10% FBS-PBS) in the dark for 1 h. The cells were then stained with DAPI in the dark for 10 min. The images were captured using a fluorescence microscope (Olympus, Tokyo, Japan).

**Proteins extraction**

Total and nuclear proteins in H9c2 cardiomyocytes were extracted using the RIPA lysis buffer solution or the nuclear and cytoplasmic protein extraction kit according to the supplier’s instructions. The treated cells were lysed on ice with RIPA buffer for 3 min and then centrifuged at 10000×g for 5 min. The supernatant was collected as total proteins. For nuclear protein extraction, treated cells were exposed to 200 μL buffer solution A containing 1 mM PMFS. After vortex for 5 s and incubation on ice for 15 min, 10 μL buffer solution B was added. After vortex and incubation, centrifugation was carried out at 16000×g and 4°C for 5 min. The supernatant was then removed. And the pellet was incubated with 50 μL nuclear protein extraction solution containing PMSF on ice for 30 min. Meanwhile, the vortex was performed for 30 s every 2 min. After centrifugation, the supernatant was collected as nuclear proteins.

**Western blot analysis**

After quantification using the BCA protein assay kit, the proteins were separated on 10% SDS-PAGE and then transferred to PVDF membranes. After blocking with defatted milk, the membranes were incubated with primary antibodies including cleaved caspase-3 (1:1000), Bcl-2 (1:1000), Bax (1:1000), Nrf2 (1:1000), β-actin (1:1000) and lamin B1 (1:1000) at 4°C overnight. The membranes were incubated with horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 h and visualized using an ECL substrate on a Bio-Rad imaging system (Hercules, CA). β-actin and lamin B1 were used as an internal control. Densitometric analysis was performed using ImageJ software (NIH, Bethesda, MD).

**Dual luciferase reporter gene assay**

The H9c2 cardiomyocytes were transfected with the ARE luciferase plasmid and the renilla luciferase plasmid using lipofectamine 2000. The cells were then treated as above and the activities of fireflies and renilla luciferase were determined by the dual luciferase reporter gene assay system. Firefly luciferase activity was normalized to renilla luciferase activity, and the induction of ARE luciferase activity was determined as a ratio compared to the control group.
Saprirearine improves the survival of H9c2 cardiomyocytes induced by hypoxia/reoxygenation

As shown in Fig. 1B, up to 200 μM there is no cytotoxic effect of saprirearine on H9c2 cardiomyocytes. After treatment with hypoxia/reoxygenation viability was markedly reduced (46.3±6.0%), while in the presence of saprirearine at 5, 10 and 20 μM, the cell viability was elevated to 67.0±7.2%, 77.9±9.1%, and 90.2±6.3%, respectively (Fig. 1C). Further investigation of LDH activity revealed that saprirearine resulted in increased extracellular LDH activity (299.2±12.4 U/L) compared to the control group (277.1±10.8 U/L). However, after exposure to saprirearine, calcium-induced fluorescence was reduced to 247.4±12.3%, 204.8±12.5% and 175.1±11.1% in a dose-dependent manner (Fig. 2A). Meanwhile, the mitochondrial membrane potential was observed to collapse after hypoxia/reoxygenation treatment (44.2±5.5%) compared to the control group (100.0±11.1%). But saprirearine at 5, 10 and 20 μM reversed the decline of mitochondrial membrane potential (56.8±4.4%, 73.3±7.3% and 88.1±6.7%) (Fig. 2B). Similarly, the opening of the mitochondrial permeability transition pore was detected under hypoxia/reoxygenation by monitoring the fluorescence intensity of mitochondrial calcein (53.5±6.3%), which was attenuated by saprirearine (67.4±4.7%, 76.3±5.4% and 90.3±5.1%) (Fig. 2C). These findings indicated that saprirearine relieved mitochondrial dysfunction in H9c2 cardiomyocytes treated with hypoxia/reoxygenation.

Saprirearine ameliorates hypoxia/reoxygenation-induced oxidative stress in H9c2 cardiomyocytes

To reveal the redox status in cardiomyocytes, we explored intracellular ROS herein. As a result, unlike the control group (100.0±18.0%), overproduction was found in cardiomyocytes after hypoxia/reoxygenation induction (298.1±13.0%), while saprirearine at 5, 10 and 20 μM diminished the intracellular ROS level (251.4±11.6%, 199.1±12.6% and 160.1±13.5%) (Fig. 3A). As a product of lipid peroxidation, the content of MDA has been elevated by hypoxia/reoxygenation (175.4±7.1%). After exposure to saprirearine, calcium-induced fluorescence was reduced to 247.4±12.3%, 204.8±12.5% and 175.1±11.1% in a dose-dependent manner (Fig. 2A). Meanwhile, the mitochondrial membrane potential was observed to collapse after hypoxia/reoxygenation treatment (44.2±5.5%) compared to the control group (100.0±11.1%). But saprirearine at 5, 10 and 20 μM reversed the decline of mitochondrial membrane potential (56.8±4.4%, 73.3±7.3% and 88.1±6.7%) (Fig. 2B). Similarly, the opening of the mitochondrial permeability transition pore was detected under hypoxia/reoxygenation by monitoring the fluorescence intensity of mitochondrial calcein (53.5±6.3%), which was attenuated by saprirearine (67.4±4.7%, 76.3±5.4% and 90.3±5.1%) (Fig. 2C). These findings indicated that saprirearine relieved mitochondrial dysfunction in H9c2 cardiomyocytes treated with hypoxia/reoxygenation.

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Saprirearine activates Nrf2 to inhibit cardiomyocyte apoptosis

in the presence of saprirearine. Densitometric analysis also revealed similar results (Fig. 4B). Meanwhile, anti-apoptotic Bcl-2 was up-regulated, and pro-apoptotic Bax was down-regulated after being exposed to saprirearine (Fig. 4A). Accordingly, the ratio of Bcl2/Bax gave a clear trend for the effects of saprirearine on cardiomyocyte apoptosis.

Saprirearine activates Nrf2 in H9c2 cardiomyocytes induced by hypoxia/reoxygenation

To unravel the mechanism of saprirearine against apoptosis of cardiomyocytes induced by hypoxia/reoxygenation, we have explored the activation of Nrf2. Immunofluorescence staining has indicated that saprirearine elevated Nrf2 as well as promoted its translocation to nuclei from the cytosol (Fig. 5A). Western blot analysis together with densitometric analysis showed that saprirearine resulted in up-regulation of nuclear Nrf2, which further implied that its translocation was promoted (Fig. 5B and C). Meanwhile, total Nrf2 was also up-regulated (Fig. 5B and D), indicating that saprirearine improved Nrf2 stability against its degradation. Furthermore, the dual luciferase reporter assay revealed that Nrf2 binding capacity to ARE

Figure 3. Effects of saprirearine on oxidative stress in H9c2 cardiomyocytes.

(A) The intracellular ROS level in H9c2 cardiomyocytes was increased by hypoxia/reoxygenation and decreased by saprirearine. (B) The MDA content in H9c2 cardiomyocytes was excessively generated by hypoxia/reoxygenation and reversed by saprirearine. (C-D) SOD and CAT activity in H9c2 cardiomyocytes was inhibited by hypoxia/reoxygenation and activated by saprirearine. Data were expressed as means ± standard deviation, n=3, ***P<0.001 vs control group, *P<0.05, **P<0.01, and ***P<0.001 vs H/G group.

Figure 4. Effects of saprirearine on apoptosis of H9c2 cardiomyocytes induced by hypoxia/reoxygenation.

(A) Western blot analysis of proteins related to apoptosis in H9c2 cardiomyocytes induced by hypoxia/reoxygenation with or without saprirearine. (B) Densitometric analysis of cleaved caspase-3 in H9c2 cardiomyocytes induced by hypoxia/reoxygenation with or without saprirearine. (C) Relative ratio of Bcl-2/Bax in H9c2 cardiomyocytes. Data were expressed as means ± standard deviation, n=3, ***P<0.001 vs control group, **P<0.01 vs control group.
was significantly improved to regulate transcription after treatment with saprirearine (Fig. 5E). Collectively, these observations revealed saprirearine-activated Nrf2 in H9c2 cardiomyocytes.

**Nrf2 activation is involved in the protective effects of saprirearine**

To clarify the role of Nrf2 activation in the protective effects of saprirearine, siRNA interference was performed. After transfected with NC-siRNA or Nrf2-siRNA, Western blot together with densitometric analysis has validated that transfection was successfully (Fig. 6A and B). Then the MTT was implemented. As shown in Fig. 6C, in H9c2 cardiomyocytes transfected with NC-siRNA, saprirearine was observed to promote cell viability, while in cells transfected with Nrf2-siRNA, saprirearine did not enhance cell viability (Fig. 6C). These results demonstrated that saprirearine protected H9c2 cardiomyocytes against hypoxia/reoxygenation-induced injury by activating Nrf2.

**DISCUSSION**

Myocardial infarction remains the leading cause of mortality and disability worldwide. As primary treatment, timely reperfusion itself causes major cardiac injury, commonly referred to as myocardial ischemia/reperfusion injury (Zhao et al., 2016). Therefore, the discovery of novel therapy targeting myocardial ischemia/reperfusion is imperative. Here, we have found that saprirearine improved the survival of hypoxia/reoxygenation-induced H9c2 cardiomyocytes by increasing cell viability and inhibiting intracellular LDH release.

In the pathogenesis of myocardial ischemia/reperfusion injury, oxidative stress plays a crucial role due to the overproduction of ROS in reperfusion (Loor et al., 2011). To reduce molecular oxygen, enzymes such as xanthine oxidase and NADPH oxidase, as well as the mitochondrial electron transport chain in reperfusion myocardial tissue, will accelerate ROS production (Granger & Kvetys, 2015). As one of ROS, superoxide is generated from one electron reduction of molecular oxygen.
and converted to hydrogen peroxide by SOD, which is decomposed as water under the catalysis of CAT (Dickinson & Chang, 2011). In the present investigations, hypoxia/reoxygenation was observed to promote the overproduction of ROS and MDA in H9c2 cardiomyocytes. Meanwhile, the activity of SOD and CAT was inhibited. This poor oxidative state was reversed in the presence of saprirearine.

As the main site of ROS production, mitochondria are sensitive to ROS-related injury (Brand, 2016). Following ROS overproduction, calcium overloads as an important second messenger due to its interaction (Görlich et al., 2015). In ischemia reperfusion injury, calcium promotes the opening of the mitochondrial permeability transition pore (Hurst et al., 2017). When the mitochondrial permeability transition pore opens, its large conductance can result in collapse of the mitochondrial membrane potential as the early event of apoptosis (Halstead, 2010). Meanwhile, the poor potential of the mitochondrial membrane affects the influx of mitochondrial calcium and leads to the release of calcium into the cytosol (Hausenloy & Yellon, 2013). From current studies, saprirearine was found to inhibit calcium release, attenuated the collapse of the mitochondrial membrane, and suppressed the opening of the mitochondrial permeability transition pore induced by hypoxia/reperfusion.

In apoptosis, caspases play a central role as cysteinyl aspartate specific proteases, which are activated by cleavage at specific sites (Budihardjo et al., 1999). Of the caspases, caspase-3 is the key effector enzyme closely associated with cell apoptosis and morphological changes (Uchiyama et al., 2002). Bel-2 and Bax are members of the Bcl-2 protein family and participate in mitochondrial-mediated apoptosis. Bel-2 inhibits apoptosis and inactivates caspase-3, while Bax promotes cell apoptosis (Youle & Strasser, 2008). The formation of heterodimers with Bel-2 and Bax prevents the oligomerization of Bax which will trigger the apoptotic cascade (Olta et al., 2011). In our investigations, the results showed that saprirearine inhibited the activation of caspas-3, up-regulated Bel-2, and down-regulated Bax, which implied that it repressed apoptosis of H9c2 cardiomyocytes induced by hypoxia/reoxygenation.

As a transcription factor, Nrf2 enhances the expression of genes that encode antioxidant enzymes (Cuadra et al., 2018). Structural analysis of the Keap1-Nrf2 complex has indicated that the Keap1 homodimer interacts with the DLG and ETGE motifs of Nrf2 using their Kelch domains, respectively, which results in the retention of Nrf2 in the cytosol (Madden & Itzhaki, 2020). Due to the lower affinity for binding for binding of DLG to Kelch compared to ETGE, the complex forms a “hinge and latch” model (Tong et al., 2007). Oxidants or electrophiles can react with thiol groups in Keap1 cysteine residues through the formation of covalent bonds to alter Keap1 formation and lead to the dissociation of DLG from Keap1 (Yamamoto et al., 2008). Therefore, this interaction could prevent the ubiquitination and degradation of Nrf2 involving Keap1. Michael acceptors are typical electrophiles such as soft Lewis acids, which can react with critical cysteine thiolate (soft base) groups in Keap1 through the Michael addition reaction (Magesh et al., 2012). From the structure of saprirearine, we can find the coumaroyl moiety, which may indicate that it can activate Nrf2. In our investigations, Nrf2 is activated by saprirearine in H9c2 cardiomyocytes, which is closely associated with its protective effects against hypoxia/reoxygenation-induced apoptosis.

**CONCLUSION**

In conclusion, we have evaluated the protective effects of saprirearine against hypoxia/reoxygenation-induced apoptosis using H9c2 cardiomyocytes and explored the underlying mechanisms. The results showed that saprirearine could protect hypoxia/reoxygenated H9c2 cardiomyocytes against mitochondria-mediated apoptosis by inhibiting oxidative stress and improving mitochondrial dysfunction. And activation of Nrf2 was involved in those protective effects. These findings can provide evidence for the discovery of new therapeutic approaches to myocardial infarction and the application of saprirearine in clinical practice.

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