Picroside II inhibits hypoxia/reoxygenation-induced cardiomyocyte apoptosis by ameliorating mitochondrial function through a mechanism involving a decrease in reactive oxygen species production

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Abstract. Reactive oxygen species (ROS)-induced mitochondrial dysfunction plays an important role in cardiomyocyte apoptosis during myocardial ischemia/reperfusion (I/R) injury. Picroside II, isolated from *Picrorhiza scrophulariiflora* Pennell (Scrophulariaceae), has been reported to protect cardiomyocytes from hypoxia/reoxygenation (H/R)-induced apoptosis, but the exact mechanism is not fully clear. The aim of the present study was to explore the protective effects of picroside II on H/R-induced cardiomyocyte apoptosis and the underlying mechanism. In the H9c2 rat cardiomyocyte cell line, picroside II (100 μg/ml) was added for 48 h prior to H/R. The results showed that picroside II markedly inhibited H/R-induced cardiomyocyte apoptosis. In addition, picroside II was also able to decrease the opening degree of mitochondrial permeability transition pore (mPTP), increase the mitochondrial membrane potential, inhibit cytochrome *c* release from mitochondria to cytosol and downregulate caspase-3 expression and activity concomitantly with the decreased ROS production. These results suggested that picroside II inhibited H/R-induced cardiomyocyte apoptosis by ameliorating mitochondrial function through a mechanism involving a decrease in ROS production.

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Introduction

Myocardial ischemia/reperfusion (I/R) injury refers to cardiac dysfunction induced by the restoration of blood flow following a period of ischemia or lack of oxygen (1). Myocardial I/R injury is a common clinical problem and frequently found in the process of coronary thrombolysis, coronary artery bypass graft and heart transplantation (2-4). Cardiomyocyte apoptosis is well-established as one of the major mechanisms for I/R-induced injury (5,6). Currently, the majority of studies supported that I/R-induced cardiomyocyte apoptosis was closely associated with excessive production of reactive oxygen species (ROS) (7,8).

ROS are chemically-reactive molecules containing the element oxygen, including superoxide anion (O_2^-), hydroxyl radical (•OH) and hydrogen peroxide (H_2O_2) (9). Under physiological conditions, there is a low concentration of ROS in the heart. They are mainly produced as a by-product of mitochondrial oxidative phosphorylation. However, multiple pathological conditions, including I/R, can result in the accumulation of ROS (10,11). Mitochondrion has been reported as the main target of ROS damage (12). By contrast, increased ROS can directly lead to the oxidative damage of mitochondrial DNA, membrane phospholipids and respiratory chain proteins (13,14). Increased ROS are able to enhance the opening degree of mitochondrial permeability transition pore (mPTP) (15). The opening of mPTP leads to the increase in mitochondrial permeability, mitochondrial swelling, mitochondrial membrane potential collapse and cytochrome *c* release from mitochondria to cytosol, which ultimately results in cardiomyocyte apoptosis (16). The above studies suggested that ROS-induced mitochondrial dysfunction plays an important role in cardiomyocyte apoptosis and a decrease in ROS production may be a promising strategy for the inhibition of I/R-induced cardiomyocyte apoptosis.

Picroside II (β-D-glucopyranoside, 1a,1b,2,5a,6,6a-hexahydro-6-[(4-hydroxy-3-methoxybenzoyl)oxyl]-1a-(hydroxy methyl)oxireno[4,5]cyclopenta[1,2-c]pyran-2-yl) is a primary active ingredient extracted from *Picrorhiza scrophulariiflora*

Pennell (Scrophulariaceae), which is a traditional Chinese herbal medicine and has been extensively used in China for >1000 years (17). In recent years, it has been reported that picroside II has an antioxidant property. The study by Li *et al* (18) demonstrated that pretreatment of PC12 cells with picroside II significantly prevented glutamate-induced cell apoptosis by inhibition of ROS production. In addition, treatment with picroside II evidently ameliorated liver damage induced by carbon tetrachloride, D-galactosamine and acetaminophen. The hepatoprotective effect of picroside II was associated with scavenging free radicals and protecting normal constructions of mitochondria membrane (19). Recently, it has been reported that picroside II has the protective effect on H/R-induced cardiomyocyte apoptosis (20), but the exact mechanism is not fully understood.

The present study aimed to investigated the important role of ROS-induced mitochondrial dysfunction in cardiomyocyte apoptosis during I/R and the antioxidant property of picroside II. Using the hypoxia/reoxygenation (H/R) model in the H9c2 cardiomyocyte cell line to simulate I/R injury, the study explored whether picroside II has a protective effect on H/R-induced cardiomyocyte apoptosis, and whether the protective effect is associated with ameliorating mitochondrial function by inhibition of ROS production.

Materials and methods

Materials. H9c2 embryonic rat heart-derived cells were obtained from Academia Sinica (Shanghai, China). Picroside II (purity>99%) was purchased from the Chinese National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China), and dissolved in sterile distilled water. Dulbecco's modified Eagle medium (DMEM) and fetal bovine serum (FBS) were provided from Gibco RBL (Grand Island, NY, USA). Trizol reagent was a product of Invitrogen Life Technologies (Carlsbad, CA, USA). The First Strand cDNA Synthesis kit was purchased from MBI Fermentas, Inc. (Vilnius, Lithuania). Cytochrome *c* and β-actin antibodies were from Abcam (Cambridge, UK). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Hoechst 33342, caspase-3 activity assay kits and reactive oxygen species (ROS) detection kit were from Beyotime Biotechnology (Jiangsu, China). Lactate dehydrogenase (LDH) and creatine kinase (CK) assay kits were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Cells culture and treatment. H9c2 cells were cultured in DMEM supplemented with 15% (v/v) FBS, 100 U/ml penicillin and 100 μg/ml streptomycin at 37˚C in a humidified atmosphere of 5% CO_2, with medium changed every 2-3 days. The cells were passaged regularly. When they reached 80% confluence, cells were made quiescent by serum starvation (0.5% FBS) for 12 h, and subsequently were treated with picroside II (100 μg/ml) for 48 h prior to H/R. For the H/R experiment, a hypoxic incubator was used to produce an *in vitro* hypoxia challenge. Cells in the hypoxic incubator were subjected to hypoxia (<0.5% O_2) using pre-conditioned hypoxic medium [8 g NaCl, 0.4 g KCl, 0.14 g $CaCl_2$, 0.2 g $MgSO_4 \cdot 7H_2O$, 1.2 g $K_2HPO_4 \cdot 12H_2O$, 0.08 g KH_2PO_4 (pH 7.4)] at 37˚C. Hypoxic medium was changed to fresh DMEM medium upon initiation of reoxygenation. The cells were subsequently cultured in the incubator under an atmosphere of 5% (v/v) CO_2 at 37˚C. The selected period of hypoxia and reoxygenation time was based on the preliminary time course studies. The most suitable period of H/R was found to be 3 h hypoxia followed by 12 h reoxygenation. Control cells were cultured in normoxic conditions.

Evaluation of cell injury. Cardiomyocyte viability was measured by the MTT quantitative colorimetric assay. After picroside II treatment and H/R, the medium was removed and the cardiomyocytes were washed twice with phosphate-buffered saline (PBS), subsequently 10 μl MTT solution was added to each well and incubated for an additional 4 h at 37˚C. Following this, 100 μl dimethysulfoxide was added to dissolve the formazan product. The absorbance at 490 nm was read on a microplate reader and the data were expressed as a percentage of the control, which was considered 100% viable. CK and LDH, the indicators of cardiomyocyte injury, were detected by the commercially available colorimetric assay kits according to the manufacturer's instructions.

Apoptosis analysis. Apoptotic cells were determined by fluorescent microscopy using the fluorescent dye Hoechst 33342. Briefly, H9c2 cells were seeded at a density of $1x10^5$ cells/well in 12-well plates and cultured as described above. Following picroside II treatment and H/R, the medium was aspirated and the cells were washed twice with PBS. Subsequently, Hoeshst 33342 solution (0.1 mg/ml) was added into each well of the 12-well plate for 20 min at 37˚C in dark, followed by another three washes with PBS. Nuclear DNA staining was observed by a fluoresence microscope at 521 nm emission wavelength. The percent of apoptosis was expressed as a ratio of apoptotic to total cells. Caspase-3 is a key enzyme in the apoptosis cell-signaling cascade. The activity of caspase-3 was measured by the commercially available colorimetric assay kit according to the manufacturer's instructions. The data were expressed as a percentage of the control.

Quantitative polymerase chain reaction (qPCR). Following picroside II treatment and H/R, total RNA was isolated from the H9c2 cells by the Trizol reagent and quantified by measuring the absorbance at 260 nm. A 1 μg RNA aliquot from each sample was reverse-transcripted to cDNA using the M-MLV Reverse transcriptase kit. The cDNA was used for qPCR. Quantitative analysis of mRNA expression was performed by the ABI 7300 real-time PCR system with the Power SYBR Green PCR Master Mix kit. PCR primers were as follows: caspase-3 primers (sense, 5'-CAAGTCGATGGACTCTGGAA-3' and anti-sense, 5'-GTACCATTGCGAGCTGACAT-3'); *GAPDH* primers (sense, 5'-TGGCCTCCAAGGAGTAAGAAAC-3' and anti-sense, 5'-GGCCTCTCTCTTGCTCTCAGTATC-3'). The PCR amplification profiles consisted of denaturation at 95˚C for 10 min, followed by 40 cycles of denaturation at 95˚C for 15 sec and annealing at 60˚C for 60 sec. All the amplification reactions for each sample were carried out 4 times, and the relative expression values were normalized to the expression value of *GAPDH*.

Preparation of subcellular fractions. H9c2 cells were cultured as described above. Following picroside II treatment and H/R, the cells were collected and the preparation of subcellular fractions was isolated using the Cell Mitochondria Isolation kit. The method was carried out according to the manufacturer's instructions. Briefly, cells were washed in PBS and centrifuged at 600 x g for 10 min at 4˚C. The supernatant was removed and the cells were resuspended in the mitochondria isolation reagent. Following a 15 min incubation on ice, lysates were homogenized and centrifuged at 600 x g for 10 min at 4˚C. The supernatant was subsequently centrifuged at 11,000 x g for 10 min to isolate the mitochondria fraction in the pellet, while the supernatant was used to isolate the cytosol. The supernatant was subjected to centrifugation at 12,000 x g for 10 min. The resulting supernatant was designated as the cytosolic fraction, which was used for the detection of cytochrome c.

Measurement of intracellular ROS. The levels of ROS were measured by oxidative conversion of cell permeable 2',7'-dichlorofluorescein diacetate to fluorescent dichlorofluorescein. 2',7'-dichlorofluorescein was added at a final concentration of 10 μmol/l and incubated for 20 min at 37˚C. Fluorescence intensity was detected using a fluorescence plate reader at an excitation wavelength at 488 nm and an emission wavelength at 525 nm. The results were expressed as a percentage of the control.

Measurement of mPTP opening. The mPTP opening of cardiomyocytes was measured with calcein-acetoxymethylester (calcein-AM) in the presence of cobalt chloride using the mPTP fluorescence assay kit according to the manufacturer's instructions. In brief, cardiomyocytes were washed with Reagent A, and subsequently the cells were incubated with Reagents B and C for 20 min at 37˚C, prior to washing twice with Reagent A again. Fluorescence intensity was determined by a SprctraMax M5 microplate reader at an excitation wavelength at 488 nm and an emission wavelength at 505 nm. Fluorescence intensity was normalized to total protein concentration in the corresponding cell and the data were expressed as normalized relative fluorescence units (U/mg protein).

Determination of mitochondrial membrane potential. Mitochondrial membrane potential was determined by the tetramethylrhodamine ethyl ester (TMRE) fluorescent dye. TMRE is positively charged and highly permeable across the mitochondrial membrane. Mitochondrial depolarization is able to result in the spread of TMRE from mitochondria to cytoplasm, and enhance the whole-cell fluorescence intensity. TMRE was added at a final concentration of $1x10^{-7}$ mol/l and incubated in the dark for 20 min at room temperature. Fluorescence intensity was determined (excitation wavelength at 514 nm/emission wavelength at 590 nm). The data were expressed as a percentage of the control.

Western blotting analysis. Protein concentration was determined by a Bradford protein assay. Equal amounts of protein were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. After being blocked with 5% milk powder in TBST for 1 h at room temperature, the membranes were incubated with the primary antibody for cytochrome *c* (diluted 1 μg/ml, Cat. no. ab90529) and β-actin (diluted 1:1,000, Cat. no. ab1801) (both rabbit polyclonal antibodies; Abcam) at 4˚C overnight, and followed by incubation with the corresponding horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit; (diluted 1:5,000, Cat. no. ab175773; Abcam) at room temperature for 1 h. Detection was performed using an ECL kit according to the manufacturer's instructions. The results were normalized to β-actin expression.

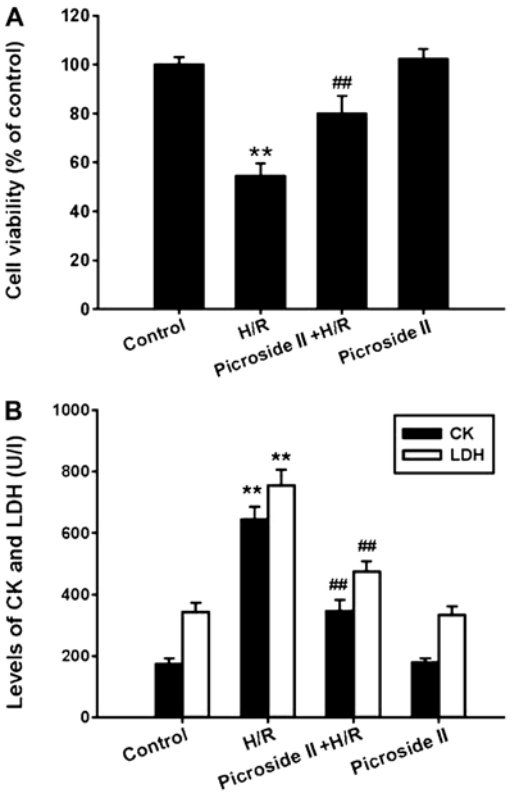

Figure 1. Effect of picroside II on H/R-induced cell injury in H9c2 cells. (A) Cell viability and (B) levels of CK and LDH. Data are expressed as mean ± standard error of the mean and represent four independent experiments. **P<0.01 vs. control; $^{\#\#}$P<0.01 vs. H/R. H/R, hypoxia/reoxygenation; CK, creatine kinase; LDH, lactate dehydrogenase.

Statistical analysis. Data are expressed as the means ± standard error of the mean. All the values were analyzed using analysis of variance and the Newman-Keuls Student's *t*-test. P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of picroside II on H/R-induced cell injury. The MTT assay showed that the cell viability was significantly decreased after hypoxia for 3 h and reoxygenation for 12 h. Pretreatment with picroside II for 48 h prior to H/R was able to markedly inhibit the decrease in cell viability (Fig. 1A). In line with the MTT assay, the levels of CK and LDH, which are the indicators of cardiomyocyte injury, were significantly increased in the H/R group, which was also inhibited by pretreatment of picroside II (Fig. 1B). However, picroside II alone had no effect on cell viability and the production of CK and LDH.

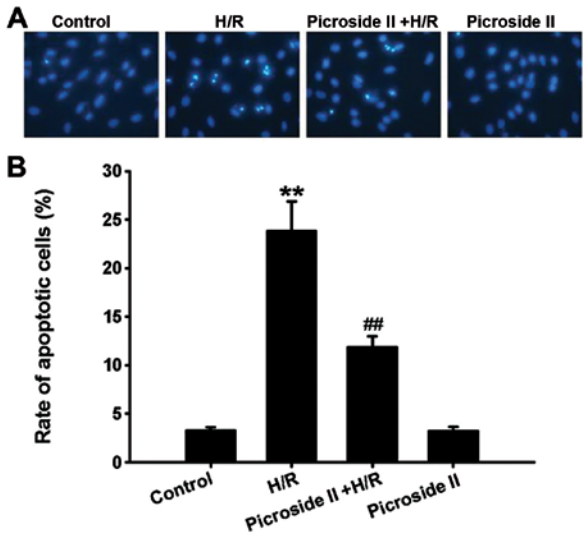

Figure 2. Effect of picroside II on H/R-induced cell apoptosis in H9c2 cells. (A) Representative image of Hoechst staining. (B) Rate of apoptotic cells. Data are expressed as mean ± standard error of the mean and represent four independent experiments. **P<0.01 vs. control; $^{\#\#}$P<0.01 vs. H/R. H/R, hypoxia/reoxygenation.

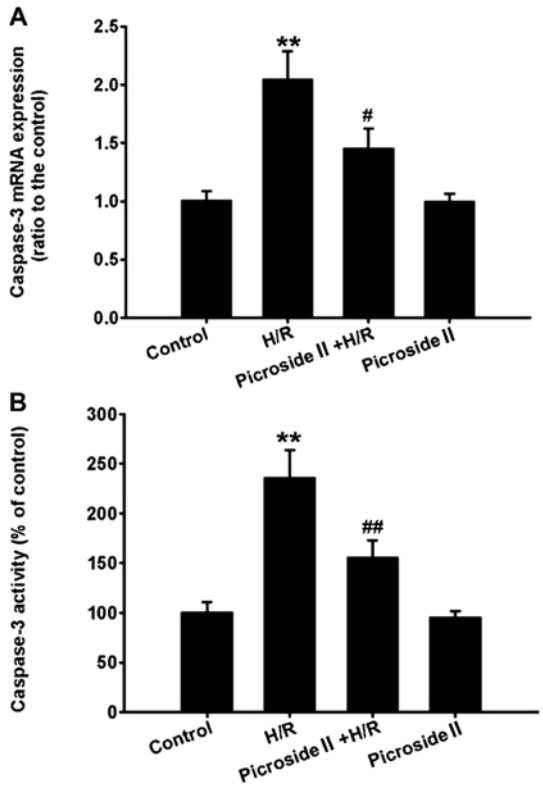

Figure 3. Effect of picroside II on H/R-induced caspase-3 mRNA expression and activity in H9c2 cells. (A) Caspase-3 mRNA expression and (B) caspase-3 activity. Data are expressed as mean ± standard error of the mean and represent four independent experiments. **P<0.01 vs. control; $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. H/R. H/R, hypoxia/reoxygenation.

Effect of picroside II on H/R-induced cell apoptosis. The Hoechst 33342 staining assay showed that the percentage of apoptotic cells was significantly increased after hypoxia for 3 h and reoxygenation for 12 h compared to the control group. Consistent with the results of Hoechst 33342 staining, the mRNA expression and activity of caspase-3, the effector caspase of the mitochondrial apoptosis pathway, were also markedly increased in the H/R group. These effects induced by H/R were significantly inhibited by pretreatment of picroside II (Figs. 2 and 3). However, picroside II alone had no effect on cardiomyocyte apoptosis and the mRNA expression and activity of caspase-3.

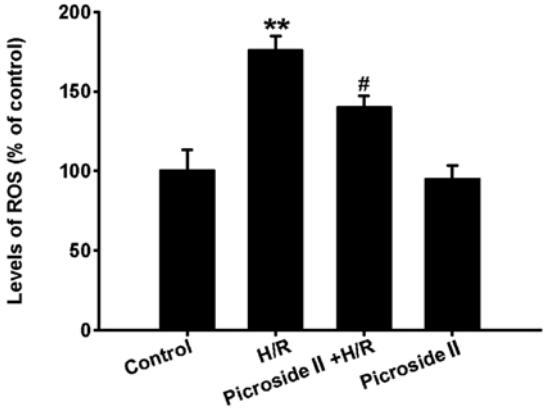

Figure 4. Effect of picroside II on H/R-induced intracellular ROS production in H9c2 cells. Data are expressed as mean ± standard error of the mean and represent four independent experiments. **P<0.01 vs. control; $^{\#}$P<0.05 vs. H/R. H/R, hypoxia/reoxygenation; ROS, reactive oxygen species.

Effect of picroside II on H/R-induced intracellular ROS production. Compared to the control group, the levels of intracellular ROS were clearly increased after hypoxia for 3 h and reoxygenation for 12 h, which was indicated by the increased fluorescence intensity. However, pretreatment of picroside II significantly inhibited the production of intracellular ROS induced by H/R (Fig. 4). Picroside II alone had no effect on the production of intracellular ROS.

Effect of picroside II on H/R-induced mPTP opening. The opening of mPTP was determined with calcein-AM in the presence of cobalt chloride. Calcein-AM fluorescent dye is able to move freely between mitochondrial and cytosol via the opening of mPTP. Cobalt chloride can quench calcein-AM fluorescence, but it cannot eliminate calcein-AM fluorescence in mitochondria when the mPTP closed. Due to this property, the fluorescence intensity of calcein-AM following treatment with cobalt chloride can reflect the extent of mPTP opening. The results showed that the fluorescence intensity was significantly decreased in the H/R group, indicating that the extent of mPTP opening is enhanced following H/R. However, pretreatment of picroside II was able to markedly increase the fluorescence intensity compared to the H/R group (Fig. 5). Picroside II alone had no effect on the fluorescence intensity. These results indicated that picroside II decreased the degree of mPTP opening in response of H/R-induced injury.

Effect of picroside II on mitochondrial membrane potential after H/R. The results showed that mitochondrial membrane

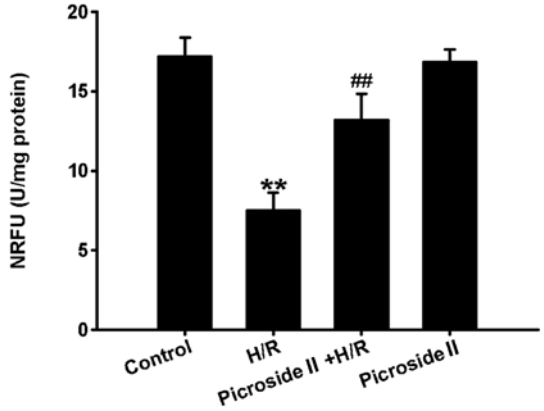

Figure 5. Effect of picroside II on H/R-induced opening of mPTP in H9c2 cells. Data are expressed as mean ± standard error of the mean and represent four independent experiments. **P<0.01 vs. control; ##P<0.01 vs. H/R. H/R, hypoxia/reoxygenation; mPTP, mitochondrial permeability transition pore.

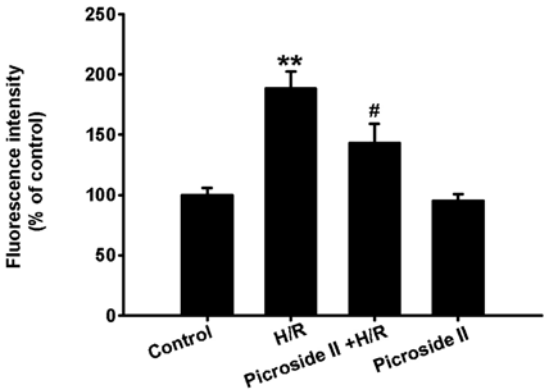

Figure 6. Effect of picroside II on H/R-induced mitochondrial membrane potential in H9c2 cells. Data are expressed as mean ± standard error of the mean and represent four independent experiments. **P<0.01 vs. control; #P<0.05 vs. H/R. H/R, hypoxia/reoxygenation.

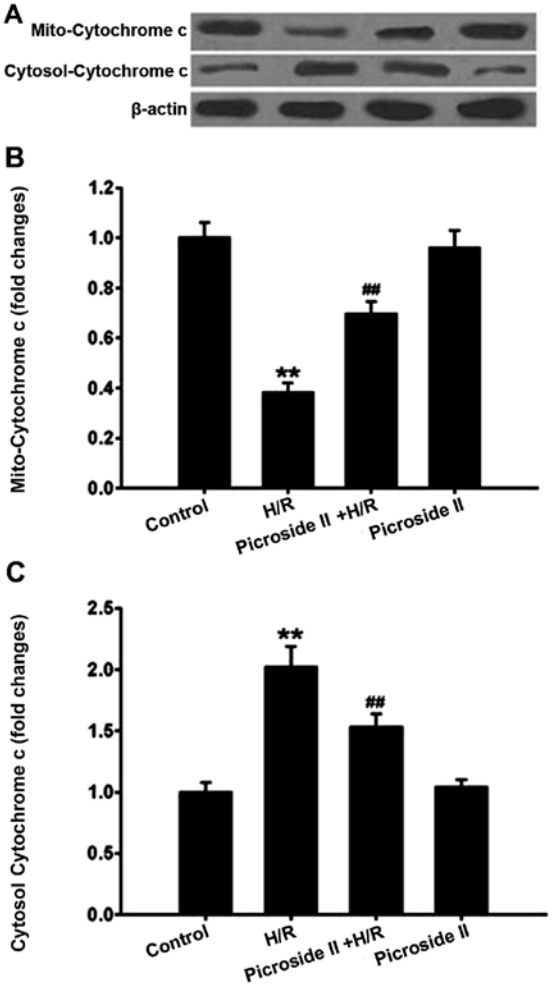

Figure 7. Effect of picroside II on H/R-induced protein expression of cytochrome *c* in the mitochondria and cytosol in H9c2 cells. (A) Representative image of cytochrome *c* protein expression in the mitochondria and cytosol by western blotting. Optical density of cytochrome *c* protein bands in (B) the mitochondria and (C) cytosol. Data are expressed as mean ± standard error of the mean and represents four independent experiments. **P<0.01 vs. control; ##P<0.01 vs. H/R. H/R, hypoxia/reoxygenation.

potential was depolarized after hypoxia for 3 h and reoxygenation for 12 h, which was indicated by the increased fluorescence intensity of TMRE. Pretreatment of picroside II was able to markedly decrease the fluorescence intensity of TMRE compared to the H/R group (Fig. 6). However, picroside II alone had no effect on the fluorescence intensity.

Effect of picroside II on H/R-induced mitochondrial cytochrome c release. In mitochondria, there was a high level of cytochrome *c* expression in the control group. However, the protein expression of cytochrome *c* in mitochondria was notably decreased following H/R. This H/R-induced effect was significantly inhibited by pretreatment of picroside II. By contrast, the cytochrome *c* expression in the cytosol was lower in the control group compared to the H/R group. Pretreatment of picroside II was able to significantly reduce the increase in cytosolic cytochrome *c* expression induced by H/R (Fig. 7). Picroside II alone had no effect on cytochrome *c* expression in the mitochondria and cytosol. Taken together, these results suggested that picroside II has the ability to inhibit cytochrome *c* release from the mitochondria to cytosol.

Discussion

In the present study, the protective effect of picroside II on H/R-induced cardiomyocyte apoptosis and the underlying mechanisms in the H9c2 rat cardiomyocyte cell line were investigated. The results indicated that pretreatment with picroside II was able to protect cardiomyocytes from H/R-induced apoptosis, which was associated with decreasing the opening degree of mPTP, increasing mitochondrial membrane potential, inhibiting cytochrome *c* release from the mitochondria to the cytosol and downregulating caspase-3 expression and activity through a mechanism involving a decrease in ROS production.

Myocardial I/R injury is a common clinical problem and may result in serious consequences, including heart failure and mortality. Although the exact pathophysiological mechanism leading to myocardial I/R injury is not fully understood, oxidative damage has long been considered a key factor in the initiation of I/R injury (21). Preclinical studies also consistently show that inhibition of ROS production can significantly alleviate I/R-induced injury (22,23). Cardiomyocyte apoptosis

is well-recognized to play a crucial role in I/R injury (5,6). Therefore, inhibition of cardiomyocyte apoptosis may be a potential strategy to prevent I/R-induced injury. Although the causes of cardiomyocyte apoptosis induced by I/R are complicated, it is widely accepted that excessive production of ROS during I/R play an important role in cardiomyocyte apoptosis (7,8).

The mitochondrial compartment is the main target of intracellular ROS as they are particularly rich in polyunsaturated fatty acids (24,25). Increased ROS can result in serious dysfunction of mitochondrion, which is the main cause of I/R-induced cardiomyocyte apoptosis (6). A pivotal factor mediating mitochondrial dysfunction is the long-lasting opening of mPTP, a large non-selective conductance pore located in the inner membrane of mitochondria (15,26). Under physiological conditions, the transient mPTP opening may occur in normal cells (27). However, under pathological conditions the long-lasting mPTP opening allows protons into mitochondria, and subsequently results in the uncoupling of the electron respiratory chain and the decrease in mitochondrial membrane potential (26). The decrease in mitochondrial membrane potential is one of the earliest events in cell apoptosis (28). The long-lasting mPTP opening increases mitochondrial permeability and allows ions and solutes with molecular weights of <1.5 kDa in cytoplasm freely entering the mitochondria, leading to mitochondrial matrix swelling and loss of critical electrochemical gradients (29). Another adverse consequence of the mPTP opening is the cytochrome *c* release from the mitochondria to the cytosol. Cytochrome *c* is a well-known pro-apoptotic factor, and the translocation plays an important role in apoptosis by activating caspase cascade reactions. In the cytosol, cytochrome *c* binds the apoptotic factor, Apaf-1, and induces Apaf-1 oligomerization, resulting in recruitment and activation of procaspase-9 (30). In turn, activated caspase-9 cleaves and activates caspase-3 (a classical executioner caspase), which terminally leads to cardiomyocyte apoptosis (31). Inhibition of the mPTP opening by cyclosporine A (an inhibitor of mPTP) or genetic deletion of the gene encoding CypD can protect against I/R-induced myocardial injury (27,32). The above studies indicated that the sustained opening of the mPTP plays an important role in cell apoptosis. Strong evidence indicates that overproduction of ROS in the I/R period is the primary triggers of the mPTP opening (15). Decrease in ROS production is able to markedly inhibit the opening of mPTP (26). Therefore, inhibition of ROS overgeneration may be a promising strategy for prevention of I/R-induced cardiomyocyte apoptosis through suppressing the mPTP opening.

Picroside II, an iridoid glucoside extracted from *Picrorhiza scrophulariiflora* Pennell, has been reported to have multiple pharmacological actions, such as anti-inflammation (33) and neuroprotective effect (34). Most recently, it has been reported that picroside II has the protective effect on H/R-induced cardiomyocyte apoptosis (20), but the underlying mechanisms are not fully understood. As mentioned previously, picroside II scavenged oxygen free radical, protected normal constructions of mitochondria membrane (19,35) and increased ROS-induced mitochondrial dysfunction during I/R, which plays an important role in cardiomyocyte apoptosis. Therefore, we hypothesized that the protective mechanism of picroside II on H/R-induced cardiomyocyte apoptosis may be associated with ameliorating mitochondrial function by a decrease in ROS production. In the present study, pretreatment of picroside II markedly inhibited H/R-induced cardiomyocyte apoptosis concomitantly with a decrease in ROS production. The further study showed that mitochondrial function was also significantly ameliorated, which was indicated by the decreased mPTP opening, increased mitochondrial membrane potential and decreased cytochrome *c* release from mitochondria to cytosol.

In conclusion, the present study suggests that picroside II is able to inhibit H/R-induced cardiomyocyte apoptosis through ameliorating mitochondrial function involving a mechanism of decrease in ROS production.

Acknowledgements

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