Gypenosides protect against cardiac ischemia-reperfusion injury by inhibiting mitochondria-dependent apoptosis

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Abstract

Purpose: To investigate the effect of gypenoside (Gyp) on myocardial ischemia-reperfusion (I/R), focusing on mitochondrial function and oxidative stress.

Methods: A 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay was employed to measure the protective effect of Gyp pre-treatment against I/R injury. Flow cytometry was used to detect cellular reactive oxygen species (ROS) content and mitochondrial membrane potential (MMP) levels. Additionally, cytochrome C release was observed by laser scanning confocal microscopy. Finally, Annexin V staining and western blot were applied to analyse cell apoptosis.

Results: MTT assay results showed that Gyp pre-treatment protected H9C2 cells against I/R injury in a Gyp concentration-dependent manner. Moreover, Gyp treatment inhibited intracellular ROS production, repressed cytochrome C transposition induced by I/R treatment, and recovered MMP to almost normal levels. Furthermore, the expression of apoptosis-related proteins included cleaved caspase-3, -9 and Bax which were decreased by Gyp treatment after I/R injury.

Conclusion: These results suggest that Gyp treatment prior to injury can help maintain normal mitochondrial function and inhibit ROS production during I/R injury, ultimately leading to the suppression of I/R-induced cell apoptosis. Thus, Gyp may be a promising drug for the treatment of myocardial I/R.

Keywords: Gynostemma pentaphyllum, Ischemia-reperfusion, Mitochondria damage, Oxidative stress, Apoptosis

INTRODUCTION

Cardiovascular dysfunction, including acute myocardial infarction and cardiac failure, is a leading cause of morbidity and mortality worldwide. Common treatment strategies for cardiovascular disease include pharmacological intervention, mechanical coronary reperfusion, and surgery [1]. Transient occlusion of a coronary artery damages myocardial cells because of ischemic insult and energy exhaustion; however, the reperfusion of blood to myocardium also causes severe tissue injury, called ischemia-reperfusion (I/R) injury [1-3]. And I/R injury is a complex pathological event, which is still not well-characterized. Research indicates that several external stimuli, such as...
mitochondrial dysfunction, reactive oxygen species (ROS) release, loss of energy supply, inflammation, microcirculatory disorder, oedema, and cell apoptosis, contribute to I/R injury [4-6]. Because of the complexity of this phenomenon, any treatment that targets a single contributing factor will be insufficient to provide comprehensive therapy.

Recent research suggested that components used in traditional Chinese medicine are protective in I/R injury [7,8]. One such medicinal herb, Gynostemma pentaphyllum, is popularly used to treat chronic inflammation, cancer, cardiovascular disease, and hepatitis. In I/R injured rats, G. pentaphyllum was found to reduce impairment of cardiac function. Yu et al reported that G. pentaphyllum treatment prior to cardiac insult inhibits iκB-α phosphorylation, translocation of the nuclear factor kappa B (NFκB) subunit into nuclei, and MAPK pathway activity [9]. Ye et al showed that gypenoside (Gyp), an important component of G. pentaphyllum, protects the kidneys from I/R injury by inhibiting inflammation, apoptosis, and ERK signalling [10]. Gyp has various biological activities, including roles in anti-inflammatory reactions, insulin resistance, reducing memory deficits, and decreasing liver fibrosis [11,12]. Moreover, Gyp IX inhibits NFκB transcriptional activity and reduced the expression of several inflammatory factors, including nitric oxide synthase (iNOS), tumour necrosis factor-α (TNF-α), and interleukin-1β (IL-1β) [11]. Qin and colleagues showed that Gyp influences lipid metabolism, reduces oxidative stress, inhibits mitochondrial damage, and prevents degeneration in fatty liver disease [13]. Also, Gyp is reported to protect the brain against I/R damage.

Preliminary evidence suggests that Gyp is a promising drug candidate for I/R therapy, but the role of Gyp in cardiac I/R injury has not been rigorously explored. In this paper, the effects of Gyp were investigated in cardiac I/R, focusing on mitochondrial function and oxidative stress.

**EXPERIMENTAL**

**Cell culture**

H9C2 cells (CRL-1446) were purchased from ATCC. Dulbecco's modified Eagle medium (HyClone, USA) with 10% foetal bovine serum (HyClone, USA) and 1% penicillin and streptomycin (HyClone, USA) was used to culture H9C2 cells.

**Chemicals**

Gyp was purchased from Jiatian Biotechnology Co. Ltd (Xi’an, Shaanxi Province, China). Dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide tetrazolium (MTT), and rhodamine-123 (Rh123) were obtained from Sigma-Aldrich. The 2',7'-dichlorodihydrofluorescein-diacetate (DCFH-DA) probe was purchased from Invitrogen (USA), and the Annexin V–FITC Apoptosis Detection kit was purchased from KeyGEN BioTECH Corp, Ltd.

**Cell preparation and Gyp pre-treatment**

To imitate I/R injury, cells were cultured in glycoprival medium without serum and exposed to 0.5% O₂ (oxygen deprivation), followed by re-oxygenation under normal oxygen conditions in complete culture medium for 24 h to mimic myocardial I/R. For Gyp treatment groups, Gyp (5, 10, 20, and 40 μg/mL) was added to the medium 12 h before the I/R treatment, and untreated cells were used as the control group.

**Cell viability assessment**

After 24 and 48 h of reperfusion treatment, cell viability was detected by MTT assay. H9C2 cells were seeded in 96-well plates overnight, and then the old culture medium was replaced by the new culture medium containing the indicated Gyp concentration. After another 12-h incubation, the medium was replaced with glycoprival DMEM with Gyp. After a 4-h incubation at 0.5% O₂, the glycoprival DMEM was removed, and complete culture medium that also contained Gyp was added. Cells were then subjected to I/R stimulus. Then, 24 and 48 h after I/R, 50 μg MTT was added to each well. After 4 h, the mixed cell culture medium was replaced with DMSO to dissolve formazan crystals, and was used to calculate the cell survival rate (C) spectrophotometrically as in Eq 1.

\[
C(\%) = \frac{(At/Ac)}{100} \quad \text{(1)}
\]

where At and Ac are the absorbance of treatment and control groups, respectively.

**Intracellular ROS determination**

DCFH-DA was used to measure ROS levels in the different cell groups, as previously described [14]. Two hours after I/R treatment, cells in each group were cleaned with phosphate buffered saline (PBS) three times, then stained with 4 μM DCFH-DA diluted in DMEM at 37 °C for 20 min. Then, the cells were harvested and cleaned with
PBS. Cell samples were analysed by flow cytometry.

**Mitochondrial membrane potential (MMP) determination**

Rhodamine 123 (Rho 123) staining was used to measure cell MMP, as previously described [15]. Two hours after treatment, cells were harvested and stained with 1 μg/mL Rho 123 at 37 °C for 20 min. After staining, all samples were analysed by using flow cytometry.

**Cytochrome c release analysis**

Cells were seeded in 6-well plates, grown overnight, pre-incubated with Gyp at the indicated concentration, and then subjected to treatment to mimic I/R injury. After 2 h, the cells were cleaned with PBS and stained with 0.5 mM Mito-Tracker Red (MTR, Molecular Probes). After 20 min, the cells were fixed with 4 % paraformaldehyde, followed by the addition of 0.1 % Triton X-100 at 4 °C. After being permeabilized, samples were blocked with goat serum for 1 h at 37 °C, then incubated with anti-cytochrome c antibody at 4 °C overnight. Cytochrome c antibody was labelled by fluorescein isothiocyanate (FITC) conjugated secondary antibody. Cell nucleus were stained with Hoechst 33258 and imaged with a confocal laser scanning microscope.

**Western blot analysis**

After I/R treatment, cells were lysed with radioimmunoprecipitation assay (RIPA) buffer, and then total protein content in the lysate was measured using a Bradford assay. Proteins levels for each treatment group were determined according to western blot standard procedures. Quantification of protein expression was normalized to a β-actin loading control.

**Apoptosis assay**

Cell apoptosis was investigated using an Annexin V/FITC-propidium iodide (PI) kit. Briefly, cells for all treatment groups were harvested and then washed twice with PBS. Then, cells were stained with Annexin V–FITC and PI at 37°C for 20 min. After staining, all samples were detected by flow cytometry.

**Statistical analysis**

All data are expressed as mean ± standard deviation (SD, n = 3). Statistical significance was calculated using one-way analysis of variance. A value of *p* < 0.05 was used to determine a significant difference between groups. SPSS 19.0 software was used for statistical analysis.

**RESULTS**

**Cell viability**

The MTT assay was applied to evaluate the cytotoxicity of the different treatments. As shown in Figure 1, 24 h after I/R injury, cell viability was 35.00 %. Pre-treatment with Gyp rescued the cytotoxicity induced by I/R-mimic treatment. In cells pre-treated with Gyp at 5, 10, 20, and 40 μg/mL, cell viability 24 h after I/R-mimic treatment was 38.67, 49.00, 71.67, and 81.06 %, respectively. When the incubation time was increased to 48 h, the cell survival was slightly higher than that 24 h after treatment. After 48 h, cell viability increased to 57.67, 67.00, 79.28, and 91.63 % for Gyp concentrations of 5, 10, 20, and 40 μg/mL, respectively. These results suggest that pre-treatment with Gyp protects H9C2 cells from I/R injury in a time- and concentration-dependent manner.

**Figure 1**: H9C2 cell viability after I/R-mimic treatment. Cell viability was measured by MTT assay 24 and 48 h after Gyp treatment at the indicated concentrations. Results are expressed as mean ± SD (n = 3)

**ROS**

Intracellular ROS in H9C2 cells was measured 2 h after the different treatments. As shown in Figure 2, I/R-mimic treatment increased the intracellular ROS production to 54.5 % from 19.7 % that was observed in the control group. Gyp treatment decreased the ROS production that resulted from I/R-mimic treatment: treatment with 10 μg/mL Gyp reduced the subset of cells with high ROS production to 48.7 %. When Gyp concentration was increased to 20 and 40 μg/mL, the percentage of cells with high ROS production further decreased to 36.7 % and 21.8 %, respectively.
Figure 2: Intracellular ROS production after I/R injury at different concentrations of Gyp treatment

Figure 3: The effect of Gyp treatment on mitochondrial damage in I/R mimic-treated myocardial cells. (A) Measurement of mitochondrial membrane potential by Rho 123 staining. (B) COX IV protein expression detected by western blot

Gyp treatment protects cell mitochondria

I/R injury damages cell mitochondria and disturbs the process of ATP generation, resulting in cell death. In this study, cell MMP was measured 2 h after I/R-mimic treatment. As shown in Figure 3A, MMP decreased in the I/R mimic-treated group; 50.3 % cells showed lower MMP following I/R injury. Treatment with 10 µg/ml Gyp rescued the loss of MMP, as only 12.9 % of cells showed lower MMP. Furthermore, higher Gyp concentrations further inhibited the MMP decrease that resulted from I/R-mimic treatment. Specifically, 40 µg/ml Gyp treatment nearly recovered MMP to a normal level. In order to further assess mitochondrial function, we measured expression of the cytochrome oxidase complex IV (COX IV), which is an important component of the respiratory chain. I/R-mimic treatment decreased COX IV expression (Figure 3B), indicating serious mitochondrial damage. COX IV expression levels increased with increasing concentrations of Gyp treatment (Figure 3B).

Cytochrome C release

Cytochrome C release was also observed in
each group after treatment (Figure 4). In control cells, MTR labelling with red fluorescence and cytochrome c labelling with green fluorescence showed considerable overlap. Although cells in the I/R-mimetic treatment group showed partial release of cytochrome C green fluorescence from the mitochondria to the cytoplasm, cells treated with 40 µg/mL Gyp exhibited decreased cytochrome c green fluorescence in the cytoplasm after I/R-mimetic treatment.

Figure 4: The effect of Gyp on cytochrome c transposition in myocardial cells after I/R-mimetic treatment. After Gyp treatment, I/R mimic-treated myocardial cells were stained with Mito-Tracker Red (MTR; mitochondrial probe, red channel), cytochrome c antibody (green channel), and DAPI (blue channel). After staining, cells were observed by confocal microscopy.

Gyp treatment on cell apoptosis

Mitochondrial damage, evidenced by cytochrome c release, can initiate mitochondria-dependent apoptosis [16]. Annexin V staining indicates both early and late apoptosis, in contrast to PI staining, which only labels necrotic cells. We found that the control group exhibited 93.2 % viable cells and 6.34 % apoptotic cells (Figure 5A). In the I/R mimic-treated group, the cell viability decreased to 37.3 %, with an increased apoptosis rate of 53.84 % and debris rate of 8.9 %. In cells treated with 10 µg/ml Gyp, cell viability increased to 63.1%, and apoptosis decreased to 35.0 %. In the cells treated with 20 and 40 µg/mL Gyp, we observed that cell viability further increased to 71.1 and 78.5 %, respectively, and the apoptosis frequency decreased to 25.59 and 20.76 %, respectively. In addition, the apoptosis-related proteins cleaved caspase-3, -9, and Bax, which were significantly upregulated in the I/R mimic-treated group. We found that Gyp treatment decreased expression of these proteins in a concentration-dependent manner. In contrast, Gyp treatment resulted in an increase in expression of the anti-apoptosis protein Bcl-2. These data indicate that an I/R stimulus can cause cell apoptosis and that Gyp treatment can rescue these apoptosis effects.

Figure 5: The effect of Gyp treatment on apoptosis in I/R mimic-treated myocardial cells. (A) Frequency of cell apoptosis in I/R mimic-treated myocardial cells by Annexin V–FITC/PI staining. (B) Cleaved caspase-3, -9, Bax, and Bcl-2 in I/R mimic-treated myocardial cells after Gyp pre-treatment. Representative images of western blots are shown.

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DISCUSSION

The I/R-mimic treatment used in this study caused cellular injury, including increased ROS production, mitochondrial damage, and energy depletion. Mitochondrial dysfunction is one of the most important causes of tissue damage, especially in myocardial cells [17]. Gyp treatment is known to relieve oxidative stress and protect mitochondria [18]. This study focused on the effect of Gyp pre-treatment on protecting heart tissue against an I/R-mimic treatment. Results showed that Gyp inhibited cell damage associated with I/R-mimic treatment in a manner dependent on Gyp concentration, indicating that Gyp has potential to protect myocardial cells against I/R injury.

I/R injury induces severe oxidative stress [19], which can cause abnormal platelet function and dysfunctional endothelial-dependent vasodilatation [20]. Abundant ROS also cause oxidative damage of cell lipids, proteins, and DNA as well as mitochondrial dysfunction and loss of membrane integrity. Much research has been conducted on the effect of reducing ROS production during I/R injury [21]. Wicha et al reported that treatment with hexahydrocurcumin protected cerebral against I/R injury by attenuating oxidative stress, increasing nuclear factor (erythroid-derived 2)-like 2 (Nrf2) and heme oxygenase-1 (HO-1) expression levels, and improving the activity of antioxidant enzymes [22]. Zhen et al found that exendin-4 activated the Nrf2 anti-oxidative signalling pathway and alleviated kidney I/R injury by suppressing oxidative stress [23]. In the present study, abundant ROS was found in H9C2 cells after I/R-mimic treatment. Gyp pre-treatment decreased cellular ROS levels in a concentration-dependent manner, consistent with our cell viability data. This result highlights the important role of ROS in myocardial cell death caused by I/R injury.

Mitochondria contribute to energy production and are involved in cell stress pathways. In myocardial I/R injury, mitochondria are key targets of damage and important regulators of the response to damage. During ischemia, the electron transport chain is firstly severely damaged, then the mitochondrial respiration chain complexes are compromised, and finally oxidative phosphorylation is decreased [22]. During the ischemic stage, both excessive ROS and calcium dysregulation cause mitochondria permeation changes, concomitant with swelling and disruption of the mitochondrial permeability transition pore. After an extended period of ischemia, I/R injury can also cause morphological changes and loss of mitochondria [22]. In cells receiving the I/R-mimic treatment, MMP and expression of COX IV decreased, indicating serious mitochondrial injury. With Gyp pre-treatment at increasing concentrations, decreased MMP and expression of COX IV were gradually rescued.

Several reports have indicated that the damaged mitochondria may subsequently drive cells to initiate the apoptotic process [24]. We found that the mitochondrial marker MTR co-localized with cytochrome c in the control cells, and after I/R-mimic treatment, cytochrome c was released from the mitochondria to the cytoplasm. The Annexin V–FITC/PI assay revealed that 53.84 % of the cells were apoptotic after I/R-mimic treatment, in accord with caspase-3 and -9 activation. Gyp treatment decreased apoptosis frequency and expression of apoptosis-related proteins in the I/R mimic-treated group. As mentioned above, I/R-mimic treatment induced mitochondria-dependent apoptosis in H9C2 cells, and Gyp pre-treatment decreased this I/R-associated apoptosis.

CONCLUSION

The findings of this study demonstrate that Gyp pre-treatment protects myocardial cells against I/R-mimic treatment. Gyp pre-treatment helps maintain mitochondrial function and inhibits ROS production during I/R-mimic stimulus, resulting in suppression of cell apoptosis caused by I/R. Thus, Gyp treatment provides a promising strategy for the management of cardiac I/R injury.

DECLARATIONS

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Xinwen Liu designed all the experiments and revised the paper. Song Qiao and Longsheng Chen performed the experiments, and Song Qiao wrote the paper.

REFERENCES


