The protective effects of total paeony glycoside on ischemia/reperfusion injury in H9C2 cells via inhibition of the PI3K/Akt signaling pathway

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Abstract. At present, cardiovascular disease is the global leading cause of mortality. Total paeony glycoside (TPG) is a traditional Chinese medicine, which serves a pivotal role in the cardiovascular system. In the present study, the effects and underlying mechanisms of TPG on ischemia/reperfusion (I/R) injury-induced apoptosis of cardiomyocytes were investigated in vitro. Cell Counting kit-8 and flow cytometry were used to assess the viability, reactive oxygen species (ROS) content and apoptosis of H9C2 cells. The activities of lactate dehydrogenase (LDH), malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione peroxidase (GPX) were analyzed by commercial detection kits. Reverse transcription-quantitative polymerase chain reaction and western blot analysis were conducted to evaluate the expression levels of various factors. The results demonstrated that the viability of H9C2 cells was not significantly altered in response to various concentrations of TPG. However, following I/R injury, TPG markedly enhanced cell viability in a time- and dose-dependent manner. Furthermore, TPG decreased the rate of apoptosis and ROS levels, and reduced the activities of MDA and LDH. Conversely, TPG increased SOD and GPX activities. In addition, TPG upregulated the expression levels of pro-caspase-3 and B-cell lymphoma2 (Bcl-2), whereas it downregulated cleaved-caspase-3, poly (ADP-ribose) polymerase 1, Bcl-2-associated X protein, phosphorylated (p)-phosphatidylinositol 3 kinase (PI3K) and p-protein kinase B (Akt) expression. Treatment with insulin-like growth factor-1 increased the apoptosis of H9C2 cells, thus suggesting that activation of the PI3K/Akt signaling pathway reversed the protective effects of TPG. Taken together, TPG may suppress I/R-induced apoptosis and oxidative stress of H9C2 cells possibly by inhibiting the PI3K/Akt signaling pathway; such a phenomenon may have a therapeutic effect on cardiovascular disease.

Introduction

Cardiovascular disease is currently the global leading cause of mortality (1). It has previously been reported that acute myocardial infarction is a major cause of cardiovascular disease (2). When coronary artery occlusion occurs, in order to save the myocardial tissues, the infarct-associated artery is promptly opened and blood supply is restored to the ischemic myocardium. However, reperfusion causes damage to the cardiac structure, leading to the occurrence of cardiac arrest, decreased cardiac function and malignant cardiac arrhythmia. Such a phenomenon is known as ischemia/reperfusion (I/R) injury (3). I/R injury is a clinicopathological process in which varying levels of cardiomyocyte apoptosis can occur (4). I/R injury also induces oxidative stress (5,6); therefore, inhibition of apoptosis and oxidative stress may reduce damage.

Recently, studies have demonstrated that traditional Chinese medicine may be effective in the prevention and treatment of myocardial I/R injury (7,8). According to traditional Chinese medicine, Radix Paeoniae Rubra, the dried root of Paeonia lactiflora Pall, is able to reduce heat and cool blood, disperse stasis and relieve pain (9-11). Total paeony glycoside (TPG) is a major component of Radix Paeoniae Rubra, and the effective component of TPG is a monoterpene compound (12). Modern pharmacological studies have demonstrated that Radix Paeoniae Rubra has various pharmacological functions that mainly produce effects on the cardiovascular system (9,13,14). In addition, it exerts antitumor and anti-endotoxin effects, as well as other functions (9,13,14). Furthermore, TPG has been used to treat cancer, atherosclerosis and ischemic cerebrovascular disease in China (15,16). Previous studies have demonstrated that TPG is capable of preventing thrombosis, of inhibiting and scavenging oxygen free radicals, and of suppressing cell apoptosis (15,17,18); it also has the potential
to effectively protect liver and brain cells (15,19,20). However, whether it has such a protective effect on myocardial I/R injury remains unknown.

The phosphatidylinositol 3 kinase/protein kinase B (PI3K/Akt) signal transduction pathway is a critical pathway that serves a pivotal role in cell proliferation, apoptosis and differentiation (21,22). Furthermore, studies have demonstrated that the PI3K/Akt pathway is involved in I/R injury in various organs, including liver, brain and heart (23-25). However, whether the role of TPG in I/R injury is associated with this pathway remains unknown.

In the present study, the rat cardiac myoblast cell line H9C2 was selected to establish an I/R injury model in vitro. The effects and mechanism of TPG on the I/R-induced apoptosis and oxidative stress of H9C2 cells were subsequently investigated.

Materials and methods

Drug preparation. TPG powder was obtained from Haoxuan Biological Technology Co., Ltd. (Xi’an, China), which had been approved by the State Food and Drug Administration for clinical trials. The powder was dissolved in PBS, and the solution was filtered and sterilized using a 0.22-µm filter membrane (EMD Millipore, Billerica, MA, USA). Following filtration and sterilization, the solution was diluted to 10, 20, 40, 80, 160 and 320 µg/ml using PBS. Insulin-like growth factor (IGF)-1 was purchased from Sigma-Aldrich; Merck KGaA, Darmstadt, Germany; the final concentration used to activate PI3K/Akt was 100 ng/ml (26,27).

Cell culture. The H9C2 rat cardiac myoblast cell line was acquired from Cobioer Biosciences Co., Ltd. (Nanjing, China). Cells were maintained in complete high-glucose Dulbecco's Modified Eagle Medium (DMEM; Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) supplemented with 10% fetal bovine serum (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) and 1% penicillin-streptomycin (Beijing Leagene Biotechnology Co., Ltd., Beijing, China) in an incubator containing 95% humidified air and 5% CO₂ at 37°C.

Establishment of a myocardial I/R injury model. H9C2 cells were selected to establish an I/R model, according to a previous study (28). Briefly, cells were cultured at 37°C for 48 h (95% humidified air and 5% CO₂). After cell culture, cells were incubated in serum- and glucose-free DMEM and were maintained in a low-oxygen incubator at 37°C (95% N₂, 5% CO₂ and 1% O₂) for 10 h, in order to mimic hypoxia. Subsequently, cells were cultured in complete high-glucose DMEM and were maintained in an incubator containing 95% humidified air and 5% CO₂ at 37°C for 2 h, in order to mimic re-oxygenation (reperfusion). Cells in the control group were cultured without any treatment at 37°C (95% humidified air and 5% CO₂).

Cell Counting kit-8 (CCK-8) assay. Cell viability was evaluated using CCK-8 (Wuhan Merck Biotechnology Co., Ltd., Wuhan, China), according to the manufacturer's protocol. Briefly, cells were incubated in 96-well plates (2.5x10⁴ cells/well) for 24 h at 37°C. Subsequently, a portion of cells was treated with various concentrations of TPG (10, 20, 40, 80, 160 and 320 µg/ml) for 12, 24 and 48 h at 37°C. Another portion of cells was used to establish the I/R injury model and were treated with low, medium or high concentrations of TPG (10, 40 and 160 µg/ml) for 12, 24 and 48 h at 37°C. Subsequently, CCK-8 reagent was added to the cells, which were incubated for 4 h at 37°C. The optical density (OD) value was measured at 450 nm using a light absorption microplate reader (ELx808; BioTek Instruments, Inc., Winooski, VT, USA). The concentrations of TPG used were adopted according to previous studies (18,29).

Reactive oxygen species (ROS) assay. ROS production was assessed using 2',7'-dichlorofluorescein diacetate (DCFH-DA; Sigma-Aldrich; Merck KGaA), according to the manufacturer's protocol. DCFH-DA can be oxidized by ROS into fluorescent DCF, and the fluorescent signal indicates ROS production. Briefly, cells were seeded at a density of 2.5x10⁴ cells/well into a 96-well plate, after which they were subjected to I/R injury and then treated with various concentrations of TPG (10, 40 and 160 µg/ml) for 24 h at 37°C. Cells were subsequently incubated with 25 µM DCFH-DA at 37°C for 30 min and were washed three times with PBS. ROS production was measured using a FACSCalibur flow cytometer with Cell Quest 3.3 software (BD Biosciences, Franklin Lakes, NJ, USA).

Cell apoptosis assay. The Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit (Shanghai Qbicio Science & Technologies Co., Ltd., Shanghai, China) was performed to determine cell apoptosis, according to the manufacturer's protocol. Briefly, cells were seeded at a density of 6x10⁵ cells/well in 6-well plates, after which they were subjected to I/R injury and then treated with 10 µg/ml TPG and 100 ng/ml IGF-1. Following cell treatment, the cells were rinsed three times with PBS. Subsequently, the cells were stained with Annexin V-FITC and PI solution in the dark at room temperature for 20 min. Finally, 1X Binding Buffer was added to the cells on ice and cell apoptosis was measured using flow cytometry.

Lactate dehydrogenase (LDH) assay. LDH activity was examined using a LDH detection kit (Shanghai Genmed Pharmaceutical Technology Co Ltd., Shanghai, China), according to the manufacturer's protocol. Following cell treatment, the cells were exposed to reagent A for 5 min at room temperature, and then to reagent B for 10 min at room temperature. Subsequently, cells were centrifuged at 300 x g for 5 min and the supernatant was added to 96-well plates, and fixed with reagent C and reagent D in the dark for 30 min at room temperature. Subsequently, reagent E was added to the mixture. The OD value was measured at 490 nm using a light absorption microplate reader.

Malondialdehyde (MDA) assay. The MDA detection kit (Beijing Leagene Biotechnology Co., Ltd.) was performed to detect MDA activity, according to the manufacturer's protocol. Following cell treatment, the cells were lysed on ice using an ATPIO-4000SD ultrasonic cell breaker (Nanjing ATPIO Instruments Manufature Co., Ltd., Nanjing, China). Subsequently, the cell lysate was mixed with TAB solution; the mixture was heated in boiling water for 10 min.
and centrifuged at 800 x g at room temperature for 5 min. Subsequently, the precipitate was resuspended in PBS. The OD value was measured at 532 nm using a light absorption microplate reader.

Superoxide dismutase (SOD) assay. SOD activity was identified using the SOD detection kit (Beyotime Institute of Biotechnology, Haimen, China), according to the manufacturer’s protocol. Following cell treatment, the cells were lysed on ice using an ATPIO-400SD ultrasonic cell breaker. Subsequently, the cell lysate was mixed with hydroxylamine working fluid at 37˚C for 30 min. Subsequently, chromogenic reagent was added to the mixture to 16 min at room temperature. The OD value was measured at 550 nm using a light absorption microplate reader.

Glutathione peroxidase (GPX) assay. GPX activity was analyzed using a GPX detection kit (Beyotime Institute of Biotechnology), according to the manufacturer’s protocol. Following cell treatment, the cells were lysed on ice using an ATPIO-400SD ultrasonic cell breaker. Subsequently, cells were mixed with peroxide solution at 25˚C for 10 min. The OD value at 430 nm was measured every 30 sec at 25˚C using a light absorption microplate reader.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted using the TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). RNA (1 µg) was used to synthesize cDNA by RevertAid™ cDNA Synthesis kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA). RNA (1 µg) was used to synthesize cDNA by RevertAid™ cDNA Synthesis kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The primer sequences are listed in Table I. The 2−ΔΔCq method was used to compare the gene expression levels.

Western blot analysis. Cells were lysed with enhanced radioimmunoprecipitation assay buffer (Beijing Leagene Biotechnology Co., Ltd.) and total protein was extracted. The protein concentration was measured by bicinchoninic acid protein assay (Beyotime Institute of Biotechnology). A total of 25 µg protein was separated by 10% SDS-PAGE and bound to nitrocellulose membranes (Shanghai Kang Kang Biological Technology Co., Ltd., Shanghai, China). Membranes were blocked with 5% non-fat milk at 37˚C for 1 h. Subsequently, membranes were incubated with anti-pro-caspase-3 (cat. no. AF835; 1:1,000; R&D Systems, Inc., Minneapolis, MN, USA), anti-cleaved caspase-3 (cat. no. MAB835; 1:1,000; R&D Systems, Inc.), anti-cleaved poly (ADP-ribose) polymerase (PARP) 1 (cat. no. ab32561; 1:800; Abcam, Cambridge, UK), anti-B-cell lymphoma 2 (Bcl-2)-associated X protein (Bax; cat. no. MA5-14003; 1:1,000), anti-Bcl-2 (cat. no. MA5-11757; 1:1,000; both Invitrogen; Thermo Fisher Scientific, Inc.), anti-phosphorylated (p) -Akt (cat. no. AA329), anti-Akt (cat. no. AA326; 1:1,500; both Beyotime Institute of Biotechnology), anti-phosphorylated (p) -PI3K (cat. no. PA5-12799; 1:1,600), anti-PI3K (cat. no. MA5-17149; 1:1,000; both Invitrogen; Thermo Fisher Scientific, Inc.) and anti-β-actin (cat. no. MAB8969; 1:1,000; R&D Systems, Inc.) at 4˚C overnight. Subsequently, membranes were washed 2-4 times in TBS with 2% Tween-20 and were incubated with the following secondary antibodies: Mouse anti-rabbit immunoglobulin G (IgG) (cat. no. 3678; 1:8,000; Cell Signaling Technologies, Inc., Danvers, MA, USA), rabbit anti-mouse IgG (cat. no. 58802; 1:7,000; Cell Signaling Technologies, Inc.), and rabbit anti-goat IgG (cat. no. ab6741; 1:8,000; Abcam) for 1.5 h at room temperature. Proteins were detected using the iBright™ imaging system (A32749; Thermo Fisher Scientific, Inc.).

Statistical analysis. GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA, USA) was used to perform the data analysis. The experimental data are presented as the means ± standard deviation. The differences between groups were assessed by one-way analysis of variance followed by Tukey’s test. Each experiment was repeated at least three times. P<0.05 was considered to indicate a statistically significant difference.

### Table I. Primer sequences.

| Primer name       | Sequence (5'-3') | Product size (bp) |
|-------------------|------------------|-------------------|
| Caspase-3-forward | TGGATGTTCAGCTGCAATG | 224               |
| Caspase-3-reverse | CAGGTCCGTTCGTTCCA | 187               |
| Bax-forward       | GAGACACCTCAGCTGACC | 221               |
| Bax-reverse       | GCTCTGCACAACTGTCAGC | 205               |
| PARP1-forward     | CCAAGGCAGCATGTAATC | 232               |
| PARP1-reverse     | TTGTGTGTCCTTGATGCC |                   |
| β-actin-forward   | AATGCACGCACGATTTCCC |                 |
| β-actin-reverse   |                   |                   |

Bax, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma 2; PARP, poly (ADP-ribose) polymerase.
Results

**TPG enhances the viability of H9C2 cells following I/R.** CCK-8 was used to determine the effects of TPG on the viability of H9C2 cells. The CCK-8 assay results demonstrated that there was no alteration in viability when cells were treated with TPG alone (Fig. 1A). Additionally, when cells were impaired by I/R, cell viability was attenuated compared with in the control group. Conversely, TPG could significantly enhance the viability of cells inhibited by I/R in a time- and concentration-dependent manner compared with in the model group (P<0.05; Fig. 1B).

**TPG inhibits I/R-induced apoptosis of H9C2 cells.** To investigate the effects of TPG on the apoptosis of H9C2 cells, the Annexin V-FITC/PI apoptosis detection kit, RT-qPCR and western blotting were performed to measure the rates of apoptosis, and the mRNA and the protein expression levels of apoptosis-associated factors, respectively. The results of flow cytometry revealed that the rates of apoptosis were 4.93 and 19.00% in the control and model groups, respectively. In the 10, 40 and 160 µg/ml TPG groups the rates of apoptosis were 9.88, 6.94 and 5.27%, respectively. Compared with in the model group, apoptosis was reduced by TPG (P<0.05; Fig. 2). In addition, TPG markedly promoted the expression levels of pro-caspase-3 and Bcl-2, and suppressed the expression levels of cleaved-caspase-3, cleaved-PARP1 and Bax in a concentration-dependent manner, compared within the model group (P<0.05; Fig. 3).

**TPG suppresses I/R-induced oxidative stress in H9C2 cells.** To analyze whether TPG affects the oxidative stress of H9C2 cells, ROS levels and the activities of LDH, MDA, SOD and GPX were determined using various kits. The ROS assay results demonstrated that the ROS levels according to mean fluorescence intensity were 59.69, 195.34, 142.44, 109.22, and 75.95 in the control, model and TPG (10, 40 and 160 µg/ml) groups, respectively. ROS levels were increased in the model group compared with in the control group. Conversely, the ROS levels were decreased by 52.9, 86.12 and 119.39% in the TPG (10, 40 and 160 µg/ml) groups compared with in the model group (P<0.05; Fig. 4). Furthermore, TPG markedly reduced the activities of LDH and MDA; however, it promoted SOD and GPX activities in a concentration-dependent manner compared with in the model group (P<0.05; Fig. 5).

**TPG suppresses the PI3K/Akt signaling pathway in H9C2 cells following I/R.** In order to further investigate the molecular mechanism underlying the inhibitory effects of TPG on I/R-induced apoptosis of H9C2 cells, the protein expression levels of p-PI3K, PI3K, p-Akt and Akt were detected by western blot analysis. The results revealed that compared with in the model group, the expression levels of p-PI3K and p-Akt were attenuated in response to TPG (10, 40 and 160 µg/ml; P<0.05). However, no changes were detected in total PI3K and Akt protein expression among the various groups (Fig. 6).

**IGF-1 reverses the effects of TPG on I/R injury.** As previously described, IGF-1 is able to activate the PI3K/Akt signaling pathway (31). In the present study, IGF-1 was used to investigate the association of the PI3K/Akt signaling pathway with the protective effects of TPG against I/R injury. I/R-induced apoptosis was attenuated by TPG. Conversely, the number of apoptotic cells in the TPG + IGF-1 group was increased compared with in the TPG group, thus suggesting that IGF-1 treatment reversed the effects of TPG (Fig. 7).

Discussion

Traditional Chinese medicine has been widely used to treat various diseases in medical practice. TPG is a traditional Chinese medicine, which possesses antitumor effects and is able to target cardiovascular and cerebrovascular diseases (15,16). Furthermore, previous studies have reported that TPG regulates cell proliferation and apoptosis (17,18). Therefore, in the present study, the effects of TPG on the viability and apoptosis of I/R-induced H9C2 cells were examined. The present results demonstrated that TPG alone did not affect H9C2 cell viability. However, TPG significantly promoted the
viability of H9C2 cells following I/R, and restrained apoptosis in a dose-dependent manner, thus suggesting that TPG exerts protective effects on an I/R injury model in vitro.

It has been reported that myocardial I/R is closely associated with myocardial cell apoptosis (32,33). Among the apoptosis-associated genes, Bcl-2 is an inhibitor of apoptosis
and Bax is a pro-apoptotic gene; the interaction between these two proteins regulates apoptosis (34,35). Furthermore, Bcl-2 can inhibit the activity of caspase-3, thus suppressing cell apoptosis (35). In addition, PARP1 is the most important substrate of caspase-3, which is involved in DNA repair and gene integrity monitoring; cleaved-PARP1 is a marker of apoptosis (36,37). Therefore, the role of TPG in inhibiting apoptosis induced by I/R was investigated by detecting...
the expression levels of these apoptosis-associated factors. The results demonstrated that TPG markedly upregulated the expression levels of pro-caspase-3 and Bcl-2, whereas it downregulated cleaved-caspase-3, cleaved-PARP1 and Bax.
expression. Therefore, it was suggested that TPG inhibited I/R injury-induced apoptosis in vitro.

Oxidative stress is another important factor in the process of myocardial injury, which is characterized by the accumulation of ROS, increased nicotinamide adenine dinucleotide phosphate-oxidase and decreased antioxidant enzymes (38,39). A previous study demonstrated that sodium thiosulfate conspicuously reduces I/R damage-induced oxidative stress in rat hearts, and decreases the ROS levels, and LDH and MDA activities; however, it enhances SOD and GPX activities (40). Similar to previous studies, the present data revealed that TPG markedly reduced ROS levels, and LDH and MDA activities, whereas it increased the activities of SOD and GPX. Therefore, the present results indicated that TPG may reduce I/R-induced oxidative stress.

Previous studies have suggested that the PI3K/Akt signaling pathway is involved in the apoptosis of various tumor cells (41,42). Furthermore, it has been revealed that certain drugs could decrease apoptosis and oxidative stress of cells via regulation of the PI3K/Akt signaling pathway (41,43,44). Numerous drugs and proteins exert potent protective effects on myocardial injury via regulation of the PI3K/Akt signaling pathway (45-47). As expected, the present study observed that TPG significantly downregulated the phosphorylation levels of PI3K and Akt. Furthermore, apoptosis was increased by IGF-1, thus suggesting that the effects of TPG may be reversed by the activation of PI3K/Akt. Therefore, it may be suggested that TPG inhibited apoptosis and oxidative stress induced by myocardial I/R injury via restraining PI3K/Akt signaling; however, this requires further validation.

In conclusion, TPG facilitated cell viability, and suppressed I/R-induced apoptosis and oxidative stress in H9C2 cells, possibly via inhibiting the PI3K/Akt signaling pathway. Therefore, TPG may be of clinical significance in the treatment of cardiovascular diseases; however, this requires further investigation.

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Availability of data and materials
All data generated or analyzed during this study are included in this published article.

Authors’ contributions
PS and MP conceived and designed the study. JC analyzed and interpreted the data. PS drafted the manuscript. All authors were responsible for approving the final version of the manuscript.

Ethics approval and consent to participate
Not applicable.

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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