Calycosin-7-O-β-D-glucoside attenuates ischemia-reperfusion injury in vivo via activation of the PI3K/Akt pathway

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Abstract. The aim of the present study was to investigate the effects and mechanisms of calycosin-7-O-β-D-glucoside (CG) on ischemia-reperfusion (I/R) injury in vivo. Hemodynamic parameters, including ejection fraction (EF), fractional shortening (FS), left ventricular end-systolic pressure (LVESP) and left ventricular end-diastolic pressure (LVEDP) were monitored using an ultrasound system, and infarct size was measured using Evans blue/tetr唑olium chloride double staining. The activities of serum creatine kinase (CK), lactate dehydrogenase (LDH) and superoxide dismutase (SOD), and the levels of malondialdehyde (MDA) were determined to assess the degree of myocardial injury and oxidative stress-induced damage. The protein expression levels of cleaved-caspase-3, cleaved-caspase-9, phosphorylated (p)-phosphatidylinositol 3-kinase (PI3K) p85, PI3K p85, p-Akt and Akt were determined using western blotting. The results demonstrated that pretreatment with high dose (H)-CG markedly improved cardiac function, as evidenced by upregulated EF, FS and LVESP, and downregulated LVEDP. In addition, administration of CG resulted in significant decreases in infarct size in the I/R+low dose-CG and I/R+H-CG groups, compared with the I/R group. The activities of CK and LDH, and the levels of MDA in the I/R+H-CG group were reduced, compared with those in the I/R group. MDA contents were significantly lower in the I/R+H-CG group, compared with those in the I/R group. Notably, administration of the PI3K inhibitor, LY294002, markedly lowered the levels of p-PI3K p85/p-Akt, and eradicated the inhibitory effects of H-CG on infarct size, myocardial injury and oxidative stress-induced damage. Taken together, the results suggested that CG may alleviate I/R injury by activating the PI3K/Akt signaling pathway.

Introduction

Acute myocardial infarction (AMI) is associated with high mortality rates worldwide (1). AMI often occurs due to rupture of an atherosclerotic plaque in a coronary artery, which may induce thrombosis and artery occlusion, resulting in loss of blood supply to the affected area and necrosis. Annually, over 3,000,000 individuals suffer from acute ST-elevation myocardial infarction, and over 4,000,000 individuals suffer from non-ST-elevation myocardial infarction (2). Currently, the therapeutic strategies considered most effective are mechanical revascularization by percutaneous coronary intervention (3), thrombolytic therapy (4), primary angioplasty (5), coronary artery bypass grafting and antithrombotic therapy combined with timely reperfusion (6). However, these treatments are unable to improve cardiac function (7). In addition, tissue ischemia followed by reperfusion initiates systemic inflammation, which may aggravate local injury and induce remote multi-organ dysfunction (8). Therefore, the development of a safer, more effective strategy for reducing I/R injury and improving postoperative survival rates is required.

Astragalus Radix (AR), the root of Astragalus membranaceus and Astragalus membranaceus var. mongholicus (9), is a traditional Chinese medicine (10). AR exerts various bioactivities, including antioxidant, enhancement of cardiovascular function, hepatoprotection, immunostimulation and myocardial protection in diabetic nephropathy (11). AR has also been reported to reduce myocardial ischemic injury (12), and AR extracts efficiently protect MRC-5 cells from H2O2-induced oxidative damage via the inhibition of superoxide dismutase (SOD) and catalase (13). A previous clinical report indicated that AR may be a promising agent in the treatment of acute cerebral infarction (14). AR contains various active components, including polysaccharides, flavonoids, astragalosides I-VII (saponins), amino acids and trace elements (15,16). Calycosin-7-O-β-D-glucoside (CG) is a predominant flavonoid of AR (17-19), which is known to possess anti-inflammatory (20) and anti-osteoarthritic properties (21). A previous study has shown that CG significantly reduces cerebral infarct size and histological damage in a rat model of I/R. In addition, CG protects blood-brain barrier integrity by inhibiting...
the activities of matrix metalloproteinases, scavenging nitric oxide and promoting the expression of caveolin-1 (22). However, the effects of CG on myocardial I/R injury and the underlying mechanisms remain to be fully elucidated.

In the present study, a rat model of myocardial I/R injury was treated with CG, and the underlying molecular mechanisms of CG on myocardial I/R injury were evaluated.

Materials and methods

Animals. Male Wistar rats (8-week-old) were purchased from Charles River Laboratories (Beijing, China). Experiments were performed according to the guidelines for the animal care and use of laboratory animal protocols, and were approved by the Ethics Committee of The Second Affiliated Hospital of Harbin Medical University (Harbin, China; approval no. SCXK-2012-0001). The rats were maintained in an air-conditioned room with a constant temperature of 22°C and an alternating 12 h light/12 h dark cycle. The rats were provided with access to water and a standard diet ad libitum.

In vivo myocardial I/R model and experimental groups. The rats were anesthetized with 10% chloral hydrate (3 ml/kg body weight; Sinopharm Medicine, Shenyang, China) by intraperitoneal injection. The rats were intubated, and mechanical ventilation was achieved by connecting the endotracheal tube to a scientific ventilator (HX-300S; Chengdu Technology & Market Co., Ltd, Chengdu, China) at a respiratory rate of 80 breaths/min with a tidal volume of 6-8 ml/kg body weight. A left thoracotomy was performed, in order to expose the heart and the root of the large blood vessel. The left anterior descending (LAD) coronary artery was subsequently transiently ligated with a nylon suture for a 45 min ischemic period. Microsurgical scissors were used to release the ligature, and the heart was reperfused for 3 h.

The rats were randomly divided into five groups (12 animals per group). In the sham group, the rats underwent the described anesthetic and surgical procedures without ligation of the LAD coronary artery; in the I/R group, the rats underwent myocardial ischemia for 45 min and reperfusion for 3 h by ligation of the LAD coronary artery; in the I/R+H-CG group, the rats were pretreated with a high dose of CG (H-CG; 30 mg/kg body weight; Dalian Meilun Biological Technology Co., Ltd, Dalian, China) via intravenous injection 30 min prior to ligation of the LAD coronary artery; in the I/R+L-CG group, the rats were pretreated with a low dose of CG (L-CG; 15 mg/kg body weight) via intravenous injection 30 min prior to ligation of the LAD coronary artery; in the sham+H-CG group, the rats were pretreated with H-CG via intravenous injection, and then underwent the described anesthetic and surgical procedures without ligation of the LAD coronary artery.

Detection of cardiac function. Following reperfusion, an ultrasound system (IE33; Philips GmbH, Herrsching, Germany) was used to collect hemodynamic parameters, including ejection fraction (EF), fractional shortening (FS), left ventricular end-systolic pressure (LVESP) and left ventricular end-diastolic pressure (LVEDP). Blood samples were obtained for biochemical investigations and the hearts were removed for Evans blue/tetrazolium chloride (TTC) staining and western blotting.

Tissue staining. The LAD coronary artery was religated following I/R and 2-3 ml 2% Evans blue solution (Wokai, Shanghai, China) was transcardially perfused. The rats were administered with KCl solution via a marginal ear vein, and the heart was stopped in diastole. The heart was subsequently removed, washed with saline, and maintained at -20°C for 30-60 min, prior to being divided into six 2-mm sections. The sections were stained with 1% TTC (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) at 37°C and images were captured using a digital camera (D3000; Nikon, Tokyo, Japan). The area at risk (AAR; red staining) indicating the ischemic area, the infarct area (IA; white staining) and non-ischemic area (blue staining) were analyzed using an Image Analysis system (Image Pro Plus 6.0; Media Cybernetics, Inc., Rockville, MD, USA). Infarct size was defined as a percentage of IA to AAR (%).

Detection of creatine kinase (CK), lactate dehydrogenase (LDH), SOD and malondialdehyde (MDA). Following reperfusion, blood samples (5-8 ml/mouse) were collected from the carotid artery and serum was obtained by centrifugation (1,111 x g, 10 min, 4°C). Commercial assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) were used to detect the activities of CK (cat. no. A032), SOD (cat. no. A003-1), LDH (cat. no. A020-1) and MDA (cat. no. A003-1) in the serum, according to the manufacturer's protocol.

Western blot analysis. Total proteins were extracted from the AAR tissues using radioimmunoprecipitation assay lysis buffer (50 mM Tris, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, pH 7.4) (Beyotime Institute of Biotechnology, Haimen, China) and protein concentrations were determined using a Bichinchoninic Acid Protein Assay protocol. Subsequently, 40 µg protein was separated by 10 or 13% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes (EMD Millipore, Bedford, MA, USA). The membranes were blocked with 5% nonfat milk or 1% bovine serum albumin (Amresco, Framingham, MA, USA), and then incubated with the following primary antibodies at 4°C overnight: Rabbit anti-rat cleaved-caspase-3 polyclonal antibody (pAb) (1:1,000 dilution; cat. no. WL0146); rabbit anti-rat cleaved-caspase-9 pAb (1:1,000 dilution; cat. no. WL0191); rabbit anti-rat phosphatidylinositol 3-kinase (PI3K) p85 pAb (1:1,000 dilution; cat. no. WL0191); rabbit anti-rat phosphorylated (p)-Akt pAb (1:1,000 dilution; cat. no. WLP001); rabbit anti-rat Akt pAb (1:1,000 dilution; cat. no. WL0003) (all Wanleibio, Shenyang, China) and rabbit anti-rat p-PI3K p85 pAb (1:500 dilution; cat. no. bs-5538R; Bioss, Beijing, China). The membranes were then washed with Tris-buffered saline containing Tween 20 (Beijing Solarbio Science & Technology Co., Ltd.), and incubated with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (1:5,000, Beyotime Institute of Biotechnology) at 37°C for 45 min. Band densities were analyzed using Gel-Pro Analyzer software 4.0 (Media Cybernetics, Inc.) and normalized to β-actin.
Detection of caspase-3/9 activity. Caspase-3/9 activity was measured using a Caspase Activity Assay kit (Beyotime Institute of Biotechnology), according to the manufacturer's protocol. Briefly, the total cellular proteins were quantified and reacted with the corresponding substrates: Ac-DEVD-pNA or Ac-LEHD-pNA. Caspase-3/9 activity was subsequently measured as the optical density of the cleaved substrate pNA at 405 nm using a microplate reader (ELX-800; Bio-Tek Instruments, Inc., Winooski, VT, USA).

PI3K/Akt signaling pathway. The rats were randomly divided into three groups of 12: The I/R group, I/R+H-CG group and I/R+H-CG+LY294002 group. The PI3K inhibitor, LY294002, (0.3 mg/kg body weight; Sigma-Aldrich) was administered to the rats in the I/R+H-CG+LY294002 group via intravenous injection 30 min prior to the administration of H-CG. The rats were then subjected to I/R. Heart tissues from the AAR was lysed with lysis buffer and the expression levels of PI3K p85, p-PI3K p85, Akt and p-Akt were measured using western blot analysis. Infarct size was assessed using Evans blue/TTC double staining. The serum was obtained and levels of CK, SOD, LDH and MDA were analyzed using commercial kits (Nanjing Jiancheng Bioengineering Institute) as described above.

Statistical analysis. GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA) was used for statistical analysis and image processing. Data are expressed as the mean ± standard deviation. Comparisons between the experimental groups were conducted using one-way analysis of variance, followed by a Bonferroni post-hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

CG ameliorates I/R-induced cardiac dysfunction. Ultrasound analysis was performed to detect cardiac function. As shown in Fig. 1, H-CG had no effect on EF (Fig. 1A), FS (Fig. 1B), LVEDP (Fig. 1C) or LVESP (Fig. 1D), in the sham+H-CG group, compared with the sham group. However, EF, FS and LVESP levels were markedly lower in the I/R group, compared with those in the sham group (P<0.01), whereas LVEDP was significantly higher, compared with the sham group (P<0.01). Following treatment with H-CG, EF, FS and LVESP (P<0.05) were significantly increased, whereas the LVEDP was decreased (P<0.01).

CG reduces myocardial infarct size. To determine whether CG affected myocardial infarct size, the rats were pretreated with L-CG or H-CG, and then subjected to I/R. As shown in Fig. 2, no ischemic and necrotic areas were detected in the sham or the sham+H-CG groups. I/R significantly increased the infarct size (29.98±5.28%, vs. 0%; P<0.01). As expected, compared with the I/R group (29.98±5.28%), the infarct size was significantly smaller in the I/R+L-CG group (22.74±4.00; P<0.05) and the I/R+H-CG group (16.22±5.15%; P<0.01).

CG attenuates I/R-induced myocardial injury and oxidative stress-induced damage. The effects of CG were also evaluated on I/R-induced myocardial injury and damaged from oxidative stress. The activities of serum CK (Fig. 3A; P<0.01) and LDH (Fig. 3B; P<0.01) were markedly elevated in the I/R group, compared with those in the sham group. Following treatment with L-CG, only CK activity was inhibited (P<0.01); however, treatment with H-CG markedly inhibited the activities of the two
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CG reduces the I/R-induced increased expression levels and activities of pro-apoptotic factors. The results of the present study demonstrated that caspase cleavage (Fig. 4A; P<0.01), and the activities of caspase-3 (Fig. 4B; P<0.01) and caspase-9 (Fig. 4C; P<0.01) were enhanced in the I/R group, compared with those in the sham group. Treatment with L-CG and H-CG markedly downregulated the levels of cleaved-caspase-3 (P<0.01) and cleaved-caspase-9 (L-CG, P<0.05; H-CG, P<0.01). In addition, caspase activity was significantly inhibited following treatment with L-CG (caspase-3, P<0.01; caspase-9, P<0.05) or H-CG (P<0.01).

CG increases the phosphorylation of PI3K p85 and Akt. The protein expression levels of p-PI3K p85 (Fig. 5A; P<0.01) and p-Akt (Fig. 5B, P<0.01) were downregulated in the I/R group, compared with the sham group, and were upregulated in the I/R+L-CG group (p-PI3K p85, P<0.05; p-Akt, P>0.05).
and I/R+H-CG group (P<0.01). No statistical differences were observed between the groups in the expression levels of total PI3K p85 or total Akt.

LY294002 inhibits H-CG-induced activation of the PI3K/Akt pathway. To confirm that CG attenuated I/R injury in vivo via activation of the PI3K/Akt pathway, the PI3K inhibitor, LY294002, was administered to the rats prior to H-CG. Treatment with H-CG significantly increased the phosphorylation of PI3K and Akt (Fig. 6A; P<0.01); however, suppressing PI3K activity with LY294002 effectively inhibited H-CG-induced PI3K/Akt phosphorylation (P<0.01). Treatment with H-CG significantly decreased infarct size, compared with the I/R group (15.67±3.28, vs. 35.46±5.33%, respectively; P<0.01), as shown in Fig. 6B, however, infarct size was significantly higher in the I/R+H-CG+LY294002 group, compared with that in the I/R+H-CG group (27.81±4.10, vs. 15.67±3.28%, respectively; P<0.01). Treatment with H-CG significantly decreased CK activity (Fig. 6C; P<0.01), LDH activity (P<0.01) and MDA content (P<0.01), and significantly increased SOD activity.
However, co-treatment with LY294002 attenuated these effects (CK, P<0.01; LDH, P<0.05; MDA, P<0.05; SOD, P<0.05).

**Discussion**

AMI is a leading contributor to morbidity and mortality rates worldwide (23-25). Reperfusion improves clinical symptoms in patients with AMI (26); however, restoration of blood flow following ischemia may result in I/R injury (27,28), which is involved in the development of myocardial necrosis, arrhythmia, myocardial stunning, endothelial dysfunction and microvascular complications (26,29). The present study demonstrated that CG may exert a cardioprotective effect in a rat model of I/R-induced injury via the PI3K/Akt signaling pathway.

Previous studies have indicated that myocardial I/R injury alters hemodynamic parameters and affects cardiac function (30,31). In addition, levels of EF, FS (32,33) and LVSP are lower in I/R groups than in sham groups, whereas, LVEDP levels are higher (34,35), which is in agreement with the results of the present study. The present study also demonstrated that treatment with H-CG significantly restored the I/R-induced downregulation of EF, FS and LVESP; and markedly lowered the levels of LVEDP in the I/R+H-CG group, compared with the...
I/R group. These results suggested that CG improved cardiac function in the rat model of I/R.

Myocardial infarct size is an indicator of myocardial injury, and I/R has been reported to result in infarction in MI/R groups, compared with sham groups (36,37). Treatment with CG has been shown to significantly reduce infarct volume in a rat model of middle cerebral artery occlusion cerebral I/R injury (22). Consistently, the present study observed that L-CG and H-CG efficiently decreased infarct size.

LDH (38) and CK (39) are often elevated in MI, and are used to assess the degree of myocardial injury. Numerous evidence has demonstrated that I/R often induces the generation of reactive oxygen species (ROS) and oxidative stress (40-42). Subsequently, ROS interacts with cell membrane lipids and produces MDA, which impairs cardiac function and induces myocardial cell injury (43,44). Therefore, reducing oxidative stress is an advantageous strategy for the alleviation of I/R injury. In the present study, CK and LDH activity, and MDA content were increased in the I/R group, compared with the sham group; however, SOD activity was decreased, which was consistent with the results of previous studies (37,45). These results indicated that CG exerted its cardioprotective effects by notably decreasing CK, LDH and MDA, and increasing SOD activity.

Apoptosis is important in development and tissue homeostasis (46), and caspases are considered the executioners of apoptosis (47). Once cells receive apoptotic stimuli, the mitochondrial outer membrane becomes permeabilized and cytochrome c is released from the mitochondria into the cytosol (48). Cytochrome c interacts with apoptotic protease activating factor 1 and procaspase-9, which is cleaved into caspase-9 and initiates the activation of caspase-3, caspase-6 and caspase-7 (49). Previous studies have reported that I/R injury is associated with the apoptosis of cardiomyocytes (50,51). The present study demonstrated that the expression levels of cleaved-caspase-3 and cleaved-caspase-9, and caspase activity were downregulated in the I/R group, compared with the I/R group. Therefore, CG may alleviate I/R injury by suppressing caspase activity and inhibiting cardiomyocyte apoptosis.

PI3K consists of a catalytic subunit (p110) and a regulatory subunit (p85) (52,53). Akt is a serine-threonine kinase and, following phosphorylation, performs its antiapoptotic effects via the activation of B-cell lymphoma-2-associated death promoter and caspases (54). Previous studies have reported that the PI3K/Akt signaling pathway is crucial in protecting the myocardium from MI/R injury (55), and the activation of PI3K/Akt significantly reduces cardiomyocyte apoptosis (56). The present study examined the expression levels of PI3K p85, p110 and Akt, and Akt. Pretreatment with CG effectively activated and phosphorylated PI3K and Akt, whereas the levels of total PI3K p85 and Akt were not changed. The PI3K inhibitor, LY294002, was used to determine whether the PI3K/Akt pathway was involved in the CG-mediated alleviation of I/R injury. Suppressing PI3K activity with LY294002 reversed the beneficial effects of CG. Based on the above results, it was hypothesized that CG alleviates I/R injury by activating the PI3K/Akt signaling pathway.

In conclusion, the results of the present study demonstrated that CG attenuated myocardial I/R injury in the rat model. The protective effects may be associated with activation of the PI3K/Akt pathway, and the inhibition of oxidative stress and pro-apoptotic factors.

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