Ginsenoside Rg3 attenuates myocardial ischemia/reperfusion injury via Akt/endothelial nitric oxide synthase signaling and the B-cell lymphoma/B-cell lymphoma-associated X protein pathway

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Abstract. Previous studies have suggested that ginsenoside Rg3 (GSRg3) extract from the medicinal plant *Panax ginseng*, may increase nitric oxide production via increases in the phosphorylation and expression of endothelial nitric oxide synthase (eNOS). The present study used an *in vitro* neonatal rat cardiomyocyte (NRC) model of anoxia-reoxygenation injury and an *in vivo* rat model of myocardial ischemia/reperfusion (MI/R) injury. Hemodynamic, histopathological and biochemical assessment of the myocardial injury was performed and the expression levels of lactate dehydrogenase (LDH), superoxide dismutase and creatine kinase (CK) were measured in serum from the animal model, which may reflect myocardial injury. NRC injury was determined using a Cell Counting kit-8. The GSRg3 anti-apoptotic effects were assessed using flow cytometry to investigate the number of early-late apoptotic cells and western blot analysis was performed to analyze the protein expression levels of caspase-3, caspase-9, B-cell lymphoma-2 (Bcl-2), phosphorylated (p-)Akt and eNOS. The results suggested that pretreatment with GSRg3 (60 mg/kg) significantly improved rat cardiac function, as demonstrated by increased left ventricular systolic pressure, heart rate and first derivative of left ventricular pressure. GSRg3 also reduced the size of the myocardial infarct and LDH/CK levels in the blood following MI/R. *In vitro* investigations revealed that GSRg3 (10 nM) decreased NRC apoptosis through inhibiting the activation of caspase-3 and caspase-9, and decreasing the expression levels of p-Akt, eNOS and the ratio of Bcl-2/Bcl-2-associated X protein (Bax). Overall, the present study revealed that GSRg3 mediated a cardioprotective effect against MI/R-induced apoptosis via Akt/eNOS signaling and the Bcl-2/Bax pathway.

Introduction

There is evidence suggesting that myocardial ischemia (MI) is a major risk factor of myocardial infarction, which induces myocardial remodeling. The predominant morphological changes of ventricular remodeling are the exhibition of an infarction area, ventricular hypertrophy and ventricular expansion (1,2). The mechanisms underlying ventricular remodeling remain to be fully elucidated, however, it is generally accepted that following myocardial injury, the molecules, cells and mechanisms change due to altered gene expression levels and the imbalance between cell apoptosis and proliferation, which is important in the entire disease process (1).

It is important to develop novel treatments to inhibit or slow the disease process to enable more time for subsequent treatment. Traditional Chinese medicine has gained increased attention for the treatment of various diseases. Although the use of Chinese medicine in the treatment of ventricular remodeling has been investigated only relatively recently, it has been demonstrated that using traditional Chinese medicine can affect the occurrence and development of ventricular remodeling in a number of aspects (3). This is also supported by previous reports suggesting that certain agents, including statins, angiotensin converting enzyme inhibitors and angiotensin II receptor inhibitors, can improve ventricular remodeling by increasing cell apoptosis and preventing cell proliferation in smooth muscle cells in hypertensive animal models (4,5).

Ginsenoside Rg3 (GSRg3), extracted from *Panax ginseng*, is a traditional Chinese herbal medicine used widely in clinical treatment and may significantly improve basilar artery hypertrophic remodeling through the prevention of artery smooth muscle cell proliferation (6). The present study predominantly investigated how GSRg3 attenuates MI/reperfusion (MI/R) injury and examined the main pathways involved.

Materials and methods

Animals and drugs. The present study was performed in accordance with the National Institutes for Food and Drug Control (http://www.nicpbp.org.cn) for the Use of Laboratory Animals and was approved by the Tongji Hospital of Tongji University (Shanghai, China) Committee on Animal Care. A total of 30 male eight-week-old Sprague-Dawley rats (Experimental Animal Center, Tongji University) weighing between 260 and 280 g
were housed in diurnal lighting conditions (12 h light/12 h dark; 22-24°C) and allowed free access to food and water for 7 days prior to performing the investigation. GSRg3 (purity>98%; Fig. 1) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China).

MI/R procedure in the rat hearts. The cardiac MI/R surgery was performed, as described previously (5). Briefly, the rats were anaesthetized via intraperitoneal injection of pentobarbital sodium (60 mg/kg body weight; Sigma-Aldrich, St. Louis, MO, USA) and were placed on a warm board (25°C) to control the body temperature at 37°C for surgery. The neck was opened with a ventral midline incision and the animals were ventilated with room air using a rodent respirator (tidal volume 8 ml/kg body weight; 60-80 breaths/min; Shanghai Alcott Biotech Co., Ltd., Shanghai, China). An electrocardiogram in lead II was recorded through needle electrodes attached to the limbs. Following the adjustment of the respiratory rate and the tidal volume of gases, the chest was opened by a middle thoracotomy. Following peri-cardiotomy, a 4-0 black silk thread (Millar, Inc., Houston, TX, USA) was passed behind the left anterior descending coronary artery and was occluded by a knot for 30 min to cause ischemia. Subsequently, the knot was released and reperfusion was performed for 3 h. For the sham control group, the black silk was placed under the left anterior descending coronary artery without occlusion.

Hemodynamic measurements. The right common carotid artery was exposed and cannulated with a Millar vessel (Millar, Inc.) into the left ventricular cavity of the rat through the ascending aorta. The heart function, including the left ventricular systolic pressure (LVSP), heart rate (HR) and first derivative (dPV/dt) of the left ventricular pressure of the rats in each group (sham, I/R and I/R+Rg3) were recorded and programmed using a biotic signal collection and processing system (PowerLab; AD Instruments, New South Wales, Australia), as described previously (6).

Determination of the serum levels of SOD, LDH and CK. Following 3 h reperfusion, blood samples (5 ml) were collected through the ventral aorta using a scalp vein set, and the serum was frozen at -80°C until subsequent analysis. The activities of LDH, CK and SOD were determined using an ELISA kit, according to manufacturer's instructions (BioAssay Systems, Hayward, CA, USA).

Infarct size measurement. Following reperfusion, the hearts were rapidly extracted from the rats using surgical scissors and frozen at -20°C. The left ventricular area was sliced into six 2-3 mm-thick slices perpendicular to the base-apex and incubated in 2% triphenyltetrazolium chloride (TTC; pH 7.4; Xiya Reagent, Chengdu, China) buffer for 15 min at 37°C. The viable tissues were stained dark red with TTC, while the infarcted portion remained grayish-white. The area of infarction was measured using an image analysis system [National Institutes of Health (NIH) image software, version 1.60; NIH, Bethesda, MA, USA].

Isolation of primary neonatal rat cardiomyocytes (NRCs) and anoxia-reoxygenation injury. Primary neonatal Sprague-Dawley rats (1-3 days-old) were purchased from the experimental Animal center of Tongji University (Shanghai, China). Cardiac myocytes were cultured from 1-3 day-old Sprague-Dawley rats with Dulbecco's modified Eagle's medium (DMEM; Gibco Life Technologies, Carlsbad, CA, USA) at a density of 5x10⁵ for three days, as described previously (1,2). The primary neonatal rats were anaesthetized with sodium pentobarbital and decapitated, and then immersed in 75% ethanol (20 ml) for 30 sec. The chest was opened and the heart ventricles were dissected rapidly and immersed in ice-cold Krebs-Ringer buffer containing 137 mM NaCl, 2.6 mM CaCl₂, 5.4 mM KCl, 11.1 mM MgCl₂·6H₂O, 0.4 mM NaHPO₄, 2H₂O, 11.9 mM NaHCO₃, and 5.6 mM glucose (pH 7.4), and the ventricles were minced into small sections using eye scissors and digested with trypsin (0.1%; Beyotime Institute of Biotechnology, Guangzhou, China). The cardiomyocytes were cultured in DMEM containing 10% newborn calf serum (NCS; Gibco Life Technologies), in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. The neonatal rat cardiomyocytes (NRCs) were used and the ginsenoside-Rg3 and solvent were preincubated with cells for 30 min prior to I/R injury.

Simulated I/R (SI/R) was performed, as described previously (1,2). Briefly, simulated ischemia buffer, containing 98.5 mM NaCl, 1.2 mM MgSO₄, 10 mM KCl, 1 mM CaCl₂, 40 mM sodium lactate and 20 mM HEPES (pH 6.8), and simulated reoxygenation buffer, containing 20 mM HCO₃, 0.9 mM NaH₂PO₄, 1 mM CaCl₂, 1.2 mM MgSO₄, 20 mM HEPES, 5 mM KCl, 129.5 mM NaCl and 5.5 mM glucose (pH 7.4), were prepared in advance. The medium of the NRCs was replaced with 1 ml simulated ischemia buffer, incubated in a hypoxic chamber (humidified atmosphere 5% CO₂/0% O₂ balanced with N₂ at 37°C) for 3 h, and then reoxygenated in a standard incubator for 2 h with reoxygenation buffer. The cells subjected to control conditions were cultured with normal Tyrode solution (pH 7.4; Beijing Leagene Biotech Co., Ltd., Beijing, China) in a humidified atmosphere of 5% CO₂/21% O₂ balanced with N₂ at 37°C for 5 h (4). The cells were divided randomly into four groups: Control group, incubated with Tyrode solution during the entire experimental period; SI/R group, incubated with simulated ischemia buffer for 3 h hypoxia, followed by 2 h re-oxygenation; Vehicle group, subjected to 0.2% (v/v) dimethyl sulfoxide administration 30 min prior to SI/R; SI/R+GSRg3 group, subjected to GSRg3 (10 mM) administration 30 min prior to SI/R (5).

Cell viability. The cell viability was assessed using a Cell Counting Assay kit-8 (CCK-8, Beyotime Institute of Biotechnology) according to the manufacturer's instructions. The NRCs (100 µl) were plated into 96-well plates at a density of 1x10⁵ cells/well, followed by 30 min pre-incubation with different concentrations of GSRg3 (0.1-100 µM). Following treatment, the cells were exposed to 10 µl CCK-8 solution for a further 2 h and the absorbance at 450 nm was measured using a microplate reader (ELx808; Bio-Tek Instruments, Winooski, VT, USA) (1).

Determination of apoptosis by flow cytometry. The apoptotic rate of the NRCs was determined by flow cytometry using annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) staining according to the manufacturer's instructions. Briefly, the NRCs were pretreated with 10 µM GSRg3 for 30 min followed by SI/R treatment. The cells were digested with trypsin
(0.25%) and were then resuspended in phosphate-buffered saline (PBS) containing 10% NCS. The cells were centrifuged at 200 x g for 10 min at 4˚C and then washed twice with cold PBS. The cells were then treated with 5 µl annexin V-FITC (1:80) and 10 µl PI (1:40) (Bioworld Technology Co., Ltd., Nanjing, China), and incubated in the dark at room temperature for 15 min. Each sample was analyzed using a Beckton-Dickinson flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Western blot analysis. The NRCs were lysed in lysis buffer (Beyotime Institute of Biotechnology) containing Protease Inhibitor Cocktail (Merck Millipore, Billerica, MA, USA) for 30 min on ice. The cellular proteins were collected using a cell scraper. Following centrifugation for 15 min at 12,000 rpm, a Bicinchoninic acid Protein Assay kit (Beyotime Institute of Biotechnology) was used to determine the protein concentrations. Equal quantities of protein were separated using 10% SDS-PAGE gels and transferred onto polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked using 5% non-fat milk in Tris-buffered saline (8 g NaCl and 6 g Tris), containing 1% Tween-20 (TBST), for 1 h at room temperature. The membranes were then incubated with primary antibodies against eNOS (610297; IgG1; polyclonal; 1:3,000; BD Biosciences), p-Akt (2920; mouse monoclonal; 1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA), Akt (4691; rabbit monoclonal; 1:1,000; Cell Signaling Technology, Inc.) Bcl-2 (2870; mouse polyclonal; 1:10000; Cell Signaling Technology, Inc.), Bax (2772; mouse polyclonal; 1:2,000; Cell Signaling Technology, Inc.) and PARP (5625; rabbit monoclonal; 1:1,000; Cell Signaling Technology, Inc.) overnight at 4˚C. Following washing three times for 5 min with TBST, the membranes were incubated with secondary antibodies, including alkaline phosphatase-linked anti-mouse (7056; IgG; 1:5,000; Cell Signaling Technology, Inc.) or horseradish peroxidase-linked anti-rabbit antibodies at room temperature for 1 h. β-actin (7074; IgG; 1:5,000; Cell Signaling Technology, Inc.) was used as an internal control. The protein bands were visualized using a Chemiluminescence Electrophoretic Mobility Shift Assay kit (Beyotime Institute of Biotechnology) and X-ray film (Kodak BioMax MS Film; Kodak. Corp., Rochester, NY, USA). The band density was statistically analyzed using Image J software (National Institutes of Health, Bethesda, MD, USA).

Figure 1. Chemical structure of ginsenoside Rg3. The molecular formula is C42H72O13 and the molecular weight is 785.01.

Figure 2. GSRg3 improves rat cardiac function following 30 min ischemia and 3 h reperfusion. Improvements were observed in (A) LVSP, (B) LVEDP, (C) +dP/dt max and (D) -dP/dt max. Data are expressed as the mean ± standard error of the mean (n=8; *P<0.05, vs. sham, **P<0.05 and ***P<0.01, vs. I/R). LVSP, left ventricular systolic pressure; LVEDP, left ventricular end diastolic pressure; ±dP/dt max, instantaneous first derivation of left ventricle pressure; sham, untreated; I/R, ischemia/reperfusion; I/R+Rg3, I/R+Rg3 administration 3 days prior to surgery; baseline, immediately following stabilization; R0, pre-ischemic treatment; R30, 30 min after reperfusion; R60, 60 min after reperfusion; R180, 180 min after reperfusion. GSRg3, ginsenoside Rg3.
Statistical analysis. Data are expressed as the mean ± standard error of the mean. The statistical comparisons between groups were performed using one-way analysis of variance. SPSS version 19.0 was used to perform all statistical analyses (SPSS Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of GSRg3 on cardiac function. GSRg3 had no effect on blood glucose, cardiac function or blood pressure normality and no significant differences were observed between the groups at the baseline conditions. Pretreatment with GSRg3 increased LVSP and +dp/dt max, and decreased LVEDP and -dp/dt max following 3 h reperfusion, compared with the MI/R group (P<0.05; Fig. 2). Treatment with GSRd markedly increased the mean heart rate compared with the MI/R group (P<0.05; Fig. 3).

The hemodynamic data demonstrated that GSRg3 improved rat cardiac systolic and diastolic function following MI/R.

GSRg3 reduced rat myocardial injury (infarct size, necrosis, and apoptosis) post MI/R. The infarcted areas and areas at risk are shown in Fig. 4. No MI was observed in the hearts from the sham group. Pretreatment with GSRg3 significantly decreased the infarct size compared with the MI/R group (P<0.05). To determine whether GSRg3 attenuated MI/R induced cardiomyocyte necrosis, the plasma levels of CK, LDH and SOD were measured following reperfusion. GSRg3 treatment markedly decreased the levels of CK and LDH, and increased the levels of SOD compared with the MI/R group (P<0.05). These data demonstrated that GSRg3 reduced myocardial necrosis following MI/R.

GSRg3 improves SI/R-induced in vitro cell injury, increasing viability and decreasing apoptosis. The NRCs were treated with different concentrations of GSRg3 (0.1-100 mM) to determine the effects of GSRg3 alone. Treatment with these concentrations of GSRg3 for 24 h were not cytotoxic, as demonstrated by the CCK-8 assay (Fig. 5A). As shown in Fig. 5B, the concentration response curves determined cellular viability, and this was observed at dosage of 10 mM GSRg3.

Flow cytometric analysis was performed to assess the cellular apoptosis (Fig. 5C). Annexin V/PI double staining revealed a significant increase in apoptosis in the sham group compared with the control post-SI/R (8.6±0.3%, vs. 3.1±0.2%; P<0.05), and treatment with 10 mM GSRg3 markedly decreased cellular apoptosis (4.6±0.1%; P<0.01; Fig. 5D). Overall, these in vitro results suggested that GSRg3 protected cardiomyocytes, which was in accordance with the in vivo data.
GSRg3 modulates the expression levels of Bcl-2 and Bax in NRCs subjected to SI/R. The present study aimed to determine whether GSRg3 inhibited the apoptosis of NRCs induced by SI/R by modulating the Bcl-2 family proteins. SI/R treatment reduced the expression of Bcl-2 and increased the expression of Bax, therefore, downregulating the Bcl-2/Bax ratio (Fig. 6A). Pretreating NRCs with 10 mM GsRg3 prior to SI/R induced the expression of Bcl-2 and inhibited the expression of Bax, therefore, increasing the Bcl-2/Bax ratio (Fig. 6A).
GSRg3 decreases the activities of caspase-3 and caspase-9 in NRCs following SI/R. The caspase family of proteins regulate cellular apoptosis. Caspase-9 is activated by cytochrome c, which activates caspase-3, causing cell apoptosis (4). SI/R significantly increased the protein expression levels of cleaved caspase-9 and caspase-3, however, pretreating NRCs with 10 mM GsRg3 significantly attenuated the expression levels of cleaved caspase-9 and caspase-3 (Fig. 6B).

GSRg3 increases the phosphorylation of Akt and eNOS in NRCs subjected to SI/R. To further investigate the molecular mechanism underlying GsRg3-mediated cardioprotection,
western blot analysis was performed to determine the protein expression levels of phosphorylated (p)-Akt/Akt and p-eNOS in NRCs following SI/R. No significant differences were observed in the expression levels of Akt and eNOS between the treatment groups at the baseline (Fig. 7A and B). Consistent with previous reports, pretreatment with 10 mM GSRg3 significantly increased the expression levels of p-Akt and p-eNOS, and consequently increased the ratios of p-Akt/Akt and p-eNOS/eNOS (P<0.01).

Treatment with the phosphoinositide 3-kinase inhibitor, LY294002, inhibited the GSRg3-mediated phosphorylation of Akt (Fig. 7B).

Discussion

The present study revealed that GSRg3 significantly attenuated MI/R injury in the rat model, as demonstrated by the reduced myocardial infarct size, improved rat cardiac functions, CK/LDH levels in blood following MI/R and decreased NRC apoptosis. The in vitro investigation revealed that treatment with GSRg3 (10 mM) reduced the NRC apoptotic response by inhibiting the activation of caspase-3 and caspase-9 and by increasing the phosphorylation of Akt/eNOS and the Bcl-2/Bax ratio.

As one of the most popular Chinese herbal medicines, ginseng has been used for the treatment of diabetes, cancer and cardiovascular diseases for thousands of years (7,8). Over 40 ginsenosides have been isolated and identified (9). Previous studies have demonstrated that ginsenosides exerts significant protective effects on the cardiovascular system (9-11). MI/R injury is predominantly caused by cardiomyocyte apoptosis (12) and GSRg3 is able to directly depress cardiomyocytes contraction by increasing the production of nitric oxide (NO) (13). Yang et al demonstrated that the NO produced by eNOS has a direct impact on cardiac remodeling (14). In addition, it has also been suggested that GSRg1 is important in the improvement of the cardiovascular system. In a tumor necrosis factor-α stimulated HUVEs culture model, GSRg1 increases the production of NO and the mRNA expression of eNOS (15). In vitro, GSRg1 is capable of reducing homocysteine-induced endothelial dysfunction and free radical production in porcine coronary arteries (16-18). Therefore, the present study aimed to determine whether pretreatment with GSRg1 reduced myocardial infarction following MI/R, and whether this had significant clinical importance. A previous study suggested that GSRg1 may induce the production of NO and regulate the acute activation of eNOS in human aortic endothelial cells (19).

GSRg3, an important ginsenoside in the extract of ginseng, is used in herbal medicine as a tonic and restorative agent. However, the molecular mechanism underlying the beneficial effects of GSRg3 remains to be elucidated. The present study demonstrated, using an in vivo rat model, that pretreatment with GSRg3 significantly decreased the infarct size and plasma stress of CK/LDH. The levels of CK/LDH and oxidative stress in the myocardium were also significantly suppressed in the GSRg3 treated group, whereas the level of SOD was improved. In vitro, SI/R treatment increased the expression of Bax, a pro-apoptotic protein, decreased the expression of Bcl-2, an anti-apoptotic protein, reduced the Bcl-2/Bax ratio and activated caspase-3 and caspase-9. GSRg3 upregulated the phosphorylation of eNOS and increased the expression of p-Akt.

In conclusion, GSRg3 exerted cardioprotective effects in MI/R injury and may have a positive significance for clinical treatment.

References