Effects of Panax Quinquefolium Saponin on Phosphatidylinositol 3-Kinase/Serine Threonine Kinase Pathway of Neonatal Rat Myocardial Cells Subjected to Hypoxia

ABSTRACT  
Objective: To explore the effects of Panax Quinquefolium Saponin (PQS) on phosphatidylinositol 3-kinase/serine threonine kinase (PI3K/Akt) pathway of neonatal rat myocardial cells subjected to hypoxia.

Methods: Neonatal rat myocardial cells were cultured in vitro. After the myocardial cell injury was induced by hypoxia, the cells were randomized into 5 groups: the normal group, the model group, the positive control group (Ciclosporin A, 2 μmol/L), the low-dose PQS group (PQSL, 25mg/L), and the high-dose PQS group (PQSH, 50 mg/L). Morphology and behavior of myocardial cells were observed under an inverted microscope. Apoptosis rate and lactate dehydrogenase (LDH) leakage rate of myocardial cells were determined by colorimetry. Mitochondrial transmembrane potential was assessed using a fluorexon laser. Phospho-glycogen synthase kinase (GSK)-3β and phospho-Akt as well as cytochrome C were determined by Western blot.

Results: LDH leakage in the Ciclosporin A group, PQSH group and PQSL group reduced progressively compared with the model group (P<0.05). Akt and GSK-3β was strongly phosphorylated after treatment with Ciclosporin A and PQS compared with the model group (P<0.05, P<0.01). Compared with the model group (16.41 ± 1.74; 35.28 ± 6.30), both the integrated optical density of mitochondrial permeability transition pore (MPTP) and the mitochondrial transmembrane potential significantly increased in the PQSH group (42.74 ± 2.12; 71.36 ± 6.54) and the PQSL group (39.58 ± 1.49; 66.99 ± 5.45; P<0.05, P<0.01). However, the protein of cytochrome C outside the mitochondrion decreased in the PQSH group (273.66 ± 14.61) and the PQSL group (259.62 ± 17.31) compared with the model group (502.41 ± 17.76; P<0.05).

Conclusion: Through activation of the PI3K/Akt pathway and inhibition of the MPTP, PQS might protect the heart against ischemia injury and apoptosis of myocardial cells.

KEYWORDS  Panax Quinquefolium Saponin, ischemic cardiomyocytes, phosphatidylinositol 3-kinase/serine threonine kinase, mitochondrial permeability transition pore.

Coronary heart disease (CHD) is a major cause of death and disability. Despite extensive investigation, we still do not fully understand the cellular and molecular mechanisms that are involved in the initiation and propagation of myocardial injury. In 2000, researchers treated CHD with Panax Quinquefolium Saponin (PQS) combined with modern routine medication at nine AAA hospitals (the highest standard) in Beijing. The results showed that PQS could effectively reduce the mortality rate and the incidence. We have previously reported that PQS played an important role in realizing the therapeutic effects such as energy supply, neovascularization and anti-oxidation injury in ischemic myocardium after acute myocardial infarction (AMI). We therefore suggest that PQS protects myocardium from ischemia/reperfusion injury through different pathological mechanisms. Phosphatidylinositol 3-kinase/serine threonine kinase (PI3K/Akt) pathway is an intracellular signalling pathway important in apoptosis and become one of the major targets for the study of myocardial ischemia. Up to now, we still don't know whether myocardium protection of PQS is through PI3K/Akt pathway and what the exact target point is. The present study, therefore, explored the effects of PQS on PI3K/Akt pathway of neonatal rat myocardial cells subjected to hypoxia.

METHODS

Experimental Animals
Neonatal (1–2 days) Sprague-Dawley (SD)
rats, male or female, were provided by Beijing Weitong Lihua Experimental Animal Technique Company [Certification of Experimental Animal: No. SCXX(Jing)2006-2009].

**Drugs and Reagents**

Xinyue Capsule (心悦胶囊), containing PQS 50 mg, was provided by Yisheng Pharmaceutical Co. Ltd., China, certification No. Z20030073. Methylthiazolyl-tetrazolium (MTT) and Ciclosporin A (CsA) was from Beijing Solarbio Science & Technology Co., Ltd., China. Cytochrome C and mitochondrial permeability transition pore (MPTP) kits were provided by Scientific Inc. USA. Primary antibodies of glycogen synthase kinase (p-GSK)-3β and PI3K-Akt (CST, USA) were rabbit polyclonal antibodies, and goat anti-rabbit horse radish peroxidase labeled secondary antibodies were from Beijing Zhongshan-Golden Bridge Biological Technology Co. Ltd., China.

**Primary Culture of Neonatal Rat Cardiomyocytes**

Primary myocyte cultures were prepared from neonatal (1–2 days) SD rat ventricles as described previously. In brief, the hearts were isolated and ventricles were digested with collagenase (Sigma, C7661, USA) and pancreatin (Sigma, P-3232; 73 U/mL and 0.6 mg/50 mL final concentration), and incubated at 37 °C for 30 min with gentle shaking. After discarding the initial supernatant, the subsequent 20 min digestions were pooled and suspended in Dulbecco’s modified eagle medium (DMEM) supplemented with 15% fetal calf serum, and incubated at 37 °C for 30 min with gentle shaking. After discarding the initial supernatant, the subsequent 20 min digestions were pooled and suspended in Dulbecco’s modified eagle medium (DMEM) supplemented with 15% fetal calf serum. The supernatant was centrifuged at 500 × g and the pellet was resuspended in the medium. The mixed cell population was centrifuged at low speed over a percoll gradient. The fraction of cells migrating at a density of 1.050 and 1.062 was washed twice in the medium and was plated and maintained at 37 °C in a 5% CO₂ environment for 4 days.

**Hypoxia Cell Culture**

To mimic hypoxia, the cardiomyocytes were incubated at 37 °C in a modular incubator chamber for 3 h, where normal air was replaced by 95% N₂–5% CO₂ after cell treatment.

**Grouping and Medication**

The cardiomyocytes were randomly assigned to 5 groups: a normal group; a model group; a positive control group (CosA, treated with Ciclosporin A, 2 μ mol/L); a high-dose PQS group (PQSH, 50 mg/L) and a low-dose PQS group (PQSL, 25 mg/L). All treatments were administered before the cardiomyocytes were subjected to hypoxia.

**Beating Frequencies and Morphology of Cultured Cardiomyocytes**

The spontaneous beating frequency and rhythm was measured by counting the pulsation rate of cardiomyocytes incubated in DMEM at 37 °C heating plate of phase-contrast inverted microscope (Olympus, Japan). Morphological characteristics of cultured cardiacmyocytes were observed at the same time.

**Analysis of Lactate Dehydrogenase Leakage and Cell Survival**

Cell survival was assessed by MTT assay. Cells were seeded in 96-well plates at a density of 5 × 10⁵ cells per well. After treatments, cells were incubated with 5 mg/mL MTT for approximately 4 h. The medium was aspirated and the formazan product solubilized with 150 μL dimethylsulfoxide. Cell survival was assessed by microplate spectrophotometer at 492 nm absorbance using a 96-well plate reader. The ratio of MTT to normal group was calculated. Lactate dehydrogenase (LDH) activity was determined with LDH assay kits.

**Western Blot Analysis**

Following exposure to hypoxia, cells were harvested and prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting. The phosphorylation states of Akt and GSK-3β were evaluated by immunoblotting whole cell lysates with antibodies that specifically recognize phospho-Ser⁴⁷³ Akt (1:1000), and phospho-Ser⁹⁸ GSK-3α/β (1:1000), all from Cell Signaling Technology Inc., USA. Antibodies that equally recognize the phospho- and dephospho-states of these proteins were also obtained from Cell Signaling Technology. Immunoreactivity was detected by electrochemical luminescence (Amersham, USA) and quantified using densitometric analysis with an ImagePro digital analysis system.

**MPTP and Mitochondrial Membrane Potential**

To monitor MPTP, cells (5 × 10⁵ cells/well) plated in a 24-well primaria culture dish were incubated for 30 min according to the operational guidelines. Then they were visualized with an Olympus AX-70