Panax Quinquefolium Saponin Attenuates Cardiomyocyte Apoptosis and Opening of the Mitochondrial Permeability Transition Pore in a Rat Model of Ischemia/Reperfusion

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Key Words
Panax quinquefolium saponin • Ischemia/reperfusion • Cardiomyocyte • Apoptosis • Mitochondrial permeability transition pore

Abstract
Aims: Opening of the mitochondrial permeability transition pore (mPTP) is a critical event during ischemia/reperfusion (I/R) injury. Recently, we showed that Panax quinquefolium saponin (PQS) alleviates apoptosis of cardiomyocytes by suppressing excessive endoplasmic reticulum stress (ERS) during I/R injury. Here, we hypothesized that this anti-apoptotic effect might be mediated through inhibition of mPTP and the mitochondrial apoptotic pathway. Methods: Ninety-six healthy male Sprague-Dawley rats were randomly divided into sham, I/R, I/R+PQS (200 mg/kg/d), Cyclosporine A (CsA, 10 mg/kg), I/R+CsA (10 mg/kg), and I/R+PQS+CsA. I/R was modeled in rats by ligating the left anterior descending artery (LAD) for 30 min followed by 120 min of reperfusion. To evaluate the cardioprotective function of PQS, we measured hemodynamics, serum content of creatine kinase-MB (CK-MB), myocardial infarct size, and myocardial apoptotic index (AI). We investigated the underlying mechanism by examining changes in the mitochondrial ultrastructure and membrane potential (ΔΨm), dynamics of mPTP opening, expression of cleaved caspase-3, cleaved caspase-9 in the myocardium, Bcl-2 and Bax in the mitochondria versus cytosol, and translocation of cytochrome c. Results: Administration of PQS to I/R rats significantly reduced serum CK-MB level, infarct size and AI. In addition, PQS protected the mitochondrial structure, markedly inhibited mPTP opening and ΔΨm depolarization, led to upregulation of Bcl-2 and downregulation of Bax in the mitochondria versus cytosol, and translocation of cytochrome c. Conclusion: Our results show that PQS can alleviate apoptosis of cardiomyocytes during I/R injury, possibly due to repressed mitochondrial apoptotic pathway associated with the opening of mPTP induced by myocardial I/R injury.
Introduction

Apoptosis is a highly regulated mode of cell death, known to play an essential role in the pathogenesis and prognosis of different cardiac diseases, including chronic heart failure and ischemia [1]. In ischemia/reperfusion (I/R) injury cardiomyocytes undergo apoptosis, a process that can be initiated by multiple factors, including oxidative stress and Ca\(^{2+}\) overload [2]. Enhanced cell death results in myocardial contractile dysfunction, compensatory hypertrophy and reparative fibrosis, all of which contribute to myocardial injury [3]. Therefore, elucidating the mechanisms underlying apoptosis of cardiomyocytes, and developing therapeutic strategies to reduce this effect following I/R injury, is crucial to the treatment of cardiac disorders [4].

Apoptosis can be induced by several signal transduction pathways, including at least three that were recently shown to play a significant role: mitochondrial pathway, death receptor pathway, and endoplasmic reticulum pathway [5]. The mitochondria, which are central to apoptotic signaling, are also involved in the other two pathways, and contribute to apoptotic modulation. There is increasing evidence that opening of the mitochondrial permeability transition pore (mPTP) plays a key role in triggering apoptosis of cardiomyocytes following I/R [6]. mPTP is a large, nonselective conductance pore located in the inner mitochondrial membrane [7]. Opening of mPTP is induced, during the early stages of reperfusion, by Ca\(^{2+}\) overload, a massive production of reactive oxygen species (ROS) and abrupt restoration of pH. This in turn causes the dissipation of the mitochondrial membrane potential (ΔΨm), uncoupling of oxidative phosphorylation, inhibition of ATP production, and mitochondrial swelling [8]. In addition, mPTP opening leads to the release of pro-apoptotic proteins including cytochrome c (Cyt-C) from the mitochondria into the cytoplasm. The released Cyt-C in turn activates the mitochondrial apoptotic pathway and aggravates myocardial injury [9]. Hence, inhibition of mPTP opening and the subsequent activation of the mitochondrial apoptotic pathway is a promising therapeutic strategy to protect the heart from I/R injury [10].

Panax quinquefolium saponin (PQS) is extracted from the stems and leaves of Radix panacisquinquefolii (American ginseng). Previous studies have shown that PQS can increase ATP content and energy charge (EC), upregulate the expression of the anti-apoptotic protein Bcl-2, and downregulate Fas expression in the myocardium of AMI rats. These studies, however, did not explore its association with the regulation of mPTP opening [11, 12]. In addition, in the cerebral cortex of ischemic rats, PQS was shown to upregulate the anti-apoptotic protein Bcl-2, and reduce the levels of cleaved caspase-9 and cleaved caspase-3 [13, 14]. Although these studies demonstrated the anti-apoptotic effects of PQS, the underlying mechanism was not explored. Recently, our group showed that PQS can reduce the extent of myocardial I/R injury and apoptosis of cardiomyocytes both in vivo as well as in vitro, which mechanistically, is associated with a reduction in the level of endoplasmic reticulum stress (ERS) [15, 16]. Furthermore, we demonstrated that PQS can also alleviate cardiac ventricular remodeling following myocardial infarction by suppressing ERS-related apoptosis [17]. However, whether the anti-apoptotic property of PQS is related to the regulation of mPTP opening and inhibition of the mitochondrial apoptotic pathway during I/R, remains unclear.

In the current study, we hypothesized that PQS induces cardioprotection by inhibiting the opening of mPTP and consequently, the mitochondrial apoptotic pathway in I/R injured myocardium. To address this question, we investigated the effect of PQS on mPTP and the expression of pro- and anti-apoptotic factors in the myocardium in rat models of I/R injury.

Materials and Methods

Materials

Panax quinquefolium saponin was provided by Yisheng Pharmaceutical Co, Ltd, (Jilin, China). Cyclosporine A (CsA) was purchased from Novartis (Basel, Switzerland); DeadEnd™ Fluorometric TUNEL
System was purchased from Promega (Madison, CA, USA); antibodies against Bcl-2, Bax, caspase-9, cytochrome c, GAPDH and COX IV were purchased from Cell Signaling Technology (Boston, MA, USA); antibodies against caspase-3 was purchased from Merck Millipore (Darmstadt, Germany); All chemicals, including Evans blue, triphenyltetrazolium chloride (TTC) and pentobarbital sodium were purchased from Sigma-Aldrich Corporation (St. Louis, Mo, USA).

Animals

Adult male Sprague-Dawley rats weighing 150-160 g were purchased from the animal experimental center of the General Hospital of People’s Liberation Army (Beijing, China). All rats were housed (five animals per cage) at 23°C with 12h light/dark luminosity cycles, and were allowed free access to food and water. All experiments complied with the Animal Management Rule of the Ministry of Health, People’s Republic of China (Document 55, 2001).

Induction of myocardial I/R injury

Rats were anesthetized with pentobarbital sodium (2.3 ml/kg, administered intraperitoneally (i.p.)) and fixed on the operating table. The electrocardiogram (ECG) was recorded using MFL Lab 200 (Fudan University, Shanghai, China). Artificial ventilation was provided using a rodent ventilator (DW-2000, Jiapeng Inc., Shanghai, China). Under these conditions, the hearts were exposed through a left thoracotomy in the third or fourth intercostal space. The pericardium was opened, and a 6-0 prolene suture was tied around the proximal left anterior descending (LAD) coronary artery. The LAD was occluded for 30 min followed by 120 min of reperfusion that was carried out by releasing the tie, as previously described [18]. Ischemia was confirmed based on a significant ST-segment elevation in lead II of ECG following ligation of the LAD. Before occlusion, rats were allowed 15 min for hemodynamic stabilization.

Experimental design

Ninety-six healthy male Sprague-Dawley rats were used in this study. All rats allowed free access to food and water for four weeks and were randomly assigned to the following six groups (n=16 per group): 1. Sham group- rats subjected to the same surgical procedure as the experimental groups but without occlusion; 2. I/R group- rats subjected to 30 min occlusion followed by 120 min reperfusion; 3. I/R+PQS group- rats gavaged for four weeks with PQS solution in water (200 mg/kg/d, as determined from our previous study [15]), then subjected to the same surgical procedure as the I/R group; 4. I/R+CsA group- rats subjected to I/R, followed by infusion of CsA (10 mg/kg) i.p. 10 min before reperfusion; 5. I/R+PQS+CsA group- rats gavaged with PQS, subjected to I/R surgery, followed by i.p. infusion of CsA 10 min before reperfusion; 6. CsA group- rats subjected to CsA infusion, but without occlusion.

Immediately following reperfusion, all rats were intubated in order to carry out hemodynamic measurements. Blood samples were collected to measure the serum concentration of CK-MB. The left ventricles (LV) from six rats in each group were isolated to determine the size of the infarction. The rest were randomly divided into assays for apoptosis (TUNEL), mitochondrial experiments and western blot.

Hemodynamic measurements

After reperfusion, a polyethylene tube (PE 50, Becton-Dickinson) filled with heparinized saline was inserted into the left ventricular cavity via the right carotid artery. Mean arterial pressure (MAP), left ventricular systolic pressure (LVSP), left ventricular end diastolic pressure (LVEDP), ±dp/dt max were measured using a polygraph system (AP601G, Nihon Koden). In addition, left ventricular developed pressure (LVPD) was calculated as the difference between LVSP and LVEDP, and rate-pressure product (RPP) was estimated as the product of MAP and heart rate (HR).

Measurement of serum level of CK-MB

The serum fraction was separated from blood samples (approx. 10 mL) collected from the right common carotid artery, and the concentration of CK-MB was determined using an automatic biochemical analyzer (Cobas8000, Roche).

Measurement of infarct size

The myocardial infarct size was measured according to a previously established method [19]. Immediately following reperfusion, the LAD was ligated completely and 4 mL of 1% Evans blue dye was injected retrogradely into the aorta to delineate the region of myocardial perfusion. The hearts were rapidly
excised and cooled in saline at -20°C for 15 min. The atria were removed, and the ventricles were sliced transversely into five cross-sectional slices with uniform thickness. The viable myocardium in these slices was then stained at 37°C for 30 min with 1% 2,3,5-triphenyltetrazoliumchloride (TTC). The area of the white zone (unstained by Evans blue and TTC) was determined as the infarct size (IS), while the area unstained by Evans blue was estimated to be AAR. Infarct size was determined as the percentage of unstained LV in the slices, IS/AAR, as described previously. The extent of ischemic myocardium was calculated as the ratio AAR/LV [20].

Assessment of apoptosis

To measure the level of apoptosis, the hearts were first excised and washed in saline solution. Myocardial tissues from risk area were transected (approximately 2 mm thick) and fixed in 4% formaldehyde for 1h, processed through an ethanol gradient, embedded in paraffin wax and sectioned into 3μm thick slices. Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay was performed using an In Situ Cell Death Detection Kit, according to the manufacturer’s protocol. Stained sections were observed in an Olympus laser confocal microscope. At least 10 randomly chosen fields in the non-infarcted myocardium, containing approx. 100 cells, were scored. The level of apoptotic cell death is expressed as the number of TUNEL-positive cells over the total number of cells.

Examination of mitochondrial structure by Transmission electron microscopy (TEM)

Following reperfusion, hearts were harvested; the apical tissue of LV was isolated and sliced into 1 mm³ pieces, and fixed in 3% glutaraldehyde and 1% osmium tetroxide for 2h. The tissues were then dehydrated in acetone, embedded in resin and sectioned into 60 nm thick slices. These ultrathin LV sections were placed on uncoated 200-mesh copper grids, stained with 4% uranyl acetate and 0.2% lead citrate in 0.1 N NaOH, and examined in a Hitachi 7650 TEM (Hitachi, Tokyo, Japan) [21]. The mitochondria were imaged at 30,000×.

Isolation of mitochondria

Mitochondrial fractions were isolated by differential centrifugation, as described previously [22]. All experiments were carried out in cold conditions. Briefly, the LV tissue (150 mg) was first suspended in isolation buffer (70 mM sucrose, 210 mM mannitol, and 1 mM EGTA in Tris HCl, pH=7.4), finely minced with scissors and homogenized (10 mL/g). The homogenates were centrifuged at 1000 g for 5 min; the supernatant was centrifuged again at 10,000 g for 10 min. The supernatant from this centrifugation corresponded to the cytosolic fraction, and was collected and immediately frozen at -80°C until ready for protein estimation and analysis of western blot. The precipitate (substrate) was resuspended in ice-cold isolation buffer and centrifuged at 10,000 g for 5 min. The mitochondrial pellet obtained as a result, was suspended in 2 ml isolation buffer. The concentration of mitochondrial proteins was measured using the bicinchonic acid (BCA) assay [23].

Measurement of CaCl2-induced mPTP opening

The opening of mPTP was assessed using a method described previously [24]. Briefly, 20 μl isolated mitochondria (200 μg protein) was resuspended in 170 μl buffer (150 mM sucrose, 50 mM KCl, 2 mM KH₂PO₄, 5 mM succinic acid, and 20 mM Tris-HCl, pH 7.4), then preincubated for 1 min in 96-well plates at 37°C. CaCl₂ pulses (10 nM) were applied to these samples at the start of the experiment. The initial absorbance of mitochondria at 540 nm (Initial A540) was recorded using a microplate reader (TECAN Infinite F200 Pro, Switzerland). Subsequently, A540 was measured every 60 s until the value did not change any further. The absorbance ratio (A540/Initial A540) was calculated. A reduction in this ratio was indicative of increased opening of mPTP.

Measurement of ΔΨm

The dye 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1) was used to determine the change in mitochondrial membrane potential (ΔΨm) [25]. JC-1 is a cationic carbocyanine dye that remains within the mitochondrial matrix in a monomeric form. When the ΔΨm in the mitochondria is high, JC-1 monomers aggregate, resulting in red fluorescence. To measure ΔΨm the isolated mitochondria (0.4 mg/mL) were incubated in 310 nM JC-1 at 37°C for 30 min [26]. Fluorescence intensity was measured
on a spectrofluorometer (Hitachi F-4010, Hitachi Ltd, Tokyo, Japan). To measure the red fluorescence of JC-1 aggregates, the dye was excited at 485 nm and, the emission was detected at 590 nm. The decrease in ΔΨm was reflected by a decline in relative fluorescence units (RFU) of the red fluorescence [27]. In addition, 50μl of this reaction was transferred onto a glass slide, and observed under a laser confocal microscope (FV1000, OLYMPUS). The same excitation and emission wavelengths were used. The reduction in the intensity of red fluorescence indicated depolarization of the mitochondrial membrane.

Western blot analysis
Mitochondrial and cytosolic protein samples, obtained by methods described above, were used for this assay. Total cellular protein was extracted from the left ventricular posterior wall (AAR), as described previously [28]. Protein concentration was estimated using the BCA method, and separated on 10% sodium dodecyl sulfate- polyacrylamide gels (80μg/lane). After electrophoresis, the protein bands were electrophoretically transferred to nitrocellulose membranes, and blocked with 5% bovine serum albumin (BSA) in Tris-buffered saline containing 0.1% Tween 20, at room temperature for 1 h. The membranes were then incubated overnight at 4°C in the following primary antibodies against Bcl-2, Bax, caspase-9, cytochrome c, GAPDH and COX IV (all 1:1000), and antibodies against caspase-3 (1:100). The antibody-tagged membranes were then probed with corresponding secondary antibodies. An enhanced chemiluminescent detection system was used to detect the separated immunolabeled protein bands. The optical density of the bands (measured in arbitrary densitometry units) was determined using Image-Pro Plus (Roper Industries, New York, USA). The densitometric value of target proteins was normalized against GADPH or COX IV.

Statistics
All data are expressed as Mean ± standard error of mean (SEM). A value of $P<0.05$ was considered statistically significant. For multiple-group comparisons, we performed one-way analysis of variance (ANOVA) followed by Newman-Keuls post hoc analysis. To compare proportions, we used the Chi-square test. Statistical analyses were performed on the SPSS 17.0 software (SPSS Inc., Chicago, IL, USA).

Results
PQS improves cardiac function after I/R injury
We first examined the effects of PQS treatment on the cardiovascular physiology of rats subjected to I/R injury, by measuring various hemodynamic parameters. The results obtained from surviving rats in each control and experimental group after 120 min of reperfusion, are shown in Table 1. We found no significant change in heart rate (HR) between the different groups ($P>0.05$). As expected, the rats in the I/R group displayed a marked increase in LVEDP, decrease in MAP, RPP, LVSP, LVDP and ±dp/dtmax in comparison to the sham group ($P<0.05$). Interestingly, pretreatment with PQS significantly increased MAP, RPP, LVSP, LVDP and ±dp/dtmax, and decreased LVEDP in comparison to the I/R group ($P<0.05$). Similar changes were also observed in the I/R+PQS+CsA group of animals, with no significant difference between the latter and the I/R+PQS group ($P>0.05$). Moreover, I/R rats treated with CsA alone did not exhibit a significantly better recovery in cardiac function in comparison to the I/R group ($P>0.05$) (Table 1). These results suggest that pretreatment with PQS could increase cardiac function after I/R injury.

Table 1. Effect of PQS on hemodynamics in I/R rats. Data are expressed as mean ± SEM. n=10. *$P<0.05$ vs sham; +$P<0.05$ vs I/R; $P<0.05$ vs I/R+PQS
PQS treatment reduces serum CK-MB level after I/R injury

To further evaluate and validate the protective function of PQS during I/R injury, we measured the levels of CK-MB in the serum at 120 min after reperfusion. We found that while I/R rats exhibited a small but significant increase in CK-MB level (1.1-fold) than the sham group (P<0.05), this up-regulation was significantly diminished in I/R+PQS, I/R+CsA and I/R+PQS+CsA groups by 45.3%, 42.8% and 46.6%, respectively (P<0.05). Interestingly, the combination of PQS and CsA did not have an additive effect on the CK-MB level, when compared with rats treated with PQS alone (P>0.05) (Fig. 1A). These findings suggest that pretreatment with PQS could decrease cardiac injury in I/R rats.

PQS decreases myocardial infarct size after I/R injury

Next, we determined whether PQS treatment could alleviate the severity of myocardial infarction, and measure the ratio of AAR to LV (%AAR/LV). We found no significant difference...
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in this ratio between the different groups (sham: 43.4 ± 1.1%, I/R: 47.1 ± 1.3%, I/R+PQS: 42.2 ± 1.7%, CsA: 43.1 ± 2.0%, I/R+CsA: 45.5 ± 1.0%, I/R+PQS+CsA: 43.6 ± 1.3%). This suggests that all control and experimental rats were exposed to a comparable degree of ischemic risk (P>0.05). However, the ratio of the infarct size to AAR (%IS/AAR) was significantly reduced in I/R rats treated with PQS and/or CsA (I/R: 42.3 ± 1.6%, I/R+PQS: 21.1 ± 1.1%, I/R+CsA: 26.1 ± 2.5%, and I/R+PQS+CsA: 25.6 ± 1.7%) (P<0.05 versus I/R). Both PQS as well as CsA treatment decreased the infarct size significantly compared with the I/R group (P>0.05). Interestingly, however, the combination of PQS and CsA did not have a greater effect on the infarct size when compared to I/R rats treated with PQS alone (P>0.05) (Fig. 1B). These results indicate that pretreatment with PQS could reduce the size of myocardial infarct in I/R rats.

PQS reduces the apoptotic index (AI) of cardiomyocytes after I/R injury

To determine the cellular mechanism underlying the beneficial effects of PQS on ischemic injury and cardiac function, we analyzed the extent of apoptosis in cardiomyocytes using the TUNEL assay. The apoptotic index (AI) of cardiomyocytes in the non-infarcted myocardium was 9.8 ± 2.3% in sham group and 13.9 ± 3.4% in CsA group. This value was significantly increased in the I/R group after 120 min reperfusion (55.8 ± 6.3%), in comparison to the sham group (P<0.05). Treatment with both PQS as well as CsA resulted in a significant decrease in the AI of cardiomyocytes (I/R+PQS: 29.8 ± 3.5%, I/R+CsA: 34.1 ± 2.2%), compared to the I/R group (P<0.05). Consistent with the results from previous experiments, we found no apparent difference between the AI values of the I/R+PQS+CsA and I/R+PQS groups (P>0.05) (Fig. 2). These findings indicate that pretreatment with PQS could protect the myocardium against I/R induced apoptosis.

PQS treatment can protect mitochondrial structure after I/R injury

Using the transmission electron microscope (TEM), we found that there is significant damage to the ultrastructure of the mitochondria in response to I/R injury. Specifically, some mitochondria suffered high-amplitude swelling, the matrix became clear accompanied by
a loss of dense granules, the inner membrane became irregularly arrayed, and part of the outer membrane was disrupted. Some mitochondria in the I/R rats were characterized by sparse cristae and cavitation. Pretreatment with PQS decreased mitochondrial damage. We identified a significant decrease in vacuolar degeneration in the mitochondria, accompanied by a lack of swelling, in comparison to the I/R group. The ultrastructure of the mitochondria was similar in the I/R+PQS, I/R+CsA and I/R+PQS+CsA groups. Moreover, apart from a slight decrease in the scarcity of cristae, we did not observe a significant difference in the mitochondrial structure between the CsA and sham groups (Fig. 3A). Our data suggests that pretreatment with PQS can protect the mitochondrial structure following I/R injury.

**PQS treatment inhibits mPTP opening during reperfusion**

Alterations in mitochondrial permeability have been shown to be an important mechanism mediating apoptotic cell death in many cellular systems. Much research has focused on targeting the opening of mPTP upon reperfusion to reduce subsequent apoptosis in cardiomyocytes. In Fig. 3B, we show a representative recording of mitochondrial absorbance during exposure and increasing permeability to CaCl$_2$. In comparison to the sham
group (0.96 ± 0.01), the mitochondria in I/R rats displayed a rapid and significant decrease in the A540/Initial A540 ratio (0.73 ± 0.03), implying increased mPTP opening (P<0.05). In contrast, this ratio declined gradually in the I/R+PQS group (0.92 ± 0.01) after exposure to CaCl₂ when compared to the I/R group (P<0.05). We found no apparent difference in CaCl₂ permeability between I/R+PQS and I/R+CsA (0.94 ± 0.02) groups (P>0.05). These results suggest that pretreatment with PQS can decrease the sensitivity of mPTP to calcium, and inhibit the opening of mPTP to a level comparable to the CsA-treated I/R rats. Finally, the I/R+PQS+CsA group (0.93 ± 0.02) did not display an effect greater than the I/R+PQS group (P>0.05), implying the lack of a synergistic function between PQS and CsA (Fig. 3B).

PQS treatment prevents the loss of mitochondrial membrane potential during reperfusion
To further determine the effect of PQS on mitochondrial function, we next determined changes in the mitochondrial membrane potential. We measured ΔΨm in the different control and experimental groups using JC-1 staining, and observed the cells under a fluorescence microscope (Fig. 3C). The sham group showed bright and punctate fluorescent JC-1-positive aggregates within the mitochondria, representing normal hyperpolarized ΔΨm. In contrast, the mitochondria in the I/R group displayed a decrease in the intensity of JC-1 fluorescence, possibly due to a reduction in the formation of JC-1 aggregates and consequently, the diffusion of JC-1 monomers into the cytoplasm. This would be a consequence of ΔΨm depolarization. Both PQS and CsA treatment groups maintained the fluorescence observed in the sham group, in contrast to the I/R rats. The dissipation of ΔΨm was further confirmed on a spectrofluorometer, where we recorded the decrease in RFU in the mitochondria (Fig. 3D). Consistent with results from the JC-1 staining, the RFU in the I/R group (8.9 ± 2.2) was significantly reduced in comparison to the sham group (23.2 ± 4.0), indicating the dissipation of ΔΨm in response to I/R injury (P<0.05). As expected, a significant increase in RFU was observed following treatment with PQS (14.7 ± 3.3) and CsA (16.9 ± 0.9) in comparison to the I/R group (P<0.05). Consistent with previous findings, we detected no significant difference in RFU between the I/R+PQS+CsA and I/R+PQS groups (P>0.05). Altogether, our findings suggest that pretreatment with PQS could alleviate the loss of ΔΨm in I/R rats.

PQS rescues the downregulation of Bcl-2 and upregulation of Bax in mitochondria versus cytosol after I/R injury
In order to elucidate the molecular mechanism underlying the reduction in apoptosis in response to PQS treatment, we evaluated the expression level of Bcl-2 and Bax in cytosolic and mitochondrial fractions, by Western blot. We found that while Bcl-2 is localized to both the mitochondrial and the cytoplasm, Bax is present mainly in the cytosolic fraction, in both sham and CsA groups. In rats subjected to I/R injury, the mitochondrial to cytosolic ratio of Bcl-2 and Bax was reduced by 67.3% and increased by a factor of 8.7, respectively, in comparison to the sham group (P<0.05). Interestingly, pretreatment with PQS led to an increase in this ratio by a factor of 1.3 for Bcl-2, and decreased this ratio by 35.7% for Bax, when compared to the I/R group (P<0.05). We did not find a significant difference between the PQS- pretreated and CsA- treated groups (P>0.05). Also, the combined treatment of PQS and CsA did not cause a significantly greater alteration in their expression in comparison with I/R+PQS (P>0.05) (Fig. 4A, 4B and 4C). These findings indicate that PQS might block apoptosis through a mitochondrial pathway mediated by the relative ratio of expression of Bcl-2 and Bax in the mitochondria.

PQS rescues elevated cleaved caspase-9/caspase-3 expression in the myocardium and cytosolic translocation of cytochrome c
As shown in Fig. 4, in the I/R group, the levels of cleaved caspase-9 and cleaved caspase-3 were significantly increased by a factor of 2.1 and 2.6, when compared with sham group (P<0.05). Pretreatment with PQS effectively decreased the levels of cleaved caspase-9 and cleaved caspase-3 by 27.0% and 32.5%, respectively, in comparison to the I/R group (P<0.05). Furthermore, we show that while cytochrome c is present predominantly in the
mitochondrial fraction in the sham and CsA groups, after I/R injury, the ratio of mitochondrial to cytosolic cytochrome c was reduced by 86.6% compared with sham group (P<0.05). More importantly, pretreatment with PQS led to an increase in this ratio by a factor of 1.9, when compared to the I/R group (P<0.05). Finally, we found no significant difference in these results between the I/R+CsA and I/R+PQS groups, or between the I/R+PQS+CsA and I/R+PQS groups (P>0.05) (Fig. 4A, 4D, 4E and 4F). Our findings suggest that pretreatment with PQS might protect the myocardium against I/R injury through a mitochondrial signaling pathway.

Discussion

In our study, we chose to explore the relationship between PQS and mPTP in order to elucidate the mechanism underlying the cardioprotective function of PQS. Our findings demonstrate, for the first time, that (a) Pretreatment with PQS can protect the heart against I/R injury in rats, mitigate the apoptosis of cardiomyocytes, and inhibit the opening of mPTP and expression of mitochondria-related pro-apoptotic proteins; (b) The positive effects of PQS are similar to that of CsA, a potent inhibitor of mPTP. These findings suggest that the anti-apoptotic function of PQS is likely to be partially mediated through inhibition of the opening of mPTP and the activation of the mitochondrial apoptotic pathway during I/R injury.

Apoptosis in cardiomyocytes in response to I/R was first reported by Gottlieb et al [29]. A large body of experimental evidence suggests that apoptosis plays a critical role in cardiac injury during myocardial I/R [30]. Furthermore, reperfusion was shown to accelerate apoptotic cell death in cardiomyocytes [31]. In I/R injured myocardium, while very few apoptotic cells were found in the non-infarcted area, apoptotic myocytes were mostly localized to the border regions surrounding the infarct [32]. Therefore, in our study, we used the myocardial AAR in TUNEL assays to determine the extent of apoptosis of cardiomyocytes in the different experimental groups. Our data shows that apoptosis is dramatically increased in the myocardium in response to I/R injury, which is partially but significantly rescued on administration of PQS. This is consistent with findings from our previous study [17].

There is increasing evidence that reperfusion is also responsible for inducing additional mitochondrial dysfunction. Under physiological conditions, the inner membrane of the mitochondria (IMM) is impenetrable to almost all metabolites and ions, and mPTP is found to exist in a closed conformation [33]. During the early stages of reperfusion, Ca\textsuperscript{2+} overload, excessive production of reactive oxygen species (ROS) and the abrupt restoration of pH can trigger the opening of mPTP [34]. This results in dissipation of the ΔΨm, uncoupling of the respiratory chain, inhibition of ATP production and mitochondrial swelling and rupture [35]. Loss of ΔΨm can also subsequently induce the opening of additional mPTP resulting in further dissipation of ΔΨm, which would facilitate persistent opening of mPTP [36]. Hence, ΔΨm is an important parameter regulating mitochondrial function, and is used as an indicator of the status of mPTP [37]. In this study, we show that the opening of mPTP, ΔΨm depolarization and the consequent damage to the mitochondrial structure, induced by I/R injury, can be significantly alleviated by pretreatment with PQS. These results suggest that PQS exerts a protective effect on the mitochondria by inhibiting mPTP opening and maintain the integrity of mitochondrial structure during I/R.

Some studies have implicated the opening of mPTP during reperfusion in cell death, including necrosis and apoptosis [38]. If mPTP remains open, it can lead to the release of the pro-apoptotic protein cytochrome c from the mitochondrial matrix to the cytoplasm, where it interacts with the apoptosis protease activating factor-1 (APAF-1) and causes activation of caspase-9, which in turn activates caspase-3 [39, 40]. Caspase-3 is fast emerging as the central ‘executioner’ or ‘downstream’ caspase, whose activated form, cleaved caspase-3, has been shown to play a crucial role in apoptotic signaling, and is responsible for promoting cell death [41]. Here, we demonstrate that pretreatment with PQS can effectively down-regulate
the level of cleaved caspase-9, cleaved caspase-3 in the myocardium and translocation of cytochrome c from mitochondria to cytosol, suggesting that the protective function of PQS in the myocardium is likely to be mediated through decreased activity of mitochondria-related apoptotic pathways. Others have suggested that mPTP initiates cell death through necrosis rather than apoptosis [42]. Although in our study we did not investigate the occurrence or the extent of necrosis and associated causes in the heart, we cannot rule out the possibility that the myocardial protection offered by PQS is mediated through multiple mechanisms, in addition to mPTP opening-associated reduction in apoptosis. The potential relationship between necrosis and mPTP opening induced by I/R after PQS treatment will be explored in future studies.

The Bcl-2 family of proteins, comprised of both pro-apoptotic and anti-apoptotic members, constitutes a critical intracellular checkpoint for apoptosis within a common cell death pathway [43]. It includes proteins that predispose cells to apoptosis, such as Bax, Bad and Bak, as well as proteins such as Bcl-2 and Bcl-X, that antagonize apoptotic signaling [44]. Researchers have shown that the anti-apoptotic gene Bcl-2 and the pro-apoptotic gene Bax are accurate indicators of the level of apoptosis during myocardial I/R injury [45]. In addition, these two factors are also the critical regulators of mPTP opening and the mitochondrial apoptotic pathway [46, 47]. While Bax is mostly localized to the cytosol, it can clearly translocate to the mitochondrial outer membrane (OMM) where it could interact with VDAC (one of the components of OMM) to induce opening of mPTP [48]. On the other hand, the anti-apoptotic protein Bcl-2 is normally found in the cytoplasm in the form of a complex with Bad. The dissociation of Bcl-2 from this complex followed by translocation to the mitochondria might lead to phosphorylation of Bad in I/R injury [49]. In this study, we found that pretreatment with PQS caused an increase in Bcl-2 levels and a decrease in Bax levels in the mitochondria relative to the cytosol, after I/R injury. Although previous studies have shown an upregulation of Bcl-2 and downregulation of Bax in the myocardium of PQS-treated rats during I/R injury [12, 15, 17], our work was focused specifically on the effect of these two proteins on the dynamics of mPTP. We have demonstrated, for the first time, the independent expression levels of Bcl-2 and Bax in mitochondria and cytosol in PQS-treated I/R rats. Our results suggest that the protective effect of PQS mediated by inhibition of mPTP opening might in turn lead to the inhibition of mitochondria-mediated apoptotic signaling.

CsA is one of the most potent inhibitors of mPTP. It exerts its action by directly preventing calcium-induced interaction of cyclophilin D (CyP-D), a crucial component of mPTP [50]. There is increasing evidence that CsA is a cardioprotective agent that can rescue cardiomyocytes from reperfusion injury both in vivo as well as in vitro [51-53]. Recently, its safety and efficacy were tested in small scale clinical trials [54]. CsA is also frequently used as an immunosuppressant to prevent acute rejection of allografts. However, its long-term use has several potentially detrimental side-effects, including high blood pressure, potassium retention, renal and hepatic toxicity, and increased vulnerability to infections and cancer [55]. Because of these undesirable side-effects of CsA, it is imperative to find a safe but effective pharmacological agent that can inhibit mPTP function and offer cardioprotection. PQS, an extract of Panax quinquefolium and a popular nutritional supplement, has been widely used for many years with no known detrimental side-effects. In the present study, we have demonstrated that PQS is as potent as CsA in inhibiting the opening of mPTP and the subsequent activation of the mitochondrial apoptotic pathway. Notably, we show that a combined treatment with both PQS and CsA has no apparent additive therapeutic effect on myocardial I/R injury. Therefore, we propose that PQS might have a similar mechanism of action as CsA in exerting its cardioprotective effect during I/R injury. Additional research is needed to determine whether PQS interacts with CyP-D to inhibit mPTP opening. Furthermore, with the exception of CyP-D, CsA is likely to bind to other cyclophilins like CyP-A. This latter event can lead to the inhibition of the Ca$^{2+}$-sensitive protein phosphatase calcineurin (CaN), which can in turn directly influence cardiac function [56]. Based on the above mechanism, we could speculate that the inability of CsA to improve on cardiac function recovery after I/R in our study, might be due to CsA-mediated inhibition of CaN. Our results
are consistent with previous reports where CsA pretreatment was shown to fail to improve cardiac function after I/R, despite decreased infarct size [57]. These results strongly suggest that PQS could be a safe and effective drug, and a suitable alternative to CsA, for inducing cardioprotection during I/R injury.

Although this report is the first to demonstrate the effect of PQS on mPTP function, our study has certain limitations. First, it is not clear whether PQS-mediated cardioprotective effects can be totally abrogated by injecting an mPTP agonist. Second, little is known about the precise site of action of PQS in the mPTP structure. Therefore, further studies are required to elucidate the mechanism underlying of the effect of PQS on mPTP.

In conclusion, the present study demonstrates for the first time that the myocardial protective effect of PQS during I/R injury is partially mediated by inhibiting the opening of mPTP, as evidenced by the $\Delta \Psi_m$ depolarization and mitochondrial swelling, a reduction in I/R-induced upregulation of Bax in mitochondria, translocation of cytochrome c from mitochondria to the cytosol, cleaved caspase-9 and cleaved caspase-3 expression in the myocardium, and by the elevated levels of Bcl-2 in the mitochondria. These effects lead to a reduction in mitochondria-mediated apoptosis of cardiomyocytes. Our findings suggest a novel mechanism of the cardioprotective action of PQS against I/R injury, and reveal a potential pharmacological agent for use in clinical therapy.

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