Sasanquasaponin promotes cellular chloride efflux and elicits cardioprotection via the PKCε pathway

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Received March 17, 2015; Accepted February 6, 2016

DOI: 10.3892/mmr.2016.4984

Abstract. Sasanquasaponin (SQS) is an active component of Camellia oleifera Abel. A recent study by our group demonstrated that SQS was able to inhibit ischemia/reperfusion-induced elevation of the intracellular chloride ion concentration ([Cl\textsuperscript{-}]) and exerted cardioprotective effects; however, the underlying intracellular signal transduction mechanisms have yet to be elucidated. As protein kinase C ε (PKCε) is able to mediate Cl\textsuperscript{-} homeostasis, the present study investigated its possible involvement in the effects of SQS on cardiomyocytes subjected to ischemia/reperfusion injury. Cardiomyocytes were pre-treated with or without SQS or SQS plus εV1-2, a selective PKCε inhibitor, followed by simulated ischemia/reperfusion (sI/R). The effects on cell viability, PKCε phosphorylation levels, [Cl\textsuperscript{-}], mitochondrial membrane potential and reactive oxygen species (ROS) production were assessed using an MTS assay, western blot analysis, colorimetric assays and flow cytometry. The results revealed that treatment with SQS prior to sI/R increased the viability of cardiomyocytes, and efficiently attenuated lactate dehydrogenase and creatine phosphokinase release induced by sI/R. In addition, SQS promoted PKCε phosphorylation and inhibited sI/R-induced elevation of [Cl\textsuperscript{-}], paralleled by the attenuation of mitochondrial membrane potential loss and ROS generation. However, when the cardiomyocytes were treated with εV1-2 prior to SQS pre-conditioning, the cardioprotection induced by SQS was reduced and the inhibitory effects of SQS on sI/R-induced elevation of [Cl\textsuperscript{-}], production of ROS and loss of mitochondrial membrane potential were also attenuated. These findings indicated that SQS may inhibit sI/R-induced elevation of [Cl\textsuperscript{-}] through the PKCε signaling pathway to elicit cardioprotection in cultured cardiomyocytes.

Introduction

Myocardial ischemia/reperfusion (I/R) injury is the most common cause of cardiac morbidity and mortality and can lead to arrhythmia, heart hypofunction, cardiocyte apoptosis and other disorders (1). Therefore, identification of therapeutic approaches and molecular mechanisms responsible for I/R injury are important for the improvement of ischemic heart diseases associated with I/R.

It has been suggested that elevation of the intracellular chloride ion concentration ([Cl\textsuperscript{-}]) is an important pathophysiological factor of I/R injury. Increased [Cl\textsuperscript{-}], can activate Cl\textsuperscript{-} - OH exchange activity (2,3), thereby increasing the intracellular concentration of OH\textsuperscript{-}, which is an important member of the reactive oxygen species (ROS) family. Alternatively, the increased [Cl\textsuperscript{-}], may induce the opening of the mitochondrial permeability transition pore (mPTP), which results in a ROS burst and subsequent ROS-dependent apoptosis (4,5). Therefore, the inhibition of the elevation of [Cl\textsuperscript{-}], induced by I/R has been considered to be a reasonable therapeutic strategy to alleviate I/R injury.

Sasanquasaponin (SQS) is an active component of Camellia oleifera Abel, which is used in Traditional Chinese Medicine. SQS is a triterpenoid with structural similarity to certain ginseng saponins (6,7), and is known to exert potent cardioprotective effects against I/R injury (8). A previous study by our group indicated that these beneficial effects of SQS may be attributed to the inhibition of I/R-induced elevation of [Cl\textsuperscript{-}], (9); however, the intracellular signal transduction mechanisms underlying these effects have yet to be elucidated.

Protein kinase C epsilon (PKCε), a novel PKC isotype characterized as a calcium-independent and phorbol ester/diacylglycerol-sensitive serine/threonine kinase (10,11), has been well documented to have an important role in cardioprotection (12-15). Several recent studies have reported that PKCε signaling is involved in maintaining intracellular chloride homeostasis (2,16).

Considering that PKCε signaling has important roles in the regulation of [Cl\textsuperscript{-}], and in cardioprotection, and that SQS can effectively inhibit I/R-induced elevation of [Cl\textsuperscript{-}], the present study hypothesized that the cardioprotective effects of SQS against I/R injury are mediated by PKCε via attenuation of [Cl\textsuperscript{-}], increases following I/R. To verify this hypothesis neonatal rat cardiomyocytes were subjected to simulated (s)

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Key words: sasanquasaponin, cardioprotection, protein kinase Cε signaling pathway, chloride
I/R as an in vitro I/R model, and the effects of SQS on cell viability, PKCe phosphorylation levels, [Cl\textsubscript{i}], mitochondrial membrane potential (Δψm) and ROS production were assessed.

Materials and methods

Reagents. SQS was kindly provided by Professor Yongming Luo from Jiangxi Chinese Medical University (Nanchang, China), and its identity and purity (>99%) were determined by nuclear magnetic resonance spectroscopy and high-performance liquid chromatography tandem mass spectrometry analyses. eV1-2, a specific inhibitor of PKCe, was purchased from AnaSpec (cat. no. AS-62186; Fremont, CA, USA). Anti-PKC\textepsilon and anti-phosphorylated (p)-PKCe (Ser 729) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Anti-β-actin antibody was purchased from the Jiancheng Bioengineering Institute of Nanjing (Nanjing, China). The horseradish peroxidase-labeled immunoglobulin G secondary antibody was purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA).

Cell culture. Cardiomyocytes from 30 Sprague-Dawley rats (weight, 10-15 g; age, 1-3 days; Nanchang University School of Medicine, Nanchang, China) were prepared according to the protocol of a previous study (17). The rats were treated in accordance with the National Institute of Health's Guide for the Care and Use of Laboratory Animals, and all procedures were approved by the ethics committee of Nanchang University (Nanchang, China). The rats were anesthetized by intraperitoneal injection with 10% chloral hydrate (0.03 ml/kg; Sigma-Aldrich, St. Louis, MO, USA), after which they were sacrificed by cervical dislocation prior to the removal of the rat hearts. The hearts were removed and placed in phosphate-buffered saline (PBS). The ventricles were digested with 0.1% trypsin (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) and then collected by centrifugation at 60 x g for 10 min, re-suspended in plating medium, including 85% Eagle’s minimum essential medium, 15% fetal calf serum and 100 U/ml penicillin and streptomycin (Beijing Solarbio Science & Technology Co., Ltd.), seeded in a culture dish and incubated for 2 h in an atmosphere containing 95% air and 5% CO\textsubscript{2} to remove non-myocytes. The supernatant was collected and plated in 60-mm gelatin-coated culture dishes at 1x10\textsuperscript{5} cells per dish. After 24 h, cardiomyocytes were washed and the medium was replaced, followed by incubation for three days prior to the experiments.

Experimental groups and treatments. The neonatal primary rat cardiomyocytes were divided into various experimental groups as follows: i) In the control group, cardiomyocytes were incubated under normal conditions for an additional 24 h; ii) in the sl/R group, cardiomyocytes were subjected to anoxia by incubating them in fresh anoxic medium (0.9 mM NaH\textsubscript{2}PO\textsubscript{4}, 6.0 mM NaHCO\textsubscript{3}, 1.8 mM CaCl\textsubscript{2}, 1.2 mM MgSO\textsubscript{4}, 40 mM sodium lactate, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 98.5 mM NaCl, 10.0 mM KCl, pH 6.8; Sigma-Aldrich) at 37°C in a chamber containing 95% N\textsubscript{2} and 5% CO\textsubscript{2} for 3 h. Subsequently, the medium was replaced with re-oxygenation medium (129.5 mM NaCl, 5.0 mM KCl, 0.9 mM NaH\textsubscript{2}PO\textsubscript{4}, 20 mM NaHCO\textsubscript{3}, 1.8 mM CaCl\textsubscript{2}, 1.2 mM MgSO\textsubscript{4}, 5.5 mM glucose, 20 mM HEPES, pH 7.4; Sigma-Aldrich) and the cells were incubated at 37°C in an atmosphere of 95% air and 5% CO\textsubscript{2} for 2 h; iii) In the SQS+sl/R group, cells were pre-treated with SQS at 10 µM for 24 h prior to sl/R; iv) In the SQS+eV1-2+sl/R group, cells were pre-treated with eV1-2 (1 µM) and SQS (10 µM) for 24 h prior to sl/R. The doses of SQS and eV1-2 used were selected on the basis of a preliminary study by our group and previous reports (8,18,19).

Cell viability assay. A colorimetric MTS assay was used to assess the viability of cardiomyocytes subjected to sl/R. MTS is a pale yellow substrate that is converted into a dark blue formazan product by living cells. Primary cardiomyocytes were seeded into 96-well plates at 1x10\textsuperscript{4} cells/well. After sl/R treatment, cells were incubated with 20 µl MTS (5 mg/ml; Promega Corp., Madison, WI, USA) in 100 µl medium at 37°C for 2 h. The absorbance of each well at a wavelength of 490 nm was then measured using a microplate reader (no. 680; Bio-Rad Laboratories, Inc., Hercules, CA, USA). The results were expressed as a percentage of the control.

Biochemical parameters. Following sl/R treatment, the culture media of the control groups and the supernatants of cell lysates from each group were collected to the activities of creatine phosphokinase (CPK) and lactate dehydrogenase (LDH) were assessed using kits (A020-2 and A032, respectively; Jiancheng Bioengineering Institute of Nanjing), according to the manufacturer’s instructions.

Measurement of intracellular ROS. ROS levels were determined using the fluorescent probe 2’7’-dichlorodihydrofluorescein diacetate (DCFH-DA; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), which is cleaved by cellular esterases to non-fluorescent DCFH and oxidized by intracellular ROS to the fluorescent product dichlorofluorescein (DCF). ROS production is proportional to the fluorescence ratio of the treatment vs control group. After the indicated treatments, the cells were incubated with 10 µM DCFH-DA for 20 min at 37°C prior to being harvested and analyzed for green and red fluorescence intensity using a flow cytometer (FACSCalibur; BD Biosciences, Franklin Lakes, NJ, USA) at wavelengths of 485 and 528 nm, respectively.

Determination of [Cl\textsubscript{i}]. The measurement of [Cl\textsubscript{i}], was performed according to the method described in a previous study by our group with minor modifications (8). Briefly, after the indicated treatments, cardiomyocytes were washed twice with Cl\textsubscript{-}-free solution (NaCl was replaced by an equimolar amount of d-glucuronic acid, MgCl\textsubscript{2} by MgSO\textsubscript{4} and KCl by potassium gluconate), and incubated with 10 mM N-(ethoxycarbonylmethyl)-6-methoxyquinolinium (MQAE; Thermo Fisher Scientific, Inc.) in the dark for 20 min at 37°C. Cl\textsubscript{-}-free solution was then used to remove any excess dye. Cells were suspended in Cl\textsubscript{-}-free solution immediately subjected to flow cytometric analysis (FACSCalibur).

Assessment of Δψm. The fluorescent dye JC-1 was used to assess the mitochondrial membrane potential as described in the study by Tang et al (18). Briefly, after the indicated treatments, cardiomyocytes were incubated with JC-1 (200 µM;
Beijing Solarbio Science & Technology Co., Ltd.) at 37°C for 20 min. Cells were then washed with ice-cold PBS to remove remaining dye. Fluorescence was measured by flow cytometry (FACSCalibur) with excitation/emission wavelengths of 530/580 nm (red) and at 485/550 nm (green). The ratio of red to green fluorescence intensity of cells reflected the ΔΨm.

Western blot analysis. Western blotting was performed as described previously (18). The protein concentrations were measured using the DC Protein Assay kit II (cat. no. 500-0112; Bio-Rad Laboratories, Inc.). Equal quantities of protein (30 μl/lane) were then separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes. After blocking with 5% non-fat milk in Tris-buffered saline containing 0.2% Tween 20 (TBST), the blots were probed with rabbit anti-PKCε (C-15; 1:500; sc-214), goat anti-p-PKCε (Ser 729; 1:500; sc-12355) and rabbit anti-β-actin (1:1,000; #21338) polyclonal antibodies overnight at 4°C. After washing with TBST, the blots were incubated with horseradish peroxidase-conjugated goat anti-rabbit (1:5,000; #7074) and rabbit anti-goat (1:5,000; #7075) secondary antibodies for 1 h at room temperature. After washing, the blots were saturated with enhanced chemiluminescence mixture (Pierce Biotechnology, Inc., Rockford, IL, USA) for 1 min, and images of the blot were captured by exposure to pre-flashed X-ray film (Kodak, Rochester, NY, USA) for 300 sec. Densitometric scanning was performed using Quantity One software, version 4.62 (Bio-Rad Laboratories, Inc.). Results were expressed as a percentage of the control. The bands were normalized to β-actin.

Statistical analysis. Data are expressed as the mean ± standard error of the mean. Statistical analyses were performed using SPSS software, version 11.0 (SPSS, Inc., Chicago, IL, USA). One-way analysis of variance was applied to test the significance of differences between groups, followed by post-hoc testing for individual differences. P<0.05 was considered to indicate a statistically significant difference between values. For each assessment, at least three independent experiments were performed.

Results

SQS activates PKCe in cardiomyocytes following sI/R. Primary cardiomyocytes were incubated with or without 10 μM SQS for 24 h, followed by sI/R, and the levels of total and phosphorylated PKCe (Ser 729) were assessed by western blotting. As shown in Fig. 1, the levels of p-PKCe Ser 729 were significantly increased in the SQS pre-treated group (P<0.01 vs. control group; P<0.01 vs. sI/R group), whereas the levels of total PKCe remained unchanged. These results suggested that SQS treatment led to the activation of PKCe in cardiomyocytes following sI/R.

SQS reduces cardiomyocyte death following sI/R via PKCe. The release of CPK and LDH into the culture medium was analyzed to evaluate the effects of SQS and PKCe inhibitor εV1-2 on cardiomyocyte death. As shown in Fig. 2, a significant increase in CPK and LDH levels was observed in the sI/R group compared to that in the control group (P<0.01), which was significantly inhibited by pre-treatment with SQS (P<0.01 vs. sI/R group). Of note, the release of CPK and LDH in the group additionally pre-treated with εV1-2 prior to sI/R was enhanced when compared with that in the SQS+sI/R group (P<0.01) (Fig. 2), indicating that SQS may exert its protective effects via PKCe. Furthermore, the results of the MTS assay showed that SQS significantly increased cardiomyocyte viability following sI/R (P<0.01), which was significantly inhibited by co-treatment with εV1-2 (P<0.01 vs. SQS+sI/R group) (Fig. 3). These data suggested that PKCe is required for SQS to elicit its cardioprotective effects.
SQS reduces sI/R-induced increases in [Cl\(_i\)] in cardiomyocytes via PKCe. To investigate whether PKCe is implicated in the inhibitory effects of SQS on the elevation of [Cl\(_i\)] induced by sI/R, cardiomyocytes were treated with PKCe inhibitor εV1-2 in combination with SQS and [Cl\(_i\)], was assessed via MRAE staining and flow cytometric analysis. As shown in Fig. 4, SQS pre-treatment abrogated the increases in [Cl\(_i\)] in cardiomyocytes following sI/R (P<0.01 vs. sI/R group), which was inhibited by the selective PKCe inhibitor εV1-2 (P<0.01 vs. SQS+sI/R group). These results indicated that activation of PKCe is required for SQS to inhibit sI/R-induced elevation of [Cl\(_i\)].

SQS attenuates increases of the Δψ\(_m\) in cardiomyocytes following sI/R via PKCe. As the decrease of Δψ\(_m\) induced by Cl\(_i\) influx reflects the opening of the mPTP, which results in a ROS burst, the present study assessed the effects of SQS on sI/R-induced changes in Δψ\(_m\) in cardiomyocytes as well as the possible involvement of PKCe. As shown in Fig. 5, sI/R decreased the Δψ\(_m\) as indicated by a decrease in the ratio of red to green fluorescence intensity (P<0.01 vs. control group). However, pre-treatment with SQS significantly attenuated the loss of Δψ\(_m\) (P<0.01 vs. sI/R group). As expected, the restorative effect of SQS on the Δψ\(_m\) was inhibited by εV1-2 (P<0.01 vs. SQS+sI/R group), indicating that PKCe is required for SQS-mediated attenuation of Δψ\(_m\) loss following sI/R in cardiomyocytes.

SQS inhibits ROS generation in cardiomyocytes undergoing sI/R via PKCe. Intracellular ROS levels were assessed by measuring cDCF fluorescence intensity. As shown in Fig. 6, sI/R induced marked intracellular ROS production, while pre-treatment with SQS inhibited ROS production induced by sI/R. Co-treatment with εV1-2 attenuated the inhibitory effects of SQS on ROS production induced by sI/R, indicating that PKCe is required for SQS to inhibit sI/R-induced ROS production.

**Discussion**

SQS is an active component extracted from the Chinese medicinal herb *Camellia oleifera* Abel and has gained considerable attention due to its wide range of biological and pharmacological properties (19-21). Previous studies by our group showed that SQS effectively protected against myocardial injury in an isoproterenol-induced rat model of ischemia *in vivo* and in a hypoxia and reoxygenation model in Langendorff-perfused rat hearts *in vitro* (8,22,23). Another previous study by our group revealed that SQS exerts its cardioprotective effects by suppressing intracellular Cl\(_i\) accumulation induced by sI/R (9). The present study investigated the molecular mechanisms underlying SQS-induced effects, focusing on the link between the PKC\(_e\) signalling pathway and the variations of [Cl\(_i\)].

It is well known that Cl\(_i\) is the primary intracellular anion and that changes in [Cl\(_i\)], can affect a variety of basic cellular functions, including ionic conductances, membrane potential, intracellular pH, apoptosis, cell volume and Ca\(^{2+}\) homeostasis (2,24). Studies on ischemic myocardial injury have revealed that I/R-induced elevation of [Cl\(_i\)], is an important pathophysiological factor. Huang et al (4) found that increases in [Cl\(_i\)], can lead to the opening of the mPTP, which results in ROS burst and subsequent ROS-dependent cell injury. In line with this result, the present study found that cardiomyocytes subjected to sI/R show a rapid and significant increase in intracellular Cl\(_i\) levels accompanied by loss of Δψ\(_m\), mPTP opening and ROS production. This was also accompanied by cardiomyocyte injury as indicated by a marked increase of LDH and CPK release as well as reduction of cell viability. These results further suggested that dysregulation of intracellular Cl\(_i\) homeostasis has a significant role in myocardial cell injury induced by ischemia/reperfusion. In addition, SQS was shown to suppress the elevation of [Cl\(_i\)], induced by sI/R and induce cardioprotection from sI/R injury in an *in vitro* cell model. As the underlying molecular mechanisms had remained elusive, the present study assessed the possible role of...
of PKCε in the inhibition of sI/R-induced [Cl⁻], by SQS in cardiomyocytes.

PKCε is a member of a novel group of the PKC family of serine and threonine kinases, which are involved in a wide range of physiological processes, including cell survival under stressful conditions, mitogenesis, transcriptional regulation and metastasis (12). It has been confirmed that PKCε-associated signaling exerts cardioprotective functions (25-27), and that the PKCε pathway is involved in maintaining intracellular chloride homeostasis (2,16). On this basis, the present study hypothesized that activation of the PKCε pathway may be responsible for the cardioprotective effects of SQS and inhibition of I/R-induced increases in [Cl⁻] by SQS. To test this hypothesis, the present study first determined the effects of SQS on the phosphorylation

Figure 5. Effects of SQS alone or in combination with protein kinase C epsilon inhibitor εV1-2 on the mitochondrial membrane potential in cardiomyocytes subjected to sI/R. Cardiomyocytes were incubated for 24 h with SQS (10 µM) in the absence or presence of εV1-2 (1 µM) followed by sI/R. Representative flow cytometric dot plots are shown. Quantitative evaluation of flow cytometry data provided the red/green fluorescence ratio of the cells to represent the mitochondrial membrane potential. Values are expressed as the mean ± standard error of the mean of four individual experiments. *P<0.01 vs. control group; **P<0.01 vs. sI/R group; ***P<0.01 vs. SQS+sI/R group. sI/R, simulated ischemia/reperfusion; SQS, sasanquasaponin.

Figure 6. Effects of SQS alone and in combination with protein kinase C epsilon inhibitor εV1-2 on the generation of ROS in cardiomyocytes subjected to sI/R. Cardiomyocytes were incubated for 24 h with SQS (10 µM) in the absence or presence of εV1-2 (1 µM) followed by sI/R. The generation of ROS was determined by flow cytometric analysis of the fluorescence of DCF. A representative flow cytometric histogram of DCF fluorescence is shown. The bar graph shows DCF fluorescence representing ROS levels in all groups. Values are expressed as the mean ± standard error of the mean for four individual experiments. *P<0.01 vs. control group; **P<0.01 vs. sI/R group; ***P<0.01 vs. SQS+sI/R group. DCF, 2'7'-dichlorodihydrofluorescein diacetate; sI/R, simulated ischemia/reperfusion; SQS, sasanquasaponin.
of PKCe in cardiomyocytes. Of note, pre-treatment with SQS significantly increased the levels of p-PKCε, indicating that SQS can activate the PKCe pathway in cardiomyocytes subjected to sI/R. To further explore the association between PKCe activation and the cardioprotective effects of SQS, the selective PKCe inhibitor εv1-2 was employed. The results revealed that SQS reduced cardiomyocyte death following sI/R injury, which was inhibited by εv1-2. Furthermore, SQS inhibited sI/R-induced elevation of [Cl−], which was attenuated by εv1-2. These results suggested that activation of PKCe is required for SQS to elicit its cardioprotective effects.

Due to the fact that the elevation of [Cl−], induced by sI/R contributes to loss of Δψm, mPTP opening and ROS production, the present study further detected the effects of SQS on Δψm and ROS production in the absence or presence of εv1-2. As expected, it was observed that SQS attenuated the sI/R-induced loss of Δψm as well as ROS production in cardiomyocytes, which was inhibited by εv1-2, suggesting that the activation of PKCe is also required for SQS to attenuate sI/R-induced Δψm loss and ROS production.

Although the present study demonstrated that activation of PKCe is required for the inhibitory effects of SQS on the elevation of [Cl−], following sI/R injury, the detailed molecular interactions deserve further investigation. Anion exchanger 3 (AE3), a member of the solute carrier 4 protein family, mediates the reversible electroneutral exchange of Cl− for HCO3− across the plasma membrane (28). A previous study by our group showed that AE3 is closely linked with the inhibitory effects of SQS on sI/R-induced elevation of [Cl−] (9). Of note, Alvarez et al (29) showed that AE3 is the PKCe-sensitive anion exchange protein of the heart, and that the PKCe-dependent phosphorylation of serine 67 on AE3 can cause an increase in anion transport. Therefore, it may be speculated that SQS inhibits sI/R-induced elevation of [Cl−], and induces cardioprotection through activation of the PKCe pathway and consequent PKCe-dependent phosphorylation of serine 67 on AE3. However, this notion requires experimental validation.

In conclusion, the present study was the first to report that activation of PKCe is required for SQS to exert its cardioprotective effects against sI/R injury. It was demonstrated that activation of PKCe is crucial for SQS-mediated inhibition of sI/R-induced elevation of [Cl−], Δψm loss and ROS production. The present study enhanced the current understanding of the molecular mechanisms of the cardioprotective effects of SQS, suggesting that it may be efficient for reducing I/R-induced injury.

Acknowledgements

The present study was supported by grants from the Natural Scientific Foundation of China (nos. 30660209 and 81260491).

References


