

Sasanquasaponin induces increase of $\text{Cl}^-/\text{HCO}_3^-$ exchange of anion exchanger 3 and promotes intracellular Cl^- efflux in hypoxia/reoxygenation cardiomyocytes

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Abstract. Anion exchanger 3 (AE3) is known to serve crucial roles in maintaining intracellular chloride homeostasis by facilitating the reversible electroneutral exchange of Cl^- for HCO_3^- across the plasma membrane. Our previous studies reported that sasanquasaponin (SQS) can inhibit hypoxia/reoxygenation (H/R)-induced elevation of intracellular Cl^- concentration ($[\text{Cl}^-]_i$) and elicit cardioprotection by favoring $\text{Cl}^-/\text{HCO}_3^-$ exchange of AE3. However, the molecular basis for SQS-induced increase of $\text{Cl}^-/\text{HCO}_3^-$ exchange of AE3 remains unclear. The present study demonstrated that SQS activates protein kinase C ϵ (PKC ϵ) and stimulates the phosphorylation of AE3 in H9c2 cells. Notably, SQS-induced AE3 phosphorylation was blocked by the PKC ϵ selective inhibitor ϵ V1-2, and a S67A mutation of AE3, indicating that SQS could promote phosphorylation of Ser67 of AE3 via a PKC ϵ -dependent regulatory signaling pathway. Additionally, both inhibition of PKC ϵ by ϵ V1-2 and S67A mutation of AE3 eradicated the SQS-induced increase of AE3 activity, reversed the inhibitory effect of SQS on H/R-induced elevation of $[\text{Cl}^-]_i$, Ca^{2+} overload and generation of reactive oxygen species, and eliminated SQS-induced cardioprotection. In conclusion, PKC ϵ -dependent phosphorylation of serine 67 on AE3 may be responsible for the increase of

$\text{Cl}^-/\text{HCO}_3^-$ exchange of AE3 and intracellular chloride efflux by SQS, and contributes to the cardioprotection of SQS against H/R in H9c2 cells.

Introduction

The chloride ion (Cl^-) is a primary intracellular anion, and is involved in a wide variety of cell and intracellular organelle functions, including regulation of electrical activity, intracellular pH, cell volume, apoptosis and Ca^{2+} homeostasis (1,2). Previous studies have demonstrated that an increase of intracellular chloride ion concentration ($[\text{Cl}^-]_i$) serves an important role in the pathogenesis of myocardial ischemia/reperfusion (I/R) injury (3-8). However, increased $[\text{Cl}^-]_i$ can promote the release of intracellular Ca^{2+} to elicit Ca^{2+} overload through Cl^- -increase-induced Ca^{2+} release from intracellular stores (3,9,10); on the other hand, the increased $[\text{Cl}^-]_i$ can also activate the Cl^-/OH^- exchanger to increase intracellular reactive oxygen species (ROS) (3,10). Furthermore, the increased $[\text{Cl}^-]_i$ can induce the mitochondrial permeability transition pore opening, which results in ROS burst and subsequent oxidative stress (11). Therefore, inhibition of increased $[\text{Cl}^-]_i$ has been considered to be a reasonable therapeutic strategy to alleviate I/R injury.

Anion exchange protein 3 (AE3), an embedded membrane protein, belongs to the solute carrier 4 (SLC4) protein family and is differentially expressed in excitable tissues (e.g. the brain, heart and retina) (12). The main function of AE3 is to mediate the reversible electroneutral exchange of Cl^- for HCO_3^- across the plasma membrane (13-15). It means that AE3 protein has two operating modes-the forward exchange mode and the reverse exchange mode. The former is mainly an acidifying mechanism that promotes the electroneutral efflux of bicarbonate in exchange for chloride influx. However, the later contributes to sustaining intracellular chloride homeostasis by promoting excess chloride efflux, which is subject to regulation by intracellular pH, transmembrane $\text{Cl}^-/\text{HCO}_3^-$ gradient and phosphorylation state near the AE3 N-terminal region (13,16,17). Notably, Alvarez *et al* (16,18) demonstrated that AE3 is the protein kinase C (PKC)-sensitive anion exchange protein of the heart, and that PKC ϵ -dependent

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Abbreviations: SQS, sasanquasaponin; AE3, anion exchanger 3; H/R, hypoxia/reoxygenation; I/R, ischemia/reperfusion; $[\text{Cl}^-]_i$, intracellular Cl^- concentration; PKC ϵ , protein kinase C ϵ

Key words: sasanquasaponin, anion exchanger 3, intracellular Cl^- concentration, cardioprotection, protein kinase C ϵ

phosphorylation of serine 67 on AE3 can promote $\text{Cl}^-/\text{HCO}_3^-$ reverse exchange activity.

Sasanguasaponin (SQS; 22-O-angeloyl camelliagenin C 3-O-[b-D-glucopyranosyl (1,2)] [b-D-glucopyranosyl (1,2)-a-L-arabinopyranosyl (1,3)]-b-D-glucopyranosiduronic acid, $\text{C}_{58}\text{H}_{92}\text{O}_{26}$) is a biologically active ingredient extracted from the Chinese medicinal herb *Camellia oleifera* Abel and has gained considerable attention due to its wide range of biological and pharmacological properties; in particular, its cardioprotective effect. Previous studies have demonstrated that SQS effectively protects cardiomyocytes against I/R injury by suppressing I/R-induced elevation of $[\text{Cl}^-]_i$ (19). Our previous study further demonstrated that AE3 is required for SQS to elicit cardioprotective effects against I/R injury, and the inhibitory effect of SQS on I/R-induced elevation of $[\text{Cl}^-]_i$ is involved in the increase of $\text{Cl}^-/\text{HCO}_3^-$ reverse exchange activity of AE3 (20). However, the molecular basis for SQS-induced increase of $\text{Cl}^-/\text{HCO}_3^-$ reverse exchange of AE3 remains to be fully elucidated.

Therefore, the aim of the present study was to identify the molecular basis for SQS activation of AE3 in H9c2 cells. It was revealed that SQS could promote phosphorylation of the Ser67 of AE3 through activating PKC ϵ in H9c2 cells undergoing hypoxia/reoxygenation (H/R). Importantly, the PKC ϵ -dependent phosphorylation of serine 67 on AE3 was responsible for the increase of $\text{Cl}^-/\text{HCO}_3^-$ exchange of AE3 and intracellular chloride efflux by SQS.

Materials and methods

Chemicals and reagents. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2-tetrazolium bromide (MTT) were purchased from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). 5(6)-carboxy-20, 70-dichlorofluorescein diacetate (cDCFH-DA) and N-ethoxycarbonyl-methyl-6-methoxyquinolinium bromide (MQAE) were from Invitrogen (Thermo Fisher Scientific, Inc.) ϵ V1-2 (PKC ϵ inhibitor) and 8-[2-(2-pentyl-cyclopropyl-methyl)-cyclopropyl]-octanoic acid (DCP-LA; PKC ϵ activator) were purchased from Merck KGaA (Darmstadt, Germany). 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF) and 1-[2-Amino-5-(2,7-dichloro-6-acetoxy-methoxy-3-oxo-9-xanthenyl) phenoxy]-2-(2-amino-5-methylphenoxy) ethane-N,N,N', N'-tetraacetic acid, tetra (acetoxymethyl) ester (Fluo-3/AM) were purchased from Beyotime Institute of Biotechnology (Shanghai, China). The primary antibodies against β -Actin (rabbit, pAb; sc-130656; 1:1,000), PKC ϵ (C-15; rabbit, pAb; sc-214; 1:500), p-PKC ϵ Ser 729 (goat, pAb; sc-12355; 1:1,000) and a horseradish peroxidase (HRP)-conjugated secondary antibody (mouse anti-rabbit IgG-HRP; sc-2357; 1:5,000) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Antibodies against AE3 (rabbit, pAb; LS-C20639; 1:1,000), were from LifeSpan BioScience, Inc. (Seattle, WA, USA), and anti-phosphoserine (anti-p-ser; rabbit, pAb; AB1603; 1:500) were from EMD Millipore (Billerica, MA, USA). SQS was kindly provided by Professor Yongming Luo from Jiangxi Chinese Medical University (Nanchang, China). Fluo-3/AM and all other

chemicals were from Sigma-Aldrich (Merck KGaA), unless otherwise stated.

Cell culture. H9c2 cells, a clonal line derived from embryonic rat hearts, and HEK293 cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in DMEM supplemented with 10% (v/v) FBS, 10 mM L-glutamine and 5 mg/ml penicillin/streptomycin, in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

Adenovirus and cell infections. The adenoviral vector expressing rat wild-type AE3 (Ad-AE3) and AE3 S67A mutant (Ad-AE3-S67A) were produced by Shanghai GeneChem, Co. Ltd. (Shanghai, China). For cell infections, 80% confluent dishes of H9c2 or HEK293 cells were used. Cells were infected at a multiplicity of infection of 25 for 24 h with recombinant adenoviruses Ad-AE3 or Ad-AE3-S67A. The adenoviruses were removed and cells were left to recover for 24 h in complete medium. These conditions resulted in uniform expression of the transgenes in close to 95% of the cells, as estimated by control vector expressing only green fluorescent protein.

Cellular model of H/R injury. H/R was achieved as described previously (21-23). Briefly, hypoxia was achieved by incubating the cells for 2 h in an airtight chamber in which O_2 was replaced by N_2 with glucose-free Tyrode's solution containing 139 mM NaCl, 4.7 mM KCl, 0.5 mM MgCl_2 , 1.0 mM CaCl_2 and 5 mM HEPES, pH 7.4, at 37°C. Following incubation in hypoxic conditions, the cells were provided with fresh medium and then moved to normoxic conditions (5% CO_2 , 37°C) for 60 min for reoxygenation.

Quantification of cell damage. Cell damage was determined by measuring cell viability and the release of lactate dehydrogenase (LDH) and creatine phosphokinase (CPK) into the cell culture medium. The release of LDH and CPK were quantified using the CytoTox-ONE™ Homogenous Membrane Integrity assay (Promega Corporation, Madison, WI, USA) according to the manufacturer's protocol. Cell viability was determined by MTT assay. Briefly, cells were seeded into 96-well plates at a density of 1×10^5 cells/well. Following treatment, cells were washed with warm phosphate buffered saline (PBS) and incubated with 0.5 mg/ml MTT in PBS for 4 h at 37°C. The reaction was stopped by the addition of 150 μ l diphenylamine solution and the absorbance of the blue formazan derivative was read at a wavelength of 570 nm using a spectrophotometer.

Determination of anion exchange activity of AE3. Anion exchange activity was determined by 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF) according to previously described protocols (18,24). Briefly, H9c2 cells were cultured on coverslips in 60 mm dishes. Following treatment, coverslips were incubated in 4 ml serum-free medium containing 2 μ M BCECF-AM (37°C, 30 min) and mounted in a fluorescence cuvette. The cuvette was perfused at 3.5 ml/min alternately with Ringer's buffer (5 mM glucose, 5 mM potassium gluconate, 1 mM calcium gluconate, 1 mM MgSO_4 , 2.5 mM NaH_2PO_4 , 25 mM NaHCO_3 , 10 mM HEPES, pH 7.4) containing 140 mM sodium chloride (Cl $^-$ buffer) or 140 mM sodium gluconate (Cl $^-$ -free buffer). The two buffers were

bubbled continuously with air containing 5% CO₂. Intracellular pH was monitored by measuring fluorescence at excitation wavelengths of 440 and 502 nm and an emission wavelength of 529 nm, using a spectrofluorometer. Fluorescence data were converted to pH_i by calibration using the nigericin/high potassium method (25), with pH values of 6.4, 6.8 and 7.2. Transport rates were determined by linear regression of the initial linear rate of the change of pH_i.

Determination of [Cl⁻]_i. The levels of [Cl⁻]_i were measured using the Cl⁻-specific fluorescence probe MQAE, as previous described (11,19,20). Briefly, following treatment, cells were washed twice with Cl⁻-free Tyrode solution (NaCl was replaced by equimolar amounts of D-glucuronic acid; MgCl₂ by MgSO₄; KCl by potassium gluconate), and loaded with 10 mM of MQAE in the dark for 60 min at 37°C. Then the excess dye was washed off and the cells were resuspended in Cl⁻-free Tyrode solution. The fluorescence intensity of each group was determined by flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA) at excitation and emission wavelengths of 355 and 460 nm and analyzed with FlowJo software version 7.6 (FlowJo LLC, Ashland, OR, USA). Finally, [Cl⁻]_i was calculated in the light of the calibration curve for the mean fluorescence intensity of MQAE which changed with [Cl⁻]_i.

Measurement of ROS generation. ROS generation was determined using the cell-permeable probe cDCFH-DA, which is cleaved by cellular esterases to nonfluorescent 2',7'-dichlorofluorescein (DCFH) and oxidized by intracellular ROS to a fluorescent product dichlorofluorescein (DCF). Following the indicated treatments, the cells were harvested and washed with cold PBS. Washed cells were further incubated with 10 μM cDCFH-DA at 37°C for 20 min. The excess dye was washed off and the cells were resuspended in PBS. The fluorescent intensity was measured using a fluorescence microscope (Olympus BX51; Olympus Corporation, Tokyo, Japan) at an excitation wavelength of 488 nm and an emission wavelength of 525 nm (26).

Determination of [Ca²⁺]_i. The change in [Ca²⁺]_i of cells was determined by the fluorescence of the calcium-sensitive dye fluo-3/AM as previously described (27). Briefly, cells in 60-mm plastic petri dishes were incubated for 30 min at 37°C in the absence of light in loading buffer [20 mM HEPES, pH 7.4, 130 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgSO₄, 0.8 mM Na₂HPO₄, 0.2 mM NaH₂PO₄, 25 mM mannose and 1 mg/ml bovine serum albumin (BSA)] containing 2 mM Fluo-3/AM and 0.008% Pluronic F-127 dissolved in DMSO. Following incubation, the monolayers were washed in detaching buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 5 mM KCl, 0.55 mM MgCl₂ and 3 mM EDTA) and incubated in the same buffer at 37°C for 10 min. Detached cells were harvested by low-speed centrifugation (1,000 x g) at room temperature for 5 min, re-suspended in assay buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 5 mM KCl, 0.55 mM MgCl₂ and 1 mM CaCl₂) and analyzed on a flow cytometer (BD Biosciences) with FlowJo software version 7.6 (FlowJo LLC).

Western blot analysis. Total proteins were extracted from cells using a Protein Extraction kit (Pierce; Thermo Fisher

Scientific, Inc.) according to the manufacturer's protocol. Protein content was determined by the Lowry method using a DC protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Immunoblotting analysis was performed as described previously (21,22). In brief, 30 μg proteins were separated by 9% SDS-PAGE and transferred to polyvinylidene difluoride membranes. Following blocking with 5% non-fat milk or BSA at room temperature for 2 h, the blots were then incubated with primary antibodies against AE3 (1:1,000), PKCε (1:1,000), p-PKCε (1:1,000), p-ser (1:500) and β-actin (1:1,000) at 4°C overnight. The bound antibodies were detected by an appropriate HRP-conjugated secondary antibody at room temperature for 1 h and visualized using an enhanced chemiluminescence system (EMD Millipore). The levels of each protein were standardized to the loading control (β-actin) and were quantified using ImageJ software version 1.41 (National Institutes of Health, Bethesda, MD, USA).

Measurement of AE3 phosphorylation. AE3 phosphorylation was detected in AE3 immunoprecipitates with an anti-phosphoserine antibody. Briefly, whole-cell lysates containing 0.5 mg proteins were pre-cleared with protein A/G plus-agarose (Santa Cruz Biotechnology, Inc.) at 4°C for 2 h. Aliquots (40 μl) of pre-clared lysates were then incubated with anti-AE3 antibodies at 4°C overnight followed by incubation with protein A/G plus-agarose for 4 h. The agarose beads were collected by centrifugation (500 x g at 4°C for 5 min), washed 4 times with cold PBS (4°C) and heated to 95°C for 5 min after adding Laemmli buffer. The resulting immunoprecipitates were separated by SDS-PAGE and probed with anti-AE3 or anti-phosphoserine antibodies in western blotting as described above. In brief, 40 μl proteins were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes. Following blocking with 5% non-fat milk or BSA in room temperature for 2 h, the blots were then incubated with primary antibodies against AE3 (rabbit, pAb; LS-C20639; 1:1,000; LifeSpan BioScience, Inc.), p-Ser (rabbit, pAb; AB1603; 1:500; EMD Millipore) and β-actin (rabbit, pAb; sc-130656; 1:1,000; Santa Cruz Biotechnology, Inc.) at 4°C overnight. The bound antibodies were detected by an appropriate HRP-conjugated secondary antibody (mouse anti-rabbit IgG-HRP; sc-2357; 1:5,000; Santa Cruz Biotechnology, Inc.) at room temperature for 1 h. The blots were analyzed densitometrically and the ratio of phosphorylated AE3 to total AE3 protein was determined to normalize for differences in loading by using ImageJ software version 1.41 (National Institutes of Health).

Statistical analysis. All data are expressed as the mean ± standard error. Statistical comparisons among groups were performed by one-way analysis of variance followed by the least significant difference post hoc test (two-tailed). Statistical calculations were performed using SPSS software version 11.0 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

SQS induces PKCε activation in H9c2 cells undergoing H/R. Initial experiments were conducted to determine the effect

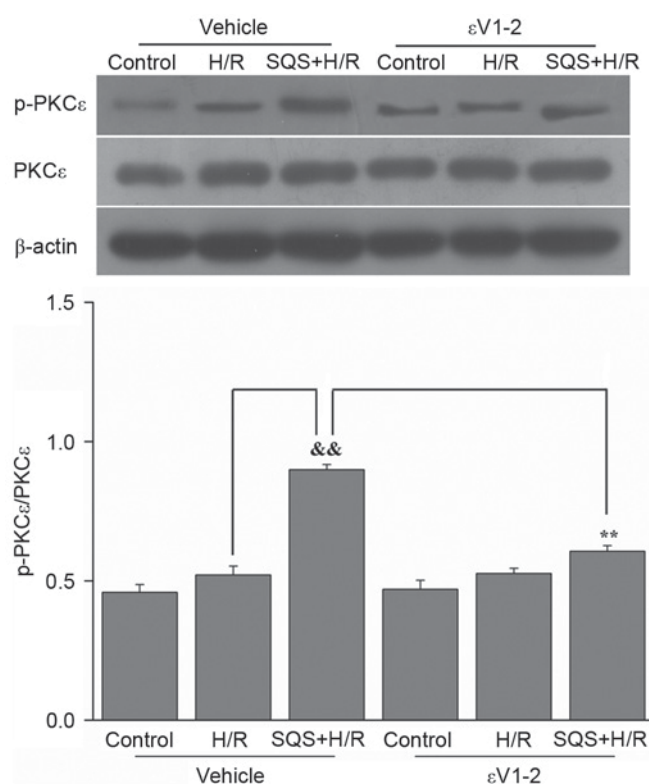


Figure 1. Effects of SQS on PKC ϵ activation in H9c2 cells undergoing H/R. H9c2 cells were incubated for 24 h with SQS (10 μ M) alone or combination with ϵ V1-2 (1.0 μ M) 60 min before and during SQS pretreatment, followed by H/R. Representative western blot images and quantification of protein expression levels of PKC ϵ and p-PKC ϵ . β -actin served as an internal control. Data are presented as the mean \pm standard error of least four independent experiments. &&P<0.01, **P<0.01. SQS, sasanquasaponin; PKC ϵ , protein kinase C ϵ ; H/R, hypoxia/reoxygenation; p, phosphorylated; ϵ V1-2, protein kinase C ϵ inhibitor.

of SQS on PKC ϵ activation. H9c2 cells were pretreated with 10 μ M SQS for 24 h, followed by H/R. Subsequently, PKC ϵ activation was determined by western blot analysis. The results demonstrated that SQS increased PKC ϵ phosphorylation, whereas the expression of total PKC ϵ proteins remained unchanged (Fig. 1). These results suggested that SQS pretreatment can activate PKC ϵ in H9c2 cells subjected to H/R. In addition, when administered 60 min before and during SQS pretreatment, 1.0 μ M ϵ V1-2 (PKC ϵ inhibitor) eradicated the activation of PKC ϵ induced by SQS (Fig. 1).

SQS promotes phosphorylation of Ser67 of AE3 via the PKC ϵ -dependent signaling pathway in H9c2 cells undergoing H/R. The effect SQS on AE3 phosphorylation in H9c2 cells undergoing H/R was examined. SQS upregulated AE3 expression and increased AE3 phosphorylation in H9c2 cells undergoing H/R. Interestingly, pretreatment with 1.0 μ M ϵ V1-2 (PKC ϵ inhibitor) blocked SQS-induced AE3 phosphorylation but did not affect AE3 expression (Fig. 2A), suggesting that SQS can promote AE3 phosphorylation via activation of PKC ϵ . Notably, in Ad-AE3-S67A-transfected H9c2 cells, the S67A mutation completely annulled the SQS-induced phosphorylation of AE3 (Fig. 2B). By contrast, the negative control virus Ad-C did not alter phosphorylation of AE3 by SQS. These data indicated that SQS-induced AE3 phosphorylation

appears to be dependent on Ser67. To further support the fact that activation of PKC ϵ and Ser67 of AE3 are required for SQS-induced AE3 phosphorylation, the effect of the PKC ϵ activator, DCP-LA, on AE3 phosphorylation was examined in Ad-AE3 and Ad-AE3-S67A-transfected H9c2 cells. As expected, it was observed that DCP-LA treatment resulted in phosphorylation of AE3 in Ad-AE3-transfected H9c2 cells but not in Ad-AE3-S67A-transfected H9c2 cells (Fig. 3). Taken together, these results demonstrated that SQS increases the phosphorylation of AE3 in a manner that is dependent on Ser67 and PKC ϵ activation.

PKC ϵ -dependent phosphorylation of serine 67 on AE3 is responsible for SQS increasing the Cl $^-$ /HCO $_3^-$ exchange activity of AE3. To investigate whether PKC ϵ -dependent phosphorylation of AE3 Ser67 is associated with SQS-induced increase of Cl $^-$ /HCO $_3^-$ exchange activity of AE3, the effect of SQS on AE3 exchange activity in H9c2 cells with or without 1.0 μ M ϵ V1-2 pretreatment or Ad-AE3-S67A transfection was detected. SQS elicited increased anion exchange activity of AE3 in H9c2 cells undergoing H/R, which was blocked by pretreatment with ϵ V1-2 (Fig. 4A). In addition, the increased transport activity elicited by SQS was also attenuated in Ad-AE3-S67A-transfected H9c2 cells (Fig. 4B). This indicated that the increase of AE3 activity on treatment with SQS is mediated via PKC ϵ -dependent phosphorylation of AE3 Ser67.

PKC ϵ -dependent phosphorylation of AE3 serine 67 is responsible for the inhibitory effect of SQS on H/R-induced elevation of [Cl $^-$] $_i$. Subsequently, the effect of SQS on H/R-induced elevation of [Cl $^-$] $_i$ in H9c2 cells with or without 1.0 μ M ϵ V1-2 pretreatment or Ad-AE3-S67A transfection was detected. As presented in Fig. 5, when cells were subjected to H/R, the [Cl $^-$] $_i$ was significantly increased and the peak of [Cl $^-$] $_i$ values was 49.7 ± 5.1 mM, compared with 26.8 ± 3.8 mM in the control (P<0.01). Treatment with 10 μ M SQS produced a significant reduction in intracellular Cl $^-$ concentration in H9c2 cells undergoing H/R, whereas pretreatment with 1.0 μ M ϵ V1-2 abrogated the inhibitory effect of SQS on H/R-induced elevation of [Cl $^-$] $_i$ (Fig. 5A). These results suggested that SQS can attenuate H/R-induced elevation of [Cl $^-$] $_i$ in a PKC ϵ -dependent manner. Additionally, in Ad-AE3-S67A-transfected H9c2 cells, SQS did not delay the H/R-induced increase in [Cl $^-$] $_i$ (Fig. 5B). These results indicated that PKC ϵ -dependent phosphorylation of AE3 Ser67 is required for SQS preconditioning to inhibit H/R-induced elevation of [Cl $^-$] $_i$.

PKC ϵ -dependent phosphorylation of serine 67 on AE3 is responsible for SQS inhibiting H/R-induced Ca $^{2+}$ overload. Subsequently, the effect of SQS on H/R-induced Ca $^{2+}$ overload in H9c2 cells with or without 1.0 μ M ϵ V1-2 pretreatment or Ad-AE3-S67A transfection was further detected. As presented in Fig. 6, when the H9c2 cells were subjected to H/R, intracellular Ca $^{2+}$ was significantly increased. Following preincubation with 10 μ M SQS for 24 h, the H/R-induced increase in [Ca $^{2+}$] $_i$ was suppressed, whereas pretreatment with 1.0 μ M ϵ V1-2 eliminated the inhibitory effect of SQS on H/R-induced Ca $^{2+}$ overload (Fig. 6A). These results suggested that SQS can attenuate H/R-induced Ca $^{2+}$ overload in a PKC ϵ -dependent manner.

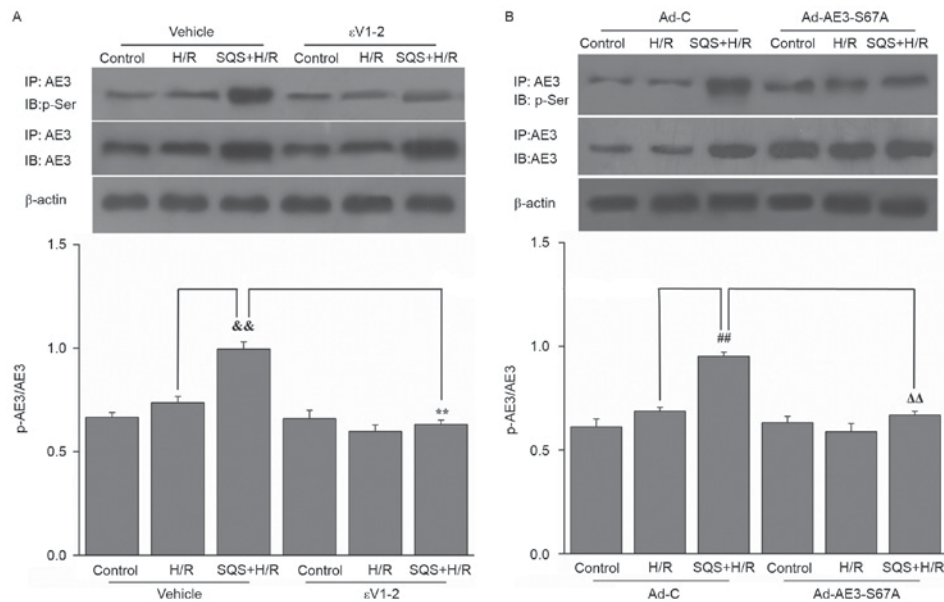


Figure 2. Effects of SQS on phosphorylation of AE3 in H9c2 cells undergoing H/R were assessed by immunoprecipitation. Cell lysates were immunoprecipitated (IP) with anti-AE3 antibody, and immunoblot (IB) analysis was performed with anti-p-Ser or anti-AE3. Representative western blot images and quantification of protein expression levels of AE3 and p-AE3 in H9c2 cells (A) incubated for 24 h with SQS (10 μ M) alone or combination with the protein kinase C ϵ inhibitor ϵ V1-2 (1.0 μ M), followed by H/R and (B) transfected with Ad-AE3-S67A 24 h before SQS (10 μ M, 24 h) pretreatments, followed by H/R. β -actin served as an internal control. Data are presented as the mean \pm standard error of least four independent experiments. $\&\&P<0.01$, $^{**}P<0.01$, $^{##}P<0.01$, $^{\Delta\Delta}P<0.01$. SQS, sasanquasaponin; AE3, anion exchanger 3; H/R, hypoxia/reoxygenation; p, phosphorylated; Ad-C, negative control adenovirus.

However, in Ad-AE3-S67A-transfected H9c2 cells, SQS did not delay the H/R-induced increase in $[Ca^{2+}]_i$ (Fig. 6B). These results indicated that SQS can attenuate H/R-induced the elevation of $[Ca^{2+}]_i$ through PKC ϵ signaling pathway and phosphorylation Ser67 of AE3.

PKC ϵ -dependent phosphorylation of AE3 serine 67 is necessary for SQS to reduce H/R-induced ROS production. In addition, the effect of SQS on H/R-induced ROS production in H9c2 cells with or without 1.0 μ M ϵ V1-2 pretreatment or Ad-AE3-S67A transfection was assessed. As presented in Fig. 7, H/R induced marked intracellular ROS production, whereas simultaneous pretreatment with 10 μ M SQS inhibited ROS production. In the presence of ϵ V1-2 (Fig. 7A) or Ad-AE3-S67A (Fig. 7B), SQS lost its inhibitory effect on ROS production induced by H/R, indicating that PKC ϵ -dependent phosphorylation of AE3 Ser67 is required for SQS preconditioning to inhibit H/R-induced ROS production.

PKC ϵ -dependent phosphorylation of AE3 serine 67 is essential for SQS to elicit its cardioprotective effect. Finally, to determine whether PKC ϵ -dependent phosphorylation of AE3 Ser67 is required for SQS-induced cardioprotection against H/R injury, H9c2 cells were pretreated with 10 μ M SQS for 24 h in the absence or presence of ϵ V1-2 or Ad-AE3-S67A, followed by H/R. CPK and LDH release and cell viability were used as indexes of cellular injury. As presented in Fig. 8, SQS pretreatment attenuated H/R-induced viability loss and CPK and LDH leakage. However, in the presence of ϵ V1-2 (Fig. 8A) or Ad-AE3-S67A (Fig. 8B), SQS did not induce cardioprotection against H/R injury, indicating that PKC ϵ -dependent phosphorylation of AE3 Ser67 is required for the cardioprotective effect elicited by SQS.

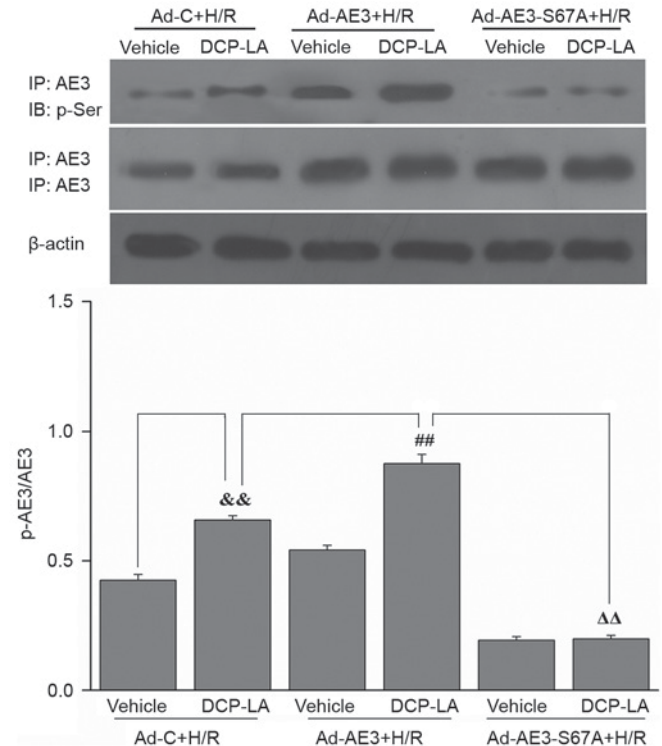


Figure 3. Effects of the PKC ϵ activator DCP-LA on phosphorylation of AE3 in Ad-AE3 or Ad-AE3-S67A-transfected H9c2 cells undergoing H/R were assessed by immunoprecipitation. Cell lysates were immunoprecipitated (IP) with anti-AE3 antibody, and immunoblot (IB) analysis was performed with anti-p-Ser or anti-AE3. Representative western blot images and quantification of protein expression levels of AE3 and p-AE3 in H9c2 cells. β -actin served as an internal control. Data are presented as the mean \pm standard error of least four independent experiments. $\&\&P<0.01$, $^{##}P<0.01$, $^{\Delta\Delta}P<0.01$. PKC ϵ , protein kinase C ϵ ; DCP-LA, 8-[2-(2-pentyl-cyclopropyl-methyl)-cyclopropyl]-octanoic acid; AE3, anion exchanger 3; H/R, hypoxia/reoxygenation; p, phosphorylated; Ad-C, negative control adenovirus.

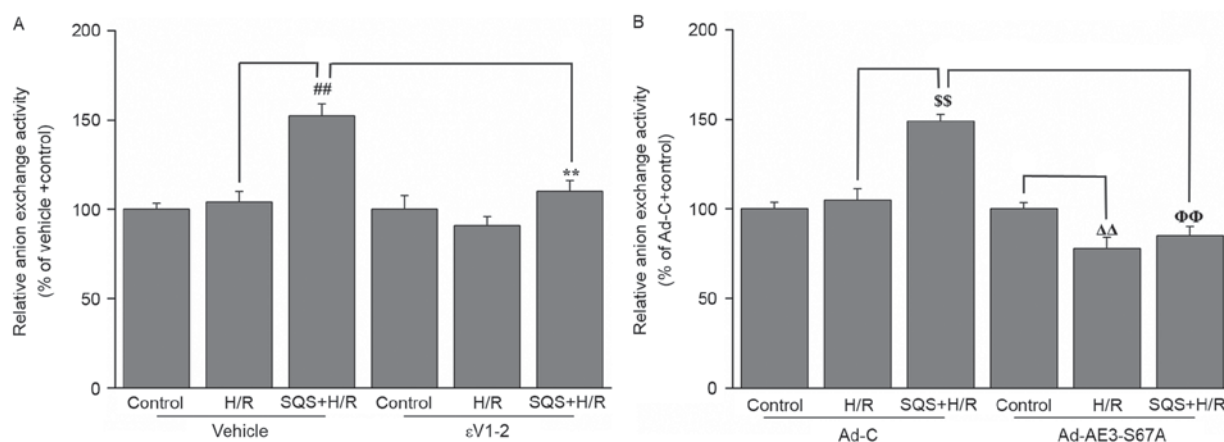


Figure 4. Effects of SQS on anion exchange activity of AE3 in εV1-2-pretreated or Ad-AE3-S67A-transfected H9c2 cells undergoing H/R. Relative anion exchange activity in H9c2 cells (A) incubated with εV1-2 and (B) transfected with Ad-AE3-S67A. Data are presented as the mean ± standard error of four independent experiments. ^{##}P<0.01, ^{**}P<0.01, ^{ΔΔ}P<0.01, ^{ss}P<0.01, ^{ΦΦ}P<0.01. SQS, sasanquasaponin; AE3, anion exchanger 3; Ad-C, negative control adenovirus; H/R, hypoxia/reoxygenation; p, phosphorylated.

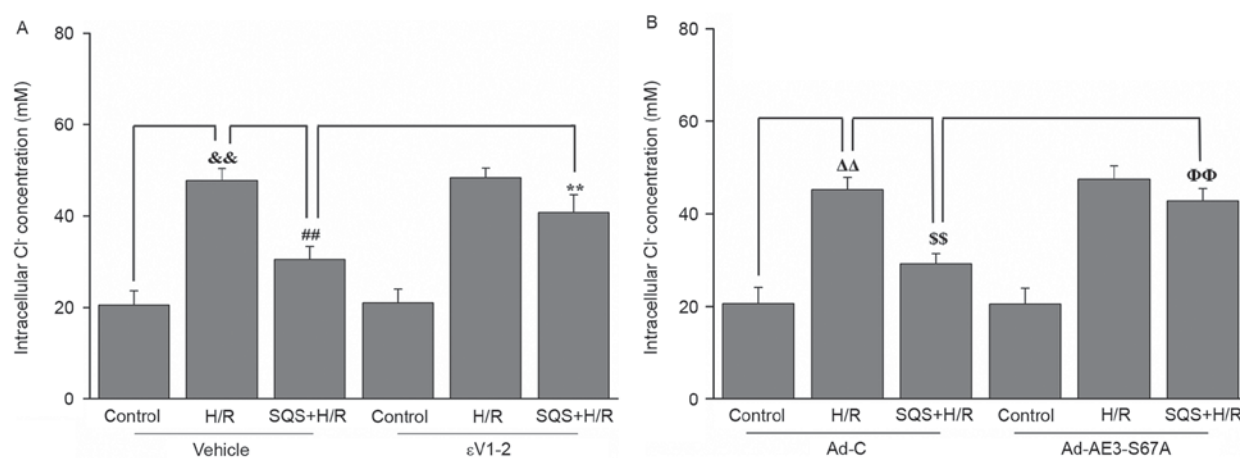


Figure 5. Effects of SQS on [Cl⁻]_i in εV1-2-pretreated or Ad-AE3-S67A-transfected H9c2 cells undergoing H/R. [Cl⁻]_i in H9c2 cells (A) incubated with εV1-2 and (B) transfected with Ad-AE3-S67A. Data are presented as the mean ± standard error of four independent experiments. ^{##}P<0.01, ^{**}P<0.01, ^{ΔΔ}P<0.01, ^{ss}P<0.01, ^{ΦΦ}P<0.01. SQS, sasanquasaponin; H/R, hypoxia/reoxygenation; p, phosphorylated; AE3, anion exchanger 3; Ad-C, negative control adenovirus; [Cl⁻]_i, intracellular Cl⁻ concentration.

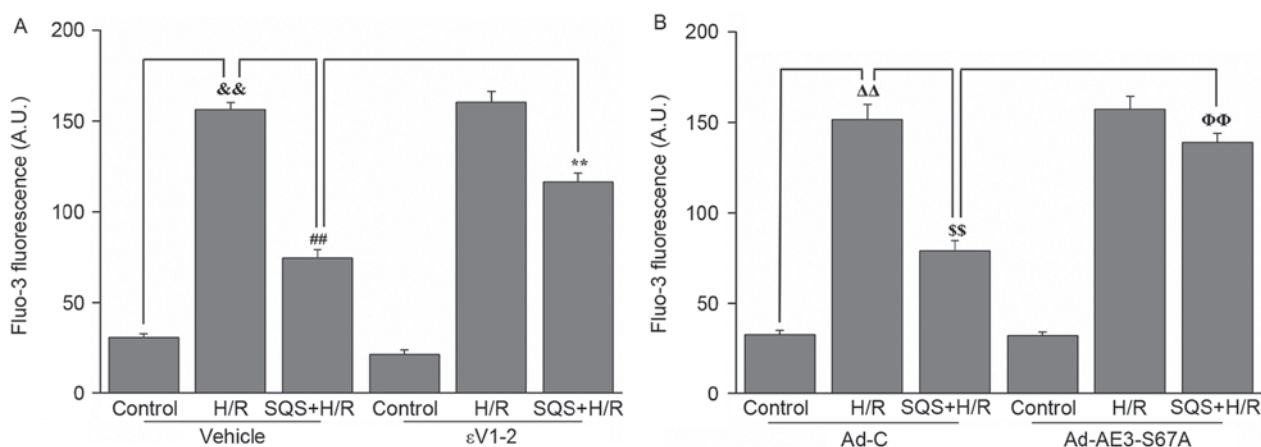


Figure 6. Effects of SQS on [Ca²⁺]_i in εV1-2-pretreated or Ad-AE3-S67A-transfected H9c2 cells undergoing H/R. [Ca²⁺]_i in H9c2 cells (A) incubated with εV1-2 and (B) transfected with Ad-AE3-S67A. Data are presented as the mean ± standard error of four independent experiments. ^{##}P<0.01, ^{**}P<0.01, ^{ΔΔ}P<0.01, ^{ss}P<0.01, ^{ΦΦ}P<0.01. SQS, sasanquasaponin; H/R, hypoxia/reoxygenation; p, phosphorylated; AE3, anion exchanger 3; Ad-C, negative control adenovirus; [Ca²⁺]_i, intracellular Ca²⁺ concentration.

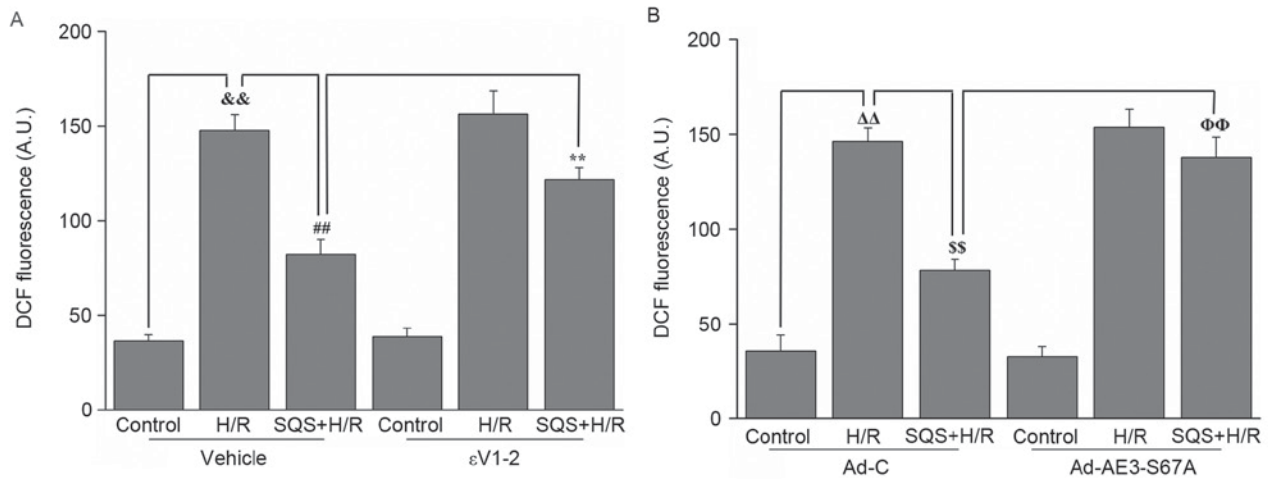


Figure 7. Effects of SQS on ROS generation in εV1-2-pretreated or Ad-AE3-S67A-transfected H9c2 cells undergoing H/R. DCF fluorescence in H9c2 cells (A) incubated with εV1-2 and (B) transfected with Ad-AE3-S67A. Data are presented as the mean ± standard error of four independent experiments. ##P<0.01, **P<0.01, ΔΔP<0.01, ΦΦP<0.01. SQS, sasanquasaponin; H/R, hypoxia/reoxygenation; p, phosphorylated; AE3, anion exchanger 3; Ad-C, negative control adenovirus; ROS, reactive oxygen species; DCF, 2,2'-dichlorofluorescein.

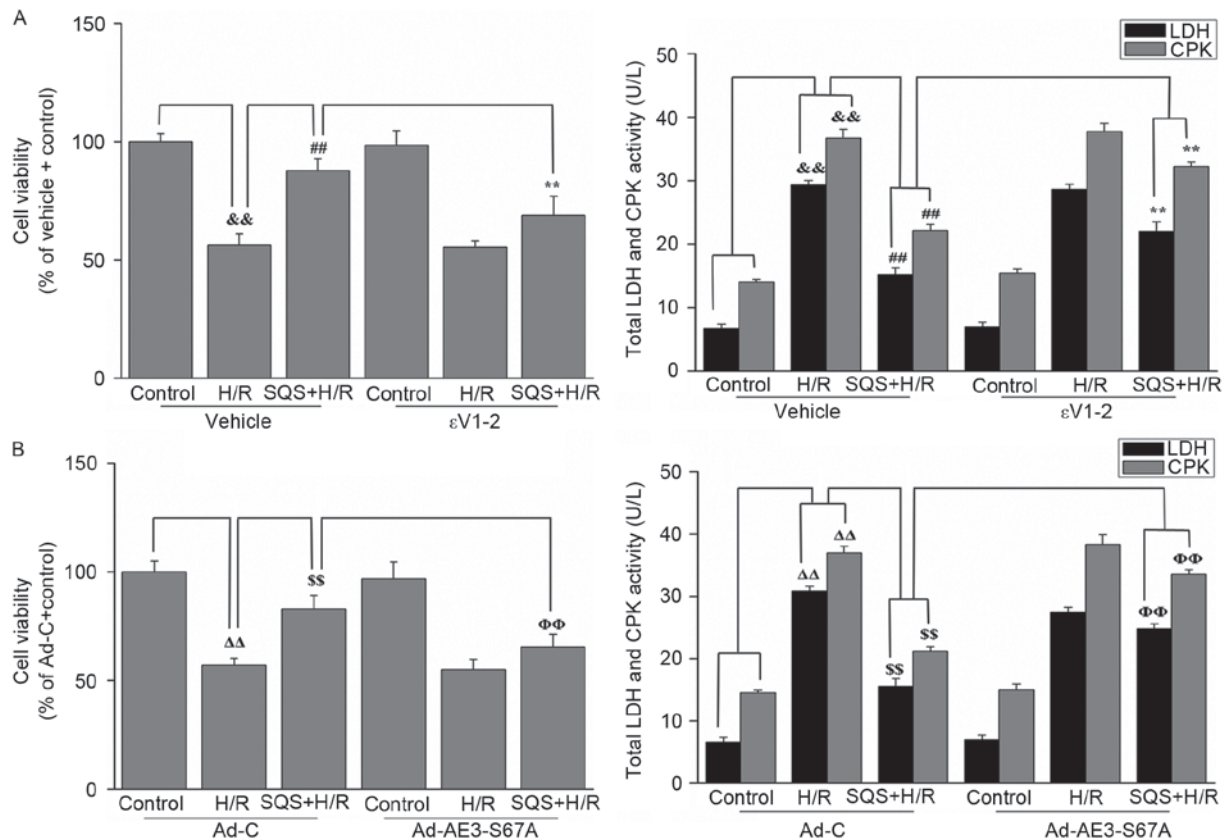


Figure 8. Effects of SQS on cell viability and release of LDH and CPK in εV1-2-pretreated or Ad-AE3-S67A-transfected H9c2 cells undergoing hypoxia/reoxygenation (H/R). Cell viability and LDH and CPK release in H9c2 cells (A) incubated with εV1-2 and (B) transfected with Ad-AE3-S67A. Data are presented as the mean ± standard error of four independent experiments. ##P<0.01, **P<0.01, ΔΔP<0.01, ΦΦP<0.01. SQS, sasanquasaponin; H/R, hypoxia/reoxygenation; p, phosphorylated; AE3, anion exchanger 3; Ad-C, negative control adenovirus; LDH, lactate dehydrogenase; CPK, creatine phosphokinase.

Discussion

The present study demonstrated that PKCε-dependent phosphorylation of serine 67 on AE3 is a critical molecular basis by which SQS increases activity of Cl⁻/HCO₃⁻ exchange of AE3, promotes the intracellular chloride efflux and elicits

cardioprotection in H9c2 cells undergoing H/R. This was indicated by results demonstrating that SQS promotes PKCε activation and enhances the level of phosphorylation of AE3 in H9c2 cells subjected to H/R. Additionally, SQS-induced AE3 phosphorylation was blocked by the PKCε selective inhibitor, εV1-2, and S67A mutation of AE3. Furthermore, inhibition

of PKC ϵ by ϵ V1-2 and the S67A mutation of AE3 abrogated SQS-induced increase of AE3 activity, reversed the inhibitory effect of SQS on H/R-induced elevation of $[Cl^-]_i$, Ca^{2+} overload and ROS production, and eliminated the cardioprotection induced by SQS in H9c2 cells. Taken together, the present study expanded the current understanding of the cardioprotective effects of SQS by identifying a novel regulatory process induced by SQS, which stimulates phosphorylation of Ser67 of AE3 via PKC ϵ activation and, as a result, increases AE3 activity and attenuates H/R-induced elevation of $[Cl^-]_i$ in H9c2 cells.

SQS has profound cardioprotective effects on ischemia/reperfusion injury (19). Our previous study reported that upregulation and activation of AE3 may contribute to SQS cardioprotection (20). The present study demonstrated that SQS can promote intracellular chloride efflux by increasing Cl^-/HCO_3^- exchange of AE3 and can elicit cardioprotection in H9c2 cells subjected to H/R. However, the molecular basis for SQS-induced increase of Cl^-/HCO_3^- exchange of AE3 remains unclear; therefore, the present study focused on elucidating the underlying mechanisms.

AE3 is known to serve crucial roles in maintaining intracellular chloride homeostasis by facilitating the reversible electroneutral exchange of Cl^- for HCO_3^- across the plasma membrane. Notably, AE3 has been reported to be a PKC ϵ -sensitive anion exchange protein of the heart, and the PKC ϵ -dependent phosphorylation of serine 67 on AE3 can obviously promote the Cl^-/HCO_3^- exchange activity (16,18). Notably, our previous study indicated that SQS exerts its cardioprotective effects in association with PKC ϵ activation (28). On this basis, it was hypothesized that PKC ϵ -dependent phosphorylation of serine 67 on AE3 may be responsible for the increase of Cl^-/HCO_3^- exchange activity of AE3 by SQS and contribute to the inhibitory effect of SQS on H/R-induced elevation of $[Cl^-]_i$ in H9c2 cells. To investigate this, the present study first determined the effects of SQS on PKC ϵ activation and subsequent AE3 phosphorylation. The results demonstrated that SQS promotes PKC ϵ activation and increases AE3 phosphorylation, whereas both inhibition of PKC ϵ by ϵ V1-2 and the S67A mutation of AE3 exhibited adverse effects. These findings suggested that SQS can promote phosphorylation of Ser67 on AE3 via the PKC ϵ -dependent signaling pathway.

To further verify the contribution of PKC ϵ -dependent phosphorylation of Ser67 on AE3 in the SQS-induced increase of Cl^-/HCO_3^- exchange activity of AE3 and intracellular chloride efflux in H9c2 cells subjected to H/R, the present study investigated the effect of inhibition of PKC ϵ by ϵ V1-2 and the S67A mutation of AE3 on AE3 exchange activity, and H/R-induced elevation of $[Cl^-]_i$ in H9c2 cells. As expected, the results demonstrated that SQS pretreatment led to a significant increase of AE3 activity and a significant reduction in $[Cl^-]_i$ in H9c2 cells undergoing H/R. However, these effects were attenuated in both ϵ V1-2-pretreated and Ad-AE3-S67A-transfected H9c2 cells, suggesting that PKC ϵ -dependent phosphorylation of AE3 Ser67 is essential for SQS to increase the Cl^-/HCO_3^- exchange activity of AE3 and to inhibit H/R-induced elevation of $[Cl^-]_i$.

In addition, as elevation of $[Cl^-]_i$ induced by H/R contributes to Ca^{2+} overload and ROS production, the present study further detected the effects of SQS on Ca^{2+} overload and ROS

production in H9c2 cells with or without ϵ V1-2 pretreatment or Ad-AE3-S67A transfection. As expected, it was observed that SQS attenuated the Ca^{2+} overload and ROS production that normally follows H/R injury, accompanied by reduction of LDH and CPK release and an increase in cell viability. However, in the presence of ϵ V1-2 or Ad-AE3-S67A, the inhibitory effects of SQS on Ca^{2+} overload and ROS production were reversed, and the cardioprotective effects of SQS were attenuated, suggesting that the PKC ϵ -dependent phosphorylation of AE3 Ser67 is a prerequisite for SQS to attenuate H/R-induced Ca^{2+} overload and ROS production and to elicit cardioprotection.

In conclusion, to the best of our knowledge, the present study was the first to investigate the underlying mechanism of regulation of AE3 activity by SQS. It was demonstrated that SQS promotes phosphorylation of Ser67 of AE3 via a PKC ϵ -dependent regulatory signaling pathway. Notably, it was revealed that PKC ϵ -dependent phosphorylation of serine 67 on AE3 is responsible for the increase of Cl^-/HCO_3^- exchange of AE3 and intracellular chloride efflux by SQS, and contributes to the cardioprotection of SQS against H/R in H9c2 cells. These findings may be beneficial in further understanding the molecular mechanism associated with SQS-induced cardioprotection, and elucidating the cellular effect and pharmacological profiles of SQS.

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