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Anion Exchanger 3 Is Required for Sasanquasaponin to Inhibit Ischemia/Reperfusion-Induced Elevation of Intracellular Cl^- Concentration and to Elicit Cardioprotection

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ABSTRACT

Recent studies have shown that the cardioprotection of sasanquasaponin (SQS) against ischemia/reperfusion injury is related to inhibiting ischemia/reperfusion-induced elevation of intracellular Cl^- concentration ($[Cl^-]_i$). However, the mechanism of inhibition remains unclear. Anion exchanger 3 (AE_3) is an important regulatory protein for $[Cl^-]_i$. This study investigated whether AE_3 plays the critical role in the inhibitory effect of SQS on elevation of $[Cl^-]_i$ induced by ischemia/reperfusion and mediates the cardioprotection of SQS in H9c2 cells. Normal and AE_3-knockdown H9c2 cells were incubated for 24 h with or without various concentrations of SQS (0.1, 1, or 10 μM) followed by simulated ischemia/reperfusion (sI/R). AE_3 expression was detected by Western blot. Flow cytometer analysis was employed to determine $[Cl^-]_i$, $[Ca^{2+}]_i$, reactive oxygen species (ROS) production, and cell apoptosis. The results showed that SQS pretreatment concentration-dependently attenuated sI/R-induced viability loss and lactate dehydrogenase leakage in normal H9c2 cells. Additionally, SQS concentration-dependently up-regulated AE_3 protein expression, and inhibited sI/R-induced the elevation of $[Cl^-]_i$ followed by the attenuation of Ca^{2+} overload, ROS production, and cell apoptosis. However, the dose-dependent cardioprotection induced by SQS was abolished in AE_3-knockdown H9c2 cells, and the inhibitory effects of SQS on $[Cl^-]_i$, Ca^{2+} overload, ROS production, and cell apoptosis were also reversed. Our data indicate that AE_3 mediates the cardioprotective effect of SQS against sI/R injury. Importantly, AE_3 is required for SQS to inhibit sI/R-induced elevation of $[Cl^-]_i$, which subsequently inhibited sI/R-induced Ca^{2+} overload, ROS production, and cell apoptosis. J. Cell. Biochem. 112: 2803–2812, 2011.

KEY WORDS: SASANQUASAPONIN; ANION EXCHANGER 3; CARDIOPROTECTION; INTRACELLULAR CL− CONCENTRATION

It is well known that Cl^- is the primary intracellular anion and plays a potentially important role in cardiac physiology and pathophysiology [Hume et al., 2000]. Numerous studies have already shown that changes in intracellular Cl^- concentrations ($[Cl^-]_i$) can affect a variety of basic cellular functions, including membrane potential, ionic conductances, intracellular pH, cell volume, apoptosis, and Ca^{2+} homeostasis, etc. [Hiraoka et al., 1998; Hume et al., 2000]. Moreover, cumulated evidence shows that an increase of myocardial $[Cl^-]_i$ induced by ischemia/reperfusion is also an important pathophysiological factor that contributes to arrhythmia, intracellular acidification, and Ca^{2+} overload [Lai et al., 1996; Lai and Nishi, 1998; Kawasaki et al., 2001; Lai, 2002; Chen et al., 2007b].

Ming He and Min Huang contributed equally to this work.

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Sasanquasaponin (SQS; 22-*O*-angeloyl camelliagenin C 3-*O*-[β-D-glucopyranosyl (1, 2)] [β-D-glucopyranosyl (1, 2)-α-L-arabinopyranosyl (1, 3)]-β-D-glucopyranosiduronic acid, $C_{58}H_{92}O_{26}$, SQS) is a biologically active ingredient extracted from the Chinese medicinal herb *Camellia oleifera* Abel, with a purity of more than 99%. Experimental data have demonstrated that SQS possesses many biological and pharmacological properties, such as anti-inflammation, anti-oxidation, anti-hyperlipidemia, anti-effusion, anti-allergy, etc. [Akagi et al., 1997; Sur et al., 2001; Chen et al., 2007a]. Recent studies have shown that SQS could effectively protect cardiomyocytes against ischemia/reperfusion injury by suppressing ischemia/reperfusion-induced elevation of $[Cl^-]_i$) [Lai et al., 2004]. However, the involved mechanisms of its inhibition effect on $[Cl^-]_i$ during ischemia/reperfusion remain unclear.

Anion exchanger 3 (AE_3), a member of the solute carrier 4 (SLC4) protein family, is differentially expressed in excitable tissues (e.g., brain, heart, and retina) and mainly exists in the cytoplasmic membrane [Romero et al., 2004]. The main function of AE_3 is to mediate the reversible electroneutral exchange of Cl^- for HCO_3^- across the plasma membrane [Alper et al., 2002]. Thus, AE_3 activity efficiently contributes to the cellular regulation of $[Cl^-]_i$, intracellular pH, and cell volume homeostasis [Cordat and Casey, 2009]. Recent studies have found that genetic ablation of AE_3 results in more rapid decompensation and heart failure in a hypertrophic cardiomyopathy model [Al Moamen et al., 2011]. Interestingly, our recent research suggested that AE_3 is involved in the cardioprotection of ischemic preconditioning by maintaining intracellular chloride homeostasis [Liu et al., 2006].

Considering that AE_3 plays important roles in regulating $[Cl^-]_i$, and that SQS can effectively inhibit ischemia/reperfusion-induced elevation of $[Cl^-]_i$. We hypothesized that AE_3 could mediate the cardioprotection of SQS against ischemia/reperfusion injury and be related to the inhibitory effect of SQS on elevation of $[Cl^-]_i$ induced by ischemia/reperfusion. The current investigation was therefore undertaken to elucidate this possibility in H9c2 cardiomyoblasts, a cell line extensively used in cardiological research since it shares most of the molecular and functional features of adult cardiomyocytes [Hescheler et al., 1991]. In this study, our data provided the first evidence in a cardiac myocyte model that AE_3 is involved in the cardioprotection of SQS. Importantly, our observations revealed AE_3 is required by SQS to inhibit simulated ischemia/reperfusion (sI/R)-induced elevation of $[Cl^-]_i$ and subsequently inhibit sI/R-induced Ca^{2+} overload, reactive oxygen species (ROS) production, and cell apoptosis.

METHODS

MATERIALS

Dulbecco's modified Eagle's medium, fetal bovine serum, 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2-tetrazolium bromide (MTT), penicillin, and streptomycin were purchased from GIBCOL (Grand Island, NY). Annexin V-FITC apoptosis detection kits, 5(6)-carboxy-2′,7′-dichlorofluorescein diacetate (cDCFH-DA), and *N*-ethoxycarbonyl-methyl-6-methoxyquinolinium bromide (MQAE) were from Invitrogen (Carlsbad, CA). Anti-AE_3 antibody was from Chemicon Biotechnology (Chemicon, Temecula, CA). SQS was kindly provided by Prof. Luo Yongming from Jiangxi Chinese Medical University (Nanchang, China), and its identity and purity (>99%) were determined by NMR spectroscopic and HPLC-MS analyses. Fluo-3/AM and all other chemicals were from Sigma Chemical Co. (St Louis, MO), unless otherwise stated.

CELL CULTURE

The rat embryonic heart-derived H9c2 cell line (American Type Culture Collection, Rockville, MD) was maintained as described previously [Pesant et al., 2006] in Dulbecco's modified Eagle's medium with 5.5 mM glucose supplemented with 10% (v/v) fetal bovine serum and 1% penicillin/streptomycin (v/v). Cells were routinely grown to 80% confluence in 75 cm^2 flasks at 37°C in an atmosphere of 5% CO_2 prior to passage and seeding for experiments. Twenty-four hours prior to experiments, the cells were made quiescent by serum starvation.

ESTABLISHMENT OF AE_3-KNOCKDOWN CELL LINES

Three candidate AE_3 siRNA target sequences and scramble (negative) control siRNA sequence were designed using the siRNA Target Finder and Design Tool available at www.ambion.com/techlib/misc/siRNA_finder.html. These siRNA sequences are 5′-AAAGC AAAGTTCTCCATTGGA-3′ (AE_3 siRNA-1), 5′-AACTCAGT-CTTGGGGAATCAC-3′ (AE_3 siRNA-2), 5′-AACTGAATAGTAGTGC-CCTGC-3′ (AE_3 siRNA-3), and 5′-AAACTACCGT TGTTATAGGTG-3′ (Negative control siRNA), respectively. Subsequently, these siRNA duplexes were synthesized and transiently transfected into H9c2 cells according to the instructions of the manufacturer. Their efficacies in extinguishing AE_3 expression were evaluated by Western blot. AE_3 siRNA-1, above, showing the highest efficacy, was selected for establishing AE_3-knockdown cell lines. Briefly, the template oligonucleotides corresponding to AE_3 siRNA-1 were synthesized and ligated into *Bam*HI–*Hin*dIII sites of pSilencer 3.1-H1 hygro vector (Ambion, Austin, TX). These plasmids were then transfected into H9c2 cells using Lipofectamine 2000 reagent (Invitrogen), and the cells were cultured in the medium in the presence of 400 μg/ml hygromycin for 14 days. Cells that were resistant to the drug were then selected, and the intrinsic expression of AE_3 was checked by Western blot with an anti-AE_3 antibody.

SIMULATED ISCHEMIA/REPERFUSION (SI/R) INJURY MODEL

Simulated ischemia was achieved as described previously [Mizukami et al., 2004]. Briefly, after cells were pretreated for 24 h with or without various concentrations of SQS (0.1, 1, or 10 μM) [Lai et al., 2004; Chen et al., 2007a], cells were washed with phosphate-buffered saline (PBS). The cells were then incubated in slightly hypotonic Hanks' balanced saline solution (1.3 mM $CaCl_2$, 5 mM KCl, 0.3 mM KH_2PO_4, 0.5 mM $MgCl_2$, 0.4 mM $MgSO_4$, 69 mM NaCl, 4 mM $NaHCO_3$, and 0.3 mM Na_2HPO_4) without glucose and serum for 2 h at 37°C. Hypoxia was achieved using an airtight incubator from which the oxygen was reduced to 1% by replacement with nitrogen. After incubation under the conditions of sI, the cells were incubated in DMEM without serum under normoxic conditions (20% O_2, 5% CO_2) at 37°C for 60 min.

ASSAY OF CELL VIABILITY

Cell viability was determined by MTT assay. Cells were seeded at the density of 4×10^4 cells/well in 24-well plates. After experiment treatment, cells were washed with warm 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 μl diphenylamine solution, and the absorbance of the blue formazan derivative was read at 570 nm using a microplate reader (Bio-Rad Laboratories, Richmond, CA).

ASSAY OF LACTATE DEHYDROGENASE (LDH) ACTIVITY

To measure lactate dehydrogenase (LDH) activity in the culture medium of cells, after experiment treatment, 0.1 ml of culture medium was taken and analyzed with an automatic biochemical analyzer (Hitachi 7060, Japan).

WESTERN BLOT ANALYSIS

Total protein was extracted from cells with lysis buffer containing 50 mM Tris–HCl (pH 7.5), 100 mM NaCl, 5 mM EDTA, 1% (v/v) TritonX-100, 1 mM NaF, 1 mM Na_3VO_4, 0.2 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. Protein content was determined using Bio-Rad DC protein assay kit (Bio-Rad). Then, 30 μg of protein was resolved on 8% gradient gels and transferred onto nitrocellulose membrane (Schleicher and Schuell, Keene, NH). After blocking with 5% nonfat milk, the blots were probed with a polyclonal anti-AE_3 antibody (1:1,000 dilution) 4°C overnight. After washing, the blots were incubated with HRP-conjugated secondary antibody (1:2,000 dilution) for 1 h at room temperature. After final washing, blots were incubated with Super-Signal West Pico chemiluminescense substrate reagent (Pierce Biotechnology, Inc. Rockford, IL) and developed using Kodak Biomax film. Developed blots were scanned, and the band intensities were determined using quantity one image analysis program (Bio-Rad). The results were normalized to the quantity of β-actin in each sample lane.

DETERMINATION OF $[Cl^-]_i$

The measurement of $[Cl^-]_i$ was performed according to the method described in our previous work with minor modifications [Lai et al., 2004; Huang et al., 2010]. First, the calibration curve for mean fluorescence intensity (MFI) of MQAE which changed with $[Cl^-]_i$ was prepared. Five solutions containing different $[Cl^-]_i$ (0.5, 5, 20, 50, and 150 mM) were individually mixed with MQAE (10 mM, final concentration) and the mixture was co-incubated in the dark for 60 min at 37°C. The MFI of MQAE was detected by a flow cytometry. As the fluorescence of MQAE is quenched by Cl^-, the relation between MFI and $[Cl^-]_i$ is inversely proportional. Next, the treated cells were harvested, washed twice with Cl^--free solution (NaCl was replaced by equimolar amounts of D-glucuronic acid; $MgCl_2$ by $MgSO_4$; 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 flushed off and the cells were resuspended in Cl^--free solution. The MFI was measured by FACS Callibur flow cytometer (Becton Dickinson, San Jose, CA) and was analyzed by CellQuest software. Finally, in the light of the calibration curve, $[Cl^-]_i$ was calculated.

DETERMINATION OF $[Ca^{2+}]_i$

The change in intracellular calcium level or $[Ca^{2+}]_i$ of cells was determined by the fluorescence of the calcium-sensitive dye fluo-3/AM as our previous described [Chen et al., 2007a]. 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 $CaCl_2$, 1 mM $MgSO_4$, 0.8 mM Na_2HPO_4, 0.2 mM NaH_2PO_4, 25 mM mannose, and 1 mg/ml BSA) containing 2 mM Fluo-3/AM and 0.008% Pluronic F-127 dissolved in DMSO. After incubation, the monolayers were washed in detaching buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 5 mM KCl, 0.55 mM $MgCl_2$, 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*g*), re-suspended in assay buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 5 mM KCl, 0.55 mM $MgCl_2$, and 1 mM $CaCl_2$), and analyzed on a flow cytometer.

DETECTION OF ROS GENERATION

ROS generation was determined using the cell-permeable probe cDCFH-DA, which is cleaved by cellular esterases to nonfluorescent 2′,7′-dichlorofluorescin (DCFH) and oxidized by intracellular ROS to a fluorescent product DCF. After the indicated treatments, the cells were harvested and washed with cold PBS. Washed cells were further incubated with10 μM of cDCFH-DA at 37°C for 20 min. Then, DCF fluorescence was monitored by flow cytometry at wavelengths of 495 nm (excitation) and 525 nm (emission). The generation of ROS was expressed as the MFI of 10,000 cells determined in each sample.

ASSAY OF APOPTOSIS

Apoptosis was determined by Annexin V and propidium iodide (PI) double staining. After experimental treatment, cells were detached with trypsin–EDTA, washed twice with PBS, and resuspended in 500 μl of binding buffer (10 mM HEPES, 140 mM NaCl, and 2.5 mM $CaCl_2$). The cells were then stained with Annexin V-FITC and PI according to the manufacturer's instructions. Apoptosis rates were determined by flow cytometry.

STATISTICAL ANALYSIS

Values are expressed as means ± SEM. One-way analysis of variance followed by the Student–Newman–Keuls test was applied to calculate the statistical significance between various groups. A value of $P < 0.05$ was considered to be statistically significant.

RESULTS

INVOLVEMENT OF AE_3 IN THE CARDIOPROTECTION OF SQS PRECONDITIONING

We first investigated the effect of SQS on the expression of AE_3 protein in H9c2 cells and found SQS concentration-dependently up-regulated AE_3 protein expression (Fig. 1). Subsequently, to determine whether AE_3 is required for SQS-induced cardioprotection against sI/R injury, normal and AE_3-knockdown H9c2 cells were pretreated with SQS (0.1, 1, or 10 μM) for 24 h, followed by sI/R. The releases of LDH and cell viability are used as indexes of cellular injury. As shown in Figures 2 and 3, SQS pretreatment concentration-dependently attenuated sI/R-induced viability loss

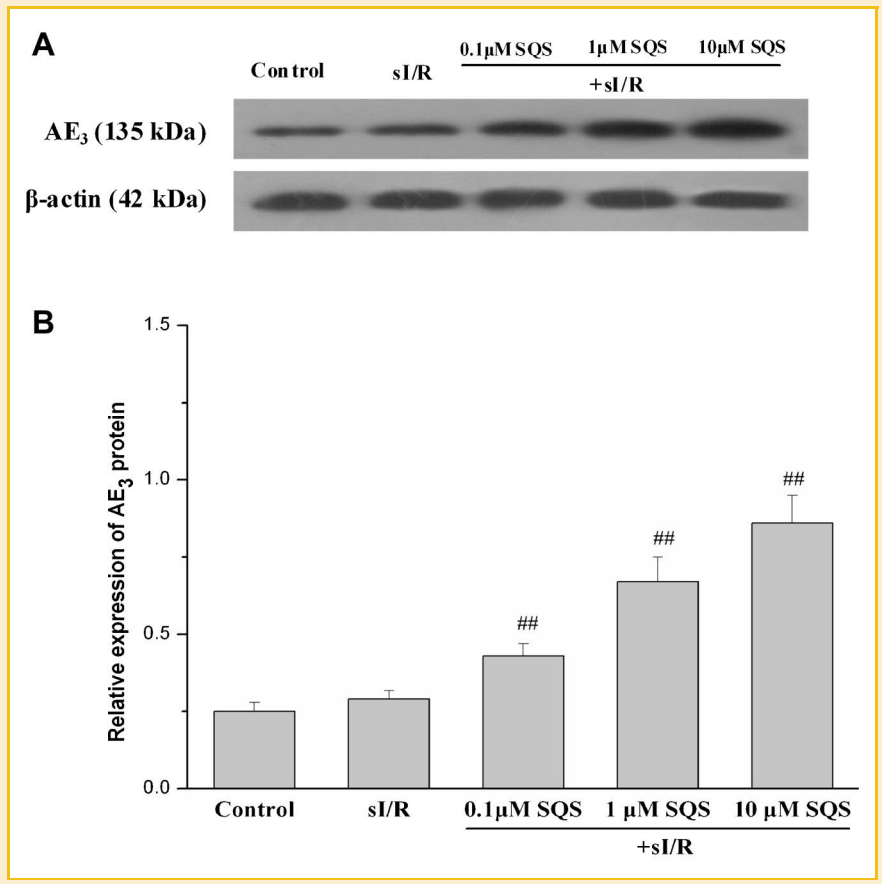

Fig. 1. Effect of SQS on the expression of AE_3 protein. H9c2 cells were incubated for 24 h with or without various concentrations of SQS (0.1, 1, or 10 μM) followed by simulated ischemia/reperfusion (sI/R). The expression of AE_3 was assessed by Western blot as described in the Materials and Methods Section. A: Representative Western blot for AE_3 protein. B: Relative levels of AE_3 protein as assessed by densitometry. Quantitations were normalized to value obtained for β-actin protein expression. Values are expressed as mean ± SEM of four independent experiments (n = 4). $^{\#\#}P < 0.01$ versus control group.

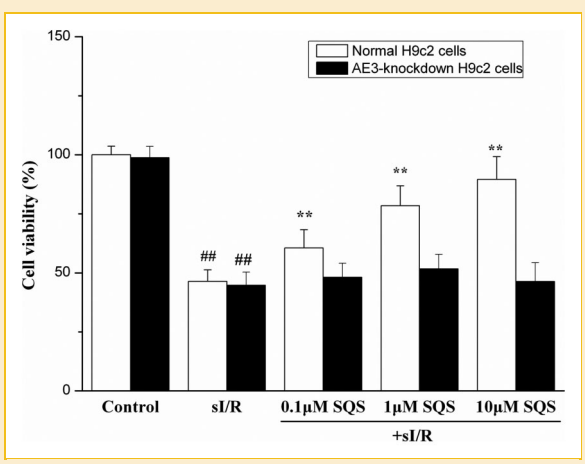

Fig. 2. Effect of SQS on cell viability of normal and AE_3-knockdown H9c2 cells subjected to simulated ischemia/reperfusion (sI/R). Normal and AE_3-knockdown H9c2 cells were preincubated for 24 h with or without various concentrations of SQS (0.1, 1, or 10 μM) followed by sI/R. Cell viability was measured by MTT assay as described in the Materials and Methods Section. All data were presented as mean ± SEM (n = 6). $^{\#\#}P < 0.01$ versus the corresponding control group; $^{**}P < 0.01$ versus the corresponding sI/R group.

(Fig. 2) and LDH leakage (Fig. 3) in normal H9c2 cells, indicating that SQS can exert direct cardioprotection from sI/R injury in a dose-dependent manner. However, in AE_3-knockdown H9c2 cells, the expression of endogenous AE_3 protein was suppressed by approximately 77% (Supplemental Fig. 1). Interestingly, SQS did not induce dose-dependent cardioprotection against sI/R injury in AE_3-knockdown cells, indicating that AE_3 is required for the cardioprotective effect elicited by SQS.

AE_3 IS REQUIRED BY SQS TO INHIBIT sI/R-INDUCED ELEVATION OF $[Cl^-]_i$

To investigate whether AE_3 is related to the inhibitory effect of SQS on elevation of $[Cl^-]_i$ induced by sI/R, we examined the effect of SQS on sI/R-induced elevation of $[Cl^-]_i$ in the normal and AE_3-knockdown H9c2 cells. As shown in Figure 4, in the normal H9c2 cells, when the cells suffered from sI/R, the $[Cl^-]_i$ was significantly increased and the peak of $[Cl^-]_i$ values was 49.7 ± 5.1 mM from 26.8 ± 3.8 mM in the control ($P < 0.01$). Treatment with SQS (0.1, 1, or 10 μM) produced a significant reduction in $[Cl^-]_i$ in normal H9c2 cells undergoing sI/R (37.34 ± 3.71, 31.87 ± 3.98, and 26.51 ± 2.77 nM, respectively), whereas SQS applied alone, under

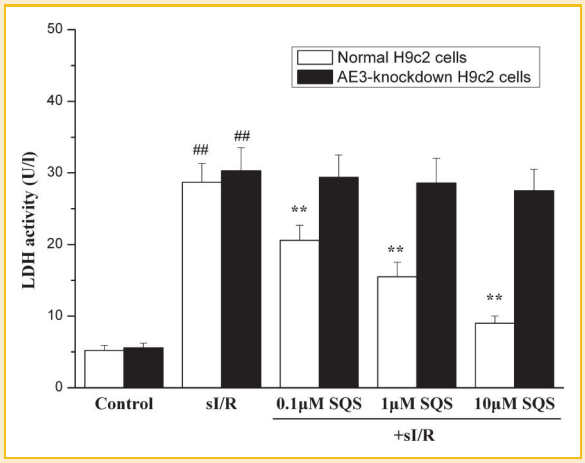

Fig. 3. Effect of SQS on LDH activity of normal and AE_3-knockdown H9c2 cells subjected to simulated ischemia/reperfusion (sI/R). Normal and AE_3-knockdown H9c2 cells were incubated for 24 h with or without various concentrations of SQS (0.1, 1, or 10 μM) followed by sI/R. Values are expressed as mean ± SEM (n = 6). $^{\#\#}P < 0.01$ versus the corresponding control group; $^{**}P < 0.01$ versus the corresponding sI/R group.

normoxic conditions, without sI/R, did not affect $[Cl^-]_i$. (data not shown). These results suggest that SQS can attenuate sI/R-induced the elevation of $[Cl^-]_i$ in a dose-dependent manner. Additionally, in AE_3-knockdown H9c2 cells, SQS did not delayed the sI/R-induced increase in $[Cl^-]_i$. These results indicate that AE_3 is required for SQS preconditioning to inhibit sI/R-induced elevation of $[Cl^-]_i$.

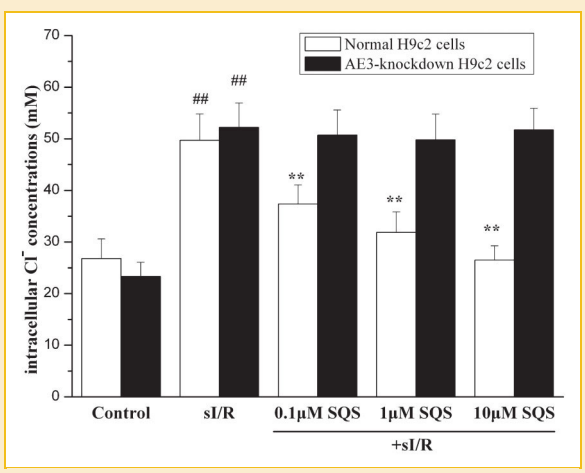

Fig. 4. Effect of SQS on intracellular Cl^- concentration ($[Cl^-]_i$) of normal and AE_3-knockdown H9c2 cells subjected to simulated ischemia/reperfusion (sI/R). Normal and AE_3-knockdown H9c2 cells were incubated for 24 h with or without various concentrations of SQS (0.1, 1, or 10 μM) followed by sI/R. The $[Cl^-]_i$ was determined by the fluorescence of MQAE with a flow cytometry as described in the Materials and Methods Section. Values are expressed as mean ± SEM (n = 4). $^{\#\#}P < 0.01$ versus the corresponding control group; $^{**}P < 0.01$ versus the corresponding sI/R group.

AE_3 IS REQUIRED BY SQS TO INHIBIT SI/R-INDUCED Ca^{2+} OVERLOAD

Next we detected the effect of SQS on sI/R-induced Ca^{2+} overload in the normal and AE_3-knockdown H9c2 cells. As shown in Figure 5, in the normal H9c2 cells, when the cells suffered from sI/R, the intracellular Ca^{2+} was significantly increased. After preincubation with 0.1, 1, and 10 μM SQS for 24 h, the sI/R-induced increase in $[Ca^{2+}]_i$ was concentration-dependently suppressed, indicating that SQS can attenuate sI/R-induced Ca^{2+} overload in a dose-dependent manner. However, in AE_3-knockdown H9c2 cells, SQS did not delayed the sI/R-induced increase in $[Ca^{2+}]_i$, indicating that AE_3 is required for SQS preconditioning to inhibit sI/R-induced elevation of $[Ca^{2+}]_i$.

AE_3 IS REQUIRED BY SQS TO REDUCE ROS PRODUCTION

In addition, we detected the effect of SQS on ROS production in the normal and AE_3-knockdown H9c2 cells following sI/R. As shown in Figure 6, in normal H9c2 cells, sI/R induced marked intracellular ROS production, while simultaneous pretreatment with 0.1, 1, and 10 μM SQS concentration-dependently inhibited ROS production, which had been induced by sI/R. Unfortunately, in AE_3-knockdown H9c2 cells, SQS lost its inhibitory effect on ROS production induced by sI/R, indicating that AE_3 is required for SQS preconditioning to inhibit sI/R-induced ROS production.

AE_3 IS REQUIRED BY SQS TO INHIBIT SI/R-INDUCED APOPTOSIS

Finally, we further investigated the effect of SQS on sI/R-induced apoptosis in the normal and AE_3-knockdown H9c2 cells. As shown in Figure 7, in normal H9c2 cells, after sI/R injury, the ratio of apoptotic cells (annexin V positive, PI negative-lower right quadrant of the dot plots in Fig. 7A) was significantly increased compared with the control ones, while SQS decreased apoptotic cells compared with sI/R in a concentration-dependent manner. However, in AE_3-knockdown H9c2 cells, SQS lost its inhibitory effect on apoptosis induced by sI/R, indicating that AE_3 is also required for SQS preconditioning to inhibit sI/R-induced apoptosis.

DISCUSSION

In the current study, we took advantage of AE_3-knockdown H9c2 cells to demonstrate for the first time that AE_3 is required as a mediator of SQS-cardioprotection, the inhibition of elevation of $[Cl^-]_i$ induced by sI/R and the subsequent attenuation of Ca^{2+} overload, ROS production, and cell apoptosis. These findings are summarized in a hypothetical scheme in Figure 8.

Recently, accumulating data of ischemic myocardial injury supports the elevation of $[Cl^-]_i$ induced by ischemia/reperfusion is an important pathophysiological factor. On one hand, the increased $[Cl^-]_i$ can activate the Cl^-–OH^- exchanger [Hume et al., 2000; Alvarez et al., 2004] to increase intracellular concentration of OH^-, an important member of the ROS family; on the other hand, the increased $[Cl^-]_i$ can also induce a increase in $[Ca^{2+}]_i$ due to the Cl^--increase induced Ca^{2+} release from intracellular stores [Campbell and Shamoo, 1980; Sukhareva et al., 1994; Lai, 2002]. Moreover, the increased $[Cl^-]_i$ can induce the mPTP opening, which results in ROS

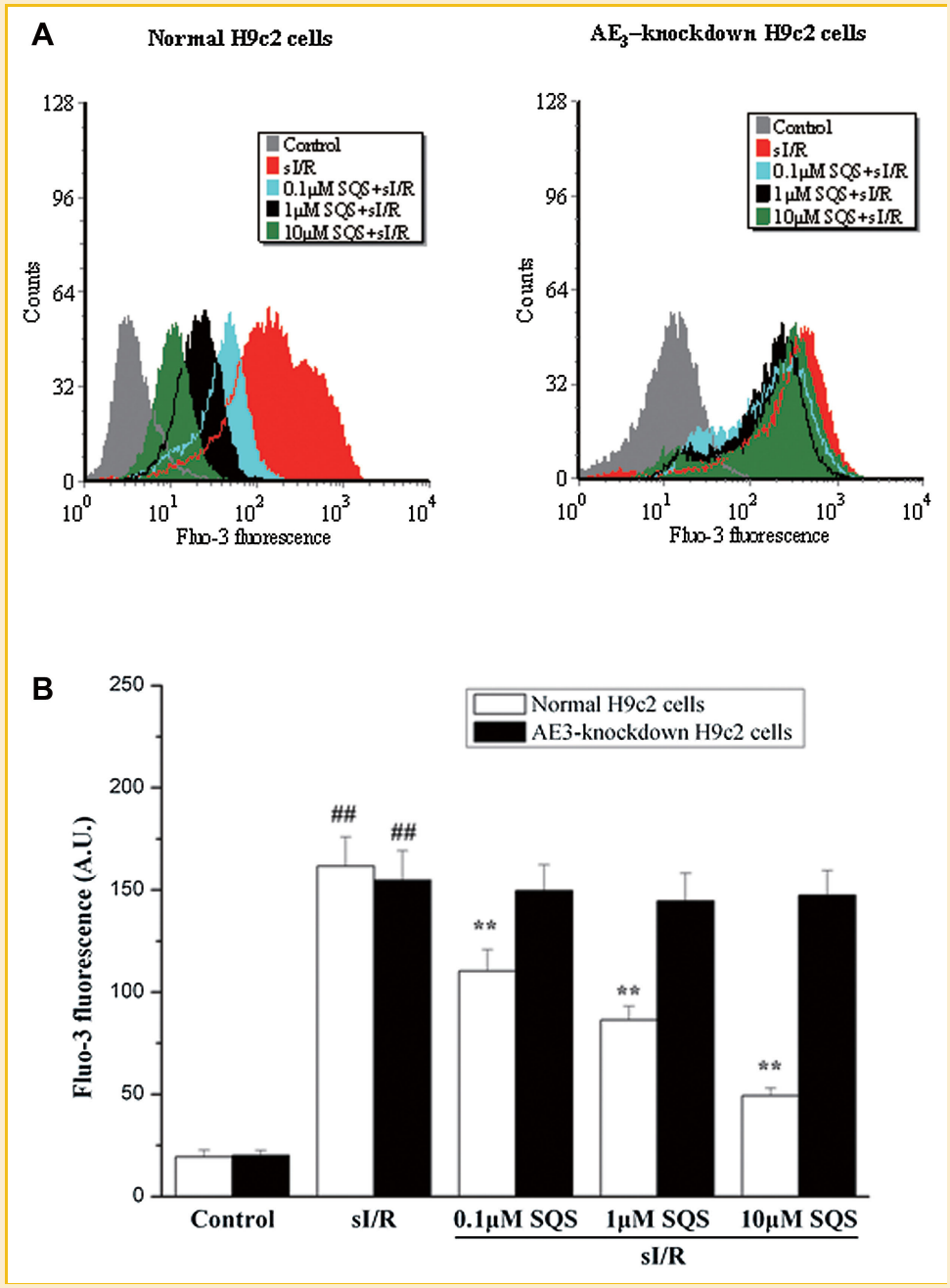

Fig. 5. Effect of SQS on intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) of normal and AE_3-knockdown H9c2 cells subjected to simulated ischemia/reperfusion (sI/R). Normal and AE_3-knockdown H9c2 cells were incubated for 24 h with or without various concentrations of SQS (0.1, 1, or 10 μM) followed by sI/R. The $[Ca^{2+}]_i$ was determined by fluorescence of Fluo-3 with a flow cytometry as described in the Materials and Methods Section. A: Flow cytometric histograms of Fluo-3. B: Column bar graph of cell fluorescence for Fluo-3. Values are expressed as mean ± SEM (n = 4). $^{\#\#}P < 0.01$ versus the corresponding control group; $^{**}P < 0.01$ versus the corresponding sI/R group. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

burst and subsequent ROS-dependent apoptosis[Huang et al., 2010]. In the present study, we also found that H9c2 cells subjected to sI/R have a rapid and significant increase in $[Cl^-]_i^-$ levels accompanied by increase of $[Ca^{2+}]_i$, ROS production, and cell apoptosis. It was also accompanied by H9c2 cells injury with marked increase of LDH activity and reduction of cell viability. These findings further suggest that dysregulation of $[Cl^-]_i^-$ homeostasis plays a significant role in the myocardial cell injury induced by ischemia/reperfusion. Therefore, the inhibition of elevation of $[Cl^-]_i$ induced by ischemia/reperfusion has been considered to be a reasonable therapeutic strategy to alleviate ischemia/reperfusion injury.

SQS is a biologically active ingredient extracted from the Chinese medicinal plant *Camellia oleifera* Abel and has gained considerable attention due to its wide range of biological and pharmacological properties. In previous studies, we have showed that SQS effectively protected against myocardium injury in an isoproterenol-induced

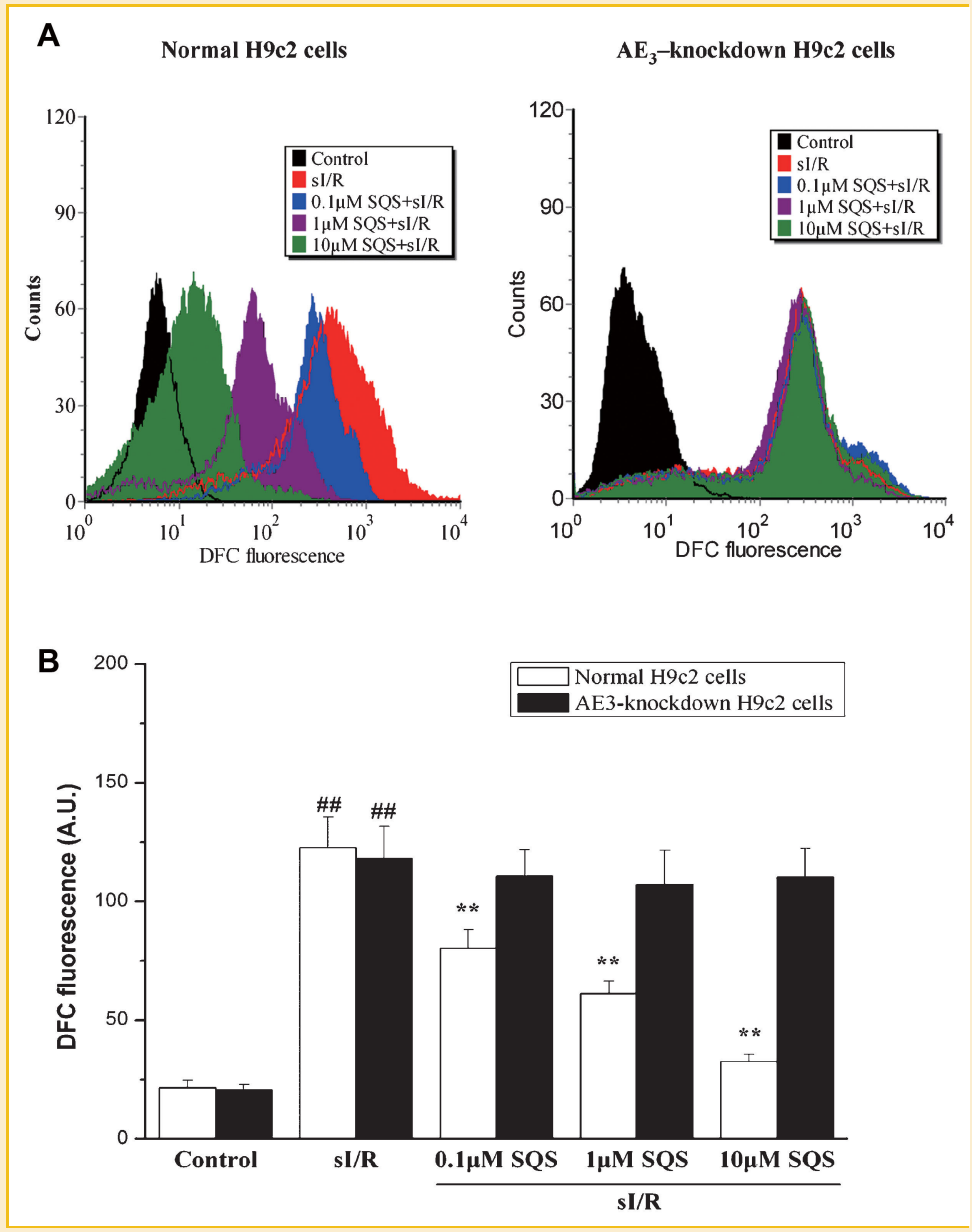

Fig. 6. Effect of SQS on ROS generation of normal and AE_3-knockdown H9c2 cells subjected to simulated ischemia/reperfusion (sI/R). Normal and AE_3-knockdown H9c2 cells were incubated for 24 h with or without various concentrations of SQS (0.1, 1, or 10 μM) followed by sI/R. The generation of ROS estimated by the fluorescence of 2′,7′-dichlorofluorescein (DCF) with a flow cytometry as described in the Materials and Methods Section. A: Flow cytometric histograms of DCF. B: Column bar graph of cell fluorescence for DCF. Values are expressed as mean ± SEM (n = 4). $^{\#\#}P < 0.01$ versus the corresponding control group; $^{**}P < 0.01$ versus the corresponding sI/R group. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

ischemia model in the rat in vivo or in a hypoxia and reoxygenation model in Langendorff-perfused rat hearts in vitro by inhibiting Ca^{2+} overload and production of ROS induced by ischemia [He et al., 1996; Li et al., 2000; Lai et al., 2002]. In a more recent study, Lai et al. [2004] have demonstrated that SQS protected myocardium against ischemia/reperfusion injury and exerted anti-arrhythmia effects by modulating $[Cl^-]_i^-$ homeostasis in mouse hearts. In the present study, we further found that SQS could suppress the elevation of $[Cl^-]_i$ induced by sI/R and induce cardioprotection from sI/R injury in a concentration-dependent manner in a H9c2 cell model. However, the molecular mechanisms by which SQS inhibits sI/R-induced elevation of $[Cl^-]_i$ remain unclear up to date. Accordingly, in the present study, we focus our attention on investigating the molecular mechanism of inhibitory effect SQS on the elevation of $[Cl^-]_i$ induced by sI/R in H9c2 cells.

It is known that $[Cl^-]_i^-$ homeostasis is predominantly modulated by membrane Cl^- channel proteins (ClCs) and ion transporters, such as AE_3 (Cl/HCO_3^- exchangers, AEs), Cl^-/OH^- exchanger, and Na^+–K^+–$2Cl^-$ cotransporter [Shennan, 2008]. Of these transporters, Na^+-independent AEs (SLC4A) exert a central role in the regulation of $[Cl^-]_i$, intracellular pH, blood pressure, and cardiovascular homeostasis [Faber et al., 1998]. To date, three

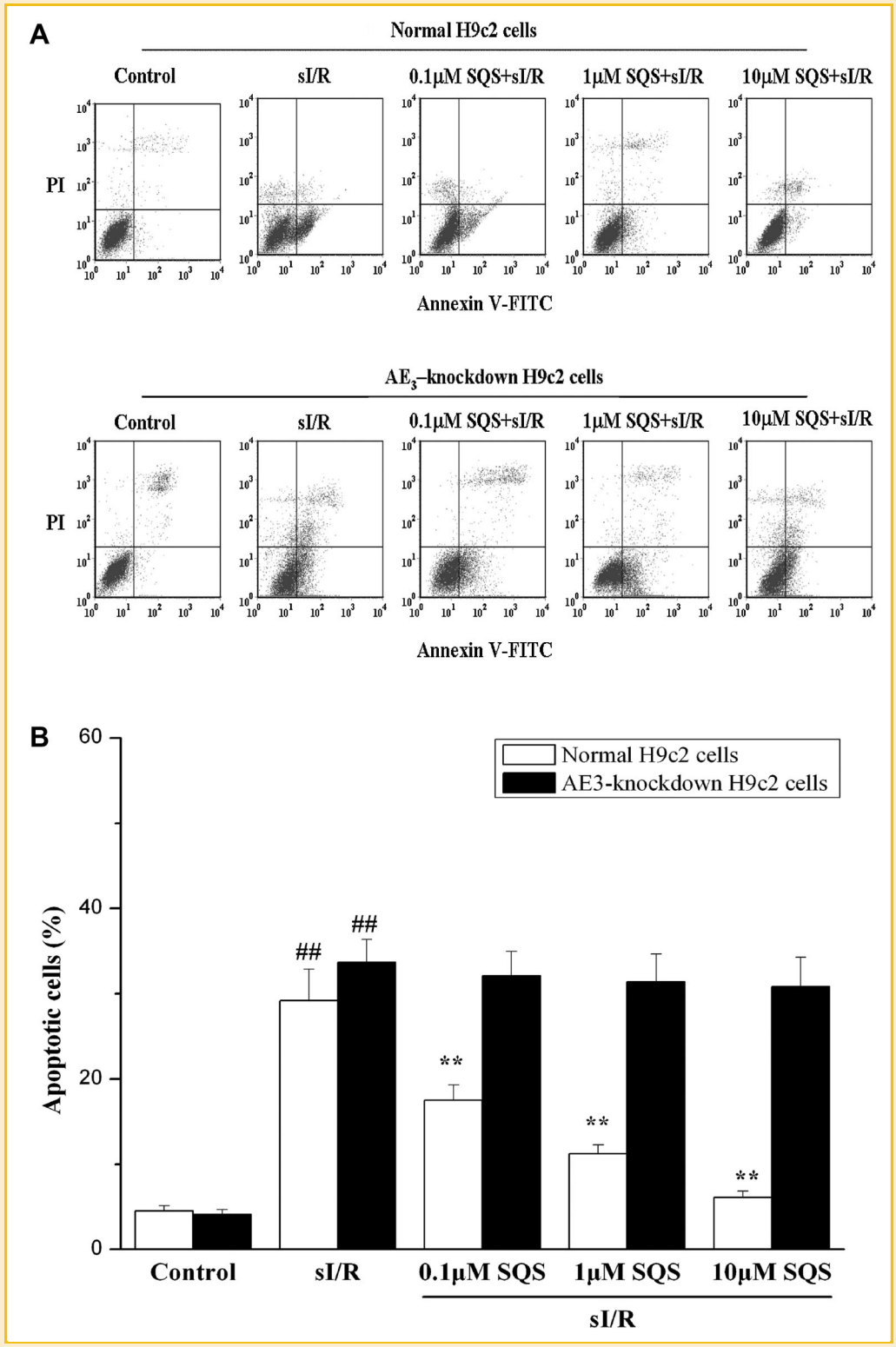

Fig. 7. Effect of SQS on cell apoptosis of normal and AE_3-knockdown H9c2 cells subjected to simulated ischemia/reperfusion (sI/R). Normal and AE_3-knockdown H9c2 cells were incubated for 24 h with or without various concentrations of SQS (0.1, 1, or 10 μM) followed by sI/R. Cell apoptosis was detected by a flow cytometry with annexin V-FITC/propidium iodide (PI) double staining. A: Representative flow cytometric dot plots (x-axis: annexin V staining/y-axis: PI staining) showing two-cell populations: annexin V/PI negative cells (normal cells, lower left quadrant) and annexin V positive/PI negative cells (early apoptotic cells, lower right quadrant) and annexin V positive/PI negative cells (late apoptotic cells, lower right quadrant). B: Quantitation of annexin V-positive/PI negative cell population as shown in (A). Values are expressed as mean ± SEM ($n = 4$). $^{\#\#}P < 0.01$ versus the corresponding control group; $^{**}P < 0.01$ versus the corresponding sI/R group.

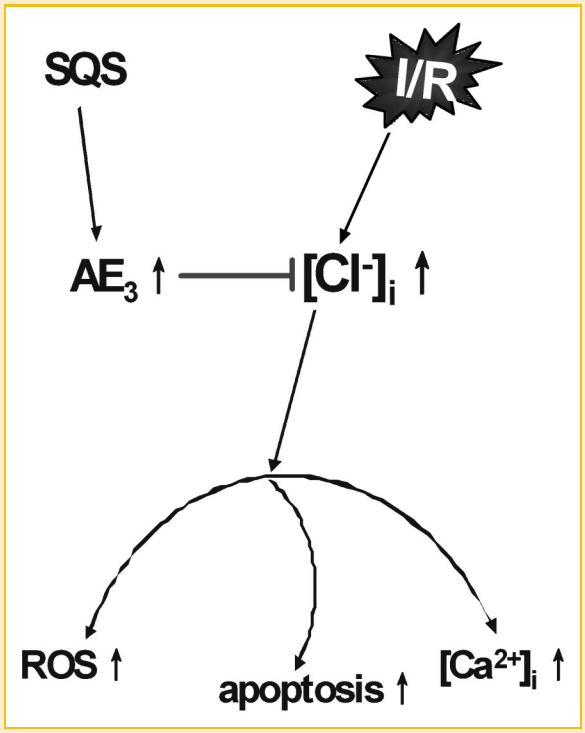

Fig. 8. Involvement of AE_3 in SQS-induced cardioprotection. AE_3 is required for SQS to inhibit sI/R-induced elevation of $[Cl^-]_i$, which subsequently inhibited sI/R-induced Ca^{2+} overload, ROS production, and cell apoptosis.

structurally and functionally related isoforms of the SLC4A/AEs family have been identified as AE_1, AE_2, and AE_3. The AE_1 (Band 3) and AE_2 are predominantly expressed in erythrocytes and epithelial cells, whereas the AE_3 is expressed at higher levels in the heart than in any other tissue [Romero et al., 2004; Alper, 2006]. The remarkable abundance of AE_3 in cardiac tissue suggests that AE_3 serves important functions in the heart, whereas the function of AE_3 is mainly to mediate both Cl^-/HCO_3^- and Cl^-/OH^- exchange. Recent studies have demonstrated that genetic ablation of AE_3 results in more rapid decompensation and heart failure in a hypertrophic cardiomyopathy model [Al Moamen et al., 2011]. Furthermore, our recent studies have found that AE_3 is involved in the cardioprotection of ischemic preconditioning by maintaining intracellular chloride homeostasis [Liu et al., 2006]. These reports indicate that AE_3 may be involved in the regulation of comprehensive cellular responses through catalyzing the reversible exchange of Cl^-/HCO_3^- across the plasma membrane in response to environmental stimuli in the heart.

Given that AE_3 plays important roles in regulating $[Cl^-]_i$, and is implicated in ischemic preconditioning, while SQS can effectively inhibit ischemia/reperfusion-induced elevation of $[Cl^-]_i$ and induce cardioprotection against ischemia/reperfusion. Accordingly, we hypothesized that AE_3 could mediate the cardioprotection of SQS against ischemia/reperfusion injury and be related to the inhibitory effect of SQS on elevation of $[Cl^-]_i$ induced by ischemia/reperfusion. To test our hypothesis we first determined, in H9c2 cells, the effect of SQS on expression of AE_3 protein, as well as LDH activity, cell viability, and $[Cl^-]_i$ after sI/R. We found that SQS concentration-dependently up-regulates AE_3 protein expression, accompanied by reduction of LDH release, increase of the viability, and prevention of the elevation of $[Cl^-]_i$ that normally follows sI/R injury. These findings suggest that the AE_3 protein may be involved in the cardioprotection of SQS. However, whether there is a causal relationship between AE_3 protein and SQS-cardioprotection deserves further exploration. Thus, we next investigated whether AE_3 is necessary for the inhibition effect of SQS on $[Cl^-]_i$ and SQS-cardioprotection using AE_3-knockdown H9c2 cells. As shown in this study, we found that SQS concentration-dependently reduced cell death following sI/R injury in normal H9c2 cells but not in AE_3-knockdown H9c2 cells, suggesting that AE_3 is required for the cardioprotective effect elicited by SQS. Crucially, we have shown that SQS inhibited sI/R-induced elevation of $[Cl^-]_i$. in a concentration-dependent manner in normal H9c2 cells but not in AE_3-knockdown H9c2 cells, suggesting that AE_3 is required for SQS to inhibit elevation of $[Cl^-]_i$. induced by sI/R.

Additionally, due to the fact that the elevation of $[Cl^-]_i$ induced by sI/R contributes to Ca^{2+} overload, ROS production, and cell apoptosis, we further detected the effects of SQS on Ca^{2+} overload, ROS production, and cell apoptosis in the normal and AE_3-knockdown H9c2 cells following sI/R. As expected, we observed SQS concentration-dependently attenuated Ca^{2+} overload, ROS production, and cell apoptosis, which had been induced by sI/R in normal H9c2 cells. However, in AE_3-knockdown H9c2 cells, the inhibitory effects of SQS on Ca^{2+} overload, ROS production, and cell apoptosis were reversed, suggesting that the AE_3 is also required for SQS to attenuate sI/R-induced Ca^{2+} overload, ROS production, and cell apoptosis.

In summary, we report for the first time that AE_3 is critical to the cardioprotective effect of SQS against sI/R injury. Importantly, we show that AE_3 is required for SQS to inhibit sI/R-induced elevation of $[Cl^-]_i$, which subsequently inhibited sI/R-induced Ca^{2+} overload, ROS production, and cell apoptosis. These findings may be beneficial in understanding the molecular mechanism associated with SQS-induced cardioprotection and lightening the cellular effect and pharmacological profiles of SQS.

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