Effects of Sasanquasaponin on Ischemia and Reperfusion Injury in Mouse Hearts

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Abstract. We investigated effects of sasanquasaponin (SQS), a traditional Chinese herb’s effective component, on ischemia and reperfusion injury in mouse hearts and the possible role of intracellular Cl⁻ homeostasis on SQS’s protective effects during ischemia and reperfusion. An in vivo experimental ischemia model was made in mice (weight 27 – 45 g) using ligation of left anterior descending coronary artery, and in vitro models were made in perfused hearts by stopping flow or in isolated ventricular myocytes by hypoxia. The in vivo results showed that SQS inhibited cardiac arrhythmias during ischemia and reperfusion. Incidence of arrhythmias during ischemia and reperfusion, including ventricular premature beats and ventricular fibrillation, was significantly decreased in the SQS-pretreated group (P<0.05). Results in perfused hearts showed that SQS suppressed the arrhythmias, prevented against ischemia-induced decrease in contract force and promoted the force recovery from reperfusion. Furthermore, intracellular Cl⁻ concentrations ([Cl⁻]i) were measured using a MQAE fluorescence method in isolated ventricular myocytes in vitro. SQS slightly decreased [Cl⁻]i in non-hypoxic myocytes and delayed the hypoxia/reoxygenation-induced increase in [Cl⁻]i during ischemia and reperfusion (P<0.05). Our results showed that SQS protected against ischemia/reperfusion-induced cardiac injury in mouse hearts and that modulation of intracellular Cl⁻ homeostasis by SQS would play a role in its anti-arrhythmia effects during ischemia and reperfusion.

Keywords: sasanquasaponin, ischemia and reperfusion injury, arrhythmia, intracellular Cl⁻ concentration, left anterior descending coronary artery

Introduction

The genetically altered mouse models provide important insights into a wide variety of processes in cardiovascular electrophysiology, pathophysiology, and development of hearts (1 – 3). Therefore, many investigators make an ischemia and reperfusion model in the mouse and use this model to estimate the cardiac physiology or electrophysiological functions in some genetically altered mice to understand the complex pathophysiology of ischemia and reperfusion injury (4 – 6). The mouse model can provide a powerful tool to further our understanding of basic mechanisms that underlie human cardiac electrophysiology or pathophysiology in some cardiac diseases (7).

Because the cardiac diseases still seriously influence human health, the cardiac injury occurring during the ischemia and reperfusion period have been a subject of investigation for many years, and the study in development of new anti-arrhythmia drugs is still an important but imminent work. According to this strategy, our group began to search for some effective anti-arrhythmia drugs from Chinese traditional medicine and herbs. Sasanquasaponin (22-O-angeloylcamelliagenin C-3-O-[β-D-glucopyranosyl(1,2)] [β-D-glucopyranosyl(1,2)-O-L-arabinopyranosyl(1,3)]-β-D-glucopyranosiduronic acid, SQS), one of the effective components of Chinese traditional herbs, was a mixture of many kinds of saponin structures origin from sasanqua Camellia oleifera ABEL (Fig. 1). The main structure of SQS is a
In the present study, we examined effects of SQS on intracellular Cl\textsuperscript{-} homeostasis in single mouse ventricular myocytes during hypoxia and reoxygenation with fluorescence dye methods. Our study provided systemic data to show that SQS provides effective protection against ischemia and reperfusion-induced cardiovascular injury in mouse hearts and suggest that the mouse model is a useful tool for investigating cardiac ischemia and reperfusion injury.

Fig. 1. Chemical structure of sasanquasaponin. Sasanquasaponin (SQS, molecular weight is 1204.0352) is a saponin obtained from a traditional medicine, Camellia oleifera Abel. The chemical structure has a triterpenoid and is similar to ginseng saponin and other saponins. The active chemical constituent is 22-O-angeloylcamelliaigenin C-3-O-[β-D-glucopyranosyl(1,2)] [β-D-glucopyranosyl(1,2)-O-β-L-arabinopyranosyl(1,3)]-β-D-glucopyranosyl-siduronic acid.

**Materials and Methods**

**Experiments in vivo**

Ischemia and reperfusion model in mouse hearts in vivo: Adult male ICR mice (Charles River Japan, Inc., Yokohama), age 12 – 18 weeks (weight 27 – 45 g), were used in this study, and all animals were handled in accordance with “Rules of the Animal Experimentation Committee, Kumamoto University, School of Medicine”. Animals were anesthetized with intraperitoneal injection of pentobarbital (50 – 60 mg/kg); and then they were tracheotomized, intubated, and ventilated with a small animal ventilator (SAR-830/P; CWE, Ardmore, PA, USA). The tidal volume and ventilation rate were 0.5 – 0.6 ml and 100 – 120 strokes per min, respectively. Thoracotomy was performed through the third intercostal space of the left side, and the pericardium was opened as previously described (12 – 14). Then, 7-0 silk threads were passed around the left anterior descending coronary artery (LAD) using a tapered needle. Electrodes were placed to record the second limb electrocardiogram (ECG) through experiments. The artificial acute myocardial ischemia was induced by ligation of LAD. Myocardial ischemia was confirmed by regional cyanosis and S-T segment elevation in the ECG. Reperfusion was induced by releasing the ligation and confirmed by a rapid color change in the surface of the myocardium. The arrhythmias were observed and the incidence of arrhythmias during ischemia and reperfusion was analyzed.

Measurements of blood pressure (BP), dP/dT and infusion of SQS: A cervical incision was made, the trachea was intubated, and the animal was connected to a volume-cycled ventilator as described above. Then, a PE 10 flame-stretched, fluid-catheter was introduced into the left common carotid artery and attached to a transducer element (AP-621G; Nihon Kohden, Tokyo) for BP recording, and the signal was transferred by EG-600G for dP/dT recording. The second cannula using a PE 10 catheter was inserted to the left femoral vein for injection of drugs. BP, dP/dT, and heart rates (HR) were monitored continuously during the following protocols and recorded on a thermal arraycorder (WR8500-UM-101; Graphtec, Yokohama) per 5 min or saved on a magneto-optical disk and analyzed by the PowerLab system (BRC, Tokyo) in a Macintosh computer.

**Experiments in vitro**

Preparation of isolated mouse hearts: Isolated heart preparation was performed using a method modified for the mouse heart as described (15). Mice (ICR, 25 – 35 g) were injected intravenously with heparin (5000
U/kg). After 30 min, the mice were anesthetized with sodium pentobarbital (50–60 mg/kg, i.p.), and the thorax was opened. The heart was quickly excised, placed in ice-cold saline, and immediately mounted, via the ascending aorta, onto a perfusion apparatus. The heart was perfused with a non-recirculating perfusate at a constant flow (1.6 ± 0.1 ml/min) and pressure of approximately 100 mmHg (Langendorff preparation). The perfusate was a modified Tyrode solution containing: 127 mM NaCl, 4.7 mM KCl, 0.25 mM MgCl\textsubscript{2}, 1.2 mM KH\textsubscript{2}PO\textsubscript{4}, 5.5 mM glucose, and 2.5 mM CaCl\textsubscript{2}. The perfusate was equilibrated with 5% CO\textsubscript{2}/95% O\textsubscript{2} at 37°C, and pH was adjusted to 7.4 with 20 or 25 mM NaHCO\textsubscript{3} to 7.4. The perfusate in the reservoir was continuously stirred in albumin-free Tyrode solution containing 0.1% bovine serum albumin (Fraction V; Sigma, St. Louis, MO, USA) followed, after which the hearts were treated with 0.06% collagenase (YK-102; Yakult, Tokyo) in the same solution as -tolerant rod-shaped cells were used for experiments. All experiments were finished within 24 h.

**Hypoxia and reoxygenation model in single myocytes:** Myocytes were perfused initially with a normal Tyrode solution equilibrated with a gas mixture of 95% O\textsubscript{2}/5% CO\textsubscript{2} and pH was adjusted to 7.4 at 37°C. To simulate hypoxia and reoxygenation, we switched to modified Tyrode solution pH 6.8 at 37°C without glucose to prevent glycolysis and aerated the cells with a gas mixture of 95% N\textsubscript{2}/5% CO\textsubscript{2} before treating them with normal Tyrode solution. Cl\textsuperscript{-} or HCO\textsubscript{3}\textsuperscript{-}-free solution was prepared by equimolar substitution with gluconate or HEPES, respectively. The perfusion rate was fixed at 1.0 ml/min with a peristaltic pump throughout the experiment, and it took approximately 30 s to change the perfusion media in the chamber.

**Measurement of [Cl\textsuperscript{-}] with MQAE fluorescence dyes:** For [Cl\textsuperscript{-}] measurements, isolated ventricular myocytes were incubated with 5 mM N-(6-methoxyquinolyl)acetoxymethyl ester (MQAE; Dojindo Laboratories, Kumamoto) for 60 min at 37°C. Then the cells were washed with the Tyrode solution, and the cells were placed on a poly-L-lysine-coated mounted coverslip dish (35-mm diameter, MatTek Corp., Ashland, MA, USA) and incubated for 30 min. Then, microfluorometric measurements were made with an image processor system (ACOUScosmos; Hamamatsu Photonics Corp., Hamamatsu) as described (17). The fluorescence intensity of MQAE was collected and stored on a magneto-optical disk every 4 s until analysis.

**Calibration of intracellular MQAE:** The myocytes were resuspended in an experimental Cl\textsuperscript{-}-free solution (NaCl was substituted by equimolar amounts of D-glucuronic acid; MgCl\textsubscript{2}, by MgSO\textsubscript{4}; KCl, by potassium gluconate), centrifuged once, and resuspended in a cuvette with 350 μl of Cl\textsuperscript{-}-free medium plus 10 mM glucose and 0.1% BSA. The fluorescence intensity was monitored using an ACOUScosmos imaging system (excitation 350 nm, emission 460 nm) at room temperature (24°C).

Calibration of intracellular MQAE intensity was performed by determining the correlation between fluorescence intensity and Cl\textsuperscript{-} concentration by a procedure modified from those described by Krapf et al. (18). The maximal fluorescence in the absence of Cl\textsuperscript{-} was obtained by depleting intracellular Cl\textsuperscript{-} by adding the ionophores nigericin (7 μM) and tributyltin acetate (10 μM) to the isolated mouse ventricular myocytes in the Cl\textsuperscript{-}-free solution. To see change of MQAE fluorescence intensity with different Cl\textsuperscript{-} concentration in perfused solution, five different concentrations of Cl\textsuperscript{-} were made (0.5, 5, 20, 50, 150 mM) and added to the dish, respectively. Then the MQAE fluorescence intensities were recorded. At the end of the experiment, 150 mM KSCN, in the

**Action potential recording in isolated ventricular papillary muscles:** Mice were stunned, and their hearts were quickly removed, placed in a cold Tyrode solution, and the cells were placed on a poly-L-lysine-coated mounted coverslip dish (35-mm diameter, MatTek Corp., Ashland, MA, USA) and incubated for 30 min. Then, microfluorometric measurements were made with an image processor system (ACOUScosmos; Hamamatsu Photonics Corp., Hamamatsu) as described (17). The fluorescence intensity of MQAE was collected and stored on a magneto-optical disk every 4 s until analysis.

**Preparation of isolated single mouse cardiomyocytes:** To obtain single mouse ventricular myocytes, mouse hearts were isolated from adult male ICR mice anesthetized with diethyl ether and Langendorff-perfused for 10 min with oxygenized Tyrode solution at 37°C. A brief perfusion with a Ca\textsuperscript{2+}-free Tyrode solution containing 0.1% bovine serum albumin (Fraction V; Sigma, St. Louis, MO, USA) followed, after which the hearts were treated with 0.06% collagenase (YK-102; Yakult, Tokyo) in the same solution as described (16). After 30-min collagenase treatment, the ventricles were minced and incubated for 20 min with stirring in albumin-free Tyrode solution containing 0.2 mM Ca\textsuperscript{2+} and 0.001% trypsin (Type 3, Sigma). The cells obtained by pipetting were filtered through 120-mm nylon mesh, centrifuged (85–100 × g for 3 min), and resuspended in the normal Tyrode solution. The Ca\textsuperscript{2+}-tolerant rod-shaped cells were used for experiments. All experiments were finished within 24 h.

**Hypoxia and reoxygenation model in single myocytes:** Myocytes were perfused initially with a normal Tyrode solution equilibrated with a gas mixture of 95% O\textsubscript{2}/5% CO\textsubscript{2} and pH was adjusted to 7.4 at 37°C. To simulate hypoxia and reoxygenation, we switched to modified Tyrode solution pH 6.8 at 37°C without glucose to prevent glycolysis and aerated the cells with a gas mixture of 95% N\textsubscript{2}/5% CO\textsubscript{2} before treating them with normal Tyrode solution. Cl\textsuperscript{-} or HCO\textsubscript{3}\textsuperscript{-}-free solution was prepared by equimolar substitution with gluconate or HEPES, respectively. The perfusion rate was fixed at 1.0 ml/min with a peristaltic pump throughout the experiment, and it took approximately 30 s to change the perfusion media in the chamber.

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presence of 5 μM of the K⁺ ionophore valinomycin, was added to quench the fluorescence of MQAE. The SCN⁻ ion has a much high affinity for the indicator than Cl⁻ ion and therefore quenches most of the MQAE fluorescence at the given Cl⁻ concentration minus F_{KSCN}. The calibration curve of intracellular MQAE was obtained from the data. The Cl⁻ free solution was made by replacement of Cl⁻ with equimolar gluconate as described previously (19).

Reagents and statistical analyses

All salts for preparing solutions and poly-L-lysine and other pharmacological agents were purchased from Sigma. Sodium gluconate, potassium gluconate, and calcium gluconate monohydrate were purchased from Wako (Osaka). Sasanquasaponin (C_{35}H_{42}O_{26}, FW is 1204.0352) was extracted and isolated from Camellia oleifera ABEL and purified by the previously described method (20). Data were analyzed by basic statistical methods, including analysis of the two-tailed Student’s t-test (unpaired) and ANOVA or Chi-square test and expressed as the arithmetic means ± S.E.M. A P<0.05 value was considered to be statistically significant.

Results

SQS slightly inhibited HR and decreased BP in non-ischemia mice

We examined effects of SQS on BP and HR in non-ischemia mice. SQS at a concentration of 0.1 mg/kg slightly decreased HR from 347 ± 24 beats/min of the control to 307 ± 40 beats/min at 10 min after application of drugs (n = 9, P<0.05). After intravenous injection of SQS at 0.1 mg/kg, BP was immediately decreased from 91 ± 7 mmHg in the control to 70 ± 12 mmHg, and this decrease was continued to 10 min after administration of the drug (Fig. 2: A and B, n = 9, P<0.05). However, even though it still inhibited HR at high concentrations (0.2, 0.4 mg/kg), SQS increased BP and got a peak level of BP at 98 ± 14 or 102 ± 10 mmHg, respectively (Fig. 2: B and C, P<0.05).

SQS inhibited incidence of arrhythmias during ischemia and reperfusion in vivo

Then we observed the effects of SQS on ischemia and reperfusion injury in vivo. The mouse received 30-min ischemia following 30-min reperfusion; then BP and HR was recorded and the incidence of arrhythmia during ischemia and reperfusion were observed. The arrhythmia type observed during the ischemia and reperfusion period included ventricular premature beats (VPB); escape beats (EB); bradycardia (BR), bradycardia and tachycardia (B and T), bigeminy (BG); salvos (SA); ectopic beats (EC); ventricular tachycardia (VT); ventricular fibrillation (VF); AV dissociation (AVD) (see Fig. 3A). The arrhythmia type in mouse in vivo was similar to that occurring in the rat (13, 21, 22), rabbit (23), or dog (24, 25). However, the incidence of arrhythmias was different from other animal types (26). In the present study, we showed that during the ischemia period, the incidence of VT and VF was relatively lower (VT, 4/13, 30.7%; VF, 2/13, 15.4%) and the incidences of BR and EB or AVD were relatively higher. Similar results were also observed in the reperfusion period (Fig. 3B).

When mice were injected with intravenous administration of SQS 10 min before ligation of LAD, the incidences of arrhythmias during both ischemia and reperfusion periods in mouse hearts were decreased (Fig. 3B, Chi-square test, P<0.05). However, there was no significantly change in systolic BP in the SQS-treated group compared with that in the non-SQS-treated group (LAD ligation induced decrease in systolic BP about 20 mmHg). Furthermore, we investigated the direct effects of SQS on arrhythmias that occurred during ischemia and reperfusion in some cases. In these experiments, the ischemia and reperfusion model was made by the same protocol as above (30-min ischemia followed by 30-min reperfusion), and the ECG, BP, and dP/dT were persistently monitored. However, SQS was not pretreated before ischemia but was immediately administrated with intravenous injection once the arrhythmia appeared. Figure 4 shows a typical recording in which SQS effectively inhibited the SA type arrhythmias during reperfusion. Similar results were obtained in another 4 animals and indicated that SQS had acute treatment effects on arrhythmias, including SA- and tachy-arrhythmias, during ischemia or reperfusion (data not shown).

SQS prevented the ischemia-induced decrease in contraction force and promoted recovery from reperfusion in Langendorff-perfused hearts

We examined possible protective effects of SQS on change of contraction force in Langendorff-perfused mouse hearts during ischemia and reperfusion. The ischemia and reperfusion model was made in Langendorff-perfused mouse hearts, and the contraction force of hearts was monitored using a PowerLab system. The contraction force of hearts gradually decreased during ischemic period and then ceased at approximately 11 min after ischemia (11.6 ± 2.5 min). When the hearts begin to reperfusion with Tyrode solution, the contraction force gradually recovered to the control level. After treated with SQS 0.1 μM, the time to cessation of contraction force during ischemia was prolonged com-
pared with that in the non-SQS group \( (P<0.05) \). The recovery of contraction force during the reperfusion period in the SQS-treated group was also significantly faster than that in the no-SQS group \( (P<0.05) \). Furthermore, the elevation in resting tension of muscles induced by ischemia was suppressed in the SQS-treated group (Fig. 5).

Similarly, when the Cl\(^-\) in perfused solution was replaced with iso-molar gluconate, or when hearts were perfused with SITS (4-acetamido-4'-isothiocyanostilbene-2,2'-disulfonic acid) at a concentration of 0.1 mM, change of contraction force during ischemia and reperfusion was the same as those obtained from SQS-treated hearts.

**SQS inhibited cardiac arrhythmias during ischemia and reperfusion in vitro**

We then analyzed whether SQS has any effects on incidence of ischemia and reperfusion induced arrhythmias in Langendorff-perfused mouse hearts. As shown in Table 1, we calculated the related incidence of arrhythmias occurring in the SQS- or SITS-treated group during ischemia and reperfusion. Results showed that both SQS and SITS (a Cl\(^-\)-transport blocker) significantly decreased the incidence of arrhythmias that occurred in both the ischemia and reperfusion periods (compared with non-drug group, Chi-square test, \( P<0.05 \)).
SQS slightly decreased [Cl\(^{-}\)] in non-ischemic myocytes and delayed the ischemia- and reperfusion-induced increase in [Cl\(^{-}\)].

To see whether SQS has any effects on intracellular Cl\(^{-}\), we measured [Cl\(^{-}\)] using MQAE fluorescence methods in isolated mouse cardiac myocytes (Fig. 6). Single mouse ventricular myocytes were isolated enzymatically from mouse ventricle and then centrifuged and resuspended in a low Ca\(^{2+}\) Tyrode solution followed by a normal Ca\(^{2+}\) solution. In non-ischemic cardiac myocytes, the value of [Cl\(^{-}\)] in mouse cardiac myocytes was in the range of 25 mM – 35 mM. This value was similar to previous reporters in human Jurkat T lymphocytes (17), but slightly higher than that reported in cardiac muscles in the guinea pig (19). However, SQS slightly decreased the [Cl\(^{-}\)] in the non-ischemic condition. In the non-ischemia group, the [Cl\(^{-}\)] was 28.6 ± 8.5 mM in the control and 18.4 ± 8.5 mM in the SQS-treated group (P<0.05). The effects of SQS were similar to the effects of SITS, a stilbene derivative, which was reported in our previous study (19). When the cells suffered from hypoxia and reoxygenation, the Cl\(^{-}\) was...
significantly increased and the peak of [Cl\textsuperscript{-}] values was 48.9 ± 9.6 mM from 28.6 ± 8.5 mM in the control (P<0.05). When the cells were pre-treated with SQS at a dose of 0.1 μM, the ischemia-induced increase in [Cl\textsuperscript{-}] was significantly suppressed (28.4 ± 4.9 mM, P<0.05). Similarly, SITS, one of the stilbene derivatives, also protected against ischemia-induced increase in [Cl\textsuperscript{-}] during ischemia and reperfusion in mouse myocytes. The time course of inhibition by SQS on [Cl\textsuperscript{-}] during ischemia and reperfusion is similar to that by SITS (Fig. 7).

**SQS shortened the action potential duration and induced hyperpolarization in isolated ventricular papillary muscle**

Because SQS decreased the BP in mice in vivo (Fig. 2) and previous reports showed that effects of SQS on isoprenaline-induced ST-elevation in rats can be suppressed by glibenclamide known as a K\textsubscript{ATP} channel blocker (10), we investigated whether SQS has any effect acting as an ATP-dependent K\textsuperscript{+} channel opener on mouse ventricular muscules and measured the action potential in isolated ventricular papillary muscles using glass microelectrode techniques. As shown in Fig. 8, SQS at a concentration of 0.1 μM shortened the action potential duration and hyperpolarized the cells. This effect of SQS can be blocked by glibenclamide at a concentration of 1 μM.

**Discussion**

Our results showed that SQS protected against ischemia- or hypoxia-induced deterioration in mouse hearts in vivo and in vitro. Because SQS also suppressed ischemia-induced elevation of [Cl\textsuperscript{-}] in isolated single mouse cardiomyocytes, we suggest that SQS would play an important role in its anti-arrhythmia effects during ischemia and reperfusion in mouse cardiac cells by modulation of intracellular Cl\textsuperscript{-} homeostasis. On the other hand, the ischemia and reperfusion model in the mouse in the present study reproduced the cardiac ischemia and reperfusion injury, including decrease in BP and contractile force, increase in incidence of arrhythmias, and elevation of [Cl\textsuperscript{-}]. It was similar to previous reports in the rat (27, 28), dog (29, 30) or guinea pig (19). Furthermore, the effect of SQS on arrhythmias in the present study was similar to that reported in the rat (10), and the effect of SITS on ischemia-induced increase in [Cl\textsuperscript{-}] was similar to that in guinea pig ventricular muscle (19). Therefore, it is apparent that the ischemia and reperfusion model in mouse is credible and useful.

The mechanism through which SQS protected against ischemia- and reperfusion-induced injuries in mouse hearts is complicated and still unclear. However, besides SQS’s suppression on Ca\textsuperscript{2+} overload as previously described (10), one possibility is that SQS acts as a K\textsubscript{ATP} channel opener to induce a pharmacological preconditioning like it did in the rat model described (11) or like nicorandil did in the rabbit (31). Indeed, in the present study, we found that intravenous bolus injection of SQS at a dose of 0.1 mg/kg transiently decreased BP, including systolic pressure and diastolic pressure. SQS at the same concentration also induced shortening of action potential duration and hyperpolarization of
Fig. 5. SQS prevented the ischemia-induced decrease in contraction force and promoted the recovery of contraction force during perfusion. A: Representative example of ischemia and reperfusion induced changes in contract force in the no-drug (upper), SQS 0.1 μM (middle), and in a Cl⁻-free condition (bottom). B: The time of contraction force cessation after pretreatment with SQS and in Cl⁻-free conditions. Open bars, control; closed bars, data obtained from SQS-treated group; hatched bars, values obtained in a Cl⁻-free condition. C: Recovery of the force from reperfusion in application of SQS or in a Cl⁻-free condition. Open circles, control; closed circles, SQS at a concentration of 0.1 μg/ml. Open squares, values obtained in a Cl⁻-free condition. *, Compared with control, P<0.05.

Table 1. Sasanquasaponin and SITS protected against cardiac arrhythmias during ischemia and reperfusion

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<th>Ischemia-induced arrhythmia (%)</th>
<th>Reperfusion-induced arrhythmia (%)</th>
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<tr>
<td></td>
<td>VPB</td>
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<tr>
<td>Control</td>
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<td>(12/12)</td>
<td>(12/12)</td>
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<td>0.1 μM SQS</td>
<td>75</td>
<td>83</td>
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<td>(9/12)</td>
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<td>100 μM SITS</td>
<td>83</td>
<td>67</td>
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Isolated mouse hearts (n = 12 per group) were subjected to a 10-min period of equilibration, 30 min of ischemia, and 30 min of reperfusion, and arrhythmias were observed. VPB, ventricular premature beats; BG, bigeminy; SA, salvos; VT, ventricular tachycardia; VF, ventricular fibrillation. *P<0.05 compared with controls (Chi-square test). Numerals in parentheses indicate numbers on hearts in which arrhythmias were occurred.
cells in isolated mouse ventricular muscles that can be blocked by glibenclamide, a $K_{ATP}$ channel antagonist (Fig. 8). Thus, it is possible that SQS at a low concentration may act as an opener of ATP-dependent $K^+$ channels and resulted in a decrease of BP in vivo and APD shortening or hyperpolarization in vitro. Therefore,
the protective effects of pretreatment with SQS at this concentration before starting ischemia in the mouse model in vivo and in vitro would result from SQS-induced effective preconditioning.

However, the present study also showed that not only pretreatment of SQS but also immediate intravenous application of the drug inhibited the arrhythmias during ischemia and reperfusion. The data in Fig. 4 clearly showed that immediate injection of SQS inhibited the SA type arrhythmias in mouse hearts. Obviously, this effect of SQS cannot be attributed to its pharmacological preconditioning only. Interestingly, data obtained from isolated single myocardial cells showed that SQS slightly decreased [Cl\textsuperscript{-}], in non-ischemia ventricular myocytes and suppressed the ischemia-induced increase in [Cl\textsuperscript{-}], in the mouse. Because both SQS and SITS have similar effects to promote the recovery of contractile function, it is possible that this suppression effect of SQS’s protective effects are exerted through the same mechanism as those by SITS, that is, by modulating intracellular Cl\textsuperscript{-} homeostasis. Furthermore, even though we considered SQS as a K\textsubscript{ATP} channel opener, it is not surprising that SQS has effects on [Cl\textsuperscript{-}], because a previous study showed that some ATP-sensitive K\textsuperscript{+} channel inhibitors, for example, U83757, might act as potent Cl\textsuperscript{-} channel blockers in the smooth muscle cell line A10 (32).

Another study also showed that glibenclamide increased [Cl\textsuperscript{-}], which was significantly blocked by the cystic fibrosis transmembrane conductance regulator (CFTR) Cl\textsuperscript{-} channel activators levamisole and bromotetramisole in HepG2 human hepatoblastoma cells (33). That means a K\textsubscript{ATP} channel opener can decrease the [Cl\textsuperscript{-}], by inhibition of the CFTR Cl\textsuperscript{-} channel or by blocking some Cl\textsuperscript{-} transporters. The later possibility may be confirmed by experiments with the stilbene derivative SITS in isolated ventricular myocytes or in perfused hearts as shown in Figs. 5 and 6. Similar results in our unpublished data also showed that cromakalim, known as a potent K\textsubscript{ATP} channel opener, dose-dependently decreased [Cl\textsuperscript{-}], in guinea pig ventricular muscles and cultured cardiac myocytes that can be blocked by glibenclamide. However, SQS at high concentrations, such as 0.2 and 0.4 mg/kg, did not decrease but increased the BP in non-ischemic mice. This elevation of BP in SQS-treated mice with high concentrations may be other effects of SQS on the vascular system via some unknown pathways.

It is known that intracellular Cl\textsuperscript{-} homeostasis plays an important role in maintaining the resting membrane potential or regulating action potential firing. During cardiac ischemia, in fact, our previous studies showed that a simulated ischemia induced a marked increase of [Cl\textsuperscript{-}], in the quiescent guinea pig ventricular papillary muscle (34). The increment in [Cl\textsuperscript{-}], via activation of the Cl\textsuperscript{-}-HCO\textsubscript{3}\textsuperscript{-} exchanger during ischemia or hypoxia would partially contribute to the generation of arrhythmias (35). Using pH-selective microelectrodes to measure intracellular pH (pH\textsubscript{i}) in ischemic guinea pig ventricular muscles, we also showed that the application of stilbene derivatives suppressed the rapid onset of ischemia-induced intracellular acidosis and delayed the onset of ischemia-induced deterioration of action potentials in cardiac muscles (34). Under a Cl\textsuperscript{-}-free condition, the time to cessation of action potentials caused by ischemia was significantly delayed, and the development of intracellular acidosis during ischemia was attenuated. These results indicate that activation of the Cl\textsuperscript{-}-HCO\textsubscript{3}\textsuperscript{-} exchange system may result in counter-transport of intracellular HCO\textsubscript{3}\textsuperscript{-} and extracellular Cl\textsuperscript{-} and, therefore, is partly involved in the development of intracellular acidosis during the early phase of cardiac ischemia. Therefore, the data in the present study may provide new evidence to support a hypothesis that the inhibition of development of intracellular acidosis by decrease in activity of Cl\textsuperscript{-}-HCO\textsubscript{3}\textsuperscript{-} exchanger is favorable for recovery of contractile function during ischemia and reperfusion. Furthermore, because SQS can attenuate the ischemia-induced Cl\textsuperscript{-} increase by activation of K\textsubscript{ATP} channels, it is possible that this suppression effect of SQS may influence the Cl\textsuperscript{-}-Ca\textsuperscript{2+} interaction by inhibition.
of the Cl\(^-\) increase-induced Ca\(^{2+}\) release from intracellular stores (36 – 38) and by suppression of the ischemia-induced membrane depolarization. Then, as a result, the incidence of arrhythmias may be suppressed.

By way of summary, our findings suggest that SQS had some protective effects on ischemia- or hypoxia-induced deterioration both in vitro and in vivo. The facts that anti-arrhythmia effects of SQS in the mouse in the present study were similar to that in rats indicated that the ischemia and reperfusion model in the mouse is a useful tool to research cardiovascular diseases. Modulation of intracellular Cl\(^-\) homeostasis by SQS would partially play a role in its anti-arrhythmia effects during ischemia and reperfusion in mouse cardiac cells. However, it is still unclear how SQS-induced-K\(_{ATP}\) channel activation results in attenuation of intracellular Cl\(^-\) accumulation during ischemia and reperfusion, and further investigations need to be undertaken.

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