

Full-length article

Schisandrin B decreases the sensitivity of mitochondria to calcium ion-induced permeability transition and protects against ischemia-reperfusion injury in rat hearts¹

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Key words

schisandrin B; mitochondria; permeability transition; calcium; myocardial ischemia-reperfusion

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Abstract

Aim: In order to elucidate the molecular mechanism underlying the cardioprotection afforded by schisandrin B (Sch B), the effect of Sch B treatment on the sensitivity of mitochondria to Ca²⁺-stimulated permeability transition (PT) was investigated in rat hearts under normal and ischemia–reperfusion (I–R) conditions. **Results:** Myocardial I–R injury caused an increase in the sensitivity of mitochondria to Ca²⁺-stimulated PT *in vitro*. The enhanced sensitivity to mitochondrial PT was associated with increases in mitochondrial Ca²⁺ content as well as the extent of reactive oxidant species production *in vitro* and cytochrome c release *in vivo*. The cardioprotection afforded by Sch B pretreatment against I–R-induced injury was paralleled by the decrease in the sensitivity of myocardial mitochondria to Ca²⁺-stimulated PT, particularly under I–R conditions. **Conclusion:** The results suggest that Sch B treatment increases the resistance of myocardial mitochondria to Ca²⁺-stimulated PT and protects against I–R-induced tissue injury.

Introduction

Schisandrin B (Sch B, Figure 1) is the most abundant, active dibenzocyclooctadiene derivative isolated from the fruit of *Schisandra chinensis* (Turcz) Baill, a traditional Chinese herb clinically used for the treatment of viral and chemical hepatitis^[1]. Previous studies in our laboratory have demonstrated the ability of Sch B to protect against myocardial ischemia-reperfusion (I–R) injury^[2]. The cardioprotection

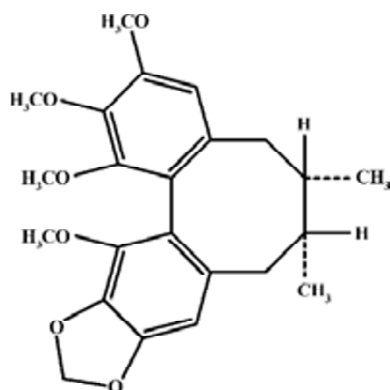


Figure 1. Chemical structure of Sch B.

afforded by Sch B pretreatment were associated with the enhancement in tissue glutathione antioxidant status, particularly in the mitochondrion^[2–4]. Recent studies also showed that Sch B protected against myocardial I–R injury partly by inducing heat shock protein (Hsp)25 and Hsp70 expression in rats^[5]. However, it is still unclear whether Sch B treatment produces any effect on the sensitivity of the mitochondria to permeability transition (PT). Growing evidence has accumulated suggesting the involvement of PT of the mitochondrial inner membrane in the pathogenesis of oxidant injury in various tissues, including myocardial I–R injury^[6,7]. One critical event of mitochondrial PT is the permeability of the inner membrane to small ions and solutes with $M_r < 1500$ daltons, with the consequent, large amplitude swelling of the mitochondria^[8]. The opening of mitochondrial PT pores plays an important role in regulating necrotic and apoptotic cell death^[9]. While the loss of ion homeostasis resulting from ATP depletion following PT can lead to necrosis^[10]. PT also causes the leakage of cytochrome c from the mitochondria to the cytosol^[11]. The released cytochrome c can trigger a cascade of events that eventually lead to apoptosis^[11,12].

Increased Ca^{2+} , reactive oxidant species (ROS), and inorganic phosphate, as well as decreased membrane potential, have been shown to activate mitochondrial PT, whereas Ca^{2+} chelator, antioxidants, such as glutathione and ubiquinone analogs, ATP/ADP and other translocase ligands, such as cyclosporin A (CsA), inhibit mitochondrial PT^[13,14]. In order to further elucidate the molecular mechanism underlying the cardioprotective action of Sch B, we endeavoured to investigate the effect of Sch B treatment on the sensitivity of the mitochondria to Ca^{2+} -stimulated PT in rat hearts under normal and I-R conditions. Changes in mitochondrial Ca^{2+} content, ROS production, and cytochrome c release were also examined in relation to alterations in the sensitivity to mitochondrial PT.

Materials and methods

Chemicals and herbal material CsA was purchased from Sigma Chemical Co (St Louis, MO, USA). All other chemicals were of analytical grade. The dried fruits of *Schisandra chinensis* were imported from mainland China. They were authenticated and supplied by a commercial dealer (Lee Hoong Kee Limited, Hongkong, China) in Hong Kong. Sch B was purified from the petroleum ether extract of *Schisandra* fruits, with the purity being higher than 95% as determined by HPLC analysis^[15].

Animal care Sprague-Dawley rats (8–10 weeks old, weighing 250–300 g) were maintained under a 12 h dark/light cycle at about 22 °C and allowed food and water *ad libitum* in the Animal Care Facilities at the Hong Kong University of Science and Technology (HKUST, Hong Kong, China). All experimental protocols were approved by the University Committee on Research Practice at HKUST.

Drug treatment The animals were randomly divided into groups of 5 animals. In the pretreatment groups, the rats were treated intragastrically with Sch B (dissolved/suspended in olive oil) at a single dose of 1.0 or 2.0 mmol/kg. Untreated controls received the vehicle only. The hearts were isolated from the rats and subjected to I-R experiment 48 h following the administration of Sch B. Previous studies indicated that the optimal single dose of Sch B for protecting against myocardial I-R injury ranged from 1 to 2 mmol/kg, and the maximum protective effect was observed at 48 h post-dosing with Sch B^[3].

Isolated-perfused rat hearts The hearts were excised quickly from the phenobarbital-anesthetized rats and immediately immersed in ice-cold and heparinized (50 unit/mL) saline. The aorta was cannulated and then transferred to a warm, moist chamber of the perfusion apparatus. The hearts were retrogradely perfused, with the coronary flow rate rang-

ing from 8 to 12 min, according to the Langendorff method as described^[16].

Myocardial I-R After an initial 30 min of perfusion for equilibration, the isolated hearts were subjected to a 40 min period of “no-flow” global ischemia followed by 20 min reperfusion. Coronary effluent was collected in 1 min fractions at increasing time intervals during the course of equilibration and reperfusion. The fractions were immediately placed on ice until assayed for lactate dehydrogenase (LDH) activity. The extent of LDH leakage during the reperfusion period, an indirect index of myocardial injury, was estimated by computing the area under the curve (AUC) of the graph plotting the percentage of LDH activity (with respect to the mean pre-ischemic value measured during the equilibration period) against the reperfusion time (1–20 min) as described^[4], and the value was expressed as arbitrary units (AU). Non-I-R hearts were perfused for 90 min. After the non-I-R or I-R procedure, ventricular tissue samples were obtained and subjected to biochemical analysis.

Preparation of mitochondrial fractions and cytosolic fractions Myocardial ventricular tissue samples were rinsed with ice-cold isotonic buffer [210 mmol/L mannitol, 70 mmol/L sucrose, 5 mmol/L HEPES, 1 mmol/L EGTA (pH 7.4) 0.2 mg/mL soybean trypsin inhibitor, 0.2 mg/mL bacitracin, and 0.16 mg/mL benzamidine]. Myocardial tissue homogenates were prepared by homogenizing 0.8 g minced tissues in 8 mL ice-cold isotonic buffer. The mitochondrial fractions were prepared by differential centrifugation as described^[17]. The homogenates were centrifuged at 600×g for 20 min. After collecting the supernatants, the pellets were resuspended with the same volume of ice-cold homogenizing buffer (without various protease inhibitors) and recentrifuged at 600×g again. The procedure was repeated twice. The pooled supernatants (a total of 4 volumes) were centrifuged at 8000×g for 30 min, and the mitochondrial pellets were collected. The supernatant was saved for the preparation of the cytosolic fraction. The mitochondrial pellets were then washed with the same volume of ice-cold sucrose buffer containing 210 mmol/L mannitol, 70 mmol/L sucrose, and 5 mmol/L HEPES–KOH (pH 7.4) and the mixtures were centrifuged at 8000×g for 30 min. The washing procedure was repeated. The mitochondrial pellets were resuspended in 0.5–1.0 mL ice-cold sucrose buffer and constituted the mitochondrial fractions. The mitochondrial fractions, prepared from various rat tissues by a similar protocol in our laboratory, were found to carry out oxidative phosphorylation in a normal manner^[18]. The cytosolic fraction was prepared by centrifuging the above supernatant at 100 000×g for 60 min at 4 °C.

Mitochondrial ROS generation The extent of mitochondrial ROS generation *in vitro* was estimated as an indirect measure of mitochondrial antioxidant capacity and structural integrity. An aliquot (50 μ L) of mitochondrial fraction (50 μ g protein/mL) and 60 μ L 2',7'-dichlorofluorescein diacetate (DCFDA; Fluka, Switzerland) solution (5 μ mol/L in incubation buffer containing 0.1% DMSO) were added into the wells of a black microtiter plate. The mixture was incubated at 37 °C for 10 min under dark condition in a Victor² Multi-Label Counter (Perkin–Elmer, Wellesley, MA, USA). After the incubation, 50 μ L incubation buffer [0.1 mmol/L EGTA, 5 mmol/LKH₂PO₄, 3 mmol/LMgCl₂, 145 mmol/LKCl, 30 mmol/L HEPES (pH 7.4)] and 50 μ L substrate solution (20 mmol/L pyruvate and 10 mmol/L malate) were added. Fluorescence intensity (excitation at 485 nm and emission at 535 nm) of the reaction mixture was monitored every 5 min for 30 min. The mitochondrial ROS generation was reflected by the fluorescence intensity of the sample after subtracting the value of a blank sample containing incubation buffer, substrate solution, and DCFDA. The extent of ROS generation over the 30 min period of incubation was estimated by computing the AUC of the graph plotting fluorescent intensity against time (0–30 min) and expressed as an AU.

Mitochondrial PT The measurement of mitochondrial PT was performed according to a procedure modified from Kowaltowski *et al*^[19]. An aliquot (1.6 mL) of the mitochondrial sample (0.5 mg protein/mL) was prepared by mixing the mitochondrial fraction with incubation buffer containing 125 mmol/L sucrose, 65 mmol/L KCl, 10 mmol/L HEPES (pH 7.2), 5 mmol/L freshly prepared succinate, and 5 μ mol/L freshly prepared rotenone. Aliquots (200 μ L) of the mitochondrial sample were mixed with 10 μ L of CsA (5 μ mol/L) in 0.5% (*w/w*) ethanol final concentration or incubation buffer. The mixtures were incubated at 30 °C for 5 min. An aliquot (10 μ L) of calcium chloride (Ca²⁺) solution (1 μ mol/L final concentration) was then added, and the mixtures were incubated at 30 °C for 5 min. Aliquots (180 μ L) of the mixtures were added into a 96-well microtiter plate, and the initial absorbance of the mixtures at 520 nm was monitored for 5 min at 30 °C. The swelling reaction was then started by adding 20 μ L potassium phosphate (0.5 mmol/L, pH 7.2), and the absorbance at 520 nm of the reaction mixtures was read every 2 min for 30 min at 30 °C using the Victor V² Multi-Label Counter. The extent of mitochondrial swelling was estimated by computing the AUC of the declining graph plotting the percentage of the initial absorbance (100% as baseline) against time (min) to obtain AUC₁. The extent of mitochondrial PT (Δ AUC₁) was estimated by subtracting the AUC₁ with CsA from the AUC₁ without CsA. Ca²⁺-induced mito-

chondrial PT was expressed in a ratio of Δ AUC₁ induced by both Ca²⁺ and PO₄³⁻ to that induced by PO₄³⁻ only.

Mitochondrial Ca²⁺ content The Ca²⁺ content was measured using a Ca²⁺-sensitive fluorescence probe Fluo-5N AM ester (Molecular Probe, OR, USA) by the Victor² V Multi-Label Counter, as described in Menze *et al*^[20]. The Ca²⁺ dissociation constant (*K*_d) was determined by using a Ca²⁺ calibration kit at a concentration range of 1–1000 μ mol/L, with the *K*_d value estimated at being ~98 μ mol/L, which is in agreement with the data provided by the manufacturer. An aliquot (25 μ L) of the mitochondrial fraction (0.5 mg/mL final concentration) was mixed with 25 μ L incubation buffer (100 mmol/L KCl and 30 mmol/L MOPS, pH 7.2) in a 96-well black microtiter plate. The mixture was incubated at 25 °C for 15 min and then added with 25 μ L digitonin (50 μ g/mL) and 25 μ L Fluo-5N AM ester (1 μ mol/L in 0.005% Pluronic F-127). Presumably, by permeating the membrane, digitonin can facilitate the entry of the fluorescence dye into the mitochondria. Experimental data indicated that the fluorescence intensity of Fluo-5N measurable in rat heart mitochondrial preparations was increased by 20-fold in the presence of digitonin. The reaction mixture was incubated at 25 °C for 30 min, and the fluorescence reading was measured at the excitation wavelength of 488 nm and emission at 532 nm. The mitochondrial Ca²⁺ content was estimated from the standard calibration curve and expressed in μ mol/mg protein.

Biochemical analysis The LDH activity of coronary effluent was measured as described^[4]. The cytosolic cytochrome c level, as an indirect measure of mitochondrial cytochrome release, was estimated by Western blot analysis using specific antibodies of cytochrome c (clone 7H8.2C12, BD PharMingen, San Diego, CA, USA) following SDS–PAGE analysis of the cytosolic fractions using a separating gel of 15% acrylamide. The extent of mitochondrial contamination in the cytosolic fractions was determined using specific antibodies against complex IV. The immunoblots were visualized by enhanced chemiluminescence reaction (Amersham ECL+, Piscataway, NJ, USA) and analyzed by densitometry (Bio-Rad). The amounts (AU) of cytochrome c were normalized with reference to actin levels (AU) in the sample. The protein concentrations of the mitochondrial fractions were determined using a Bio-Rad protein assay kit (Hercules, CA, USA).

Statistical analysis Data were analyzed by one-way ANOVA. *Post-hoc* tests for pair-wise multiple comparisons were done with Least Significant Difference, using SPSS statistical software (SPSS, Chicago, USA). *P*-values <0.05 were regarded as statistically significant.

Results

Ca^{2+} , when added at a final concentration of $1 \mu\text{mol/L}$ in the presence of PO_4^{3-} , enhanced the time-dependent decrease in absorbance at 520 nm , an indirect measure of mitochondrial swelling, in a suspension of heart mitochondria (Figure 2). The Ca^{2+} -stimulated mitochondrial swelling was largely inhibited by CsA ($5 \mu\text{mol/L}$). While mitochondria from non-I-R and I-R hearts exhibited a comparable and small extent of swelling in the absence of Ca^{2+} , they showed a larger extent of swelling in the presence of Ca^{2+} , with the extent of Ca^{2+} -induced PT of I-R mitochondria being larger than that of the non-I-R control. The extent of CsA-inhibitable mitochondrial swelling induced by Ca^{2+} was computed and served as an indirect measure of mitochondrial PT, as described in Materials and methods.

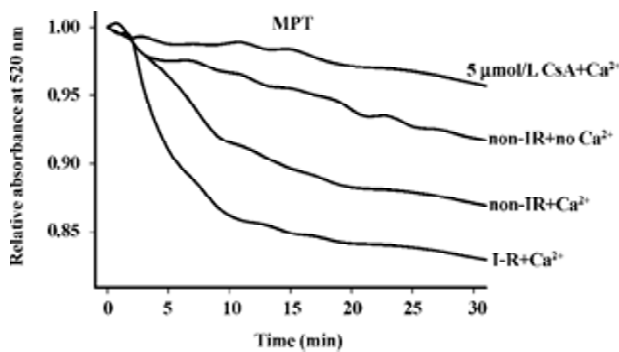


Figure 2. I-R-induced change in the sensitivity of mitochondria to Ca^{2+} -stimulated permeability transition (PT) *ex vivo*. Isolated rat hearts were subjected to 20 min of ischemia followed by 40 min of reperfusion (I-R). Myocardial mitochondria were isolated and subjected to measurement of PT induced by Ca^{2+} at a final concentration of 1 mmol/L . CsA was added at a final concentration of $5 \mu\text{mol/L}$. Each data point is the mean value from the 5 animals. Initial absorbance at 520 nm of the mitochondrial mixture was 0.587 ± 0.08 (SD).

Pretreatment with Sch B ($1\text{--}2 \text{ mmol/kg}$, *po*) protected against myocardial I-R injury in rats, as evidenced by the significant decrease in the extent of LDH leakage ($23\%\text{--}38\%$), when compared with the untreated control (Figure 3A). While Sch B treatment at the same dosage suppressed Ca^{2+} -stimulated PT in heart mitochondria ($7\%\text{--}15\%$), when compared with the untreated control, I-R caused a slight, but significant increase in the sensitivity of heart mitochondria to Ca^{2+} -stimulated PT (by 18%) in the rats (Figure 3B). The inhibitory effect of Sch B treatment on mitochondrial PT was also evident after the I-R challenge. The pretreatment with Sch B significantly reduced the extent of mitochondria PT (by $9\%\text{--}18\%$) in I-R hearts, when compared with the untreated I-R control (Figure 3B).

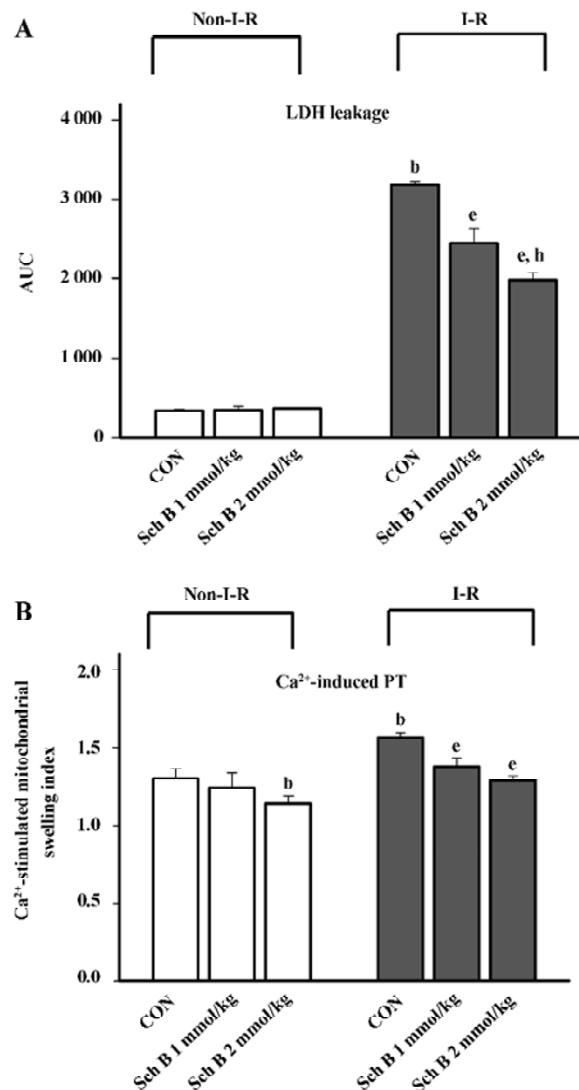


Figure 3. Effect of Sch B pretreatment on (A) I-R-induced myocardial injury and (B) change in the sensitivity of mitochondria to Ca^{2+} -stimulated PT. Rats were orally administered with Sch B at $1\text{--}2 \text{ mmol/kg}$. Forty eight hours after the dosing with Sch B, isolated hearts were subjected to I-R challenge. LDH activity in coronary effluent was measured. $n=5$. Each bar represents the mean \pm SEM. ^b $P < 0.05$ vs non-I-R Sch B untreated control (CON) group; ^c $P < 0.05$ vs I-R CON group; and ^h $P < 0.05$ vs 1 mmol/kg Sch B I-R group.

I-R caused significant increases in the mitochondrial Ca^{2+} level (21%), as well as the extent of mitochondrial ROS production (15%) and cytochrome c release (67%) in the rat hearts (Figure 4A–4C). While Sch B treatment dose-dependently decreased the mitochondrial Ca^{2+} level (by $35\%\text{--}55\%$) in non-I-R rat hearts (Figure 4A), the extent of mitochondrial ROS production and cytochrome c release remained relatively unchanged (Figure 4B,4C). Sch B pretreatment dose-

dependently suppressed the I-R-induced increases in the mitochondrial Ca^{2+} level, ROS production, and cytochrome c release to varying degrees (71%–85%, 23%–30%, and 19%–24%, respectively) when compared with the respective Sch B untreated control (Figure 4A–4C).

Discussion

Mitochondria not only generate ATP through oxidative phosphorylation, but are also an important source of ROS in mammalian cells under both physiological^[21] and pathological conditions, such as myocardial I-R injury^[22]. As far as

the maintenance of cellular structural and functional integrity is concerned, the mitochondria serve as coordinators for cell survival and death^[23]. In this regard, the opening of the mitochondrial transition pore is critically involved in the development of cellular dysfunction and cell death^[24]. In the present study, the I-R-induced myocardial injury was associated with the increase in the sensitivity of the mitochondria to Ca^{2+} -induced PT, as assessed by *in vitro* measurement of mitochondrial swelling. Core components of mitochondrial PT pore putatively consist of a voltage-dependent anion channel, an adenine nucleotide translocase (ANT), and cyclophilin D that displays peptidyl-prolyl *cis*-

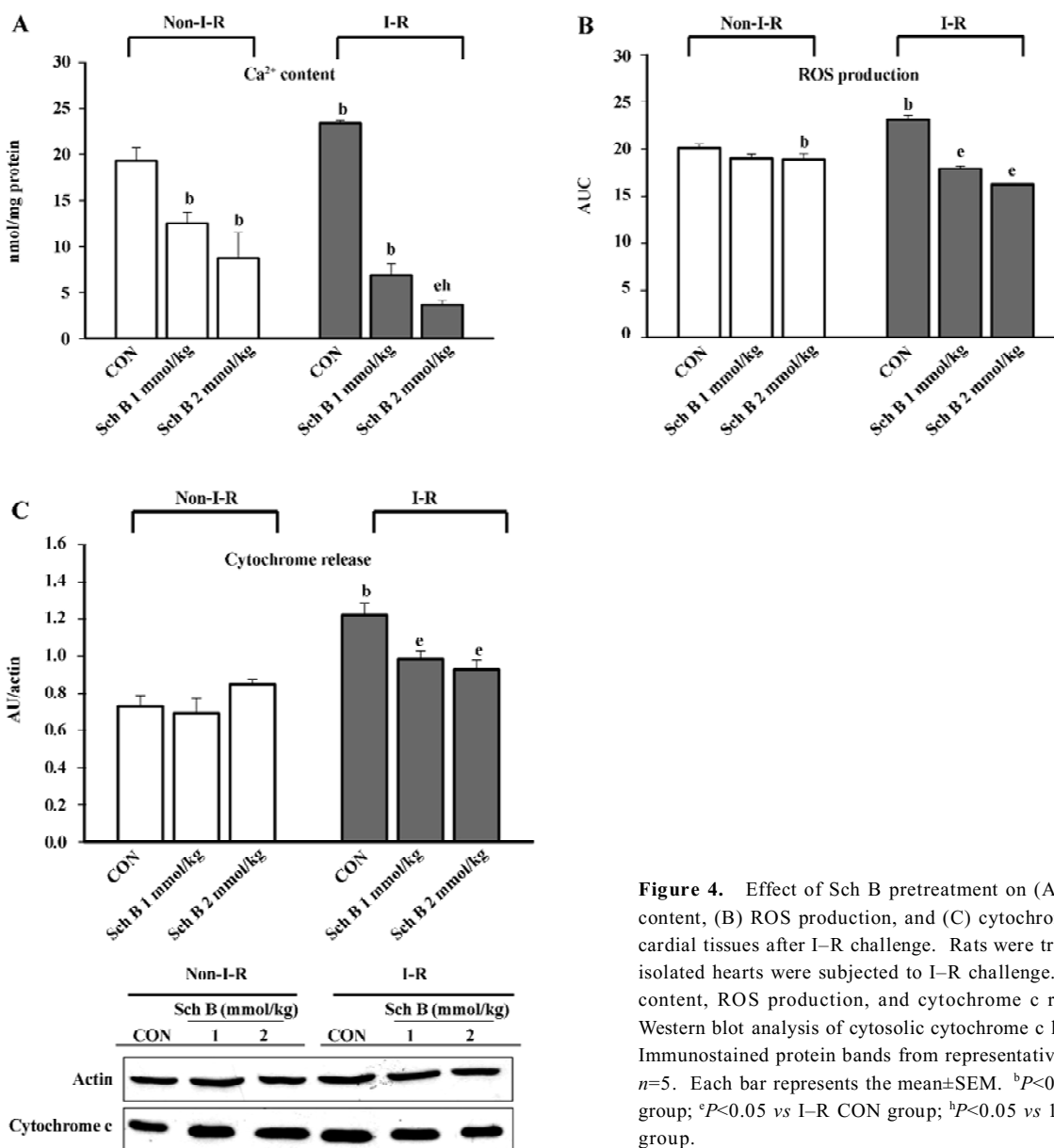


Figure 4. Effect of Sch B pretreatment on (A) mitochondrial Ca^{2+} content, (B) ROS production, and (C) cytochrome c release in myocardial tissues after I-R challenge. Rats were treated with Sch B and isolated hearts were subjected to I-R challenge. Mitochondrial Ca^{2+} content, ROS production, and cytochrome c release (indicated by Western blot analysis of cytosolic cytochrome c level) were measured. Immunostained protein bands from representative samples are shown. $n=5$. Each bar represents the mean \pm SEM. ^b $P<0.05$ vs non-I-R CON group; ^e $P<0.05$ vs I-R CON group; ^h $P<0.05$ vs 1 mmol/kg Sch B I-R group.

trans isomerase (PPIase) activity^[7]. An excess amount of Ca^{2+} would compete over ATP and activate PPIase, resulting in a conformation change that converts the ANT complex into a non-specific pore^[25]. The opening of mitochondrial PT pores, either in *in vivo* or *in vitro* conditions, is triggered by high mitochondrial Ca^{2+} content and other stimuli, including oxidants and the depletion of adenine nucleotides^[26]. Consistent with this, mitochondria isolated from I–R rat hearts were found to have increased Ca^{2+} content as well as increased extent of ROS production. The disruption of structural integrity of mitochondria, as implicated by increases in the sensitivity to mitochondria PT and the extent of mitochondrial ROS production, from I–R myocardial tissues was evidenced by the increase in cytochrome c release *in vivo*. The cardioprotection afforded by Sch B pretreatment against I–R injury was associated with the improvement in mitochondrial integrity, as indicated by decreases in the sensitivity to Ca^{2+} -induced PT, as well as the extent of ROS production and cytochrome c release. This is also corroborated by our recent findings that showed the preservation of mitochondrial ATP generation capacity by Sch B pretreatment in I–R rat hearts^[3]. The leakage of cytochrome c in non-I–R hearts, as evidenced by the presence of cytosolic cytochrome c, is likely caused by the artifact arising from tissue homogenization, as was the case for Kim *et al*^[27].

Cytosolic Ca^{2+} content increases during myocardial I–R^[28], leading to the accumulation of Ca^{2+} in the mitochondria via the uptake by the inner membrane Ca^{2+} uniporter^[29,30]. The mitochondrial Ca^{2+} overload not only generates energy-consuming futile cycles that divert the use of the inner membrane proton gradient to cation transport rather than ATP production^[30], but also predisposes the mitochondria to PT^[31]. The mitochondrial PT further jeopardizes the cellular energy status, and the consequent loss of ion homeostasis can lead to necrotic cell death. The release of cytochrome c from the mitochondrial inner membrane, which is believed to be an event secondary to the onset of mitochondrial PT^[32], is a key step leading to apoptosis^[11]. While under the present experimental conditions the relative contribution of necrotic and apoptotic cell death to I–R-induced tissue injury remains to be determined, the finding of decreased sensitivity of mitochondria isolated from Sch B-pretreated hearts to Ca^{2+} -stimulated PT suggests that the increase in mitochondrial resistance to Ca^{2+} -stimulated PT may play an important role in protecting against myocardial I–R injury. The observation that Sch B treatment decreased the mitochondrial Ca^{2+} level in both non-I–R and I–R hearts may be caused by the inhibition of the inner membrane Ca^{2+} uniporter and hence the Ca^{2+} uptake by Sch B. In this regard, Sch B was found to

inhibit the P-glycoprotein, an ATP-dependent drug transporter in cancer cells^[33].

Glutathione plays an important role in numerous cellular functions, including the regulation of Ca^{2+} homeostasis and detoxification of ROS^[34,35]. Previous studies have demonstrated the ability of Sch B to enhance mitochondrial glutathione redox status and protect against I–R-induced myocardial injury^[3]. The decrease in mitochondrial sensitivity to Ca^{2+} -stimulated PT by Sch B, as observed in the present study, may be related to the enhancement of mitochondrial glutathione redox status. In this regard, 1 of the 2 voltage-sensitive sites of mitochondrial PT pore was found to be gated by a critical dithiol that was sensitive to glutathione redox status, and the oxidation of GSH could open the pore^[36,37]. Furthermore, the modification of a specific thiol group on the ANT, either by oxidative stress or thiol reagents, also decreased adenine nucleotide binding and activated the PT pore^[38]. Increased oxidative stress enhanced cyclophilin binding and hence increased the sensitivity of mitochondrial PT^[38]. Consistently, the cardioprotection against I–R injury and the increase in the resistance of mitochondria to Ca^{2+} -induced PT afforded by Sch B pretreatment were abolished by GSH depletion (unpublished data). The observation that a smaller degree of inhibition of Ca^{2+} -induced mitochondrial PT than that of myocardial I–R injury afforded by Sch B treatment supports the notion that the decrease in the sensitivity of mitochondria to PT is an effect secondary to the enhancement of mitochondrial glutathione redox status. The direct effect of increasing the mitochondrial GSH level and/or the indirect effect on removing mitochondrial-derived ROS produced by Sch B treatment may probably increase the threshold for mitochondrial PT pore opening in the presence of Ca^{2+} . It is unlikely that Sch B can produce a direct effect on mitochondrial PT pore opening because Sch B, when being supplemented in the perfusate, could not protect against I–R injury in isolated rat hearts^[4].

In conclusion, I–R caused an increase in the sensitivity of myocardial mitochondria to Ca^{2+} -stimulated PT *in vitro*. The cardioprotection afforded by Sch B pretreatment against I–R injury was paralleled by the decrease in the sensitivity of the myocardial mitochondria to Ca^{2+} -stimulated PT, particularly under I–R conditions. The results suggest that the cardioprotection afforded by Sch B pretreatment against I–R injury may at least in part be attributed to the increase in the resistance of the myocardial mitochondria to Ca^{2+} -stimulated PT.

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