Schisandrin B Enhances Cerebral Mitochondrial Antioxidant Status and Structural Integrity, and Protects against Cerebral Ischemia/Reperfusion Injury in Rats

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Schisandrin B (Sch B), a dibenzocyclooctadiene derivative isolated from the fruit of Schisandra chinensis (FS), a traditional Chinese herb clinically used for the treatment of viral and chemical hepatitis. A recent study from our laboratory has demonstrated that long-term treatment with Sch B was able to enhance mitochondrial antioxidant status and the resistance to Ca2+-induced mitochondrial permeability transition (PT) in an age-independent manner in various rat organs including the heart and brain. The Sch B-induced enhancement of mitochondrial protective parameters in the heart was associated with the protection against myocardial ischemia/reperfusion (I/R) injury in both young and middle-aged rats. Given that the maintenance of mitochondrial antioxidant status and structural integrity is crucial for cell survival, Sch B has been proposed to be used as a universal cell protectant against tissue damage caused by endogenous and exogenous oxidants. While the protection of Sch B against I/R injury has been demonstrated in the heart, it is still unclear whether Sch B treatment can produce any beneficial effect on cerebral I/R injury.

In the present study, we investigated the effect of long-term treatment with Sch B on cerebral I/R injury in rats. To elucidate the biochemical mechanism involved in the cerebroprotection against I/R injury, cerebral mitochondrial antioxidant status as well as mitochondrial structural integrity were assessed in control and Sch B-treated rats, without or with I/R challenge.

MATERIALS AND METHODS

Chemicals Reduced glutathione (GSH), oxidized glutathione, glutathione reductase, cytochrome c, α-tocopherol (α-TOC), cyclosporin A (Cs A) and 2,3,5-triphenyl tetrazolium chloride (TTC) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other chemicals were of analytical grade. Solvents used for HPLC were of HPLC grade.

Herbal Material Dried FS was imported from mainland China. It was authenticated and supplied by a commercial dealer (Lee Hoong Kee Ltd.) in Hong Kong. Sch B was purified from the petroleum ether extract of FS, with the purity being higher than 95% as determined by HPLC analysis.

Animal Care Adult female Sprague-Dawley rats (8—10 weeks; 200—250 g) were maintained under a 12-h dark/light cycle at about 22 °C, and allowed food and water ad libitum. Experimental protocols were approved by the Research Practice Committee at the Hong Kong University of Science & Technology.

Drug Treatment Animals were randomly divided into groups, with five animals in each. In the Sch B treatment groups, rats were intragastrically administered with Sch B (dissolved/suspended in olive oil) at a daily dose of 1, 10 or 30 mg/kg from day 1 to day 15 of the experiment. This dosage regimen was found to be effective in protecting against myocardial ischemia/reperfusion injury in rats. Sch B-untreated animals received the vehicle (i.e. olive oil) only. The rat model of cerebral I/R injury was modified from that of Ishikawa and Konishi. Phenobarbital-anesthetized rats (100 mg/kg, i.v.) were subjected to 120 min of ischemia by occlusion (using aneurysm clips) of both left and right common carotid arteries, which were exposed through a middle skin incision. At the end of the ischemic period, the carotid arteries were declamped and blood reperfusion was permitted for 60 min. Non-I/R animals were sham operated. After the I/R experiment, the brain was removed and frozen for 10 min at −20 °C, and then fitted into a mould. Six coronal brain sections (6—8 mm thick) were sliced from the front pole using a razor. The sections were incubated in phosphate-buffered saline containing 2% (w/v) TTC at 37 °C for

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Preparation of Tissue Homogenate and Mitochondrial Fraction  
Minced whole brain tissues (ca. 2 g) were homogenized in 10 ml of ice-cold sucrose buffer (0.25 M sucrose, 0.1 mM Na₂EDTA, 5 mM Tris/HCl, pH 7.4), which was supplemented with a cocktail of protease inhibitors (0.2 mg/ml soybean trypsin inhibitor, 0.2 mg/ml bacitracin, 0.16 mg/ml benzamidine), using a Teflon-glass homogenizer at 200 rpm for 8—10 complete strokes. Mitochondrial pellets were prepared from tissue homogenates by centrifugation at 800 × g at 4 °C for 30 min, as described.  
Mitochondrial pellets were then resuspended in 1 ml of homogenizing buffer and constituted the mitochondrial fraction. The protein concentration of mitochondrial fractions was determined using a Bio-Rad protein assay kit.

Mitochondrial Antioxidant Status  
Mitochondrial antioxidant status was assessed by measuring the levels of GSH and α-Toc as well as the activity of Mn-SOD, as previously described. In brief, aliquots (200 μl) of mitochondrial fractions were used for measuring mitochondrial GSH and α-Toc levels by an enzymatic method and HPLC method, respectively, using GSH and α-Toc as standards. The Mn-SOD activity was measured by monitoring the oxidation of cytochrome c caused by superoxide radicals generated from the xanthine oxidase/xanthine reaction.

Mitochondrial Structural Integrity  
Mitochondrial structural integrity was assessed by measuring the extents of mitochondrial malondialdehyde (MDA) production, cytochrome c release and calcium loading, as well as the sensitivity of mitochondria to Ca²⁺-induced PT, as previously described. In brief, mitochondrial MDA level was measured by a HPLC method. Cytosolic cytochrome c level, as indirect measure of mitochondrial cytochrome c release, was estimated by Western blot analysis using specific antibodies to cytochrome c (clone 7H8.2C12, BD PharMingen, San Diego, CA, U.S.A.) following SDS-PAGE of cytosolic fractions, using a separating gel with 15% (w/v) acrylamide. The extent of mitochondrial contamination of cytosolic fractions, which was found to be negligible, was determined using specific antibodies against complex IV. The immunoblots were visualized using the enhanced chemiluminescence reaction (Amersham ECL, Piscataway, NJ, U.S.A.), and analyzed by densitometry (BioRad, Hercules, CA, U.S.A.). The amounts (arbitrary units) of cytochrome c were normalized with reference to actin levels (arbitrary units) in the sample. The Ca²⁺ content was measured using a Ca²⁺-sensitive fluorescence probe, Fluo-5N AM ester, and a Victor² V multi-Label counter. The Ca²⁺ dissociation constant (Kₐ) was determined by using a Ca²⁺ calibration kit in the concentration range 1—1000 μM, and the Kₐ value was estimated to be ca. 98 μM, in good agreement with data provided by the manufacturer. An aliquot (25 μl) of a mitochondrial fraction (0.5 mg/ml final concentration) was mixed with 25 μl of incubation buffer (100 mM KCl, 30 mM MOPS, pH 7.2) in a 96-well black microtiter plate. The mixture was incubated at 25 °C for 15 min and 25 μl digitonin (50 μg/ml), with 25 μl Fluo-5N AM ester (1 μM in 0.005% Pluronic F-127), was then added. The reaction mixture was incubated at 25 °C for 30 min, and the fluorescence reading was measured at an excitation wavelength of 488 nm and an emission wavelength of 532 nm. The mitochondrial Ca²⁺ content was estimated from a standard calibration curve and expressed in μmol/mg protein. The measurement of the sensitivity of mitochondria to Ca²⁺-induced PT was performed as follows. An aliquot (1.6 ml) of a mitochondrial sample (0.5 mg protein/ml) was prepared by mixing the mitochondrial fraction with incubation buffer containing 125 mM sucrose, 65 mM KCl, 10 mM Heps (pH 7.2), 5 mM succinate (freshly prepared) and 5 μM rotenone (freshly prepared). Aliquots (200 μl) of the mitochondrial sample were mixed with 10 μl of Cs A (5 μM in 0.5% ethanol; final concentration) or incubation buffer. The mixture was incubated at 30 °C for 5 min. An aliquot (10 μl) of calcium chloride (CaCl₂) solution (1 μM final concentration) was then added, and the mixture was incubated at 30 °C for 5 min. Aliquots (180 μl) of each mixture were pipetted to a 96-well microtiter plate, and the initial absorbances of the mixtures at 520 nm were monitored for 5 min at 30 °C. The swelling reaction was then commenced by adding 20 μl of K₂PO₄ (0.5 mM, 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 a Victor V² Multi-Label Counter. The extent of mitochondrial swelling was estimated by computing the area under the curve (AUC) of the declining graph plotting percentage initial absorbance (100%; baseline) against time (min), to obtain AUC₁. The extent of mitochondrial PT (ΔAUC₁) was estimated by subtracting the AUC₁ from Cs A from the AUC₁, without Cs A. The Ca²⁺-induced mitochondrial PT was expressed as the ratio of the ΔAUC₁ induced by both Ca²⁺ and PO₄⁻ to that induced by PO₄⁻ only (i.e., we obtained a Ca²⁺-induced mitochondrial swelling index).

Statistical Analysis  
Data were analyzed by one-way Analysis of Variance (ANOVA). Post hoc multiple comparisons were done with LSD. p-values < 0.05 were regarded as statistically significant.

RESULTS  
Long-term Sch B treatment did not produce any detectable effect on the viability of brain tissue, as assessed by measuring the percentage of TTC-stained tissue in the fourth brain slice (Fig. 1). I/R challenge caused a significant decrease (24%) in the percentage of TTC-stained tissue, an indication of brain infarction. Long-term Sch B pretreatment protected against I/R-induced brain infarction, as evidenced by a dose-dependent increase in the percentage of TTC-stained tissue, when compared with that of the Sch B untreated and I/R control, with the percent protection ranging from 10—33% (Fig. 1).

Sch B treatment enhanced the cerebral mitochondrial antioxidant status, as indicated by increases in the level/activity of GSH (13—35%), α-Toc (7—38%) and Mn-SOD (14—28%) in a dose-dependent manner (Table 1). In contrast, I/R challenge caused the impairment in cerebral mitochondrial antioxidant status, as evidenced by significant decreases in...
the level/activity of GSH (15%), α-TOC (15%) and Mn-SOD (14%). The cerebroprotection afforded by Sch B pretreatment against I/R injury was associated with dose-dependent increases in the level/activity of mitochondrial GSH (5—24%), α-TOC (9—21%) and Mn-SOD (9—24%), when compared to the Sch B-untreated and I/R control.

Sch B treatment decreased the extents of cerebral mitochondrial MDA production (8—27%), Ca²⁺ loading (5—17%) and cytochrome c release (10—25%), as well as the susceptibility of mitochondria to Ca²⁺-induced PT (9—19%) to varying extents (Figs. 2a—d), all of which are indirect measures of mitochondrial structural integrity. I/R challenge caused a disruption in cerebral mitochondrial structural integrity, as evidenced by significant increases in the extents of MDA production (45%), Ca²⁺ loading (41%) and cytochrome c release (48%), as well as the sensitivity to Ca²⁺-induced PT (16%), when compared to the Sch B-untreated and I/R control. Sch B pretreatment preserved the mitochondrial structural integrity in I/R challenged rats, as indicated by significant and dose-dependent decreases in the values of mitochondrial parameters to varying extents (7—23%), when compared to the Sch B-untreated and I/R control.

**DISCUSSION**

It is well established that I/R causes neuronal injury through multiple pathophysiological mechanisms, including intracellular calcium overload and reactive oxygen species (ROS) production, which eventually trigger necrotic and/or apoptotic cell death. In the present study, long-term Sch B treatment was found to protect against cerebral I/R injury in a dose-dependent manner in young rats. It has been shown that the same Sch B treatment regimen also protected against myocardial I/R injury in both young and middle-aged rats. In this regard, we also found that long-term Sch B treatment at a daily dose of 30 mg/kg for 15 d protected against cerebral I/R injury in 2-year old rats, with the degree of protection being larger than that of the young rats (unpublished data). Sch B may therefore be generally used as hormetic agent for preventing myocardial and cerebral I/R injury occurring at both young and old ages.

Mitochondria are important source of ROS under both physiological and pathological conditions such as cerebral I/R injury. The pathogenesis of cerebral I/R injury includes the opening of mitochondrial PT pores. This is mediated by ROS and triggered by the increased mitochondrial Ca²⁺ levels. The enhancement of tissue/mitochondrial antioxidant capacity by overexpression of Mn-SOD, or by antioxidant supplementation, has been shown to protect against oxidative stress-induced tissue injury in the brain. Consistent with this, our findings indicate that the cerebroprotection afforded by long-term Sch B treatment was associated with increases in the levels and activity of mitochondrial antioxidant components (GSH, α-TOC, and Mn-SOD), as well as preservation of mitochondrial structural integrity. Structural integrity
was assessed by measuring sensitivity to Ca\textsuperscript{2+}-induced PT and by estimating MDA production levels, Ca\textsuperscript{2+} levels and cytochrome c release.

The opening of mitochondrial PT pores plays an important role in regulating necrotic and apoptotic cell death.\textsuperscript{17} In the present study, I/R-induced damage in cerebral tissues was associated with an increased sensitivity of mitochondria to Ca\textsuperscript{2+}-induced PT, as assessed by the measurement of mitochondrial swelling \textit{in vitro}. Core components of the mitochondrial PT pore putatively include a voltage-dependent anion channel, an adenine nucleotide translocase (ANT), and a cyclophilin D displacing peptidyl–prolyl \textit{cis–trans} isomerase (PPIase) activity.\textsuperscript{18} Ca\textsuperscript{2+} competes with ATP and activates PPIase, resulting in a conformational change converting the ANT complex to a non-specific pore.\textsuperscript{19} While the loss of ion homeostasis resulting from ATP depletion following mitochondrial PT can lead to necrosis,\textsuperscript{20} PT also causes the leakage of cytochrome c from the mitochondria to the cytosol.\textsuperscript{21} The released cytochrome c can trigger a cascade of events that eventually leads to apoptosis.\textsuperscript{21,22} The opening of mitochondrial PT pores, either \textit{in vivo} or \textit{in vitro}, is stimulated by high Ca\textsuperscript{2+} levels and by other stimuli, including increases in oxidants and depletion of adenine nucleotides.\textsuperscript{23} Consistent with these observations, we found that mitochondria isolated from ischemic/reperfused cerebral tissues were more sensitive than control mitochondria to PT, as assessed by measurement of mitochondrial swelling \textit{in vitro}. Mitochondrial cytochrome c release was estimated by measuring the cytosolic cytochrome c level by Western blot analysis (d). The lower panel in (d) shows representative Western blots in various groups. Values given are mean±S.E.M., with $n$=5. *Significantly different from the Non-I/R CON; †significantly different from the 1 mg/kg Sch B group; ‡significantly different from the respective 10 mg/kg Sch B group.

Fig. 2. Effects of Long-Term Sch B Treatment on Cerebral Mitochondrial Parameters in Control and I/R Challenge Rats

Animals were treated with Sch B and then challenged by I/R as described in Fig. 1. Mitochondrial fractions were prepared from cerebral tissues and then used for the measurement of malondialdehyde (MDA) production (a), Ca\textsuperscript{2+} content (b) and (c) Ca\textsuperscript{2+}-induced permeability transition. Mitochondrial cytochrome c release was estimated by measuring the cytosolic cytochrome c level by Western blot analysis (d). The lower panel in (d) shows representative Western blots in various groups. Values given are mean±S.E.M., with $n$=5. a Significantly different from the Non-I/R CON; b significantly different from the I/R CON; c significantly different from the respective 1 mg/kg Sch B group; d significantly different from the respective 10 mg/kg Sch B group.
tioxidant response triggered by Sch B was causally related to ROS arising from the cytochrome P-450-catalyzed metabolism of Sch B in both cultured hepatocytes and cardiomyocytes (unpublished data). Conceivably, Sch B is also metabolized in cerebral tissue and subsequently leads to the ROS-mediated antioxidant response, with the resultant enhancement of mitochondrial antioxidant status. In addition, the enhanced antioxidant status may render the mitochondria less sensitive to Ca\textsuperscript{2+}-induced PT, presumably by maintaining the reduced thiol status of the voltage-sensitive sites of the mitochondrial PT pore.\textsuperscript{24,25}

In conclusion, the results indicate that long-term Sch B treatment could enhance cerebral mitochondrial antioxidant status as well as improve mitochondrial structural integrity, thereby protecting against I/R injury.

REFERENCES


