Schisandrin B-Induced Glutathione Antioxidant Response and Cardioprotection Are Mediated by Reactive Oxidant Species Production in Rat Hearts

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To investigate the involvement of reactive oxidant species (ROS), presumably arising from cytochrome P-450 (CYP)-catalyzed metabolism of schisandrin B (Sch B), in triggering glutathione antioxidant response, Sch B-induced reduced nicotinamide adenine dinucleotide phosphate (NADPH)-dependent and CYP-catalyzed reaction and associated ROS production were examined in rat heart microsomes. Sch B analogs were also studied for comparison. Using rat heart microsomes as a source of CYP, Sch B and schisandrin C (Sch C), but not schisandrin A and dimethyl diphenyl bicarboxylate (an intermediate compound derived from the synthesis of Sch C), were found to serve as co-substrate for the CYP-catalyzed NADPH oxidation reaction, with concomitant production of ROS. The stimulation of CYP-catalyzed NADPH oxidation reaction and/or ROS production by Sch B or Sch C correlated with the increase in mitochondrial reduced glutathione level and protection against ischemia/reperfusion (I/R) injury in rat hearts. The involvement of ROS in Sch B-induced cardioprotection was further confirmed by the suppressive effect produced by N-acetylcysteine or α-tocopherol pretreatment. Taken together, these results suggest that Sch B-induced glutathione antioxidant response and cardioprotection may be mediated by ROS arising from CYP-catalyzed reaction.

Key words schisandrin B; cytochrome P-450; reactive oxidant species; glutathione; mitochondrion; myocardial ischemia reperfusion injury

Schisandrin B (Sch B) (Fig. 1) is the most abundant active dibenzocyclooctadiene derivative isolated from the fruit of *Schisandra chinensis* (FS), a traditional Chinese herb commonly used as an astrigent and clinically used for the treatment of viral and chemical hepatitis. Previous studies in our laboratory have demonstrated the ability of Sch B to protect against myocardial ischemia/reperfusion (I/R) injury. The cardioprotection afforded by Sch B pretreatment was associated with enhancement in tissue glutathione antioxidant status, particularly in the mitochondrion. Recent studies also showed that Sch B protected against myocardial I/R injury partly by inducing heat shock protein (Hsp) 25 and Hsp 70 expression in rats. In regard to the action on mitochondria, Sch B treatment was found to decrease the sensitivity of myocardial mitochondria to Ca²⁺-induced permeability transition, particularly under I/R condition. However, the biochemical mechanism(s) underlying the Sch B-induced glutathione antioxidant and/or heat shock responses in the myocardium remains unclear. In this connection, it has been suggested that reactive oxidant species (ROS) generated from cytochrome P-450 (CYP)-catalyzed reaction with Sch B may trigger the glutathione antioxidant and heat shock responses in mouse livers.

CYP are a superfamily of hemoproteins that catalyze the oxidative metabolism of many endogenous (endobiotic) and xenobiotic compounds including Sch B. To explore the role of CYP in Sch B-induced glutathione antioxidant response in the myocardium, we first examined CYP-catalyzed reaction with Sch B and associated ROS production in rat heart microsomes. Sch B analogs [schisandrin A (Sch A), schisandrin C (Sch C), and dimethyl diphenyl bicarboxylate (DDB)] (see also Fig. 1), which were found to produce differential cardioprotective effects, were also studied for comparison. The in vitro studies were then paralleled by in vivo investigations on Sch B or its analogs-induced changes in myocardial mitochondrial reduced glutathione (GSH) and susceptibility to I/R injury in rats. To confirm the determinant role of ROS arising from Sch B metabolism in cardioprotection, we also examined the effect of antioxidant pretreatment on the cardioprotective action of Sch B in rats.

**MATERIALS AND METHODS**

**Chemicals** GSH, oxidized glutathione, 1-aminobenzotriazole (ABT), N-acetyl cysteine (NAC), and α-tocopherol (TOC) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). 2′,7′-Dichlorofluorescein diacetate (DCFDA)
was obtained from Fluka (Buche, Switzerland). DDB was purchased from Guangzhou Xun Xing Pharmaceutical Company (Guangzhou, China). FS was imported from China. It was authenticated and supplied by a commercial dealer (Lee Hoong Kee Ltd.) in Hong Kong. Schisandrrins (Sch A, Sch B, and Sch C) were purified from the petroleum ether extract of FS by silica gel chromatography, as previously described. The purity of schisandrrins was >90%, as determined by HPLC analysis, which was comparable to that of commercial preparations. All other chemicals were of analytical grade.

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

**Time-Course Study on Drug-Induced Changes in Myocardial Mitochondrial GSH Level** Animals were randomly divided into groups of five animals. They were treated intragastrically with Sch A, Sch B, or DDB (suspended in olive oil) at a single dose of 1.2 mmol/kg. Previous studies indicated that this dosage of Sch B was effective in protecting rats against myocardial I/R injury. At increasing periods (24, 48, or 72 h) of post-drug treatment, the hearts were harvested from phenobarbital anesthetized animals. The short duration (approximately 5 min) of phenobarbital anesthesia should not produce any marked effect on myocardial microsomal CYP and mitochondrial GSH levels. Mitochondrial fractions were prepared from myocardial homogenates and measured for GSH level. Drug-untreated animals received vehicle (8 ml/kg) only, and the hearts were obtained at 24 h post-treatment.

**Myocardial I/R Injury** Hearts isolated from Sch A, Sch B, or DDB pretreated rats were subjected to I/R challenge at 48 h post-dosing with the drug. For Sch C pretreatment, isolated hearts were subjected to I/R challenge at 24 h post-dosing when maximal stimulation of mitochondrial GSH level was observed.

Hearts were excised quickly and immediately immersed in ice-cold and heparinized (50 units/ml) saline. The aorta was cannulated and then transferred to a warm, moist chamber of the perfusion apparatus. The hearts were retrogradely perfused according to the Langendorff method as described previously. After an initial 30-min perfusion for equilibration, the isolated heart was subjected to a 40-min period of “no-flow” global ischemia followed by 20-min reperfusion. Coronary effluent was collected in 1-min fraction at increasing time intervals (10, 1 min, respectively) 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 percent LDH activity (with respect to the mean pre-ischemic value measured during the equilibration period) against reperfusion time (1—20 min), as described previously, and the value was expressed in arbitrary unit. Non-I/R hearts were perfused for 90 min.

**Antioxidant Pretreatment** To investigate the role of ROS arising from the metabolism of Sch B in the myocardial protective action, animals were pretreated with TOC (1.2 g/kg, per os (p.o.)) or NAC (400 mg/kg, intraperitoneally (i.p.)) 24 h or 30 min, respectively, prior to Sch B treatment. Preliminary studies indicated that both treatment protocols enhanced myocardial antioxidant capacity (as assessed by the susceptibility of isolated hearts to I/R injury) 24 h or 30 min post-dosing. Forty-eight hours after Sch B treatment, the hearts were isolated and subjected to I/R experiment.

**Preparation of Mitochondrial and Microsomal Fractions** Myocardial ventricular tissue samples were rinsed with ice-cold isotonic buffer (50 mM Tris, 0.32 M sucrose, 1 mM Na2 ethylenediaminetetraacetic acid (EDTA), pH 7.4). Tissue homogenates were prepared by homogenizing 0.6 g of minced tissues in 6 ml ice-cold isotonic buffer using a Teflon-glass homogenizer at 40 rpm. Mitochondrial and microsomal pellets were prepared by differential centrifugation as described previously.

**Drug-Induced Reduced Nicotinamide Adenine Dinucleotide Phosphate (NADPH)-Dependent and CYP-Catalyzed Reaction in Rat Heart Microsomes** An aliquot (100 μl) of microsomal fraction (approximately 0.6 mg protein) was mixed with 0.8 ml ABT solution (4 mM final concentration) or assay buffer (0.1 M Tris–HCl, pH 7.4), and the mixture was incubated at 37 °C for 2 h. ABT is a broad spectrum CYP inhibitor. After incubation, an aliquot (5 μl) of drug solution (Sch A, Sch B, Sch C, or DDB dissolved in dimethyl sulfoxide (DMSO)-containing buffer, 0.5%, v/v, final concentration) or vehicle (DMSO) was added. The reaction was initiated by adding 50 μl NADPH solution (0.25 mM final concentration). Absorbance at 340 nm of the reaction mixture at 37 °C was monitored for 60 min. Absorbance values at increasing time intervals were normalized with reference to the initial values (i.e. time 0). The extent of NADPH oxidation was estimated by computing the area above the curve of the graph plotting normalized absorbance values against time (0—60 min). The extent of drug-induced and ABT-inhibitable NADPH oxidation was calculated and expressed in percentage of control (DMSO).

**Drug-Induced NADPH-Mediated ROS Production in Rat Heart Microsomes** Aliquots (20 μl) of microsomal fraction (approximately 10 μg protein) were added with assay buffer (40 mM Tris–HCl, pH 7.4) and mixed with 50 μl drug solution (see above), 50 μl NADPH (0.5 mM final concentration) and 60 μl DCFDA (5 μM). The reaction mixtures were incubated at 37 °C. Oxidation of DCFDA in the reaction mixture, an indirect measure of ROS production, was monitored by measuring the fluorescence intensity for 60 min, using Victor2 V Multi-Label Counter (Perkin Elmer, Turku, Finland), with excitation and emission wavelengths being 485 and 535 nm, respectively. The fluorescence intensity of DMSO or drug-treated sample was subtracted by the value of time-matched blank (with DCFDA only). The extent of drug-induced ROS production was estimated by computing the area under the curve of the graph plotting normalized fluorescence intensity (with reference to the initial values, i.e. time 0) against time (0—60 min). The extent of drug-induced ROS production was calculated and expressed as the percentage of control (DMSO).

**Biochemical Analysis** LDH activity of coronary effluent was measured as described previously. Mitochondrial GSH
level was measured by enzymatic method described by Griffith.4,13) Protein Assay Protein concentrations of the mitochondrial and microsomal fractions were determined by Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, U.S.A.).

Statistical Analysis Data from multiple groups were analyzed by one-way ANOVA followed by least significant difference test to detect significant intergroup differences, when p<0.05.

RESULTS

Under the present experimental conditions, NADPH was spontaneously oxidized in the reaction mixture containing rat heart microsomes, with the average rate of oxidation being 0.39 nmol/min. Sch B (10 μM) stimulated the oxidation of NADPH in rat heart microsomes (by 1-fold) (Fig. 2a). The Sch B-induced NADPH oxidation was largely inhibited by ABT, indicative of CYP-catalyzed reaction. When the extents of NADPH oxidation were quantified and compared among different schisandrin/DDB, it was found that Sch B caused a larger increase in ABT-inhibitable NADPH oxidation than that of Sch C in rat heart microsomes (52% vs. 35%) (Fig. 2b). However, both Sch A and DDB did not produce detectable ABT-inhibitable NADPH oxidation.

The reaction mixture containing rat heart microsomes produced ROS under the present experimental conditions, as evidenced by the increase in fluorescence intensity (by 1.5 fold compared with the initial value) after 60 min of incubation. Sch B induced a dose-dependent increase in ROS production in rat heart microsomes, when compared with the control (Fig. 3a). When the extents of ROS production were quantified and compared among different schisandrin/DDB, both Sch B and Sch C showed a dose-dependent induction of ROS production, with the effect of Sch C being more potent (Fig. 3b). In contrast, Sch A and DDB only caused slight but not dose-dependent increases in ROS production.

Treatment with a single dose of schisandrin (Sch A, Sch B, and Sch C) caused time-dependent increases in mitochondrial GSH level in rat hearts, with the degrees of stimulation of Sch A/Sch B and Sch C being peaked at 48 and 24 h post-dosing, respectively (Fig. 4). The extents of stimulation of mitochondrial GSH by Sch B (45%) and Sch C (36%) were higher than that of Sch A (28%). In contrast, DDB treatment did not cause detectable changes in mitochondrial GSH level in rat hearts.

When schisandrin/DDB pretreated rat hearts were subjected to I/R challenge at 24 h (Sch C) or 48 h (Sch A, Sch B, Sch C, and DDB) with increasing concentrations (10—30 μM). Values given are % of control ± S.E.M., with data obtained from three separate experiments using pooled heart microsomal fractions. a Significantly different from DMSO group; b significantly different from ABT DMSO group.

Fig. 2. Drug Induced NADPH-Dependent and CYP-Catalyzed Reaction in Rat Heart Microsomes

The NADPH-dependent and cytochrome P-450 (CYP)-catalyzed reaction was measured using heart microsomes, as described in Materials and Methods. Panel (a) shows the time-dependent NADPH oxidation in the presence of vehicle (i.e., DMSO) or schisandrin B (Sch B, 10 μM), without or with 1-aminobenzotriazole (ABT, 4 μM). Blank sample contained only NADPH. The extent of NADPH oxidation was estimated, and the data were expressed in % of control and shown in Panel (b). The non-ABT and ABT control values were 250±7 arbitrary unit (AU) (S.E.M., with n=3) and 165±8 AU, respectively. Schisandrin A (Sch A), schisandrin C (Sch C), and dimethyl diphenyl bicarbonate (DDB) were also tested at 10 μM. Values given are mean±S.E.M., with data obtained from three separate experiments using pooled heart microsomal fractions.

Fig. 3. Drug-Induced NADPH-Dependent Reactive Oxidant Species Production in Rat Heart Microsomes

NADPH-dependent reactive oxidant species (ROS) production was measured using heart microsomes. Panel (a) shows the time-dependent increase in ROS production in the presence of vehicle (DMSO) or Sch B (10—30 μM). The extent of ROS production was estimated, and the data were expressed in % of control and shown in Panel (b). The control value was 5659±161 AU. Sch A, Sch C, and DDB were also tested at increasing concentrations (10—30 μM). Values given are % of control±S.E.M., with data obtained from three separate experiments using pooled heart microsomal fractions. a Significantly different from DMSO group; b significantly different from 20 μM Sch B group.
and DDB) post-dosing, significant protection against I/R injury was observed in Sch B and Sch C pretreated hearts, with the degree of protection produced by Sch C being larger (59% vs. 39%) (Fig. 5a). Sch A and DDB pretreatments did not protect against myocardial I/R injury. While I/R challenge caused a significant decrease (25%) in myocardial mitochondrial GSH level, the cardioprotection afforded by Sch B and Sch C pretreatments against I/R injury was associated with significant increases in mitochondrial GSH level (33% and 52%, respectively) in ischemic/reperfused rat hearts, when compared with drug-untreated I/R hearts (Fig. 5b).

While NAC and \( \alpha \)-TOC treatments slightly protected against I/R injury in rat hearts, they completely abrogated the cardioprotection afforded by Sch B pretreatment against I/R injury (Figs. 6a, b).

**DISCUSSION**

Previous studies from our laboratory have shown that Sch B treatment enhanced mitochondrial glutathione antioxidant status and decreased susceptibility of rat hearts to I/R injury.3) The extent of cardioprotection afforded by Sch B or its analogs pretreatment against I/R injury, as observed in the present study, correlated with the ability to stimulate CYP-catalyzed NADPH oxidation reaction and associated ROS production in rat heart microsomes. The inhibition of cardioprotective action of Sch B \textit{ex vivo} by antioxidant pretreatment further supports the causal role of ROS, presumably arising from CYP-catalyzed reaction with Sch B, in triggering glutathione antioxidant response and thereby protecting against myocardial I/R injury in rats.

Earlier studies in our laboratory showed that Sch B stimulated the ABT-inhibitable oxidation of NADPH in mouse liver microsomes, indicative of CYP-catalyzed metabolism.7) Our finding of Sch B-induced ABT-inhibitable NADPH oxidation in rat heart microsomes supports the notion that Sch B is metabolized by CYP present in rat heart tissue. While the extent of ABT-inhibitable NADPH oxidation induced by Sch B was larger than that of Sch C, no detectable stimulatory effect was produced by Sch A and DDB. The differential reac-
tivity of schisandrin/DDB in CYP-catalyzed reaction may be related to their varied chemical structures. Sch B/Sch C, which is a dibenzocyclooctadiene derivative with one or two methylenedioxy group, may undergo four possible CYP-catalyzed reactions: (a) O-demethylation of methoxy groups; (b) cleavage of the methylenedioxy group; (c) benzylid hydroxylation; and (d) methyl group hydroxylation. In this connection, both the methylenedioxy group and the cyclooctadiene ring of the schisandrin molecule are important structural determinants in mediating protection against myocardial I/R injury. Possibly, metabolism of the methylenedioxy group and the cyclooctadiene ring of Sch B/Sch C molecule by CYP-catalyzed reactions may be mechanistically linked to the cardioprotective action.

In vitro experiments using rat heart microsomes indicated that the relative potency of schisandrin/DDB in stimulating CYP-catalyzed reaction was in descending order of Sch B>Sch C>Sch A/DDB. This observation can at least partly be explained by the relative affinity of schisandrin molecule towards CYP3A4, which is mainly responsible for catalyzing the demethylenedioxy reaction. The chemical group of schisandrin molecule at carbon 6 was found to play an important role in determining the affinity of schisandrin molecules towards CYP3A4. The varied structure of schisandrin/DDB at carbon 6 may influence the extent of CYP-catalyzed metabolism. In this regard, Sch B and Sch C contain the same chemical group at carbon 6, and they showed similar extents of CYP-catalyzed metabolism in rat heart microsomes.

Sch B/Sch C caused dose-dependent stimulation of ROS production in rat heart microsomes. The dealkylation of methylenedioxy group by CYP may convert the schisandrin molecule to form derivatives of catechol, and the subsequent formation of quinone molecule can undergo redox-cycling, with resultant production of ROS. The larger extent of ROS production by Sch B/Sch C than Sch A/DDB is likely related to their relative reactivity in CYP-catalyzed reaction. In addition, DDB tends to produce lactone derivative rather than catechol metabolite in CYP-catalyzed reaction and therefore produces a lesser amount of ROS from the redox recycling of quinone moiety.

The larger extent of ROS production by Sch B/Sch C in vitro was paralleled by the higher degree of stimulation on myocardial mitochondrial GSH level in vivo. In view of the paradoxical effect of oxidants in the phenomenon of preconditioning, a low level of oxidative stress generated from CYP-catalyzed metabolism of xenobiotics can lead to protective cellular response and enhancement in cell survival, whereas a high level of oxidative stress can lead to apoptosis. In this regard, the small amount of ROS generated from the metabolism of Sch B/C in cardiomyocytes may trigger redox signaling that eventually leads to glutathione antioxidant response. While the finding of complete suppression of Sch B-induced protection against I/R injury by antioxidant pretreatment supports the involvement of ROS in the cardioprotective mechanism, this postulation is consistent with the observation that the earlier onset of glutathione antioxidant response and stronger cardioprotection induced by Sch C than those of Sch B in vivo were associated with a larger extent of ROS production in vitro. Interestingly, the more potent cardioprotection afforded by Sch C than Sch B pretreatment against I/R injury was associated with a higher level of mitochondrial GSH level in ischemic/reperfused hearts. This may probably be related to the stronger glutathione antioxidant response elicited by Sch C than Sch B, which is manifested in the enhancement of glutathione synthesis and glutathione regeneration under oxidative stress condition. The inability of Sch A to protect against myocardial I/R injury, though being able to increase mitochondrial GSH level in non/I/R hearts, may also be related to the weaker glutathione antioxidant response under oxidative stress condition.

In conclusion, the results indicate that ROS generated from CYP-catalyzed reaction with Sch B in rat heart microsomes is correlated with increase in mitochondrial GSH level and protection against I/R injury in rat hearts. The Sch B-induced glutathione antioxidant response and cardioprotection may be mediated by ROS arising from CYP-catalyzed reaction.

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