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SHORT COMMUNICATION

Methylenedioxy Group and Cyclooctadiene Ring as Structural Determinants of Schisandrin in Protecting against Myocardial Ischemia-Reperfusion Injury in Rats

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ABSTRACT. As a preliminary investigation to exploring whether the methylenedioxy group and the cyclooctadiene ring of the dibenzo[a,c]cyclooctadiene (schisandrin) molecule plays an important role in the protection against myocardial ischemia-reperfusion (IR) injury, we examined the effects of three schisandrins, namely schisandrin A (Sch A), schisandrin B (Sch B), and schisandrin C (Sch C), and the effect of dimethyl-4,-4′-dimethoxy-5,6,5′,6′-dimethylene-dioxy-biphenyl-2,2′-bicarboxylate (DDB), an intermediate compound derived from the synthesis of Sch C, on myocardial IR injury in isolated Langendorff-perfused rat hearts. While pretreating rats with Sch A or DDB at a daily oral dose of 1.2 mmol/kg for 3 days did not protect the isolated-perfused hearts against IR-induced damage, pretreatment with Sch B or Sch C at the same dosage regimen produced cardioprotective action. The extent of cardioprotection afforded by Sch B or Sch C pretreatment correlated well with the degree of enhancement in myocardial glutathione antioxidant status, as indicated by significant increases in the tissue-reduced glutathione level and Se-glutathione peroxidase (EC 1.11.1.9), glutathione transferases (EC 2.5.1.18), and glutathione reductase (EC 1.6.4.2) activities in ischemic-reperfused hearts when compared with the unpretreated IR control. Our results indicate that both the methylenedioxy group and the cyclooctadiene ring of the schisandrin molecule are important structural determinants in mediating the protection against myocardial IR injury. BIOCHEM PHARMACOL **57**;1:77–81, 1999. © 1998 Elsevier Science Inc.

KEY WORDS. schisandrin; myocardial ischemia-reperfusion; glutathione; Se-glutathione peroxidase; glutathione transferases; glutathione reductase

Fructus Schisandrae, the fruit of *Schisandra chinensis* (Turcz.) Baill., has been used as tonic and astringent in traditional Chinese medicine for centuries [1]. Sch B§ (Fig. 1b), a dibenzo[a,c]cyclooctadiene derivative isolated from FS, has been shown to protect against CCl_4-induced hepatotoxicity [2] as well as IR-induced myocardial injury [3]. Previous studies in our laboratory indicated that the methylenedioxy group of the dibenzo[a,c]cyclooctadiene (schisandrin) molecule is an important structural determinant in enhancing hepatic mitochondrial glutathione and hence protecting against CCl_4 hepatotoxicity in mice [4]. However, it is unclear whether this structure–activity relationship is still valid with regard to the cardioprotective action of schisandrins. As a preliminary investigation to exploring this relationship, we examined the effects of three schisandrins which differ structurally by the presence or absence of the methylenedioxy group, namely Sch A (Fig. 1a), Sch B, and Sch C (Fig. 1c), and DDB (Fig. 1d), an intermediate compound derived from the synthesis of Sch C lacking the cyclooctadiene ring, on myocardial IR injury in isolated Langendorff-perfused hearts prepared from drug pretreated rats. Tissue glutathione antioxidant status was also assessed in control or ischemic-reperfused rat hearts.

MATERIALS AND METHODS

Chemicals

DDB was purchased from Guangzhou Xun Xing Pharmaceutical Company. All other chemicals were of analytical grade. Solvents used for high-performance liquid chromatography were of HPLC grade. FS was imported from mainland China. It was authenticated and supplied by a commercial dealer (Lee Hoong Kee Ltd.) in Hong Kong. Schisandrins, including Sch A, Sch B, and Sch C, were

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§ *Abbreviations:* CCl_4, carbon tetrachloride; DDB, dimethyl-4,-4′-dimethoxy-5,6,5′,6′-dimethylene-dioxy-biphenyl-2,2′-bicarboxylate; GPX, Se-glutathione peroxidase; GST, glutathione transferases; GRD, glutathione reductase; IR, ischemia-reperfusion; LDH, lactate dehydrogenase; Sch A, schisandrin A; Sch B, schisandrin B; Sch C, schisandrin C; FS, *Fructus Schisandrae*; and AUC, area under the curve.

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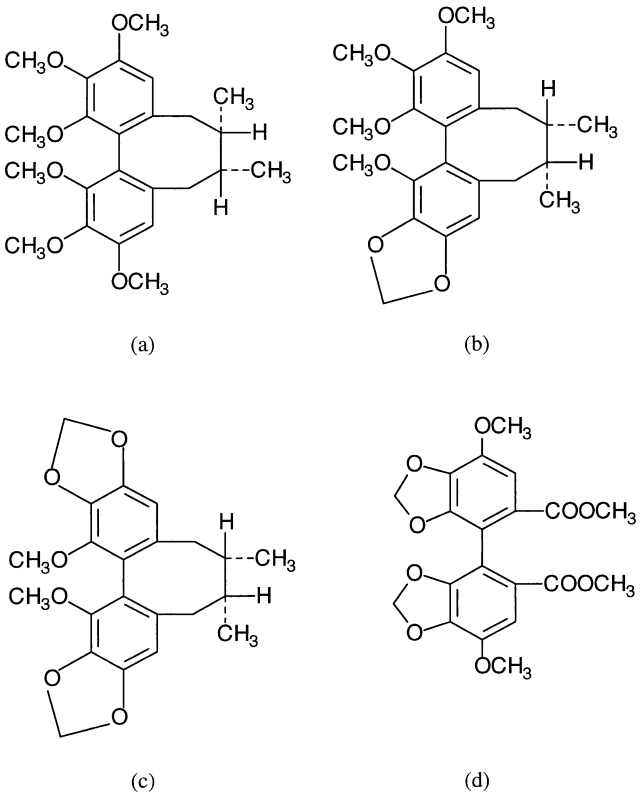

FIG. 1. Chemical structures of (a) schisandrin A; (b) schisandrin B; (c) schisandrin C; and (d) dimethyl-4,4′-dimethoxy-5,6,5′,6′-dimethylenedioxybiphenyl-2,2′-carboxylate.

purified from the petroleum ether extract of FS by silica gel column chromatography as previously described [2]. The chemical structures of schisandrins were confirmed by comparing the thin-layer chromatography and spectral characteristics (^{1}H and ^{13}C-NMR and mass spectra) with authentic standards obtained from the Institute of Materia Medica, Chinese Academy of Medical Sciences, Beijing. The purity of the compounds, as assessed by HPLC, was found to be higher than 95% (w/w).

Animal Pretreatment

Male adult Sprague–Dawley rats (240–275 g) were maintained on a 12-hr dark/light cycle at about 22° and allowed food and water *ad lib*. Animals were randomly divided into groups of at least 3 animals per group. In the pretreatment groups, rats were treated intragastrically with schisandrins or DDB for 3 days at a daily dose of 1.2 mmol/kg. All drugs were suspended/dissolved in olive oil. Control animals were given the olive oil only. Twenty-four hours after the last dosing, control or drug pretreated rats were anesthetized with diethylether. The heart was excised and subjected to IR challenge as described below.

Isolated-perfused Rat Heart

The heart was excised quickly and immediately immersed in ice-cold and heparinized (50 unit/mL) saline. The aorta was cannulated and then transferred to a warm and moist chamber of the perfusion apparatus. The heart was retrogradely perfused according to the Langendorff method as described in [5]. The apex of the heart was attached via a metal hook to an unextendable cotton thread which was connected to a force displacement transducer (Grass FT03), and the isometric contractions of the heart were recorded on a polygraph (Grass Model 7-8 P).

Myocardial IR Injury

After an initial 30-min perfusion for equilibration, the isolated heart was subjected to a 10-min period of "no-flow" normothermic global ischemia followed by a 15-min reperfusion. The nonischemic control hearts were perfused for 25 min after equilibration, without being subjected to 10 min of ischemia. Coronary effluent was collected in a 1-min fraction at increasing time intervals during the course of equilibration and reperfusion. The fraction was immediately put on ice until assay. Myocardial IR injury was assessed by measuring the extent of LDH (EC 1.1.2.3) leakage during the reperfusion period. LDH activity of the coronary effluent was assayed as described in [5]. The extent of LDH leakage was estimated by computing the AUC of the graph plotting the percent of LDH activity (with respect to the mean preischemic value measured during the equilibration period) against the reperfusion time (1–15 min) as described in [5]. The LDH activity was normalized against the preischemic value in coronary effluent in order to minimize the interanimal variation in LDH leakage prior to ischemia and reperfusion.

Reduced Glutathione and Antioxidant Enzyme Assays

Myocardial tissue samples were rinsed with ice-cold homogenizing buffer (50 mM of Tris(hydroxymethyl) aminomethane, 0.1 mM of EDTA, pH 7.6) after the IR experiment. Tissue homogenate was prepared by homogenizing 0.8 g of myocardial tissue in 8 mL of ice-cold homogenizing buffer with two 10-sec bursts of a tissue disintegrator (Ika Ultra turax T25) at 135,000 rpm. Myocardial cytosolic fraction was prepared by diluting 1 mL of tissue homogenate with 3 mL of homogenizing buffer and centrifuging at 40,000 *g* for 30 min at 4°. The GSH level was measured by an HPLC method modified from Reed *et al.* [6] as described in [7]. The activities of GRD (EC 1.6.4.2) and GPX (EC 1.11.1.9) were measured as described in [8]. GST (EC 2.5.1.18) activity was assayed by measuring the conjugation of GSH with 1-chloro-2,4-dinitrobenzene, as described by Warholm *et al.* [9].

Statistical Analysis

Data were analyzed by one-way ANOVA followed by Duncan's multiple range test to detect the intergroup difference. Differences were considered significant when $P < 0.05$.

TABLE 1. Effect of schisandrin and DDB pretreatment on myocardial ischemia-reperfusion injury in isolated Langendorff-perfused rat hearts

	The extent of LDH leakage* (AUC)	Contractile force recovery† (%)
CON-IR (N = 5)	563 ± 36	50 ± 2
Sch A-IR 1.2 mmol/kg (N = 4)	527 ± 28	50 ± 5
Sch B-IR 1.2 mmol/kg (N = 5)	260 ± 17‡ (54)	66 ± 3‡
Sch C-IR 1.2 mmol/kg (N = 4)	159 ± 21‡§ (72)	68 ± 4‡
DDB-IR 1.2 mmol/kg (N = 5)	579 ± 26	57 ± 8

Values given are mean ± SEM, with the number of animals indicated in parentheses. The italicized number in parentheses is the percent protection with respect to the CON group.

* Values obtained by subtracting the LDH leakage (640 ± 13.3, N = 5) measured from isolated perfused hearts without subjecting to IR.

† Estimated by comparing the contractile force at 15 min of reperfusion with that measured at the end of the equilibrium period.

‡ Significantly different from the CON-IR group.

§ Significantly different from the Sch B-IR group.

RESULTS AND DISCUSSION

As shown in Table 1, a 10-min period of ischemia followed by 15-min reperfusion caused an increase in LDH leakage in isolated perfused rat hearts. The increased extent of LDH leakage was associated with an incomplete (50%) recovery of contractile force at 15 min after reperfusion. It is well established that the pathogenesis of myocardial IR injury involves reactive oxidant species arising from the reperfusion process [10–12]. IR injury is a commonly used model for assessing antioxidant activities of synthetic or naturally occurring compounds, particularly in the myocardium. While all drug pretreatments failed to alter the extent of LDH leakage in non-IR hearts (data not shown), isolated hearts prepared from rats pretreated with Sch B or Sch C showed protection against IR-induced myocardial damage, with the extent of LDH leakage being decreased by 54% and 72% at daily doses of 1.2 mmol/kg, respectively. The reduction in the extent of LDH leakage was paralleled by significant improvements in contractile force recovery in hearts prepared from both Sch B and Sch C pretreated rats. Sch C pretreatment seemed to be more effective than that of Sch B in protecting against IR injury, as evidenced by the smaller extent of LDH leakage. On the other hand, neither Sch A nor DDB pretreatment at the same dosage regimen produced any detectable effect on myocardial IR injury. Since all the drugs studied are structurally related compounds with similar lipophilicity, the failure of Sch A and DDB pretreatment in protecting against myocardial IR injury is not likely due to the poorer gastrointestinal absorption of the drugs than that of Sch B and Sch C. Similarly, comparative studies between schisandrins (including Sch B) and DDB in a mouse model of CCl_4 hepatotoxicity indicated that oral Sch A and DDB pretreatment could increase hepatic mitochondrial GRD activity [4] and suppress the CCl_4-induced increase in plasma alanine aminotransferase activity [13], respectively, thus demonstrating the bioavailability of the drugs through oral administration.

The antioxidant mechanism involved in the cardioprotective action of schisandrin pretreatment was further investigated by examining the effect on myocardial glutathione antioxidant status. As shown in Table 2, all drug pretreatments failed to significantly change the glutathione antioxidant status in non-IR hearts as assessed by measuring the tissue GSH level and GSH-related antioxidant enzyme activities. The IR-induced myocardial injury was associated with an impairment in myocardial glutathione antioxidant status, as indicated by a depletion in tissue GSH (45%) as well as decreases in GPX (34%), GRD (16%), and GST (29%) activities in ischemic-reperfused hearts, when compared with the non-IR control. Treating rats with Sch B or Sch C at a daily dose of 1.2 mmol/kg protected against the IR-induced impairment in myocardial glutathione antioxidant status, as indicated by increases in the GSH level (58 and 70%, respectively), and GRD (16 and 22%), GPX (22 and 42%) and GST (20 and 40%) activities in ischemic-reperfused hearts when compared with the unpretreated IR control. In contrast, Sch A or DDB pretreatment at the same dosage regimen did not produce any detectable effect on tissue glutathione antioxidant status in ischemic-reperfused hearts.

The maintenance of myocardial glutathione antioxidant status is important in protecting against free radical-induced tissue damage [14, 15]. The glutathione antioxidant system, comprising mainly GSH, GPX, GST, and GRD, serves as an effective cellular defense against oxidative stress. In essence, the GRD-catalyzed regeneration of GSH from its oxidized form can sustain the GSH-dependent free radical scavenging activity of GPX and GST in decomposing hydrogen peroxide or other organic hydroperoxides [16]. Given that the myocardial superoxide dismutase (EC 1.15.11) and catalase (EC 1.11.1.6) activities are much lower than those in other tissues [17], the myocardium is more susceptible to oxidative damage produced by reactive oxidant species [18]. The glutathione antioxidant system is therefore crucial for scavenging reactive free radicals generated in the heart. In this regard, it has been reported that GSH-depleted rat hearts were found to be more susceptible to IR injury [19]. In addition, hearts isolated from transgenic mice with overexpressing GPX have been shown to be more resistant to IR injury [20]. The IR-induced impairment in myocardial glutathione antioxidant status, as shown in the present study (Table 2), may be caused by free radical-induced GSH depletion and enzyme inactivation. On the other hand, the decreases in the myocardial GSH level and antioxidant enzyme activities may also result from

TABLE 2. Effect of schisandrin and DDB pretreatment on myocardial glutathione antioxidant status in ischemic-reperfused rat hearts

		Antioxidant enzyme activity (mU/mg tissue)		
	GSH (nmol/mg tissue)	GPX	GRD	GST
NON-IR				
CON (N = 5)	1.74 ± 0.07	6.85 ± 0.18	0.44 ± 0.01	2.97 ± 0.21
Sch A (N = 3)	1.70 ± 0.06	6.45 ± 0.24	0.45 ± 0.01	2.85 ± 0.23
Sch B (N = 3)	1.69 ± 0.09	6.29 ± 0.19	0.44 ± 0.02	2.79 ± 0.25
Sch C (N = 3)	1.68 ± 0.03	6.48 ± 0.18	0.45 ± 0.02	2.95 ± 0.18
DDB (N = 3)	1.65 ± 0.05	6.10 ± 0.05	0.43 ± 0.02	2.89 ± 0.14
IR				
CON (N = 5)	0.95 ± 0.10*	4.49 ± 0.22*	0.37 ± 0.02*	2.11 ± 0.13*
Sch A (N = 4)	1.02 ± 0.17	4.54 ± 0.15	0.40 ± 0.02	2.18 ± 0.14
Sch B (N = 5)	1.50 ± 0.03† (*58*)	5.48 ± 0.11† (*22*)	0.43 ± 0.01† (*16*)	2.53 ± 0.08† (*20*)
Sch C (N = 4)	1.62 ± 0.02†‡ (*70*)	6.38 ± 0.12†‡ (*42*)	0.45 ± 0.02† (*22*)	2.95 ± 0.11†‡ (*40*)
DDB (N = 5)	0.97 ± 0.06	4.48 ± 0.25	0.38 ± 0.02	2.10 ± 0.16

Values given are means ± SEM, with the number of animals indicated in parentheses. The italicized number in parentheses is the percent increase with respect to the IR group.
* Significantly different from the CON group.
† Significantly different from the IR group.
‡ Significantly different from the Sch B-IR group.

the nonspecific leakage, as in the case of LDH, from the oxidatively damaged cell membranes in ischemic-reperfused hearts. However, under the present experimental conditions, the amount of LDH leakage, which was approximated to 14% of the total myocardial LDH activity (data not shown), was much lower than the extent of depletion in GPX activity (34%) in ischemic-reperfused hearts. Given that the GPX molecule has a much larger molecule size than that of LDH (84 kD vs 35 kD), it seemed unlikely that the IR-induced decreases in antioxidant enzyme activity were solely caused by nonspecific membrane leakage. The cardioprotection afforded by Sch B or Sch C pretreatment was paralleled by the enhancement in myocardial glutathione status. The degree of enhancement in myocardial glutathione antioxidant status by Sch B or Sch C pretreatment correlated with the extent of myocardial protection against IR injury. Based on the observation that Sch B or Sch C pretreatment was unable to enhance myocardial glutathione antioxidant status in non-IR hearts (Table 2), the maintenance in the functioning of the myocardial antioxidant system in the drug-pretreated and ischemic-reperfused hearts may possibly be an event secondary to a primary protective action of the drug. In this regard, the free radical scavenging action of schisandrins may be involved in protection against myocardial IR injury [21].

Regarding the structure–activity relationship, our results indicate that pretreatment with the methylenedioxy group and cyclooctadiene ring containing schisandrins, namely Sch B and Sch C, was able to enhance the myocardial glutathione antioxidant status upon IR challenge and hence protect against IR injury; however, compounds which contain the methylenedioxy group alone, as in the case of DDB, or the cyclooctadiene ring alone, as in the case of Sch A, failed to maintain the myocardial glutathione antioxidant status as well as protect against IR injury. In conclusion, our findings suggest that both the methylenedioxy group and the cyclooctadiene ring are important structural determinants of the schisandrin molecule in enhancing myocardial glutathione status in ischemic-reperfused hearts. Apparently, the possession of two methylenedioxy groups in the molecule, as in the case of Sch C, offers more potent activity.

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