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ORIGINAL ARTICLE

Schisandrin B Ameliorates Myocardial Ischemia/Reperfusion Injury Through Attenuation of Endoplasmic Reticulum Stress-Induced Apoptosis

Wei Zhang,[1] Zhiqing Sun,[2] and Fanhua Meng[3,4]

Abstract—Schisandrin B (*Sch B*), an active composition isolated from the fruit of *Schisandra chinensis*, has been proved to possess antiinflammatory, antioxidant and anti-endoplasmic reticulum (ER) stress effects in many rodent tissues. However, the exact mechanism of cardioprotective effect of *Sch B* still needs more study. Here, we detected the effects of *Sch B* on myocardial ischemia/reperfusion (I/R) injury rats. I/R injury model in this study was established by left anterior descending coronary artery ligation for 40 min followed by 1 h of reperfusion. Male healthy rats were randomly divided into five groups: the sham, I/R, *Sch B* (20 mg/kg) + I/R, and *Sch B* (40 mg/kg) + I/R, *Sch B* (80 mg/kg) + I/R, with 10 rats in each group. We showed that *Sch B* treatment significantly protected against myocardial I/R injury, as demonstrated by the decrease in the percentage of infarct formation assessed by 2,3,5-triphenyl tetrazolium chloride (TTC) staining in representative heart tissue slices, comparing with the I/R control group. The levels of creatine kinase (CK), lactate dehydrogenase (LDH), malondialdehyde (MDA), and total superoxide dismutase (T-SOD) were tested. The ER stress-related proteins such as C/EBP homologous protein (CHOP), activating transcription factor 6 (ATF6), and (PKR)-like ER kinase (PERK) were further measured by western blot, and their messenger RNA levels were measured by real-time PCR. The apoptosis of heart tissue cells was also tested through the expressions of caspase-9, caspase-3, Bcl-2, and Bax proteins. Collectively, these results revealed that *Sch B* exerts protection role on myocardial I/R injury through decreasing oxidative reaction, suppressing ATF6 and PERK pathway, and attenuating ER stress-induced apoptosis.

KEY WORDS: schisandrin B; ischemia/reperfusion injury; endoplasmic reticulum stress; apoptosis.

INTRODUCTION

Heart disease is the main cause of death in many countries in the world, and myocardial infarction contributes the most morbidity and mortality among them [1]. At present, the main therapeutic treatments against myocardial infarction are opening the infarct-related coronary artery and leading to the restoration of myocardial perfusion, which can be achieved through drug thrombolysis, percutaneous coronary intervention, and coronary artery bypass graft [2, 3]. However, these reperfusion treatments may cause ischemia/reperfusion (I/R) injury, which is not only disable to restore normal heart function but also increase heart dysfunction and structural damage, and even lead to irreversible damage [4].

I/R injury usually occurs when the blood supply to the myocardial tissue is recovered after it is interrupted a certain time [5]. Numerous studies demonstrated that prolonged reperfusion could result in serious acute or chronic

[1] Department of Electrocardiogram, Linyi People's Hospital, No. 49 Yizhou Road, Linyi, 276000, Shandong, People's Republic of China
[2] Department of Neurology, Linyi People's Hospital, No. 49 Yizhou Road, Linyi, 276000, Shandong, People's Republic of China
[3] Department of Nerve Electrophysiology Room, Linyi People's Hospital, No. 49 Yizhou Road, Linyi, 276000, Shandong, People's Republic of China
[4] To whom correspondence should be addressed at Department of Nerve Electrophysiology Room, Linyi People's Hospital, No. 49 Yizhou Road, Linyi, 276000, Shandong, People's Republic of China. E-mail: mengfh06@126.com

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myocardial damage, including myocardial ultrastructural alterations, remodeling, and systolic and diastolic dysfunction, which is severer than that produced by ischemia alone [6]. Evidence has demonstrated that ischemic cell apoptosis occurs due to the toxicity from the explosion of ensuing reactive oxygen species during reperfusion, and it plays a pivotal role in the I/R injury [7]. As a kind of programmed cell death, apoptosis is regulated by several signaling pathways. Among that, the endoplasmic reticulum (ER) stress-initiated apoptosis signaling is an important apoptosis pathway in the development of myocardial I/R injury [8, 9]. Therefore, I/R injury may be alleviated through the regulation of ERS.

The endoplasmic reticulum is an important membranous organelle in the eukaryotic cells, and its main functions include nascent polypeptide folding, assembly, modification and secretion, lipid synthesis, and calcium storage [10]. When ER is exposed to stress stimuli, such as ischemia, hypoxia free radical exposure, elevated protein synthesis, and gene mutations, the homeostasis of it is damaged, which further leads to the pathological accumulation of unfolded/misfolded proteins in the ER. Moderate ERS can be detected by the transmembrane protein sensors ((PKR)-like ER kinase (PERK), inositol-requiring protein-1 (IRE1), and activating transcription factor 6 (ATF6)) of ER and initiates the unfolded protein response (UPR) to recover the ER homeostasis [11, 12]. However, if the stress persists or strength is too strong, the effects of UPR will change to initiate apoptosis. C/EBP homologous protein (CHOP) was reported as an important molecule involved in ERS-induced apoptosis [13]. The ERS-induced apoptosis is mainly mediated by transcriptional induction of CHOP or by activation of caspase-12 or JNK signaling pathway [14].

Schisandrin B (*Sch B*), a well-known Chinese herb of traditional Chinese medicine, is the most plentiful and active ingredient isolated from the fruit of *Schisandra chinensis* [15]. Previous studies have shown that *Sch B* possesses a variety of pharmacological effects, such as hepatoprotective [16], antiinflammatory, antioxidant [17], anticancer [18], and so on. The *Sch B* has been widely applied in liver protection and treatment, cardiovascular and cerebrovascular diseases, vascular injury, and a variety of neurodegenerative diseases in clinic [19].

In spite of this, the cardioprotective effects of *Sch B* and the potential mechanism are still incomplete and need more study. The present study investigated the protective effects of *Sch B* on I/R injury and the underlying mechanism. We found that *Sch B* could affect the oxidation system, suppress the ER stress response, and attenuate ER stress-induced apoptosis, leading to the effective protection of myocardial I/R injury.

MATERIALS AND METHODS

Reagents and Antibodies

Schisandrin B was purchased from Selleckchem (Selleckchem Shanghai, China). 2,3,5-Triphenyl tetrazolium chloride (TTC) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Primary antibodies against PERK, p-PERK, CHOP, ATF-6, caspase-3, caspase-9, Bcl-2, and Bax were from CST Technology Inc. (Danvers, MA, USA). Anti-β-catenin antibody and horseradish peroxidase-conjugated second antibody were both purchased from Abcam (Cambridge, MA, USA). Primers for the detection of CHOP, ATF-6, PERK, and GAPDH were synthesized by Sangon Biotech (Shanghai) Co. Ltd. (Shanghai, China). Creatine kinase (CK), lactate dehydrogenase (LDH), malondialdehyde (MDA), and the total superoxide dismutase (T-SOD) test kit were all provided by Nanjing Jiancheng Biological Engineering institute (Nanjing, China).

Animals

Adult male Sprague-Dawley rats, weighing 200 to 250 g, were purchased from the Beijing Vital River Laboratory Animal Technology Co. Ltd. All rats were group-housed with three to four rats per cage on a 12-h light/dark cycle in a temperature-controlled room (22–25 °C) with free access to food and water for 1 week to adapt to the environment. After that, animals were divided into five groups (n = 10 per group): the sham-operated group, I/R group, *Sch B* (20 mg/kg) + I/R group, *Sch B* (40 mg/kg) + I/R group, and *Sch B* (80 mg/kg) + I/R group. All experimental animals used in this study were covered by a protocol approved by the Institutional Animal Care and Use Committee.

In Vivo Rat Model of I/R Injury and Drug Administration

The I/R injury animal model in this study was induced by Le anterior descending (LAD) ligation for 40 min followed by 1-h reperfusion [20]. First of all, rats weighing about 250 g were anesthetized with 1 g/kg urethane, and 2-cm incisions to the left and parallel to the sterni were made by cutting the fifth and sixth ribs. The hearts were exteriorized by applying pressure to the lateral aspects of the thoracic cage. LAD coronary artery was occluded for 40 min to simulate regional ischemia; after that, the duration of reperfusion was 1 h. Sham-operated control rats underwent the same surgical operation except that the suture placed under the left coronary artery was not tied. Different concentrations of *Sch B* dissolved in DMSO

solution were administrated by oral gavage for 5 days before operation, and an equal volume of DMSO solution was given to the control series of experiments. At the end of experiments, the rats were euthanatized and hearts were immediately removed for the following study.

Measurement of Myocardial Infarct Size

Myocardial infarct size of hearts was assessed by TTC staining. After surgery, the hearts were immediately removed and frozen at −20 °C for several hours. Then, the heart ventricles were directed and sliced transversely into 2-mm-thick sections. The slices were incubated in 2% TTC for 15–20 min in dark conditions at 37 °C and subsequently fixed in 4% paraformaldehyde phosphate buffer for 1 h. The degree of myocardial infarction was represented by the ratio between the infarcted and the total heart areas. White area was considered as the infarct area, whereas normal tissues were stained in red. The ImageJ software was used to measure the areas of infarct size digitally.

Determination of CK, LDH, MDA, and T-SOD in Serum

After reperfusion, blood samples were collected from the orbita of rats and then centrifugated at 2000 rpm for 10 min at 4 °C. The supernatant serum was used for the following study. The levels of CK, LDH, T-SOD, and MDA were measured by commercial assay kits (Jiancheng Biological Engineering Institute, Nanjing, China) using ultraviolet-visible spectrophotometer according to the manufacturer's protocols. All measurements were performed in duplicate.

Western Blot

Total proteins were isolated from myocardial infarction area tissue. Briefly, myocardial tissue samples were washed twice with PBS and lysed in RIPA buffer for 1 h on ice. The lysates were centrifuged at 13,000 rpm for 20 min, and protein concentration in the supernatants was quantified by Bradford assay. Fifty micrograms of total proteins was separated by 10% SDS-polyacrylamide gel electrophoresis and transferred onto a PVDF membrane. This membrane was blocked with 3% BSA in PBS for 1 h at 37 °C and then incubated at 4 °C overnight with the respective primary antibody at 37 °C. After incubated with a horseradish peroxidase-conjugated secondary antibody for 1 h at 37 °C, immunoreactive protein bands were visualized by enhanced ECL reagent with Tanon 5200 Chemiluminescence Imaging System (Shanghai, China).

Real-Time PCR

The RNA was extracted from myocardial infarction tissue using TRIzol® reagent (Invitrogen, Carlsbad, CA). The quality and concentration of total RNA were assessed by the ratio of A_{260}/A_{280} using a BioPhotometer (Eppendorf, Hamburg, Germany). One microgram of total RNA was reversely transcribed using First-Strand cDNA Synthesis superMix (TransGen Biotech Co., Ltd., Beijing, China). Quantitative real-time PCR (qPCR) was conducted in accordance with the protocol of SYBR Green qPCR Super Mixture (Vazyme Biotech Co., Ltd., Nanjing, China). The target messenger RNA (mRNA) expression levels were determined using the comparative threshold ($2^{-\Delta\Delta CT}$) method by normalizing to GAPDH (ΔCt). Primer sequences used in this study were GAPDH (forward 5′-ACC CAT CAC CAT CTT CCA GGA G-3′, reverse 5′-GAA GGG GCG GAG ATG ATG AC-3′), PERK (forward 5′-GAT CCG TCT CCC AAA CAG G-3′, reverse 5′-TAG CCA AGG CTT TGA CTT CC-3′), ATF6 (forward 5′-CTC ATG GAC CAG GTG AAG ACT-3′, reverse 5′-GGG CTC CAT ATG TCT GAC TCC-3′), and CHOP (forward 5′-AGC TGG AAG CCT GGT ATG AG-3′, reverse 5′-GAC CAC TCT GTT TCC GTT TC-3′).

Statistical Methods

All experiments were performed with at least triplicate independent replications, and data were expressed as means ± SDs. Comparisons were analyzed using one-way variance analysis (ANOVA). $^{*}P < 0.05$, $^{**}P < 0.01$ was considered to be statistically significant.

RESULTS

Sch B Decreased the Myocardial Damage in Rats Subjected to Myocardial I/R

To study the effect of *Sch B* on myocardial damage in rats subjected to MI/R, we established the I/R injury animal model. TTC staining of myocardial tissue slices showed the infarct size of hearts. As we could see in Fig. 1a, the I/R group in which TTC was unstained showed the noticeable increased infarction of hearts compared with the sham group ($P < 0.001$). On the other hand, the groups pretreated with *Sch B* at the concentration of 20, 40, and 80 mg/kg could ameliorate the infarction, particularly at 80 mg/kg ($P < 0.05$). The quantitative analysis of the infarct area in Fig. 1b further confirms these effects.

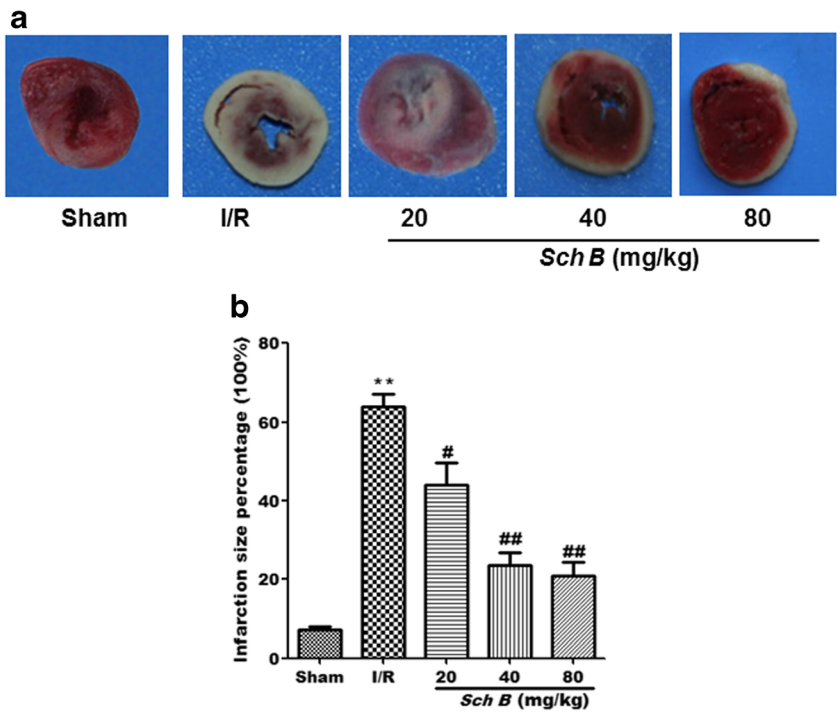

Fig. 1. TTC staining to assess the extent of myocardial necrosis. **a** Hearts in different groups were subjected to regional ischemia (40 min)/reperfusion (1 h), and TTC staining was used to assess the extent of myocardial necrosis. **b** The quantitative analysis of the infarct area. *Bars* represent the percent of ischemic area at risk in hearts. Values are means ± SEM, $n = 6$ per group. $^{\#\#\#}P < 0.001$ compared with the sham group; $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the model group.

Sch B Affected the Serum Levels of LDH, CK, MDA, and T-SOD in the I/R Injury Rats

Leakage of LDH and CK from myocardial tissues to blood is an indicator of acute myocardial infarction. As shown in Fig. 2a, b, the CK and LDH levels in serum increased significantly in the I/R model group compared with the sham control group ($P < 0.01$). However, the treatment of *Sch B* at the concentration of 40 and 80 mg/kg could markedly downregulate the levels of CK and LDH in a dose-dependent manner ($P < 0.05$).

In order to study if *Sch B* could affect the lipid peroxidation and oxidative stress in I/R injury, the levels of MDA and T-SOD were tested. As is shown in Fig. 2c, d, MDA level was increased ($P < 0.01$) and T-SOD level was decreased ($P < 0.05$) in I/R model group compared with sham group. This effect was abolished by *Sch B* treatment, particularly at 40 and 80 mg/kg ($P < 0.05$). Similarly, dose dependency was observed as well. All these results above indicated that the presence of *Sch B* downregulated the CK, LDH, and MDA levels and upregulated the T-SOD level, indicating that *Sch B* may affect the lipid peroxidation and oxidative stress in I/R injury rats' hearts.

Sch B Inhibited the ERS-Related Pathway Activated by I/R Injury

Evidences have suggested that excessive ERS enhanced the damage of I/R injury and ultimately induced cell apoptosis. This process is mediated by the ER stress transducers, named PERK, ATF6, and IRE1. Among that, PERK signaling and ATF6 signaling are proved to be proapoptotic effectors, and such effect is mediated through the induction of CHOP. To evaluate the effect of *Sch B* treatment on ERS, the mRNA levels in myocardial tissue of three ERS-related genes listed as CHOP, ATF6, and PERK were firstly studied. As detected in Fig. 3, I/R injury remarkably increased the transcription of these three genes compared with sham group ($P < 0.01$), and the levels were decreased dose-dependently in groups treated by Sch B, especially at the concentration of 40 and 80 mg/kg ($P < 0.05$).

Besides that, we further detected the protein expressions of CHOP, ATF6, p-PERK, and PERK by western blot. According to our results, the expressions of CHOP, ATF6, and p-PERK were upregulated by I/R injury ($P < 0.05$). The pretreatment of *Sch B* significantly reduced the upregulated protein expressions ($P < 0.05$). On the

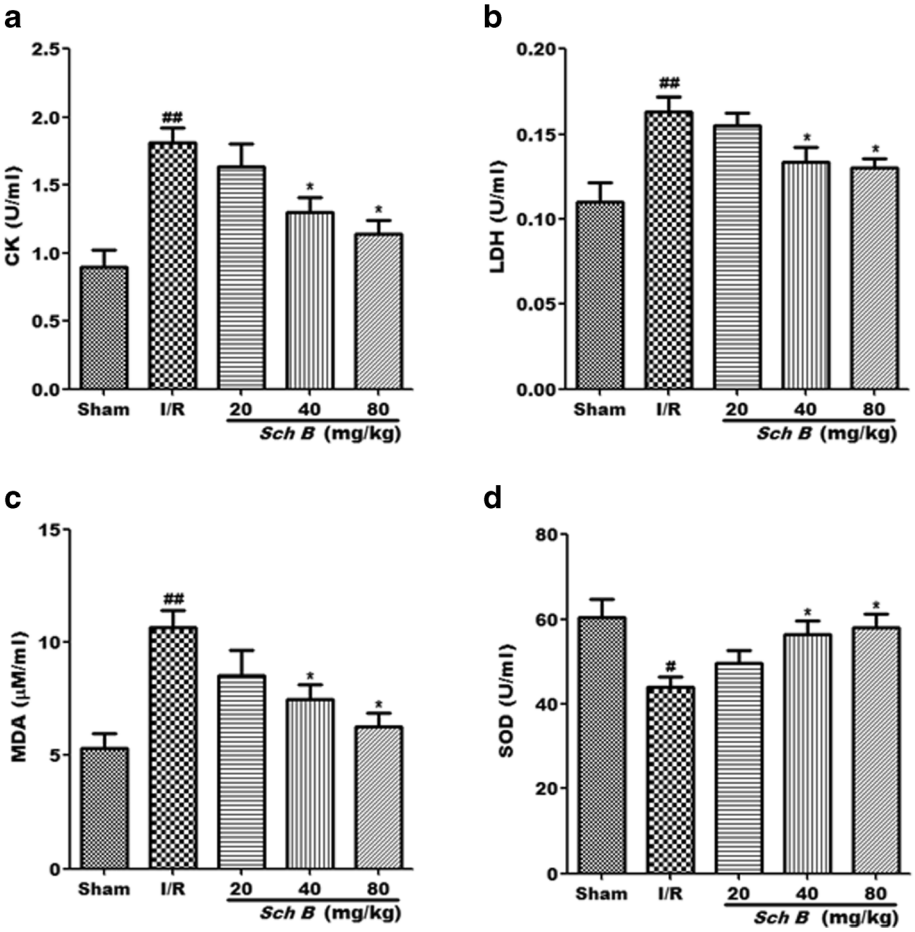

Fig. 2. Effects of *Sch B* on the biochemical parameters in serum of rats with I/R injury. Rats in *Sch B* groups were pretreated with intragastric administration of *Sch B* for 5 days before the myocardial I/R operation. Rats in the sham-operated and model groups received vehicle in an identical fashion to the drug-treated groups instead. Myocardial ischemia/reperfusion was induced by LAD ligation. **a** CK, **b** LDH, **c** MDA levels, and **d** T-SOD activities in serum were spectrophotometrically determined. Results were representative of those obtained from three independent experiments. Values are means ± SEM, $n = 6$ per group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with the sham group; $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the model group.

other hand, no significant effect of *Sch B* on PERK protein level was observed in this study. All the above results showed that the PERK and ATF6 signaling were activated by I/R injury, and the pretreatment of *Sch B* could inhibit their activation (Fig. 4).

Sch B Affects the Expression of Apoptosis-Related Proteins

To determine whether the administration of *Sch B* could affect the expressions of apoptosis-related proteins, we analyzed the levels of caspase-9, caspase-3, Bcl-2, and Bax in myocardial tissues by western blot. As we can see in Fig. 5, compared with control group, the I/R model group displayed significantly increased levels of caspase-9, caspase-3, and Bax and decreased level of Bcl-2 ($P < 0.05$). *Sch B*-treated groups displayed significantly decreased levels of caspase-9, caspase-3, and Bax and increased levels of Bcl-2 in comparison to the I/R model group ($P < 0.05$).

DISCUSSION

Ischemic heart disease is one of the most serious problems of many countries in the world, and I/R injury is considered to be the major cause of their high morbidity and mortality [21]. However, although the mechanisms of I/R injury is comprehensively understood, there are still few effective strategies for this problem up to now.

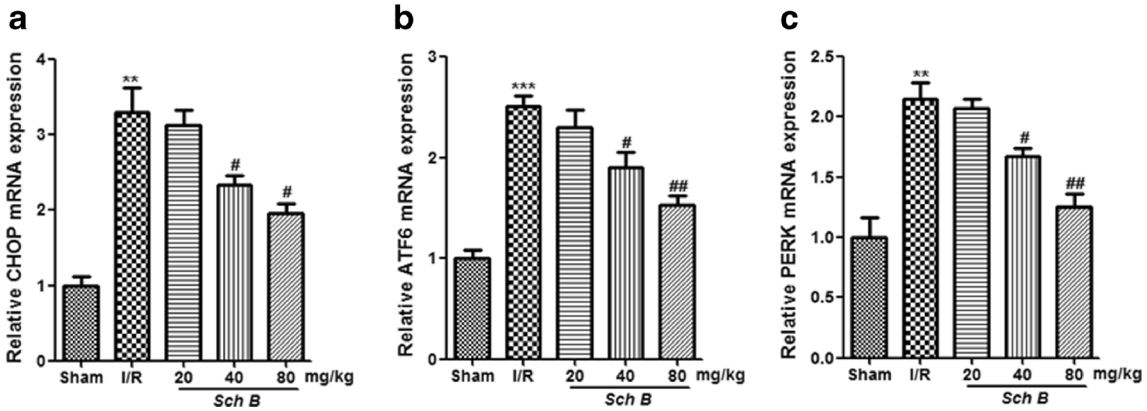

Fig. 3. Effects of *Sch B* on the mRNA expressions of CHOP, ATF6, and PERK in myocardial tissues of rats with I/R injury. The mRNA expressions of CHOP (**a**), ATF6 (**b**), and PERK (**c**) in myocardial tissues of rats were measured using real-time PCR. GAPDH was used as an endogenous housekeeping gene. Results were representative of those obtained from three independent experiments. Values are means ± SEM, $n = 6$ per group. $^{###}P < 0.001$, $^{##}P < 0.01$ compared with the sham group; $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the model group.

Therefore, it is very essential to seek for novel drug to treat myocardial I/R damage.

Numerous evidences have strongly indicated that traditional Chinese medicine has great advantages in the treatment of many diseases. *S. chinensis* is the most commonly used traditional oriental medicine and possesses diverse biological activities for human health [19]. Among many kinds of lignans that isolated from this plant, *Sch B* is

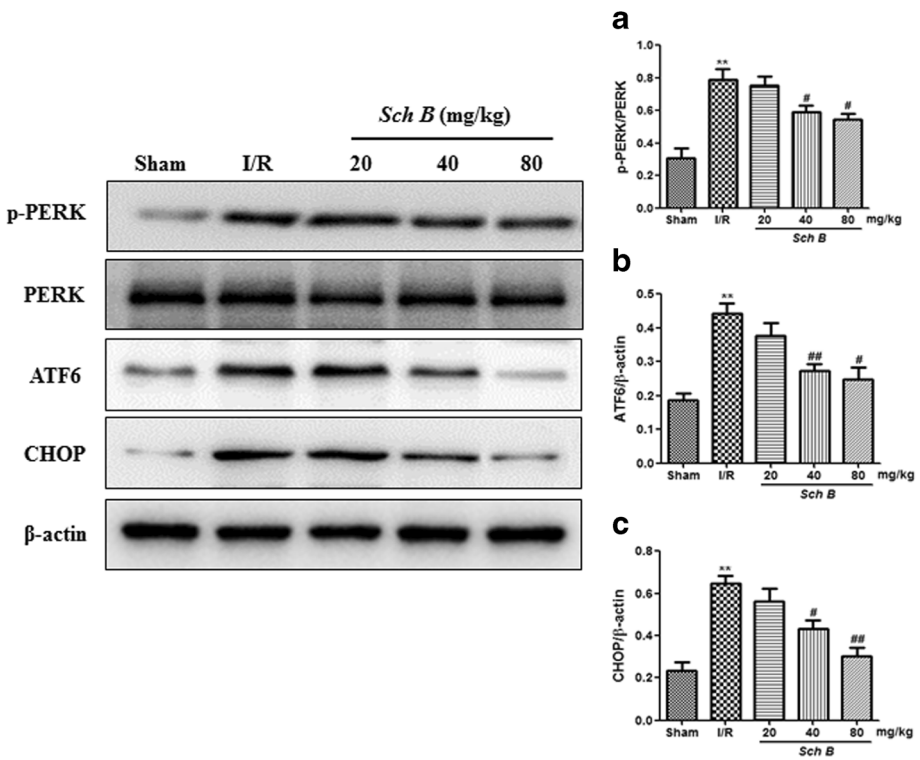

Fig. 4. Effects of *Sch B* on the proteins expressions of CHOP, ATF6, p-PERK, and PERK in myocardial tissues of rats with I/R injury. The protein expressions of PERK (**a**), ATF6 (**b**), and CHOP (**c**) in myocardial tissues of rats were measured by western blot, quantification of their expressions normalized to β-catenin. Results were representative of those obtained from three independent experiments. Values are means ± SEM, $n = 6$ per group. $^{###}P < 0.001$, $^{##}P < 0.01$ compared with the sham group; $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the model group.

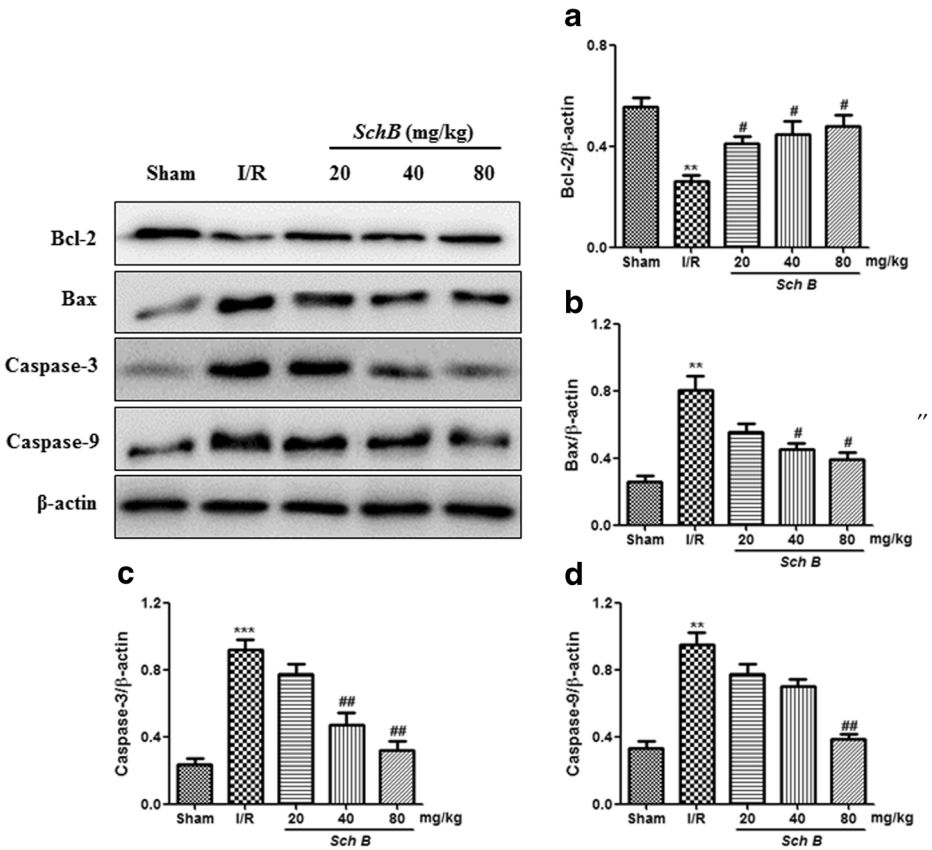

Fig. 5. Effects of *Sch B* on the expressions of apoptosis-mediating proteins in myocardial tissues of rats with I/R injury myocardial tissues. The protein expressions of Bcl-2 (**a**), Bax (**b**), caspase-3 (**c**), and caspase-3 (**d**) in myocardial tissues of rats were measured by western blot, quantification of their expressions normalized to β-catenin. Results were representative of those obtained from three independent experiments. Values are means ± SEM, $n = 6$ per group. $^{\#\#\#}P < 0.001$, $^{\#\#}P < 0.01$ compared with the sham group; $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the model group.

the most abundant and active ingredient [15]. Numerous studies have been carried out about *Sch B* in the past few years, and it is suggested that *Sch B* might be a good candidate drug for ischemic heart disease therapy. Ko *et al*. had proved that the pretreatment of *Sch B* could not only enhance the glutathione antioxidant status in the mitochondrion but also decrease the sensitivity of mitochondria to calcium ion-induced permeability transition to protect against myocardial I/R injury [22–25]. Further studies showed that *Sch B* could also protect against myocardial I/R injury partly by increasing the expressions of heat shock protein (Hsp)25 and Hsp70 in rats [26].

In addition to the above physiological characteristic changes, cell apoptosis is a widely accepted reason for myocardium I/R injury. Numerous studies had strongly indicated that ER stress plays an important role during the pathogenesis of various cardiovascular diseases including myocardial I/R injury and ischemic heart diseases [27, 28]. However, up to the known, there is still no evidence which proves if *Sch B* could affect the ER stress-induced apoptosis and protect against myocardial I/R injury.

In the present experiments, we first confirmed the cardioprotective effects of *Sch B* on I/R injury rats. It was suggested that *Sch B* effectively protected the myocardial tissue subjected to I/R injury and significantly decreased the infarct size. The trend was more obvious when *Sch B* was used at the highest concentration of 80 mg/kg. Furthermore, we tested the serum levels of LDH, CK, T-SOD, and MDA in rats. Compared with I/R group, *Sch B* group markedly decreased the activity of CK, LDH, and MDA, but significantly increased the activity of T-SOD in the serum. Our results demonstrated that *Sch B* exhibited antioxidant properties and was able to decrease the I/R injury of myocardium.

In eukaryotic cells, the ER is the structure in which most secreted and transmembrane protein folds and matures [10]. If the homeostasis of ER is disturbed by the

stimulus of a variety of physical and chemical factors, the accumulation of abnormally folded proteins will lead to ER stress, and excessive ER stress further triggered UPR. This process is mediated by the ER stress transducers. There are three different classes of ER stress transducers which include PERK, ATF6, and IRE1 that have been identified. Sustained ER stress will ultimately lead to cell apoptosis. PERK signaling and ATF6 signaling are proved to be proapoptotic effectors, and such effect is possibly through the induction of CHOP [29].

In our study, the mRNA levels of ATF6, PERK, and CHOP were remarkable upregulated in the I/R injury model group compared with sham group. This upregulation was decreased in a dose-dependent manner when rats were pretreated with *Sch B*. Besides that, the protein expressions of PERK, p-PERK, ATF6, and CHOP in the myocardial tissues were also tested, and the same changes were observed. Taken together, we demonstrated that *Sch B* could inhibit the ER stress-related signaling pathway, which may further reduce the ERS-induced apoptosis. Therefore, we next proved this idea by detecting the protein expressions in the family of apoptosis. Obviously, we found that *Sch B* downregulated the high levels of caspase-9, caspase-3, and Bax induced by I/R injury and upregulated the expression of Bcl-2 compared with I/R injury group.

CONCLUSION

In conclusion, we demonstrated that the pretreatment of *Sch B* before I/R injury could decrease the infarct size of myocardial tissue, upregulate the antioxidant ability, and attenuate the ERS-induced apoptosis, therefore protecting the myocardium against I/R injury. Our research verified the cardioprotection effects of *Sch B* and provided a new mechanism by which *Sch B* functioned.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest. The authors declare that they have no conflict of interest.

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