Heat shock protein 27 and 70 contribute to the protection of Schisandrin B against D-galactosamine-induced liver injury in mice

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<th>Journal:</th>
<th>Canadian Journal of Physiology and Pharmacology</th>
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<td>Manuscript ID</td>
<td>cjpp-2015-0419.R1</td>
</tr>
<tr>
<td>Manuscript Type:</td>
<td>Article</td>
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<tr>
<td>Date Submitted by the Author:</td>
<td>22-Sep-2015</td>
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<td>Complete List of Authors:</td>
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<td>Keyword:</td>
<td>Schisandrin B, Heat shock proteins, D-galactosamine, Liver injury, Quercetin</td>
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Heat shock protein 27 and 70 contribute to the protection of Schisandrin B against D-galactosamine-induced liver injury in mice

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Abstract: Schisandrin B is a hepatoprotective component isolated from a traditional Chinese herb *Schisandra chinensis* (Turcz.) Baill. This study determined the effect of Schisandrin B on D-galactosamine-induced liver injury and the role of Heat shock protein 27 and 70 against liver injury in mice. Acute liver injury was induced by intraperitoneal injection of D-galactosamine to mice, and Schisandrin B was given orally. The protein and gene expression of Heat shock protein 27 and 70 were detected by Western blot and real-time quantitative polymerase chain reaction respectively. Liver tissues were subjected to histological evaluation, and activities of alanine aminotransferase and aspartate aminotransferase in the serum were measured. Pretreatment of Schisandrin B significantly attenuated D-galactosamine-induced liver injury in mice. This result was evidenced by improved alteration of histopathological hepatic necrosis and reduced alanine aminotransferase and aspartate aminotransferase activities in the serum. The hepatoprotective effect was accompanied with overexpression of Heat shock protein 27 and 70 both at the protein and mRNA levels. However, the aforementioned actions of Schisandrin B were all markedly suppressed by the Heat shock protein inhibitor Quercetin. Heat shock protein 27 and 70 were involved in the protective effect of Schisandrin B against D-galactosamine-induced liver injury in mice.

Key words
Schisandrin B; Heat shock proteins; induction; protection; D-galactosamine;
Liver injury; mice; Quercetin

Introduction

Hepatitis has high incidence and mortality worldwide and remains a major public health threat. This disorder is caused by various agents, such as alcohol, viruses, environmental toxins, and drugs (Hwang et al. 2005). Liver injury is a basic pathogenesis of various forms of hepatitis. Long-term presence of liver injury leads to liver fibrosis, cirrhosis, and even hepatic carcinoma (Zou et al. 2006). Therefore, rectifying liver injury is an important strategy to cure hepatitis clinically. D-galactosamine (D-Gal)-challenged mice is a well-established animal model to investigate acute hepatic injury, which resembles clinical viral hepatitis both in morphology and function (Decker and Keppler 1972; Keppler et al. 1968). Thus, this model is widely used to evaluate hepatoprotective activity (Myagmar et al. 2004; Visen et al. 1998).

Schisandrin B (Fig. 1) is an active dibenzocyclooctadiene lignan component that is isolated from the fruit of a well-known Chinese herb Schisandra chinensis (Turcz.) Baill., and is particularly effective in viral- and chemical-induced hepatitis (Chan 2012; Liu 1989). SchB possesses a significant hepatoprotective effect against injuries caused by chemicals, such as carbon tetrachloride (CCl₄)- (Chiu et al. 2007; Ip et al. 1995) and ethanol- (Lam et al. 2010) induced hepatotoxicity, as well as injuries caused by drugs, such as tacrine- (Pan et al. 2002) and
menadione- (Ip et al. 2000) induced liver damage in animals. SchB also has beneficial effects on TNF alpha-induced hepatic apoptosis in mice (Ip et al. 2001) and on oxidative injury or lipoperoxidative damage in vitro (Chiu and Ko 2006; Zhang et al. 1992).

Heat shock proteins (HSPs) are a highly conserved family of proteins that facilitate proper folding or clearance of damaged proteins in the cytoplasm (Hartl 1996; Jäättelä 1999). HSPs are molecular chaperones that are essential in maintaining cellular homeostasis during normal cell growth and stressful conditions (Fink 1999; Hartl 1996). Among the HSPs, inducible HSP27 and HSP70 have mostly been investigated for their ability to protect cells and tissues from damage caused by pathological agents (Jäättelä 1999; Kültz 2005) including liver injury (Fujimori et al. 1997; Fujisawa et al. 2013; Mikami et al. 2004). Hepatic HSP27 and HSP70 have been recently reported to be involved in protecting against D-Gal-induced hepatocyte apoptosis (Bao and Liu 2010a, 2010b). HSPs also play a role in the hepatoprotective mechanism of SchB. Previous studies report that SchB protects against CCl₄ hepatotoxicity (Tang et al. 2003) and TNF-α-triggered hepatic apoptosis (Ip et al. 2001) in mice or rats through an HSP-inducing effect.

Our recent study has demonstrated that pretreatment with SchB significantly increases HSP27 and HSP70 expression at both the protein and gene levels in normal mice (Li et al. 2014). Furthermore, the hepatoprotection of SchB against
acetaminophen hepatotoxicity-induced liver injury is also attributed to its induction of hepatic HSP27 and HSP70 (Li et al. 2014). In the present study, we used a D-Gal-induced liver injury mouse model to determine whether the hepatoprotective effect of SchB is a result of the induction of hepatic HSPs.

**Materials and methods**

**Chemicals and reagents**

*SchB* was purchased from Push BioTechnology Co. Ltd. (Chengdu, China). *D-Gal* and *Quercetin* (*Que*) with a purity of more than 98% by HPLC were obtained from Sigma-Aldrich (St. Louis, MO, USA). *SchB* and *Que* were separately suspended in 0.5% [w/v] sodium carboxymethyl cellulose for oral administration. *D-Gal* was dissolved in physiological saline for injection. All other chemicals and reagents used were of analytical grade.

**Animals and treatment**

*Male ICR mice* (18 g to 22 g) were obtained from the National Laboratory Animal Center (Changchun, China). The animals were initially acclimated for 5 days and maintained in a 12-h light/dark cycle of 22 °C to 24 °C and a relative humidity of 50%. Food and water were given ad libitum. The animal care and experimental protocols were approved by Guide for the Care and Use of Laboratory Animals (1996, published by National Academy Press, 2101 Constitution Ave. NW,
Washington, DC 20055, USA) and the Animal Care and Ethical Committee of Qiqihar Medical University, China (Ethics no. 2011-2004-09).

Mice were randomly divided into six groups (n=12) as follows: Control, D-Gal, D-Gal + Que, SchB + D-Gal, SchB + D-Gal + Que, and Que. SchB was administered intragastrically at a dose of 200 mg/kg three times in 1 day, with an interval of 8 h. Que was given orally to mice at a dose of 200 mg/kg combined with SchB. D-Gal was intraperitoneally injected once at a dosage of 800 mg/kg 12 h before the last administration of SchB. The control mice were given equal volume of vehicles. Mice were sacrificed by decapitation 4 h after the last dose of SchB. Blood samples were then harvested to determine aminotransferase activities. Liver tissues were used for subsequent analyses.

**Western blot analysis**

A total of 30 µg of sample proteins extracted from liver tissue was separated by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) in a 12% polyacrylamide gel and then transferred to a polyvinylidene difluoride membrane (Pall Corp., Port Washington, NY, USA). The membranes were blocked with 5% (w/v) skim milk in Tris-buffered saline with Tween 20 (TBST) for 1 h at room temperature and then probed with primary antibodies at 4 °C overnight. The antibodies were rabbit anti-HSP27 (Cell Signaling, MA, USA), mouse anti-HSP70 and mouse anti-β-actin (internal control) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The following day, blots were incubated
with horseradish peroxidase-conjugated secondary antibodies in TBST for 2 h at room temperature. The protein bands were visualized using an enhanced *chemiluminescence plus kit* (Beijing Applygen Technologies, Inc., Beijing, China) and analyzed using *Gel-Pro Analyzer 4.0 software* (Media Cybernetics, Rockville, MD, USA). Each experiment was carried out in triplicate.

**Real-time quantitative PCR (qPCR) assay**

Total RNA isolated from the liver tissue was used to synthesize the complementary DNA with an *ExScript RT kit*. Messenger RNA (mRNA) levels were measured by real-time qPCR using *SYBR Premix Ex Taq* in an *ABI 7300 real-time qPCR system* (Applied Biosystems, Foster City, CA, USA). The kits and *primers* were obtained from Takara Biotechnology of China (Dalian, China). *β-actin primers* were used as the internal control. The following primer sequences were employed: HSP27 sense primer, 5′-GTCCCTGGACGTCACCCT-3′, and HSP27 anti-sense primer, 5′-GAGATGAGCCCATGTCCCT-3′; HSP70 sense primer, 5′-CAGAGGGCCAGGGCTGGATT-3′, and HSP70 anti-sense primer, 5′-ACACATGCTGGTGTCTACTTC-3′; *β-actin* sense primer, 5′-CATCCCTGGACGTCACCCT-3′, and *β-actin* anti-sense primer, 5′-ATGGAGCCACCCGATCCACA-3′. The thermal cycling parameters were as follows: 1 cycle of 95 °C for 30 s, 40 cycles of 5 s at 95 °C, and 31 s at 60 °C.

Threshold cycle (Ct) data were collected using *Sequence Detection Software*
version 1.2.3 (Applied Biosystems). Relative quantification of gene expression was analyzed by the $2^{-\Delta\Delta Ct}$ method. Ct values of the HSP genes were normalized to the Ct values of β-actin to obtain delta Ct (ΔCt). Fold change in HSP genes relative to the β-actin internal control was determined by the following equation: Fold change = $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = (Ct_{HSP\, gene} - Ct_{\beta\text{-actin}}) - (Ct_{control} - Ct_{\beta\text{-actin}})$. Each experiment was conducted in triplicate.

**Determination of the aminotransferase activities**

The activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in serum were measured using an assay kit (Beijing BHKT Clinical Reagent Co. Ltd., Beijing, China) according to the manufacturer’s instructions.

**Histopathological analysis**

The paraffin method was used to analyze hepatic histology. In brief, the fresh right lobe of the mouse livers was immediately fixed in 10% formalin. The tissue was then dehydrated with different grades of ethanol, embedded in paraffin, and cut into 5 μm sections. The paraffin was removed with xylene and ethanol, and the slide was stained with hematoxylin and eosin. Morphological evaluation was performed under a light microscope equipped with the Nikon D600 digital camera and then analyzed by *Image-Pro Plus 7.0 software* (Media Cybernetics). The pathological investigator was blinded to the treatments in the experiment.

**Statistical analysis**

All data were expressed as mean ± standard deviation (SD). *SPSS 13.0*
software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Intergroup differences were detected using one-way ANOVA, followed by Dunnett's test. \( P \)-values <0.05 were considered statistically significant.

Results

SchB increased the expression of hepatic HSP27 and HSP70 proteins in D-Gal-challenged mice

Western blot analysis showed that the hepatic HSP27 and HSP70 proteins in D-Gal-challenged mice markedly increased compared with the normal control group. Oral administration of SchB at 200 mg/kg increased the expression of hepatic HSP27 and HSP70 proteins in mice more significantly, by approximately 4.9- and 3.8-fold, respectively. This result indicates that SchB exerted more potent inducing effect on HSP27 than HSP70. However, Que inhibited the inducing effect of SchB and D-Gal both on HSP27 and HSP70. Compared with the normal control group, Que itself exhibited no noticeable effect on the expression of HSP27 and HSP70 proteins (Fig. 2).

SchB increased the expression of hepatic HSP27 and HSP70 mRNAs in D-Gal-challenged mice

Real-time qPCR analysis showed that hepatic HSP27 and HSP70 mRNAs in D-Gal-treated mice markedly increased. SchB at 200 mg/kg upregulated the
expression of hepatic HSP27 and HSP70 mRNAs by approximately 6.1- and 5.3-fold, respectively, indicating that the gene level results were in accordance with HSP27 and HSP70 proteins. Increased levels of HSP27 and HSP70 mRNAs induced by SchB (200 mg/kg) were also markedly suppressed by Que (200 mg/kg) treatment. Combined with the same observation in the protein expression, these results suggest Que is a feasible inhibitory tool in this study (Fig. 3).

SchB-induced hepatic HSP27 and HSP70 attenuated hepatotoxicity in mice treated with D-Gal

Biochemical assay showed that the serum activities of ALT and AST in D-GAL-treated mice were significantly greater than those in the control group. Oral administration with SchB (200 mg/kg) before D-GAL injection in mice significantly suppressed the serum activities of ALT and AST by 60.30% and 55.61%, respectively, showing the hepatoprotective effect of SchB. However, the activities of ALT and AST relapsed when Que was co-administered with SchB, without difference compared with the D-GAL-treated mice. By contrast, Que neither increased the serum activities of ALT and AST nor affected the increase of such activities induced by D-GAL treatment (Fig. 4).

Protective effect of hepatic HSP27 and HSP70 induced by SchB against D-Gal-induced liver injury in histology in mice

The histological assay demonstrated the normal hepatic lobular architecture and
cell structure of mice liver in the control group. Injection of D-GAL resulted in significant morphological changes in liver histology. These changes included disseminated hepatocyte necrosis, congestion, ballooning, and destruction around the central vein, as well as immigration of inflammatory cells, which were markedly attenuated by SchB pretreatment in mice. However, the hepatoprotective effect of SchB was suppressed by Que (200 mg/kg) treatment, as indicated by heavier aggravated lesions compared with the SchB + D-Gal group. Que per se exhibited neither protective nor injurious effect in mice (Fig. 5).

Discussion

We investigated a possible molecular mechanism of the hepatoprotective effect of SchB in a mouse model with D-Gal-induced acute liver injury. SchB attenuated D-Gal-induced hepatic injury by overexpression of HSP27 and HSP70 in mice.

D-Gal-induced acute liver injury is a widely used animal model that closely resembles human viral hepatitis (Decker and Keppler 1972; Keppler et al. 1968). Injection with D-Gal decreases uracil nucleotides in the liver, markedly depleting hepatic UDP-glucuronic acid. D-Gal inhibits hepatic glucuronidation, disrupting the synthesis of essential uridylate nucleotides. Reduction of these nucleotides eventually impairs protein and glycoprotein synthesis, causing progressive
damage of cellular membranes. This leads to changes in membrane permeability and ultimately enzyme leakage from the cells (Abdul-Hussain et al. 1991; Keppler et al. 1970). In this study, we used D-Gal-induced acute liver injury model to investigate the actions and mechanisms of SchB. Pretreatment with SchB protected the liver against D-Gal-induced hepatotoxicity in mice, as demonstrated by the amelioration of histopathological hepatic necrosis and the reduction in ALT and AST activities.

HSP27 and HSP70 are multidimensional proteins that are implicated in a range of diseases; thus, they serve as disease biomarkers and may be a potential drug target (Evans et al. 2010; Vidyasagar et al. 2012). Also, HSP70 has been reported to be a chaperone involved in inflammation, endoplasmic reticulum stress and apoptosis at liver level (Tarantino et al. 2012). Because HSPs have a cytoprotective effect on tissue injury, we hypothesized that the hepatoprotective effect of SchB is due to the induction of hepatic HSPs. Our previous study confirmed that SchB induces the expression of HSP27 and HSP70 in mice. Hepatic HSP27 and HSP70 production were enhanced following SchB administration. HSP27 and HSP70 mRNA and protein expression increased in a time- and dose-dependent manner. Moreover, the pattern of protein expression paralleled mRNA levels, suggesting that the induction of hepatic HSP27 and HSP70 by SchB is through the upregulation of HSP gene transcription (Li et al. 2014). The present study demonstrated that SchB
significantly induced both mRNA levels and protein expression of HSP27 and HSP70 in D-Gal-treated mice compared with D-Gal alone challenged mice, which showed higher HSP27 and HSP70 expression than the normal control group.

To investigate whether the upregulation of HSP27 and HSP70 by SchB is responsible for its hepatoprotective action against D-Gal-induced liver injury, we used Que, an inhibitor of HSP biosynthesis (Gonzalez et al. 2009; Hosokawa et al. 1990; Nagai et al. 1995). The results showed that Que significantly suppressed the induction of hepatic HSP27 and HSP70 by SchB, but Que itself did not affect the expression of hepatic HSP27 and HSP70 in the normal control or D-Gal-injected mice. In the SchB (200 mg/kg) pretreated mice, compared with D-Gal-treated mice, reduced ALT and AST activities and decreased extent of hepatic necrosis were observed. This finding suggests that SchB has a protective effect on D-Gal-induced liver injury. Simultaneous administration of Que (200 mg/kg) and SchB (200 mg/kg) in D-Gal-treated mice attenuated the protective effect of SchB on ALT and AST activities and hepatic necrosis, but Que itself exhibited neither hepatoprotective nor injurious effect on mice. Our results demonstrated that Que treatment markedly suppressed the hepatoprotective effect of SchB, suggesting that the increased HSP27 and HSP70 expression following SchB treatment contributed to mitigate the extent of D-Gal-induced liver injury.
In summary, we found that oral administration of SchB markedly alleviated D-Gal-induced liver injury in mice, as demonstrated by the reduction in ALT and AST activities and decreased hepatic necrosis, accompanied by the overexpression of HSP27 and HSP70 in the mouse liver. However, the hepatoprotective effects of SchB were significantly attenuated by Que, an inhibitor of HSP biosynthesis. Our findings revealed that SchB administration provide a significant hepatoprotective effect against D-Gal-induced liver injury in mice, and HSP27 and HSP70 contribute to the protection of SchB.

Because HSPs occur and function endogenously, the upregulation of HSP function by pharmacological approach may provide a potential prophylactic or therapeutic drug target for treatment of liver diseases (Latchman. 1998), such as inducing HSP expression in the treatment of liver injury. Therefore, SchB, with its HSP-inducing capability, might be a potential candidate compound for hepatitis or other hepatic diseases in the future.

Acknowledgements

This work was supported by the Scientific Research Fund of Heilongjiang Province Education Department of China [grant number: 1155G68]. We also thank the Basic Medical Research Section and Molecular Biological Experiment Center of Qiqihar Medical University for their help.

Conflict of interest

We declare no conflict of interest.
References


**Figure captions:**

**Fig. 1** Chemical Structure of Schisandrin B

**Fig. 2** Schisandrin B (SchB) increased protein expression of hepatic Heat shock protein (HSP) 27 and HSP70 in D-galactosamine (D-Gal)-challenged mice. Mice were orally given SchB (200 mg/kg) three times in 24 h or co-treated with Quercetin (Que) (200 mg/kg), and D-Gal (800 mg/kg) was intraperitoneally injected once 12 h before the last administration of SchB. The control animals were given the equal volume (20 mL/kg) of vehicles only. Liver tissue samples were harvested at 4 h after the last dose of SchB, whole liver proteins were extracted, and the protein expression of hepatic HSP27 and HSP70 was detected by Western blot analysis. (a) Representative Western blot analysis of HSP27 and HSP70 protein from each group. (b) Levels of hepatic HSP27 and HSP70 proteins. Protein levels were expressed as mean ± Standard Deviation (SD) (n=5) in each column. The lanes were normalized to the levels of β-actin. *P<0.05, **P<0.01 compared with corresponding control group; # P<0.05 compared with corresponding D-Gal group; && P<0.01 compared with corresponding SchB+D-Gal group. Similar results were obtained with three replications.

**Fig. 3** Schisandrin B (SchB) increased expression of hepatic Heat shock protein (HSP) 27 and HSP70 mRNA in D-galactosamine (D-Gal) -challenged mice. Animal treatment was as described as in Fig. 2. After mice sacrificed, total RNA was isolated from liver tissue samples using RNAiso reagent and then subsequently used for cDNA synthesis. The $2^{-\Delta\Delta Ct}$ analyses of quantitative
polymerase chain reaction (qPCR) of HSP27 and HSP70 were obtained from 5 mice per group. Data were expressed as mean ± Standard Deviation (SD). ** P < 0.01 compared with the corresponding vehicle-treated mice; ## P < 0.01, compared with corresponding D-Gal group; ^ P < 0.05 compared with corresponding SchB + D-Gal group. Similar results were obtained with three replications. Que, Quercetin.

Fig. 4 Schisandrin B (SchB) -induced hepatic Heat shock protein (HSP) 27 and HSP70 attenuated hepatotoxicity in mice treated with D-galactosamine (D-Gal). Mice were treated as described as in Fig. 2. Blood samples were harvested and then serum was separated for determination of (a) ALT and (b) AST activities according to the manufacturer’s instruction. Data were expressed as mean ± Standard Deviation (SD) (n=12). ** P < 0.01 compared with the corresponding control group; ## P <0.01, compared with corresponding D-Gal group; & P < 0.01 compared with corresponding SchB + D-Gal group. Que, Quercetin; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

Fig. 5 Protective effect of hepatic Heat shock protein (HSP) 27 and HSP70 induced by Schisandrin B (SchB) against D-galactosamine (D-Gal) -induced liver injury in histology in mice. Mice were treated as described as in Fig. 2. Liver sections were stained with hematoxylin-eosin for morphological evaluation (magnification: 100×)
(a) 

HSP 27 

HSP 70 

β-actin 

(b) 

HSPs / β-actin 

SchB

D-Gal

Que

182x196mm (300 x 300 DPI)