Proper Heat Shock Pretreatment Reduces Acute Liver Injury Induced by Carbon Tetrachloride and Accelerates Liver Repair in Mice

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Abstract: Whether proper heat shock preconditioning can reduce liver injury and accelerate liver repair after acute liver injury is worth study. So mice received heat shock preconditioning at 40°C for 10 minutes (min), 20 min or 30 min and recovered at room temperature for 8 hours (h) under normal feeding conditions. Then acute liver injury was induced in the heat shock-pretreated mice and unheated control mice by intraperitoneal (i.p.) injection of carbon tetrachloride (CCl4). Hematoxylin and eosin (H&E) staining, serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels and the expression levels of heat shock protein 70 (HSP70), cytochrome P450 1A2 (CYP1A2) and proliferating cell nuclear antigen (PCNA) were detected in the unheated control mice and heat shock-pretreated mice after CCl4 administration. Our results showed that heat shock preconditioning at 40°C for 20 min remarkably improved the mice's survival rate (P<0.05), lowered the levels of serum AST and ALT (P<0.05), induced HSP70 (P<0.01), CYP1A2 (P<0.01) and PCNA (P<0.05) expression, effectively reduced liver injury (P<0.05) and accelerated the liver repair (P<0.05) compared with heat shock preconditioning at 40°C for 10 min or 30 min in the mice after acute liver injury induced by CCl4 when compared with the control mice. Our results may be helpful in further investigation of heat shock pretreatment as a potential clinical approach to target liver injury (DOI: 10.1293/tox.2013-0006; J Toxicol Pathol 2013; 26: 365–373)

Key words: heat shock pretreatment, acute liver injury, CCl4, HSP70, CYP1A2, PCNA

Introduction

Acute liver failure with massive hepatocellular loss occurs due to various causes. Because liver failure still exhibits high mortality despite intensive care, effective therapeutic approaches are needed1. The carbon tetrachloride (CCl4)-induced acute liver injury model is similar to the human disease from the standpoint of morphology and biochemical aspects of collagen metabolism. A single dose or very few doses result in acute liver damage that has been widely employed to study morphological and biochemical features of cellular lesions, especially steatosis and necrosis2. At an early stage after CCl4 administration, transient increases in the expression of the stress-inducible HSP70 gene occur3. HSP70 knockout mice have a higher degree of necrosis and neutrophil infiltration than normal mice. So HSP70 could play an important role in the cytoprotection of the liver against hepatotoxic agents4. Heat shock preconditioning induces HSP72 in the rat liver with fibrosis and provides significantly increased tolerance to warm ischemia-reperfusion injury3. Hagiwara et al. (2007)5 reported that thermal pretreatment is associated with the induction of HSP70 protein synthesis, which subsequently attenuates tissue damage in experimental lung fibrosis.

Heat shock can cause cell death if cellular defense mechanisms are insufficient to cope with the stress. This is particularly obvious when the temperature increases well above that of the normal environment and/or exposure time is prolonged. An important feature of HSPs is their role in the cytoprotection and repair of cells and tissues with regard to the harmful effects of stress6,7. The major families of mammalian stress proteins, HSP90 and HSP70, as well as the smaller HSP28 family, have all been well characterized4. HSP70 overexpression confers myocardial protection, as observed by resistance to myocardial ischemic stress and...
reperfusion damage. In a rodent model for adult respiratory distress syndrome, heat shock-induced HSP70 accumulation within the lung has been associated with decreased pulmonary inflammation and prevention of lethality. The cytoprotective role of HSP70 has also been documented in the areas of metabolic disorders, and infection. These observations suggest new therapeutic strategies relying upon the development of methods that are able to increase the expression of HSPs. Furthermore, it has been shown that the production of HSPs could protect the organism against a second exposure to otherwise lethal hyperthermia, which has been described as the thermotolerance phenomenon.

Whether proper heat shock preconditioning can reduce liver injury and accelerate liver repair after acute liver failure induced by CCl4 is worth study. Our previous study showed that heat shock at a lower temperature (heat shock at 40°C for 20 min) significantly promotes hepatocyte proliferation and improves metabolic efficiency in the mouse liver, while heat shock at a higher temperature (heat shock at 46°C for 20 min) remarkably inhibits hepatocyte proliferation, promotes hepatocyte apoptosis and induces liver injury. So we selected a proper temperature and time to study whether proper heat shock preconditioning could reduce liver injury and accelerate liver repair after acute liver failure induced by CCl4.

Materials and Methods

Animals
Male BALB/c mice are sensitive to temperature, and it is easy to induce acute liver injury in them using CCl4; so male BALB/c mice (approximately 6–8 weeks old, 22 ± 2 g) were purchased from the Experimental Animals Center of Henan Province and maintained in an air-conditioned animal room at 25°C with free access to water and food under 12 h light/dark cycles for the experiments. All animals were allowed to adapt to the environment for 1 week before the experiment and were fed laboratory chow. All protocols conformed to the guidelines of the National Animal Care and Use Committee of China. All animals received care in compliance with the Principles of Laboratory Animal Care.

Heat shock preconditioning and acute liver injury induced by CCl4
Our previous work suggested that heat shock at 40°C for 20 min is a proper condition for heat shock preconditioning because it could effectively promote hepatocyte proliferation and improves the metabolic efficiency in the mouse liver. So mice were anesthetized with urethane (1.4 g/kg, i.p.) and divided into two groups. In the heat shock group (HS20 group, n=90), mice received heat shock preconditioning at 40°C for 20 min and subsequent CCl4 (analytical reagent, from Tianjin Kaitong Chemical Reagent Co., Ltd; Tianjin, China) administration. The mice in the control group (n=90) were only injected with CCl4 to induce acute liver injury. Briefly, mice in the HS20 group were placed in a temperature-controlled ventilated and humidified chamber to raise the rectal temperature to 40°C for 20 min, which was monitored by a digital thermometer in the rectum. The animals were then allowed to recover at room temperature in normal feeding conditions. At 8 h after heat shock pretreatment, 0.1% CCl4 (1 μl CCl4 in 1 ml mineral oil) was administered to the mice in group HS20 and the control group by i.p. injection of 10 mL/Kg. Blood was drawn via the orbital vein at 0, 3, 6, 12, 24, 30, 36, 42, 48 and 54 h in the mice of the two groups after CCl4 injection. The coagulated blood was left to clot at room temperature for approximately 15 to 30 min. After it was completely clotted, it was rimmed using an applicator stick and then centrifuged for approximately 5–10 min at 2500 rpm. Then the supernatant fluid was separated. Serum AST and ALT activities were determined with a commercial assay kit (Nanjing Jiancheng Biological Technology, Inc., Nanjing, China) at 0, 3, 6, 12, 24, 30, 36, 42, 48 and 54 h in the mice of the two groups after CCl4 injection. Enzyme activities were expressed as an international unit per liter (IU/L). The mice at each time point in each group were divided into three subgroups 1, 2 and 3. Each subgroup contained three mice. We also performed an experiment in which mice received heat shock preconditioning at 40°C for 10 min (HS10 group, n=90) or 30 min (HS30 group, n=90) and subsequent CCl4 administration. In addition, we performed a parallel experiment and only evaluated the survival rate in heat shock-pretreated mice and control mice after CCl4 injection.

Histologic examination
Liver specimens were obtained from 9 mice at each time point. Each liver specimen from each mouse was divided into two sections. One was used for histologic examination, and the other was used for Western blot detection. Samples of liver were fixed in 10% formaldehyde for 24 h and then dehydrated and embedded in paraffin. Six-micrometer sections were cut from each paraffin-embedded tissue and stained with hematoxylin and eosin (H&E). To evaluate the degree of necrosis after acute liver injury, we created an injury grading score (Grade 0–4) based on severity of necrotic lesions in the liver parenchyma. The grades were as follows: Grade 0, no pathological change; Grade 1, presence of degenerated hepatocytes with only rare foci of necrosis; Grade 2, small area of mild centrilobular necrosis around the central vein; Grade 3, area of mild centrilobular necrosis severer than Grade 2; and Grade 4, centrilobular necrosis severer than Grade 3. Every group contains three subgroups 1, 2 and 3. Each subgroup contains three mice.

Immunohistochemistry for HSP70, CYP1A2 and PCNA
Six-micrometer sections were cut from each paraffin-embedded tissue as prepared above. The sections of liver from the mice in different groups were immunostained with a monoclonal antibody to mouse HSP70, CYP1A2 and PCNA (dilution 1:300) (Santa Cruz) as described by Xu et al. (2000). The signal was detected using the Polink-2 plus polymer HRP detection system (Zhongshan, Beijing, China).
using DAB. A negative control was carried out on each slide by omitting the primary antibody. Sections were examined microscopically for specific staining and photographs were taken using a digital image-capture system (Olympus, Tokyo, Japan). Numbers of positive cells from the control and preheated mice were 12 mm² tissue sections counted for each mouse.

Western blot of HSP70, CYP1A2 and PCNA

HSP70 play important roles in stress response, and CYP1A2 principally participates in metabolizing chemicals and environmental toxins in the liver. PCNA has been shown to be a good marker to distinguish proliferating cells. So the expression levels of HSP70, CYP1A2 and PCNA were detected by Western blot to compare liver stress response, metabolism and regeneration in heat shock-pretreated mice and control mice after CCl₄ administration. Protein samples of 70 μg from the mice in the different groups were adjusted to the composition of the electrophoresis sample buffer (50 mM Tris, pH 6.8, 10% glycerol, 5% beta-mercaptoethanol, 2% SDS, 0.1% bromphenol blue) and boiled for 5 min prior to analysis. SDS-PAGE (10% polyacrylamide gels) in 1 mm slab gel was performed as described by Sambrook and Russell (2001). The proteins were transferred from the gel to nitrocellulose membranes. Then the membrane was probed with a monoclonal antibody to mouse HSP70, CYP1A2 or PCNA (Santa Cruz), respectively. The signal was detected by a horseradish peroxidase detection system using DAB (Sigma). Protein bands were quantified with the Gel-Pro Analyzer 4.0 software (Media Cybernetics Inc., Bethesda, MD, USA), and the intensities of the bands were normalized against β-actin. Every experiment was repeated three times.

Statistical analysis

The data were presented as means ± SEM of 9 animals per group at each time point. Statistical comparisons were made using one-way and two-way (without interaction) ANOVA with the Tukey post-hoc test for multiple comparisons. All statistical analyses were performed using SPSS 14.0 (SPSS Inc., Chicago, IL, USA). Survival rates of mice were analyzed using the log-rank test and expressed as Kaplan-Meier survival curves.

Results

Alterations in the serum AST and ALT levels and survival rate in the mice

Figure 1 shows that the maximum levels of serum AST and ALT were observed at 24 h post-CCl₄ injection in the control group, but at 30 h in the mice with heat shock pretreatment at 40°C for 20 min (HS20 group). In addition, the peak values were approximately 1.9-fold higher in the control group than in the HS20 group. In the control group, the AST and ALT values decreased from 30 h post-CCl₄ injection, reaching the basal value at 54 h, while the AST and ALT values decreased from 36 h post-CCl₄ injection, reaching the basal value at 48 h observed in the HS20 group. In addition, the AST and ALT levels in the control group were significantly higher than those in the HS20 group at each time point from 3 h to 48 h post CCl₄ injection (P<0.01). The AST and ALT levels in the HS10 group and HS30 group were lower than those in the control group at some time points post CCl₄ injection (P<0.05), but the time points at which the peak levels of serum AST and ALT were reached in the HS10 group and HS30 group were similar to those in the control group post CCl₄ injection (Fig. 1). The preheated animals showed a marked increase in survival rate (94.4%) in the HS20 group compared with the unheated control group (74.4%) at 54 h after CCl₄ injection (P<0.05). But the survival rates of the HS10 group (80%) and HS30 group
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(80%) were not significantly improved compared with the unheated control group at 54 h after CCl\textsubscript{4} injection ($P>0.05$) (Fig. 2).

**Histological changes in heat shock-pretreated mice and control mice with acute liver injury induced by CCl\textsubscript{4}**

To evaluate liver injury degree, H&E staining was performed in the liver of mice. Table 1 and Fig. 3 show that heat shock pretreatment at 40°C for 20 min significantly reduced liver injury degree compared with the control mice when acute liver injury was induced by CCl\textsubscript{4} ($P<0.01$). Heat shock pretreatment at 40°C for 20 min not only postponed the peak of liver injury but also accelerated repair of liver injury (Table 1) ($P<0.01$). But heat shock pretreatment at 40°C for 10 min or 30 min did not significantly ameliorate liver injury induced by CCl\textsubscript{4} ($P>0.05$) (Table 1 and Fig. 3).

![Fig. 2. The effect of heat shock pretreatment on the survival rate of mice in which liver injury was induced by CCl\textsubscript{4}. A, B, C and D, respectively, represent the survival rates of mice in the control group, HS10 group, HS20 group and HS30 group at 0, 3, 6, 12, 24, 30, 36, 42, 48 and 54 h after exposure to 0.1 ml/10 g 0.1% CCl\textsubscript{4} (1 \mu l CCl\textsubscript{4} in 1 ml mineral oil). The results were analyzed using the log-rank test and expressed as Kaplan-Meier survival curves. The data show that the survival rate of the HS20 group was significantly improved compared with the HS10 group and the HS30 group when compared with the control group ($P<0.05$).](image)

The expression of HSP70, CYP1A2 and PCNA in the liver of mice

The expression of HSP70 in the liver of the HS20 group was significantly higher than those in the control group ($P<0.01$) and HS10 and HS30 groups ($P<0.01$) from 0 h to 54 h after CCl\textsubscript{4} injection. But the expression of HSP70 in the HS10 and HS30 groups was only higher than that in the control group ($P<0.05$), except at 12 h and 30 h after CCl\textsubscript{4} administration. The expression of CYP1A2 in the HS20 group was always higher than that in the control group ($P<0.01$) and was higher than that in the HS10 and HS30 groups at subtotal time points ($P<0.05$), except at 12 h and 30 h after CCl\textsubscript{4} administration. The expression of CYP1A2 in the HS10 and HS30 groups was higher than that in the control group at major time points ($P<0.05$), except at 24 h and 36 h after CCl\textsubscript{4} injection (Figs. 5, 7A and 7C). The expression of PCNA in the liver of heat shock-pretreated mice reached the minimum level at 12 h and then increased at 24 h to 48 h when liver regeneration started after liver injury. On the other hand, the expression of PCNA in the liver of the
The expression level of PCNA in the heat shock-pretreated mice was significantly higher than that in the control mice after CCl4 administration at each time point from 0 h to 54 h (P<0.05). But heat shock pretreatment at 40°C for 10 min or 30 min did not significantly induce the expression of PCNA (P>0.05) after liver injury induced by CCl4 when compared with the control mice (Figs. 6, 7A and 7D).

Discussion

Our results suggested that proper heat shock pretreatment, such as 40°C for 20 min, could significantly induce HSP70, CYP1A2 and PCNA expression (Figs. 4–7), reduce liver injury and accelerate liver repair (Table 1 and Fig. 3) after liver injury induced by CCl4 in mice.

It has been reported that thermal pretreatment is associated with the induction of HSP70 protein synthesis, which subsequently attenuates acute lung injury induced by lipopolysaccharide in rats5. But whether heat shock pretreatment can reduce acute liver injury induced by CCl4 in mice has not been studied. Based on the results of analysis in a previous study15 (Li et al., 2012), we think that heat shock at 40°C for 20 min is an optimal thermal pretreatment to induce HSP70 expression and improve liver function compared with heat shock at 42°C, 44°C and 46°C for 20 min. So mice received heat shock preconditioning at 40°C for 20 min (HS20 group) and subsequent CCl4 administration. Figure 1 shows that heat shock preconditioning at 40°C for 20 min effectively lowered serum AST and ALT levels in the mice with acute liver injury induced by CCl4 compared with heat shock preconditioning at 40°C for 10 min or 30 min as compared with the control mice (P<0.01), which indicated that heat shock preconditioning at 40°C for 20 min could effectively reduce liver injury induced by CCl4 (Table 1 and Fig. 3) and improve the survival rate (Table 1 and Fig. 2) in the mice. Saad et al. (1995)14 reported that heat exposure associated with HSP induction has a significant protective effect against warm ischemic liver injury. Yang et al. (1998)20 reported that heat shock protein expression protects against death following exposure to heatstroke in rats. Although the HSPs were associated with the phenomenon of thermotolerance, cross protection against other kinds of cellular stress, the underlying mechanism of this protection is not clear. The function of HSP has been explained by an improved transportation of repair proteins across cellular and subcellular membranes, preventing intracellular calcium overload and an increased catalase activity. It has been speculated that HSPs reduce the oxidative injury caused by oxygen free radicals that occurs during the early phase of reperfusion14,21. So we think that heat shock pretreatment significantly induced HSP70 expression (Figs. 4, 7A and 7B), which is the cellular response to oxidative stress, and provided protection against oxygen distress in acute liver injury induced by CCl4 in the mice.

Our results also showed that heat shock pretreatment at 40°C for 20 min could significantly induced CYP1A2 expression compared with heat shock pretreatment at 40°C for 10 min or 30 min when compared with control mice.
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Fig. 3.

Fig. 4.

Fig. 5.

Fig. 6.
**Fig. 7.** The expression of HSP70, CYP1A2 and PCNA in the liver of unheated control mice and heat shock-pretreated mice after CCl₄ administration. The expression of HSP70, CYP1A2 and PCNA was detected by Western blot (A). The protein bands were quantified for HSP70 (B), CYP1A2 (C) and PCNA (D) with the Gel-Pro Analyzer 4.0 software (Media Cybernetics Inc.) (B), and the intensities of the bands were normalized against β-actin. AU represents arbitrary unit. Every experiment was repeated three times. All data were presented as the mean ± standard error of the mean (SEM). **## P < 0.01**; significant difference when compared with the HS10 and HS30 groups.

**Fig. 3.** Histologic examination of liver injury in mice by H&E staining at 24 h after CCl₄ administration. A, B, C and D, respectively, represent the degree of liver injury of mice in the control group, HS10 group, HS20 group and HS30 group after CCl₄ administration. E: Necrotic areas. Representative findings from counting with tissue sections that were at least 10 mm² for each mouse. Scale bar: 50 µm.

**Fig. 4.** Photomicrographs of immunohistochemical staining of HSP70 in the liver of unheated control mice and heat shock-pretreated mice at 0 h after CCl₄ administration. The arrowheads indicate the HSP70-positive cells in the liver of mice in the control group (A), HS10 group (B), HS20 group (C) and HS30 group (D). E: Numbers of HSP70⁺ cells in the liver of mice in the control group and preheated groups; tissue sections that were at least 12 mm² were counted for each mouse. (Scale bar: 50 µm).

**Fig. 5.** Photomicrographs of immunohistochemical staining of CYP1A2 in the liver of unheated control mice and heat shock-pretreated mice at 0 h after CCl₄ administration. The arrowheads indicate the CYP1A2-positive cells in the liver of mice in the control group (A), HS10 group (B), HS20 group (C) and HS30 group (D). E: Numbers of CYP1A2⁺ cells in the liver of mice in the control group and preheated groups; tissue sections that were at least 12 mm² were counted for each mouse. (Scale bar: 50 µm).

**Fig. 6.** Photomicrographs of immunohistochemical staining of PCNA in the liver of unheated control mice and heat shock-pretreated mice at 0 h after CCl₄ administration. The arrowheads indicate the PCNA-positive cells in the liver of mice in the control group (A), HS10 group (B), HS20 group (C) and HS30 group (D). E: Numbers of PCNA⁺ cells in the liver of mice in the control group and preheated groups; tissue sections that were at least 12 mm² were counted for each mouse. (Scale bar: 50 µm).
\( P \leq 0.01 \) (Figs. 5, 7A and 7C). CYP1A2 is one of the major CYPs in the human liver (approximately 13%) and metabolizes a variety of clinically important drugs. This enzyme also metabolizes several important endogenous compounds including steroids, retinoids, melatonin, uroporphyrinogen and arachidonic acid. Like many of other CYPs, CYP1A2 is subject to induction and inhibition by a number of compounds\(^{25} \). Our results demonstrated that proper heat shock preconditioning (40°C for 20 min) could effectively induce CYP1A2 expression to improve the metabolic efficiency of the liver, which may be helpful in resisting the damage induced by CCI\(_4\) in the liver of mice.

Our results indicated that heat shock preconditioning at 40°C for 20 min significantly induced PCNA expression in the liver cells of mice compared with heat shock pretreatment at 40°C for 10 min or 30 min when compared with control mice (\( P \leq 0.05 \)) (Figs. 6, 7A and 7D). PCNA is a subunit of the mammalian DNA polymerase delta and is synthesized primarily during the S phase of the cell cycle\(^{23} \). It is a relay or anchoring molecule that functions as a molecular integrator for proteins involved in control of the cell cycle, DNA replication, DNA repair and cell death\(^{24,25} \). PCNA has been shown to be a good marker to distinguish proliferating cells\(^{27,28} \). So heat shock preconditioning at 40°C for 20 min significantly promoted hepatocellular proliferation and accelerated the liver repair after acute liver failure induced by CCI\(_4\). Analysis of the above results suggested that both high levels of HSPs and proper heat shock-timing between heat shock pretreatment and CCI\(_4\)-induced liver injury were critical for optimal protection. Heat shock preconditioning at 40°C for 20 min may be helpful in improving the metabolic efficiency of the liver, lowering serum AST and ALT levels and accelerating liver repair after acute liver failure induced by CCI\(_4\). But heat shock preconditioning at 40°C for 10 min could not sufficiently induce the expression of HSP70, CYP1A2 and PCNA. The stimulation of heat shock preconditioning at 40°C for 30 min may be too heavy to induce tolerance of heat in mice.

In conclusion, our study showed that proper heat shock pretreatment ameliorated liver injury and accelerated liver repair after acute liver injury induced by CCI\(_4\) in mice, which may be helpful in further investigation of heat shock pretreatment as a potential clinical approach targeting liver injury.

**Acknowledgments:** This work was supported by grants from the National Natural Science Foundation of China (#U1204802 and #81201558) to SQL, Program for Science & Technology Innovation Talents in Universities of Henan Province (#13HASTIT025) to SQL and Foundation for Henan Province’s Key Project to Tackle Key Problems of Science and Technology (#12210231030). The authors thank all the members in the laboratory when this work was carried out. The experiments complied with the current laws of China.

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