The Protective Effect of Huperzine A Against Hepatic Ischemia Reperfusion Injury in Mice

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ABSTRACT

Background. Nowadays, hepatic ischemia reperfusion (HI/R) injury is regarded as a serious concern in clinical practices. Huperzine A (HupA) is an alkaloid isolated from the Chinese folk medicine huperzia serrate, which has possessed diverse pharmacological actions.

Methods. A mouse model of HI/R was caused by clamping the hepatic artery, the hepatoportal vein, and the bile duct with a vascular clamp for 30 minutes followed by reperfusion for 6 hours under anesthesia. The sham group experienced the identical procedure without hepatic ischemia. The HupA group received an injection into the tail vein 5 minutes prior to HI/R at the doses of 167 and 500 mg/kg. The vehicle group was injected with physiological saline instead of HupA. The liver function was assessed by determinations of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities. Tissue levels of superoxide dismutase (SOD), catalase (CAT), malondiadehyde (MDA), and glutathione (GSH) were also measured spectrophotometrically. In addition, the activities of hepatic inflammatory mediators such as nuclear factor kappa B (NF-κB) p65, tumor necrosis factors-α (TNF-α), interleukin-1β (IL-1β) and IL-6 were also measured. Furthermore, the apoptotic damage was evaluated by measuring caspase-3 activity in hepatic tissues.

Results. Treatment with HupA in mice at the doses of 167 and 500 μg/kg remarkably reduced serum ALT and AST activities in HupA-treated ischemic mice. Furthermore, HupA treatment could enhance the activities of hepatic tissue SOD, CAT, and GSH but decrease MDA tissue content. The activities of inflammatory cytokines including NF-κB p65, TNF-α, IL-1β and IL-6 were all decreased in ischemic mice treated with HupA. Colorimetric test results illustrated that a marked reduction of caspase-3 activity was found in the HupA-treated group compared with the vehicle group.

Conclusion. Our present data suggest that HupA has a protective role against HI/R injury of mice and antioxidative, anti-inflammatory, and antiapoptotic actions are involved in its protection.

IT IS WELL known that hepatic ischemia reperfusion injury (HI/R) is an important nonimmunologic factor that occurs during circulatory shock, hepatic trauma, liver transplantation, and elective liver resection [1]. Serious HI/R could lead to liver failure, remote organ failure, and even death [2,3]. Thus, HI/R has always been a major problem in the development of liver surgery. Several mechanisms exist concerning the pathophysiology of HI/R injury. Reactive oxygen species (ROS) are involved in the pathogenesis of HI/R injury. Excessive formation of ROS was previously reported during ischemic insult and it not only caused the destruction of cellular structures, but also resulted in mitochondrial dysfunction, finally activating apoptotic cascades [4]. Chandra et al [5] observed that increased H₂O₂ of tissue led to apoptotic damage by up-regulating Fas-FasL system. In fact, H₂O₂ would potentially damage the mitochondrial...
membrane, which contributes to the release of proapoptotic components located in the mitochondria. Besides, the injured mitochondria enabled some transcriptional factors and promoted them to shift into nucleus, such as p53 and nuclear factor kappa B (NF-κB). Additionally, the expression of proapoptotic genes could be facilitated by the ROS and the survival-related genes are suppressed. On the contrary, the natural antioxidants could attenuate I/R injury. The superoxide dismutase (SOD), catalase (CAT), and glutathione (GSH) reductase treatment have effectively alleviated I/R injury of animals. Taking into account the fact that ischemic injury is associated with the oxidative mechanism, it is of great interest to explore the hepatoprotective agents that may ameliorate the damage of ROS due to H/I/R injury. Apoptosis is another important mechanism during HI/R injury. Kohli et al found that 50% to 70% of sinusoidal endothelial cells in liver and 40% to 60% of hepatocytes exhibited apoptosis [6].

Huperzine A (HupA) is a novel alkaloid extracted from the Chinese folk medicine, *Huperzia serrate*, and it has good clinical prospects. It has previously been proven to provide several beneficial effects for Alzheimer’s disease (AD) patients [7] and, in China it is one of the most commonly prescribed drugs for various types of dementia, including AD [8], as a result of its inhibitory effect on acetylcholinesterase (AchE). Also, Ruan et al [9] reported that HupA dramatically decreased ROS generation and oxidative damage in D-galactose-treated rats. After renal I/R injury, HupA was also found to inhibit cellular apoptosis [10]. These investigations confirmed that HupA possessed anti-oxidative, anti-inflammatory, and antiapoptotic properties. However, it is not well understood whether HupA could alleviate HI/R of mice. Therefore, the present study was conducted to assess the hepatoprotective effects against H/I/R and further probe the potential mechanisms.

MATERIALS AND METHODS
Animals and Induction of HI/R
Male mice (30 ± 5 g) were housed in individual cages under a controlled environment (12:12 hour light/dark cycle; 50%–70% humidity; 24°C) and provided with free access to water and food. All experimental procedures were approved by the animal ethics committee of Shengjing Hospital of China Medical University in China. Great efforts were made to minimize suffering and reduce the number of animals used.

HI/R was induced according to the method described previously with minor modification [10–12]. Under the chloral hydrate (200 mg/kg) and ether anesthesia, mice underwent a median laparotomy. The hepatoporal vein, hepatic artery, and hepatic duct were separated and were clamped for 30 minutes, then following 6-hour reperfusion with an atraumatic vascular clamp. Body temperature of the animals was kept constant by a heating blanket during the reperfusion period.

Drug Administration
HupA (Sigma-Aldrich, USA, with a purity >95%) was dissolved in physiological saline and was injected into the tail vein 5 minutes prior to HI/R. The chemical structure of HupA is indicated in Figure 1. Twenty-four mice were randomly divided into 4 groups (6 mice in each group): sham, vehicle, and HupA treatments (varietal does of HupA: 167 μg/kg and 500 μg/kg). The dosage and dosing frequency of HupA were chosen as previously described [13]. The sham group was the control group and no operation was performed. The vehicle and HupA groups underwent the procedure of HI/R before injections of the same volume of physiological saline or HupA, respectively, though the tail vein. At the end of the reperfusion period, mice were humanely killed and blood and liver samples were collected. Blood samples were drawn from the suprahepatic vena cava by a fine needle and then centrifuged at the speed of 3000g for 5 minutes to collect serum for determination of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels. Liver tissue samples from each animal were stored for the measurement of hepatic tissue SOD, CAT, GSH, and malondiadehyde (MDA) levels, together with evaluating the activities of NF-κB p65, tumor necrosis factors-α (TNF-α, interleukin-1β) (IL-1β), IL-6, and caspase-3.

Measurement of Serum ALT and AST Levels
Automated analyzer autobiochemical analyzer (Toshiba, Tokyo, Japan) was used to determine serum ALT and AST levels as described previously [11,12,14].

Measurement of SOD, CAT, GSH, and MDA Activities
The enzymatic activities of SOD, GSH, GSH-PX, and MDA were measured according to the manufacturer’s instructions in different commercially available assay kits (Nanjing Jian Cheng Bioengineering Institute).

The SOD activity in hepatic tissue homogenate was estimated by calculating the rate inhibition of nucleotide oxidation. Results were expressed as U/mg protein. The CAT was assayed by quantifying the rate of oxidation of the reduced glutathione to the oxidized glutathione by H₂O₂. The result was given as U/mg protein. The content of GSH was assayed by quantifying the rate of oxidation of the reduced glutathione to the oxidized glutathione by H₂O₂. The result was indicated by milligram GSH per gram protein. The content of MDA was assayed for products of lipid peroxidation by monitoring thiobarbituric acid.
(TBA) reacting substances at the wavelength of 532 nm. The level of MDA was expressed as nanomole MDA per milligram protein.

Determinations of NF-κB p65, TNF-α, IL-1β, and IL-6 Activities

The p65 subunit was correlated with activated NF-κB pathway. Therefore, we detected the activity of NF-κB p65 unit using a commercial kit (Imgenex, United States). TNF-α, IL-1β, and IL-6 were measured using the corresponding commercial immunoassay kits (R&D Systems, California, United States).

Assay of Caspase-3 Activity

The cleavage of chromogenic caspase substrates was used to measure the activity of Caspase-3, acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA), a caspase-3 substrate. The amount of caspase-3 was measured by the colorimetric approach using a commercial kit (Beysotine Institute of Biotechnology, Natong, China). The protein samples of hepatic tissues were acquired as indicated in Western blot analysis. Approximately 50 μg protein was added to a reaction buffer involving Ac-DEVD-pNA (2 mmol/L), incubated at 37°C for 4 hours, and the absorbance of yellow pNA was calculated using a spectrometer at the wavelength of 405 nm. The specific activity of caspase-3, which was normalized for total protein in liver, was then expressed as fold of the baseline caspase-3 activity of the control group.

Statistical Analysis

Results were expressed as mean ± standard deviation (SD). Comparisons between groups were carried out by one-way analysis of variance (ANOVA) with Dunnett test using SPSS 13.0 software (SPSS, Inc., Chicago, IL). P < .05 was deemed to be statistically significant.

RESULTS

Serum ALT and AST

In the physiological saline-treated HI/R group, the levels of serum ALT, which was the marker of hepatic damage, were significantly increased (Fig. 2A) from 20.12 ± 3.65 to 1859.47 ± 234.56 U/L (P < .01; n = 6) compared with the sham group. But the HupA group (167 and 500 μg/kg) markedly reduced the ALT level from 1859.47 ± 234.56 to 257.89 ± 26.67 (P < .01; n = 6), and 233.12 ± 15.78 U/L (P < .01; n = 6), respectively, compared with the HI/R group. Similarly, the levels of serum AST of the vehicle group were notably enhanced compared with the sham group (Fig. 2B) from 15.86 ± 2.99 to 3000.78 ± 211.78 U/L (P < .01; n = 6). But the HupA group (167 and 500 μg/kg) were dramatically decreased from 3000.78 ± 211.78 to 698.78 ± 65.34 (P < .01; n = 6), and 655.67 ± 20.12 U/L (P < .01; n = 6), respectively, compared with the vehicle group.

The Activities of Antioxidative Enzymes (SOD, CAT) and the Contents of MDA and GSH in Hepatic Tissue

To explore the effects of HupA on the oxidative stress during HI/R injury of mice, the activities of anti-oxidative enzymes (SOD, CAT) and the contents of GSH and MDA in hepatic tissue were evaluated in the current investigation. Figure 3A showed that the activity of SOD, one of the most important antioxidative enzymes, in the vehicle group was significantly reduced from 215.11 ± 11.54 to 76.68 ± 11.09 U/mg protein (P < .01; n = 6) in comparison with the sham group. After administration with HupA (167 and 500 μg/kg), the activity of SOD was significantly enhanced from 76.68 ± 11.09 to 169.29 ± 31.13 (P < .01; n = 6) and 150.89 ± 10.54 U/mg protein (P < .01; n = 6) in comparison with the HI/R group. Similarly, the activity of CAT in the vehicle group was also decreased from 30.34 ± 3.11 (P < .01; n = 6) to 18.37 ± 2.34 U/mg protein (P < .01; n = 6) in comparison with the sham group. Interestingly, after treatment with HupA at the doses of 167 and 500 μg/kg, the activity of CAT was notably enhanced from 18.37 ± 2.34 to 27.11 ± 1.34 (P < .01; n = 6) and 28.94 ± 1.99 U/mg protein (P < .01; n = 6) respectively, compared with the HI/R group, as illustrated in Figure 3B. In the meantime, as displayed in Figure 3C, the quantity of GSH of the vehicle group was dramatically decreased from 57.69 ± 6.15 to 24.04 ± 4.98 μg/g protein (P < .01; n = 6) compared with the sham group. After treatment with HupA at the doses of 167 and 500 μg/kg, the content of GSH was increased to 37.74 ± 6.16 (P < .01; n = 6) and 42.4 ± 7.15 (P < .01; n = 6), respectively. Additionally, the content of MDA (Fig 3D), as a marker of lipid peroxidation, in the HI/R group was significantly increased in hepatic tissue from 3.11 ± 0.11 to 5.64 ± 0.89 mmol/mg protein (P < .01; n = 6) compared with the sham group. The dramatic
reduction of the MDA level was observed in the HupA-administrated (167 and 500 mg/kg) mice from 5.64 ± 0.89 to 3.89 ± 0.51 and 3.64 ± 0.25 nmol/mg protein (P < .01; n = 6) compared with the vehicle-treated ischemic mice.

Measurements of NF-κB p65, TNF-α, IL-1β and IL-6 Activities

Figure 4A–D show that the activities of NF-κB p65, TNF-α, IL-1β, and IL-6 were all augmented in ischemic mice (P < .01; n = 6). However, these values were all reversed by HupA treatment to ischemic mice (P < .01; n = 6).

Caspase-3 Activity

To corroborate that HupA is able to suppress caspase-3 activity, colorimetric analysis was carried out. As shown in Figure 5, caspase-3 activity in the vehicle group was markedly enhanced by 412.74% (P < .01; n = 6) compared with the sham group. In the HupA treatment (167 and 500 μg/kg) groups, there was an obvious decrease in caspase-3 activity by 60.99% (P < .01; n = 6) and 68.64% (P < .01; n = 6), respectively, compared with that in the vehicle group.

**DISCUSSION**

HupA has been widely used as the selective inhibitor of AchE to treat AD and vascular dementia in China. Besides inhibiting AchE, HupA was also reported to have neuroprotective potential against cerebral ischemic injury [15]. Recently, Wang et al revealed that HupA inhibited overexpression of proinflammatory enzymes induced by oxygen-glucose deprivation in C6 rat glioma cells, partly through a cholinergic anti-inflammatory pathway [16]. In addition, a previous investigation demonstrated that HupA could diminish the excessive production of ROS after middle cerebral artery occlusion in rats [4]. However, there is no evidence of protection from hepatic warm I/R injury. We think that administration of HupA would reduce the HI/R. Our present study demonstrated for the first time that HupA exerted protection from HI/R injury and the hepatoprotective effect might be associated with its antioxidative, anti-inflammatory, and antiapoptotic properties.

ROS is closely involved in the pathogenesis of HI/R injury [17,18]. Enhanced hepatic anti-oxidative ability can reduce
damage induced by ischemia reperfusion. A previous investigation illustrated that the mice overexpressing SOD and CAT could be significantly improved during HI/R injury compared with normal mice [19]. Besides, administration of GSH could protect hepatocytes and improve animal survival intravenously after HI/R [20]. The MDA level, the criterion of evaluating the severity of reperfusion injury, is evidently increased during ischemia reperfusion. Under physiological condition, the ROS can be quickly detoxified by endogenous antioxidative enzymes and low–molecular weight antioxidants, such as SOD, CAT, and GSH. In our study, the SOD and CAT activities as well as the GSH content were dramatically higher compared after treatment with HupA to ischemic mice, but the content of MDA was significantly lower. The present result indicated that HupA alleviated HI/R injury, at least partly through its antioxidative activity.

Hepatic damage after ischemic injury occurs via oxidative stress and/or mitochondrial dysfunction and ultimately activates NF-kB signaling and an apoptotic cascade. It is well demonstrated that caspases are a family of cystein-dependent proteases with a critical role in the initiation and execution of cellular apoptosis. Caspases are specifically activated with the apoptotic stimuli and caspase-3 is conceived of as an executioner of apoptosis [21]. Cumulative evidence has supported the up-regulation of caspase-3 after hepatic ischemia. In addition to caspases, Bcl-2 family proteins have been also shown to play a critical role in the modulation of neuronal apoptosis. Bcl-2 itself acts as an antiapoptotic protein, whereas another member of the family, Bax, functions as a proapoptotic molecule [22]. Our present study showed that HupA could dramatically cause the decreased activities of inflammatory cytokines including NF-kB, TNF-α, IL-1β, IL-6, and caspase-3 in mice induced by HI/R injury. Consistent with our investigation, HupA was also found to inhibit cellular apoptosis after renal I/R injury [10], suggesting that enhanced therapeutic effect by HupA might also be associated with its anti-inflammatory and antiapoptotic actions in ischemic mice.

In summary, our results demonstrated that HupA could attenuate HI/R injury by minimizing oxidative stress and decreasing the activities of inflammatory cytokines and caspase-3. The hepatoprotective effect of HupA might be related to its antioxidative, anti-inflammatory, and anti-apoptotic properties in HI/R injury in mice.