Huperzine A attenuates hepatic ischemia reperfusion injury via anti-oxidative and anti-apoptotic pathways

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Abstract. Hepatic ischemia reperfusion (HI/R) injury may occur during liver transplantation and remains a serious concern in clinical practice. Huperzine A (HupA), an alkaloid isolated from the Chinese traditional medicine Huperzia serrata, has been demonstrated to possess anti-oxidative and anti-apoptotic properties. In the present study, a rat model of HI/R was established by clamping the hepatic artery, the hepatoportal vein and the bile duct with a vascular clamp for 30 min followed by reperfusion for 6 h under anesthesia. HupA was injected into the tail vein 5 min prior to the induction of HI/R at doses of 167 and 500 µg/kg. The histopathological assessment of the liver was performed using hematoxylin and eosin staining. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were assayed in the serum samples. The tissue levels of superoxide dismutase (SOD), catalase (CAT), malondiadehyde (MDA) and glutathione (GSH) were also measured spectrophotometrically. Furthermore, the protein expression of caspase-3, Bcl-2 and Bax in hepatic tissues was detected via western blot analysis. Treatment of Wistar rats with HupA at doses of 167 and 500 µg/kg markedly attenuated HI/R injury as observed histologically. In addition, the significant reductions of serum ALT and AST were observed in HupA-treated ischemic rats. Furthermore, HupA treatment enhanced the activity of hepatic tissue SOD, CAT and GSH, but decreased the MDA tissue content. Western blot analysis revealed elevated levels of Bcl-2 expression but decreased Bax and caspase-3 tissue expression at the protein level in the HupA-treated group. The present data suggest that HupA attenuates the HI/R injury of rats through its anti-oxidative and anti-apoptotic signaling pathways.

Introduction

It is generally accepted that hepatic ischemia reperfusion injury (HI/R) is an important non-immunologic injury that may occur during circulatory shock, hepatic trauma, liver transplantation and elective liver resection (1). Severe HI/R contributes to liver failure, remote organ failure and even mortality (2,3). Therefore, HI/R has always been a key concern in the development of liver surgery techniques. Several mechanisms appear to be involved in the pathophysiology of HI/R injury. It is well established that reactive oxygen species (ROS) are conceived to be a critical factor in the pathogenesis of HI/R injury. Recent studies have illustrated that excessive formation of ROS during ischemic insult not only causes the destruction of cellular structures, but also results in mitochondrial dysfunction, finally activating apoptotic cascades (4). Chandra et al (5) found that increased H$_2$O$_2$ levels in tissue may lead to apoptotic damage by upregulating the Fas-FasL system. H$_2$O$_2$ may have damaged the mitochondrial membrane, thus contributing to the release of pro-apoptotic components located in the mitochondria. The injured mitochondria activated a number of transcription factors and promoted their translocation into the nucleus, including p53 and nuclear factor (NF)-κB. Additionally, the expression of pro-apoptotic genes may be facilitated by the suppression of ROS and survival-associated genes. By contrast, natural anti-oxidants may attenuate I/R injury. Superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH) reductase treatment have effectively alleviated I/R injury in animals (6). Taking into account the fact that ischemic injury is associated with oxidation, it is important to examine the hepatoprotective agents that may ameliorate the damage of ROS in HI/R injury. Apoptosis is another important mechanism involved in HI/R injury. Kohli et al (7) identified that 50-70% of sinusoidal liver endothelial cells and 40-60% of hepatocytes underwent apoptosis.

Huperzine A (HupA), a novel alkaloid extracted from the Chinese traditional medicine, Huperzia serrata, is considered to be a drug with high clinical prospects. Previous studies have demonstrated that it has several beneficial effects for patients with Alzheimer's disease (AD) (8) and, in China it is one of the most commonly prescribed drugs for various types of dementia, including AD (9), as a result of its inhibitory effect on acetylcholinesterase (AchE). Ruan et al (10) reported that HupA markedly decreased ROS generation and oxidative...
damage in D-galactosetreated rats. Following renal I/R injury, HupA was also found to inhibit cellular apoptosis (11). These studies confirmed that HupA possessed anti-oxidative and anti-apoptotic properties. However, it remains unclear whether HupA may alleviate H/I/R in rats. Therefore, the present study was conducted to assess the hepatoprotective effects against H/I/R and further examine the potential mechanisms underlying these effects.

Materials and methods

Animals and induction of H/I/R. Male Wistar rats (weight, 240±40 g) were housed in individual cages under a controlled environment (12:12 h light/dark cycle, 50-70% humidity, 24˚C) and provided with free access to water and food. All of the experimental procedures were approved by the animal ethics committee of Maanshan Municipal Health Hospital For Women and Children in China (Maanshan, China). All experimental procedures were performed in a manner that minimized suffering and reduced the number of animals used.

H/I/R was induced according to the method described previously with minor modifications (11-13). Under the chloral hydrate (200 mg/kg) and ether anesthesia, rats underwent a median laparotomy. The hepatoporal vein, hepatic arterial and hepatic duct were separated, which were clamped for 30 min followed by a 6 h reperfusion with an atraumatic vascular clamp (Hengao Company of Beijing, Beijing, China). The body temperature of the animals was maintained constantly using a heating blanket during the reperfusion period. The sham group underwent all surgery with the exception of the occlusion of the hepatoporal vein, hepatic artery and hepatic duct.

Drug administration. HupA (purity, >95%; Sigma, St. Louis, MO, USA) was dissolved in physiological saline and was injected into the tail vein 5 min prior to the induction of H/I/R. The chemical structure of HupA was indicated in Fig. 1. A total of 24 rats were randomly divided into the following four groups (n=6 per group): Sham, Vehicle and HupA treatment (varietal does of HupA: 167 µg/kg and 500 µg/kg) groups. The vehicle and HupA groups underwent the H/I/R procedure prior to injections with the same volume of physiological saline or HupA, respectively, through the tail vein. At the end of the reperfusion period, the rats were sacrificed by spinal dislocation and the blood and liver samples were collected. Separate tissue samples were quantified with microscopic scoring under light microscope (Nikon Corporation, Nikon, Tokyo, Japan) following hematoxylin and eosin (H&E) staining for the histological analysis. Blood samples were drawn from the supra-hepatic vena cava by a fine needle (Trade of Antai Company, Suzhou, China) and then centrifuged at 3,000 x g for 5 min to collect the serum for the determination of the alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels. The liver tissue samples from each animal were stored at -80˚C for the measurement of hepatic tissue SOD, CAT, GSH and malondiadehyde (MDA) levels, together with evaluating the activity of caspase-3 and the protein expression of caspase-3, Bcl-2 and Bax.

Histological examination. The liver tissue samples were fixed in 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB; pH 7.4) for 12 h, followed by two days in 30% sucrose buffer at room temperature. The serial coronal sections (6 μm-thick) were obtained using a microtome and stained with H&E and examined by light microscopy. The liver histopathological evaluation was performed in a blinded manner.

Measurement of serum ALT and AST levels. An automated autobiochemical analyzer (Toshiba, Tokyo, Japan) was employed to determine serum ALT and AST levels as described previously (12-14).

Measurement of SOD, CAT, GSH and MDA activities. The enzymatic activity of SOD, GSH, GSH-peroxidase (PX) and MDA was measured according to the manufacturer's instructions in different commercial assay kits (Nanjing Jian Cheng Bioengineering Institute, Nanjing, China). The SOD activity in the hepatic tissue homogenate was estimated by calculating the rate of inhibition of nucleotide oxidation. The results are expressed as the U/mg protein. The CAT was assayed by quantifying flaxen complex compound, configured by ammonium molybdate and the remainder peroxide, at the wavelength of 405 nm. The result are provided 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 results are indicated in mg GSH/g protein. The content of MDA was assayed for the products of lipid peroxidation by monitoring thiobarbituric acid reacting substances at a wavelength of 532 nm. The level of MDA was expressed as nmol MDA/mg protein.

Western blot assay. Western blot analysis was performed on the hepatic samples. Briefly, the samples were homogenized in an ice-cold lysis buffer [10 mM Tris (pH 8.0), 150 mM NaCl, 1% glycerol, 1% NP-40, 5 mM EDTA and protease inhibitor cocktail]. Following centrifugation at 13200 x g for 20 min at 4˚C, the supernatant was collected and the total protein levels were quantified by a bicinchoninic protein assay kit (Beyotime Institute of Biotechnology, Shanghai, China). An equal quantity of protein (50 µg) was separated by means of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5% skimmed milk for 1 h at room temperature and then probed, respectively, with the following primary antibodies: Anti-caspase-3 polyclonal rabbit antibody (1:300; Cell Signaling Technology, Inc., Beverly, MA, USA), anti-Bcl-2 monoclonal rabbit antibody (1:200; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), anti-Bcl-2 polyclonal rabbit antibody (1:200; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), anti-p53 monoclonal rabbit antibody (1:200; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), and anti-β-actin monoclonal rabbit antibody (1:200; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA).
USA), anti-Bax monoclonal rabbit antibody (1:200; Santa Cruz Biotechnology, Inc.) and anti-β-actin monoclonal rabbit antibody (1:2,000; Santa Cruz Biotechnology, Inc.), respectively, at 4°C overnight. After the membranes were washed with three changes of Tris-buffered saline with Tween-20, they were incubated for 2 h with peroxidase-labeled goat anti-rabbit IgG (1:5,000; Santa Cruz Biotechnology, Inc.). Immunodetection was conducted with enhanced chemiluminescence (Applygen, Beijing, China) and exposed on an X-ray film. Immunoblot was used as an internal reference for relative quantification. The films were digitized by a scanner (Hewlett-Packard Development Company, Beijing, China) and the grey value of the protein bands was analyzed using Quantity One software (Bio-Rad, Hercules, CA, USA).

Assay of caspase-3 activity. The reduction in the chromogenic caspase-3 substrate acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA) was used to assess the activity of caspase-3. The quantity of caspase-3 was measured using a colorimetric approach with a commercial kit (Beyotime Institute of Biotechnology). The protein samples of the hepatic tissues were acquired as indicated in the western blot analysis. Approximately 50 µg protein was added to a reaction buffer involving Ac-DEVD-pNA (2 mM), incubated at 37°C for 4 h and the absorbance of yellow pNA was calculated by a spectrometer (Shanghai CSOF Company, Shanghai, China) at a wavelength of 405 nm. The specific activity of caspase-3, which was normalized for the total protein in the liver was then expressed as the fold change of the baseline caspase-3 activity of the control group.

Statistical analysis. The results were expressed as the mean ± standard deviation. Comparisons between the groups were performed by one-way analysis of variance with
Dunnett’s test using SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Histopathological examination. As demonstrated in Fig. 2A and B, the sham group exhibited normal liver cellular structure. As observed in Fig. 2C and D, the vehicle group exhibited a mass of hepatocytes cytoplasmic color fading and nuclear condensation. When the ischemic rats were treated with HupA at the doses of 167 and 500 µg/kg, it was noted that the cytoplasmic color fading and nuclear condensation of the hepatocytes were significantly diminished, as illustrated in Fig. 2E-H.

Serum ALT and AST levels. In the physiological saline-treated HI/R group, the levels of serum ALT, which was the marker of hepatic damage, were significantly increased (Fig. 3A) from 36.10±8.37 to 2034.77±45.84 U/l (P<0.01, n=6) compared with the sham group. However, the HupA groups (167 and 500 µg/kg) markedly reduced the ALT level from 2034.77±45.84 to 342.92±38.64 (P<0.01, n=6) and 319.53±50.05 U/l (P<0.01, 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. 3B) from 72.77±11.83 to 2738.10±43.23 U/l (P<0.01, n=6). However the AST levels in the HupA groups (167 and 500 µg/kg) were markedly decreased from 2738.10±43.23 to 507.92±23.40 (P<0.01, n=6) and 422.86±38.71 U/l (P<0.01, n=6), respectively compared with the vehicle group.

Activity of anti-oxidative enzymes (SOD and CAT) and the levels of MDA and GSH in hepatic tissue. In order to examine the effects of HupA on oxidative stress during HI/R injury in rats, the activity of anti-oxidative enzymes (SOD and CAT) and the levels of GSH and MDA in hepatic tissue were investigated in the present study. Fig. 4A demonstrates that the activity of SOD, one of the most important anti-oxidative enzymes, was significantly reduced in the vehicle group from 344.44±17.41 to 113.10±14.14 U/mg protein compared with the sham group (P<0.01, n=6). Following administration of HupA (167 and 500 µg/kg), the activity of SOD was significantly enhanced from 113.10±14.14 in the HI/R group to 256.25±15.19 (P<0.01, n=6) and 297.86±12.14 U/mg protein (P<0.01, n=6) in the 167
and 500 µg/kg HupA groups, respectively. Similarly, the activity of CAT in the vehicle group was also decreased compared with the sham group from 39.10±7.58 (P<0.01, n=6) to 15.77±5.87 U/mg protein (P<0.01, n=6). Notably, following treatment with HupA at the doses of 167 and 500 µg/kg, the activity of CAT was enhanced from 15.77±5.87 in the ischemic group to 30.75±6.31 (P<0.01, n=6) and 37.03±6.82 U/mg protein (P<0.01, n=6) respectively (Fig. 4B). As revealed in Fig. 4C, the quantity of GSH in the vehicle group markedly reduced to 1.60±0.43 mg/g protein compared with the sham group (4.27±0.76, P<0.01, n=6). Following treatment with HupA at doses of 167 and 500 µg/kg, the content of GSH was increased to 2.92±0.53 (P<0.01, n=6) and 3.53±0.53 (P<0.01, n=6), respectively. Additionally, the content of MDA (Fig. 4D), a marker of lipid peroxidation, in the vehicle group was significantly increased in the hepatic tissue from 6.10±0.76 to 12.93±1.58 nmol/mg protein (P<0.01, n=6), compared with the sham group. A marked reduction in the MDA level was observed in the HupA-treated (167 and 500 µg/kg) rats from 12.93±1.58 in the vehicle-treated ischemic rats to 8.42±0.78 and 6.70±0.98 nmol/mg protein (P<0.01, n=6), respectively.

Protein expression of Bcl-2, Bax and caspase-3. Western blot analysis was further performed to examine the effect of HupA on the expression of apoptosis-regulatory proteins, including caspase-3, Bcl-2 and Bax in hepatic tissues. Fig. 5A demonstrates the western blotting results with antibodies specific to caspase-3, Bcl-2 and Bax. The protein expression of caspase-3 in ischemic rats hepatic tissues was significantly elevated from 0.60±0.15 to 1.43±0.27 (P<0.01, n=6) compared with that in the sham group. However, when treated with HupA (167 and 500 µg/kg), the caspase-3 protein level was markedly reduced to 0.82±0.08 (P<0.01, n=6) and 0.80±0.05 (P<0.01, n=6), respectively, compared with the vehicle-treated group, as demonstrated in Fig. 5B. The protein expression of Bcl-2 in the hepatic tissue of the vehicle group was markedly reduced from 1.70±0.08 to 0.73±0.10 (P<0.01, n=6) compared with the sham group. HupA treatment at doses of 167 and 500 µg/kg caused a marked elevation in the Bcl-2 protein expression level from 0.73±0.10 to 1.33±0.15 (P<0.01, n=6) and 1.51±0.09 (P<0.01, n=6) compared with the vehicle group (Fig. 5C). Additionally, in the vehicle group, the protein expression of Bax was significantly increased from 0.85±0.09 to 1.73±0.39 (P<0.01, n=6) compared with the sham group. However, the protein level of Bax was markedly decreased to 0.97±0.07 (P<0.01, n=6) and 0.94±0.03 (P<0.01, n=6), respectively, following treatment with HupA at doses of 167 and 500 µg/kg, compared with the vehicle group (Fig. 5D).

Caspase-3 activity. To identify whether HupA was able to suppress caspase-3 activity, a colorimetric analysis was performed. As revealed in Fig. 6, caspase-3 activity in the vehicle group was markedly enhanced by 181.82% (P<0.01, n=6), compared with the sham group. In the HupA treatment (167 and 500 µg/kg) groups, there was an evident reduction in activity of caspase-3 by 31.82% (P<0.01, n=6) and 58.41% (P<0.01, n=6), respectively.
caspase-3 activity by 59.68% (P<0.01, n=6) and 61.29% (P<0.01, n=6), respectively, compared with that in the vehicle group.

**Discussion**

HupA is an alkaloid isolated from the Chinese herb *Huperzia serrata*, and has been widely used as a selective inhibitor of AchE to treat AD and vascular dementia in China. As well as inhibiting AchE, HupA was also reported to have neuroprotective effects against cerebral ischemic injury (15). Recently, Wang et al (16) demonstrated that HupA inhibited the overexpression of proinflammatory enzymes induced by oxygen-glucose deprivation in C6 rat glioma cells, partly through activation of a cholinergic anti-inflammatory pathway. In addition, a previous investigation demonstrated that HupA was able to diminish the excessive production of ROS following middle cerebral artery occlusion in rats (4). However, to the best of our knowledge, there is no evidence of the protective effects of HupA against hepatic warm I/R injury. It was hypothesized that the administration of HupA may reduce HI/R. To the best of our knowledge, the present study demonstrated for the first time, that HupA exerted protective effect from HI/R injury and this hepatoprotective effect may be associated with its anti-oxidative and anti-apoptotic properties.

It is well established that the accumulation of ROS is closely correlated with the pathogenesis of HI/R injury (17,18). Enhanced hepatic anti-oxidative ability reduces the damage induced by ischemia reperfusion. A previous study demonstrated that mice overexpressing SOD and CAT exhibited significant improvements following HI/R injury compared with the normal mice (6). In another study, intravenous administration of GSH protected hepatocytes and improved animal survival following HI/R (19). The MDA level, a biomarker for evaluating the severity of reperfusion injury, is evidently increased during ischemia reperfusion. Under physiological condition, ROS levels are rapidly detoxified by endogenous anti-oxidative enzymes and low-molecular weight anti-oxidants, including SOD, CAT and GSH. In the present study, the SOD and CAT activity as well as the GSH content were markedly higher following the treatment with HupA compared with that in the ischemic rats, but the content of MDA was significantly lower. The present results indicated that HupA alleviated HI/R injury, at least partly through its anti-oxidative activity.

Hepatic damage following ischemic injury occurs via oxidative stress and/or mitochondrial dysfunction, and ultimately activates an apoptotic cascade. It is well established 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 by apoptotic stimuli and caspase-3 is conceived as an executioner of apoptosis (20). Cumulative evidence has supported the hypothesis that caspase-3 expression is upregulated following hepatic ischemia. In addition to caspases, Bcl-2 family proteins have also been demonstrated to exhibit a critical role in the modulation of neuronal apoptosis. Bcl-2 itself acts as an anti-apoptotic protein, whereas another member of the family, Bax, functions as a pro-apoptotic molecule (21). The present study demonstrated that HupA markedly decreased the protein expression levels of caspase-3 and Bax, and elevated Bcl-2 in rats induced by HI/R injury. Consistent with these data, HupA was also found to inhibit cellular apoptosis following renal I/R injury (11), suggesting that enhanced the therapeutic effect of HupA may also be associated with its anti-apoptotic action in ischemic rats.

In conclusion, the present study demonstrated that HupA attenuated HI/R injury by minimizing oxidative stress and decreasing the expression of apoptosis-associated proteins, including caspase-3, Bcl-2 and Bax. Therefore it was concluded that the hepatoprotective effect of HupA may be associated with its anti-oxidative and anti-apoptotic properties in HI/R injury in rats.

**References**