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Hemin protects against hippocampal damage following orthotopic autologous liver transplantation in adult rats

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ABSTRACT

Aims: Induction of heme oxygenase-1 (HO-1) has been widely accepted to be neuro-protective. This study aimed to examine whether hemin (a HO-1 inducer) attenuates neuronal damage in the hippocampus induced by orthotopic autologous liver transplantation (OALT) in adult rats.
Main methods: Rats were randomly allocated into four groups (n = 8 each): (i) Sham control group; (ii) OALT model group; (iii) Hemin + OALT group, with intra-peritoneal (i.p.) injection of hemin (5 mg/kg) 24 hours (h) before the OALT; and (iv) ZnPP (a HO-1 inhibitor) + OALT group, with i.p. injection of ZnPP (32 mg/kg) 24 h before the OALT. Twenty four hours after the surgery, the hippocampal tissues were collected for electron microscopic examination and terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) analysis. The levels of hippocampal HO-1 protein and serum S-100β, the concentrations of regional tumor necrosis factor-α (TNF-α) and interleukins (IL-6, IL-10), as well as the status of malondialdehyde (MDA), superoxide dismutase (SOD) and catalase (CAT) in the hippocampus were assessed.
Key findings: Rats suffered severe neuronal damage in the hippocampus after OALT, mainly in apoptosis. Pre-treatment with hemin obviously alleviated the damage; up-regulated the HO-1 protein level; inhibited the release of TNF-α, IL-6 and MDA; and promoted the activities of SOD, CAT and IL-10; however, pre-treatment with ZnPP did not exhibit the opposite effect, except that a marked increase in serum S-100β level was detected.
Significance: Hemin up-regulated the expression of HO-1 and attenuated hippocampal neuronal damage induced by OALT.

1. Introduction

The central nervous system (CNS) is a site associated with significant morbidity and mortality after orthotopic liver transplantation (OLT) [35,43]. Over 30% of allograft recipients experience some forms of post-operative neurological complications, ranging from mild encephalopathy to coma, severe headache, cerebrovascular disorder, infection, delirium and seizures [1]. Such complications commonly develop early after OLT, and negatively affect patients' long-term survival and quality of life [2,42]. A wide array of factors are responsible for the prevalence of these neurological events, and include pre-OLT factors [12], intra-operative factors [e.g. hepatic ischemia–reperfusion (I/R) injury and metabolic disturbance] [8,26,32], or post-transplant ones (e.g. immunosuppressive therapy) [35,43]. Among all these etiologies, the roles of immunosuppressant neurotoxicity, cerebral metabolic disturbance and auto-regulation impairment have been well studied during OLT [8,32], whereas little was known about the effects of hepatic I/R on pathological and biochemical alterations in the brain after OLT due to the lack of data from biopsy and animal experiments. This becomes initial motivation to carry out the experiments in the present study.

Heme oxygenase-1 (HO-1) is a rate-limiting enzyme that is responsible for the catabolism of heme into bilirubin, free iron, and carbon monoxide (CO) [29]. As an endogenous stress-responsive protein, HO-1 is widely distributed in many organ systems and highly regulated by various stimuli [23]. Either in vivo or in vitro, HO-1 was confirmed to protect cells against a wide variety of injuries, mainly through the anti-inflammatory, anti-oxidant, and anti-apoptotic effects of its three products in heme degeneration [3,28,40]. Similarly, this potential cyto-protective effect of HO-1 has also been found in neurons. Evidence shows that induction of HO-1 with hemin (a HO-1 inducer) leads to up-regulation of endogenous HO-1, and thus ameliorates neuronal inflammatory responses and oxidative damage [9,18,30]. Clinically,

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neurologists even consider HO-1 as a potential therapeutic target for some CNS diseases such as Alzheimer disease and other neurodegenerative diseases [36,37]. Therefore, here we aimed to determine: 1) whether cerebral neuronal necrosis or apoptosis can be observed in the hippocampus (an ischemia- and hypoxia-sensitive area of the brain) after OLT; 2) whether HO-1 induced by hemin attenuates OLT-related cerebral impairment; and 3) the possible underlying mechanism involved.

2. Materials and methods

2.1. Animals and experimental protocols

All experimental protocols and procedures were performed strictly according to National Institutes of Health guidelines for the use of experimental animals, and this study was approved by the Animal Care Committee of Sun Yat-sen University, Guangzhou, P. R. China.

Thirty-two adult pathogen-free male Sprague–Dawley rats, weighing 250–280 g, were used. They were provided with standard laboratory chow and sterile acidified water and housed in individual cages in a temperature-controlled (25 ± 2 °C) and humidity-controlled (45%–55%) room with regular light/dark cycles. Food was removed 8 h before animals were used; however, animals continued to have free access to water.

Animals were randomly separated into the following four groups (n = 8 each):

(1) Sham control group (Sham group), rats received only a sham surgical operation with abdomen dissection and isolation of hepatic peripheral vessels without occlusion.
(2) OALT model group (OALT group), rats underwent orthotopic autologous liver transplantation (OALT) and 20 min total hepatic ischemia together with cold heparin infusion, followed by 24 h of reperfusion.
(3) Hemin pre-treatment group (Hemin + OALT group), rats received intraperitoneal injection of hemin (5 mg/kg, Sigma, Dorset, UK) 24 h before the OALT.
(4) ZnPP pre-treatment group (ZnPP + OALT group), 32 mg/kg zinc protoporphyrin (ZnPP) (Frontier Scientific, Carnforth, Lancashire, UK) was intraperitoneally administrated 24 h before the operation. ZnPP, a competitive inhibitor of HO-1, is an endogenously molecule formed during heme biosynthesis [24], and its level often increases in chronic anemia disorders [14,15].

The time point was chosen based on the results of a previous study showing peak expression of HO-1 at 24 h after the administration of hemin [34]. The doses of hemin and ZnPP were selected based on the results of previous studies and were confirmed to effectively induce or inhibit HO-1 expression [6,34].

2.2. A rat model of OALT

The OALT model was performed as described in our previous study [10,20,21]. Briefly, all rats were anesthetized by inhaling ethyl ether continuously and fixed on a heating pad peri-operatively. A middle incision was made on the abdomen. The ligaments, vessels, and bile ducts around the liver were carefully dissociated, and the whole liver was well exposed. Four vessels including the super-hepatic vena cava (SHVC), inferior-hepatic vena cava (IHVC), hepatic artery (HA), and portal vein (PV) were clearly visualized. Before the occlusion of these vessels, heparin (50 U diluted with normal saline) was administered intravenously through the tail vein, and a cannula was then inserted into the portal vein. With these preparations, the HA and PV were clamped with atraumatic hemostatic clips, followed by occlusion of the blood flow from the SHVC and IHVC. The ischemic liver was then irrigated with cold (2–4 °C) 250 U heparin at a speed of 2.0 mL/min through a catheter in the portal vein, and a 1.0-mm hole was made on the wall of IHVC as an outflow tract. Twenty minutes later, the openings of the vessels were repaired using 8-0 sutures, and the PV, SVC, IVC and HA were unclamped to permit reperfusion. Normal saline (38 °C) was injected into the abdominal cavity to rapidly rewarm the liver before sewing up the abdominal incision.

Twenty-four hours after the operation and under anesthesia, blood samples were collected via the abdominal aorta, and the serum was separated and stored at −80 °C until analysis. At the same time, rats were killed at the time of fixation (see below), and brain tissue samples from the hippocampus were frozen immediately in liquid nitrogen and quickly transferred to a −80 °C freezer for later use in various biochemical assays.

2.3. Transmission electron microscopy (TEM)

To detect the ultrastructure alterations of neurons in hippocampal areas, five rats were randomly selected from each group. Animals were perfused through the ascending aorta with 250 mL heparinized saline solution followed by 100 mL fixative containing 2.5% glutaraldehyde and 4% paraformaldehyde in phosphate-buffered solution (PBS). The brains were carefully removed and immersed in the same fixative solution overnight. A 300-μm block of hippocampus tissue was dissected, and all sections were washed three times with PBS, fixed in 10% buffered glutaraldehyde and 1% osmic acid, dehydrated, and embedded in EPON812 (TAAB, UK). Subsequently, semi-sections (70 nm) were cut and stained with uranyl acetate and lead. A histologist who was unaware of the status of animals examined the sections using a FEI Tecnai Spirit G2 Bio Twin transmission electron microscope (FEI UK).

2.4. Lipid peroxidation assay

Lipid peroxidation has been used as an indirect measurement of cellular oxidative damage induced by oxygen free radicals (OFRs). Lipid peroxidation was commonly determined by the intracellular formation of malondialdehyde (MDA), which is one of the direct products of lipid peroxidation [11]. In contrast, superoxide dismutase (SOD) and catalase (CAT) serve as scavengers of OFRs, and thus, may exert anti-oxidant effects [17].

All reagents were purchased from Nanjing Jiancheng Biology Engineering Institute, P. R. China. Hippocampal tissues were washed with cold normal saline. Two milliliters of Tris hydrochloride (pH 7.4) buffer was added to the tissues. Each sample was homogenized and centrifuged. After stirring, some of the homogenate was transferred into Eppendorf tubes for MDA measurement. The remaining homogenate was centrifuged at 3000 rpm (rounds per minute). The supernatants obtained were used to measure SOD and CAT enzyme activities. Protein content was measured in the supernatant in which enzymatic activities were measured. The concentration of MDA is expressed as nanomoles per milligram protein (nmol/mg prot), and the activity of SOD and CAT is expressed as units per milligram protein (U/mg prot).

2.5. Enzyme-linked immunosorbent assay (ELISA)

The level of serum S-100β and the release of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and interleukin-10 (IL-10) in the hippocampal homogenates were measured using commercially available ELISA kits (KeyGen Biotech, Nanjing, P. R. China) according to the protocols provided by the manufacturer. The level of serum S-100β is expressed as picograms per milligram protein (pg/mg prot). The concentrations of hippocampal TNF-α, IL-6 and IL-10 are expressed as picograms per milliliter (pg/mL).