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Rb1 postconditioning attenuates liver warm ischemia–reperfusion injury through ROS-NO-HIF pathway

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ABSTRACT

Aims: Ginsenoside Rb1 could prevent ischemic neuronal death and focal cerebral ischemia, but its roles to liver warm I/R injury remain to be defined. We determined if Rb1 would attenuate warm I/R injury in mice.
Main methods: Mice were divided into sham, I/R, Rb1 + I/R (Rb1 postconditioning, 20 mg/kg, i.p. after ischemia), sham + L-NAME, I/R + L-NAME, and Rb1 + I/R + L-NAME groups using 60 min of the liver median and left lateral lobes ischemia. Serum levels of alanine aminotransferase (ALT) were measured and morphology changes of livers were evaluated. Contents of nitric oxide (NO) and nitric oxide synthase (NOS), malondialdehye (MDA) and activity of superoxide dismutase (SOD) were measured. Expressions of Akt, p-Akt, iNOS, HIF-1alpha, tumor necrosis factor-a (TNF-α) and intercellular adhesion molecule-1 (ICAM-1) were also determined by western blot or immunohistochemistry.
Key findings: Rb1 postconditioning attenuated the dramatically functional and morphological injuries. The levels of ALT were significantly reduced in Rb1 group ($p<0.05$). Rb1 upregulated the concentrations of NO, iNOS in serum, iNOS, and activity of SOD in hepatic tissues ($p<0.05$), while it dramatically reduced the concentration of MDA ($p<0.05$). Protein expressions of p-Akt, iNOS and HIF-1alpha were markedly enhanced in Rb1 group. Protein and mRNA expressions of TNF-α and ICAM-1 were markedly suppressed by Rb1 ($p<0.05$).
Significance: We found that Rb1 postconditioning could protect liver from I/R injury by upregulating the content of NO and NOS, and also HIF-1alpha protein expression. These protective effects could be abolished by L-NAME. These findings suggested Rb1 may have the therapeutic potential through ROS-NO-HIF pathway for management of liver warm I/R injury.

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Introduction

Ischemia reperfusion (I/R) injury is a common and important clinical problem in many different organ systems. Liver is particularly susceptible to I/R injury, which includes a great variety of sickness or pathological states, like transplantation, shock, trauma, hepatectomy, or other surgeries of the liver. To date, no specific therapy exists for the prevention or treatment of I/R injury.

Reperfusion of ischemic liver primes the cells for the more intense damage, and imposes an oxidant burden (Meldrum et al., 1998). Several studies have shown that reactive oxygen species (ROS) play a critical role in I/R injury. Upon reperfusion, activation of Kupffer cells in the liver also leads to abundant production of ROS and proinflammatory cytokines, further enhancing organ damage (Jaeschke and Leamsters, 2003). It has been reported that nitric oxide (NO) may reduce oxygen consumption that results in attenuated mitochondrial ROS formation (Xie et al., 1998; Scott et al., 2001) and reduce ROS release through inhibition of NADPH oxidase activity (Fujii et al., 1997).

Local production of tumor necrosis factor-alpha (TNF-α) also plays a role in the pathogenesis of I/R injury. Early local TNF-α production may induce various adhesion molecules, such as ICAM-1, and leukocyte activation in injured tissues (Shoskes and Halloran, 1996). Some studies have shown that TNF-α could be inhibited by NO in several conditions (Borges et al., 1998; Jiang et al., 2004). NO can also upregulate the rate of hypoxia inducible factor-1alpha (HIF-1alpha) synthesis by activating the phosphatidylinositol 3-kinase (PI3K)-Akt (Kasuno et al., 2004; Sandau et al., 2000) and blocks proline hydroxylase (PHD) activity (Kasuno et al., 2004). Activation and upregulation of HIF-1alpha have been recently found to be able to protect liver from I/R (Zhong et al., 2008; Alchera et al., 2008).

Studies have shown that many herbs have strong antioxidant characters. Among those, ginseng root has been widely prescribed to patients for a variety of ailments in Asian countries for thousands of years without apparent side effects. Ginsenoside Rb1 (Rb1) is the

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principal effective ingredient of ginseng root. Extensive studies have been conducted on Rb1, and Rb1 was found to be able to attenuate I/R-induced heart and cerebrovascular injury (Scott et al., 2001), prevent ischemic neuronal death (Yuan et al., 2007) and focal cerebral ischemia (Zhang et al., 1998), and protect spiral ganglion cells from cochlear ischemia(Fujita et al., 2007).

Several studies have suggested that NO protects organs against I/R injury (Jalowy et al., 1999; Kubota et al., 2000). The potentially protective role of endogenous NO in liver I/R injury is supported indirectly by several studies. It has also been reported that Rb1 could stimulate production of NO in human aortic endothelial cells (Yu et al., 2007) and decrease cardiac contraction through increased NO production (Scott et al., 2001). So we wonder if Rb1 treatment may have protective role against liver I/R injury through NO-mediated production. As such, the present study was undertaken to determine if Rb1 would mitigate morphological and functional dysfunction associated with liver I/R injury in mice.

Materials and methods

Ginsenoside Rb1 and N-nitro-L-arginine methylester (L-NAME)

Ginsenoside Rb1, N-nitro-L-arginine methylester (L-NAME), and a non-selective nitric oxide synthase (NOS) inhibitor, were purchased from Sigma (St. Louis, MO, USA). In this study, Ginsenoside Rb1 and L-NAME were dissolved and diluted with saline.

Animal model of 70% liver I/R injury

Male BALB/c mice (weight, 20–25 g) were used as experimental animals, maintained on a standard diet and water ad libitum, and kept in a temperature-controlled environment (20 °C to 22 °C) with alternating 12-hour cycles of light and dark. Six groups were studied (n = 8/group/time point): group I, sham group (saline, i.p. after ischemia); group II, I/R group (saline, i.p. after ischemia); group III, Rb1 + I/R group (Rb1, 20 mg/kg, i.p. after ischemia); group IV, sham + L-NAME (L-NAME, 16 mg/kg, i.v., 5 min before reperfusion); group V, I/R + L-NAME (L-NAME, 16 mg/kg, i.v. 5 min before reperfusion); and group VI, Rb1 + +I/R + L-NAME (L-NAME, 16 mg/kg, i.v. 5 min before reperfusion, Rb1, 20 mg/kg, i.p. after ischemia). After a midline laparatomy incision, an atraumatic vascular clip was placed on the vessels blocking the portal venous and hepatic arterial blood supply to the median and left lateral lobes of the liver, which results in approximately 70% mouse liver I/R injury. The animals were placed on a heating table to maintain core body temperature at 37 °C. After 60 min of ischemia, the clamp was withdrawn and prewarmed (37 °C) normal saline or Rb1 was instilled into the abdominal cavity. After 55 min of ischemia, 5 min before reperfusion, L-NAME was injected through the tail vein. Sham-operated animals went through the same surgical procedure as other animals; however, hepatic vessels clip were not applied. Animals were killed at 2, 4, and 12 h after liver I/R injury or sham surgery. Liver tissues and blood samples were taken for analysis. This study was approved by Sichuan Bioethics Committee, and all protocols were conducted under the guidelines of Animal Care and Use.

Serum alanine aminotransferase (ALT), NO, and NOS

Blood samples were obtained at the time of sacrifice. The serum concentration of alanine aminotransferase (ALT) was measured in a clinical laboratory as markers of hepatic functional damage. The serum levels of NO and NOS were determined by using an NO and NOS Kit (Jiancheng Biotech Ltd, Nanjing, China) according to the manufacture' instructions.

Histopathologic analysis

Tissue samples taken at the time of sacrifice after hepatic I/R injury were fixed in 10% buffered formalin solution and embedded in paraffin. Sections at 5 μm intervals were prepared and processed for hematoxylin–eosin staining. Histological changes were scored in a blind fashion from 0 to 3 based on the degree of cytoplasmic vacuolization, sinusoidal congestion, sinusoidal derangement, and necrosis of parenchymal cells using modified Suzuki classification as described by Takeda et al. (Takeda et al., 2003).

Determination of malondialdehye (MDA) level, total superoxide dismutase (SOD) activity, and nitricoxide synthase (NOS) in tissue

The involvement of ROS in I/R includes increased lipid peroxidation (LPO). LPO causes production of secondary products, among which MDA is used widely as a marker of oxidative stress. Levels of MDA in 2 h post-ischemic livers were measured as previously described (Peralta et al., 2002). Liver samples were homogenized and trichloroacetic acid was added to the homogenate, followed by the addition of TBA–water solution to the supernatant and boiled for 60 min. After the samples were cooled down, the optical density of supernatant at 532 nm was measured. Total SOD activity was determined by monitoring the concentration of nitroblue tetrazolium, which was reduced to a water-insoluble blue formazan dye with an absorbance maximum at about 560 nm by superoxide anion generated by xanthine–xanthine oxidase as previously described (Sun et al., 1988). Data are expressed as mean ± SD. NOS contents were assayed by using NOS assay kit (Jiancheng Biotech Ltd, Nanjing, China) according to the manufacturer's instructions.

Measurement of hepatic tumor necrosis factor-a (TNF-α) and intercellular adhesion molecule-1 (ICAM-1) mRNA levels

Total RNA was extracted from liver tissues using TRIzol reagent (Invitrogen, Carlsbad, CA). For semiquantitative PCR analysis, cDNA samples were standardized based on the content of β-actin cDNA as a housekeeping gene. RNA (1 μg) was reverse-transcribed and amplified using TaKaRa One-Step RT-PCR Kit (Takara Shuzo Co., Japan) at the following RT-PCR conditions: 95 °C for 2 min, 30 cycles at 95 °C for 1 min, 59 °C for 90 s, and 72 °C for 2 min. Primers used in PCR reactions were as follows: TNF-α 5′ primer (5′-AGCCCACGTAGCAAACCACCAA-3′) and 3′ primer (5′-ACACCCATTCCCTTCACAGAGCAAT-3′); ICAM-1 5′ primer (5′-TGGAACTGCACGTGCTGTAT-3′) and 3′ primer (5′-ACCATTCTGTT-CAAAAGCAG-3′); and β-actin 5′ primer (5′-CTGAAGTACCCCATTGAA-CATGGC-3′) and 3′ primer (5′-CAGAGCAGTAATCTCCTTCTGCAT-3′). PCR products were stained with ethidium bromide and electrophoresed in a 1.5% agarose gel. The target bands were visualized with an ultraviolet illuminator (Gel Doc EQ) (Bio-Rad Laboratories Inc., Hercules, CA) and image analysis software (QUANTITY ONE) (Bio-Rad). The mRNA expressions of TNF-α and ICAM-1 were presented as percent of β-actin.

Protein expression of HIF-1alpha, inducible NOS (iNOS), p-Akt and Akt

Proteins were extracted from hepatic tissues and quantified using the Bradford assay (Bio-Rad). Equal amounts of protein (40 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad). After overnight blocking at 4 °C, the membranes were incubated and shaken for 2 h at 37 °C with a mouse monoclonal antibody against HIF-1alpha (diluted at 1:500, AbCam, Canbridge, UK); iNOS (diluted at 1:500, AbCam, Canbridge, UK); p-Akt (diluted at 1:500, Signalway Antibody); rabbit polyclonal antibody against Akt (diluted at 1:500, Signalway Antibody); followed by secondary antibodies (diluted at 1:2000, Santa Cruz, CA). The signals were detected by using an ECL kit(Millipore, Bedford, MA,

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