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Full paper

Protective effect of *Rabdosia amethystoides* (Benth) Hara extract on acute liver injury induced by Concanavalin A in mice through inhibition of TLR4-NF-κB signaling pathway

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ABSTRACT

Extract of *Rabdosia amethystoides* (Benth) Hara (ERA), a traditional Chinese medicine has antibacterial, antiviral, anti-tumor, anti-hepatitis and anti-inflammatory properties. However, the hepatoprotective effects and molecular mechanisms of ERA on acute liver injury have not been fully elucidated. This study aims to investigate the anti-inflammatory effect and liver protection of ERA against the acute liver injury induced by Concanavalin A (Con A) and its underlying molecular mechanisms in mice. Mice received ERA (50, 100, 150 mg/kg body weight) by gavage before Con A intravenous administration. We found that ERA pretreatment was able to significantly reduce the elevated serum alanine and aspartate aminotransferase levels and liver necrosis in Con A-induced hepatitis. In addition, ERA treatment significantly decreased the myeloperoxidase, malondialdehyde levels and augmented superoxide dismutase level in the liver tissue, and also suppressed the secretion of proinflammatory cytokines in the serum, compared with Con A group by enzyme linked immunosorbent assay. Furthermore, we observed that ERA pretreatment can significantly decrease the expression level of Toll-like receptor (TLR) 4 mRNA or protein in liver tissues. Further results showed that ERA pretreatment was capable of attenuating the activation of the NF-κB pathway by inhibiting IκBα kinase and p65 phosphorylation in Con A-induced liver injury. Our results demonstrate that ERA pretreatment has hepatoprotective property against Con A-induced liver injury through inhibition of inflammatory mediators in mice. The beneficial effect of ERA may be mediated by the downregulation of TLR4 expression and the inhibition of NF-κB activation.

1. Introduction

Liver diseases are still a serious public health problem worldwide because of significant morbidity and mortality. A growing body of evidence suggests that an immune-mediated mechanism plays a central role in the development of liver disease, even in determining its prognosis (1,2). The patient undergoes the progressive destruction of the hepatic parenchyma and hypergammaglobulinemia, which is characterized by the filtration of activated T cells in liver (3). The high level of proinflammatory cytokines such as tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), and interleukin-6 (IL-6) is induced in liver diseases (4), including autoimmune hepatitis, acute liver failure and viral, bowel ischemia and postoperative liver, and sepsis (5). Excessive production of proinflammatory factors induces inflammation and is associated with the development and aggravation of hepatitis and with increased morbidity and mortality (6).

Concanavalin A (Con A)-induced specific liver injury in mice is a well reliable animal model that closely mimics the pathological processes and pathogenic mechanisms with viral and autoimmune hepatitis in human (7). Con A has the ability to activate T cells to secrete a variety of hepatotoxic cytokines (8,9), i.e., TNF-α, IFN-γ and IL-6, which contribute to the development of hepatocyte damage (10,11).

Toll-like receptors (TLRs) are widely expressed on multiple hepatocytes such as Kupffer cells, hepatic stellate cells, hepatocytes,

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sinusoidal endothelial cells, biliary epithelial cells, and hepatic dendritic cells (12), which play critical roles in the liver health (13). Recent studies indicate that TLR medicated signals have been involved in almost all liver diseases (14–16), and many therapeutic agents that abrogate liver injury might transect with the TLR4 signaling pathway (17,18). Furthermore, some studies showed that TLR4 was critically involved in the pathogenesis of Con A-induced liver damage (19,20). It had been reported that Con A also upregulated NF-κB expression in liver (21), and there was increasing evidence shown that Con A-induced liver injury was significantly attenuated via inhibiting NF-κB activation (22,23) Therefore, inhibition of the expression of TLR4 and suppressing the activation of NF-κB pathway may well represent therapeutic targets for T cell-mediated hepatitis.

Rabdosia amethystoides (Benth) Hara (usually called Wangzaozi), belonging to new rabdosia species, is a special traditional Chinese herbal medicine. Traditionally, the root from *R. amethystoides* has been widely used as a medicine for hepatitis and reported to have antiviral activity, antibacterial, anti-inflammation and anti-tumor due to the presence of triterpenoids, sterols (24,25). Modern medicines have little to offer for alleviation of hepatic disease and treatment of liver disorders. It has been only reported that, several chemical constitutes from the rabdosia species were evaluated for its possible hepatoprotective activity against chemical induced liver damage in experimental animals (26). However, the effects of Extract of *R. amethystoides* (ERA) in immune-mediated liver injury and its corresponding mechanisms have not been demonstrated yet.

In the present study, we investigated the protective effect of ERA on Con A-induced liver injury, and elucidated potential molecular mechanisms of ERA in Con A-induced hepatitis.

2. Materials and methods

2.1. Reagents

Aspartate transaminase (AST), alanine transaminase (ALT), myeloperoxidase (MPO), malondialdehyde (MDA), and superoxide dismutase (SOD) assay kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Enzyme linked immunosorbent assay (ELISA) kits for TNF-α, INF-γ, and IL-6 were purchased from R&D Systems (Minneapolis, MN, USA). Antibodies against TLR4, phospho-IκBα, IκBα, phospho-p65 and p65 were purchased from Cell Signaling Technology (Boston, MA, USA). An antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was purchased from Bioworld Technology (St. Louis Park, MN, USA). Con A was purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Preparation of ERA

Good quality root of *R. amethystoides* (Chinese herbal medicine: Wangzaozi) was purchased from Suzhou Kangyuan Pharmaceutical Co., LTD (lot number: 20140925) and dried in the shade and then ground into fine powder by pulverization. The dried powder was extracted in 10 volumes of boiled 75% ethanol for twice. The obtained extract was concentrated and passed in chromatographic column of macroporous adsorptive resins; the column was washed using 50% ethanol. The 50% ethanol suspension was evaporated under vacuum at 40 °C and stored for use (ERA). The yield of the ERA is about 7.55% weight of *R. amethystoides*. ERA fingerprinting was performed using high-performance liquid chromatography–ultraviolet detector (Supplementary Fig. 1). The two compounds were used as references as follows: oleanolic acid and ursolic acid.

2.3. Animal and experimental design

Male ICR mice (18–22 g body weight) were purchased from the Laboratory Animal Center of Yangzhou University (Yangzhou, China). The mice were housed in a controlled room temperature (23 ± 2 °C), humidity (55 ± 5%), and kept with a standard laboratory diet, allowed free access to tap water, and a 12-h light/dark cycle. All mice received humane care in compliance with the institutional animal care guidelines approved by the Experimental Animal Ethical Committee of Suzhou University (Suzhou, China).

Ninety mice were randomly divided into 6 experimental groups: (1) vehicle control (2), control + high dose of ERA (150 mg/kg) (3), Con A (Con A treated) (4), Con A (Con A treated) + low dose of ERA (50 mg/kg) (5), Con A (Con A treated) + median dose of ERA (100 mg/kg), and (6) Con A (Con A treated) + high dose of ERA (150 mg/kg). ERA (50, 100 or 150 mg/kg) was administered orally using oral gavages once daily during 7 days and 3 h before Con A injection. The mice received an intravenous Con A injection at the dose of 15 mg/ml/kg body weight. Mice were anesthetized lethally at 2, 8, and 24 h after Con A administration. The blood samples were collected using a tube and then centrifuged. The separated serum was used for analysis of hepatic enzymes and cytokines. Liver tissue was harvested for histochemical analysis, protein and gene analysis.

2.4. Serum biochemical analysis

The blood samples obtained were kept at room temperature for 2 h. Serum was then collected after centrifugation at 1000 g for 15 min. Serum ALT and AST were measured with assay kits according to the manufacture's instructions.

2.5. Histochemical analysis

The liver tissue on 8 h time point was fixed in 4% buffered paraformaldehyde for at least 24 h. Sections (4–5 μm thick) on slides were obtained and stained with hematoxylin and eosin (H&E) for histological observation.

2.6. Measurement of MPO, MDA and SOD

From the liver tissue samples on 8 h time point, the liver tissues (100 mg) were homogenized. The amounts of MPO, MDA and SOD were determined according to the manufacture's instructions.

2.7. Cytokines analysis by ELISA

Serum TNF-α, INF-γ, and IL-6 levels were determined by ELISA kits according to the manufacture's instructions.

2.8. Western blotting analysis

Liver sections were carefully homogenized in ice-cold lysis buffer (Vazyme Biotech, China). After centrifugation, protein concentration was determined by BCA protein assay kit (Beyotime biotechnology, China) with bovine serum albumin as a standard. Equal amounts of protein extracts were subjected to SDS-PAGE and then transferred onto PVDF membranes (Millipore Corporation, Billerica, MA, USA). Membranes were incubated with primary and secondary antibodies. Protein bands were visualized by ECL reagent (Vazyme Biotech, China). The densities of the bands were assessed and normalized to the GAPDH signals.