Abstract. Oxymatrine has a variety of pharmacological functions, including anti-viral, anti-liver fibrotic, anti-cancer, anti-bacterial, anti-epidemic, analgesic, anti-allergy and anti-inflammatory properties. The present study aimed to investigate the protective effects of oxymatrine against lipopolysaccharide (LPS)/D-galactosamine (D-GalN)-induced acute liver failure and the associated underlying mechanisms. Mice were administrated 4 mg/kg LPS and 600 mg/kg D-GalN. Then, mice in the Oxymatrine group were treated with 120 mg/kg of oxymatrine for 4 weeks. Oxymatrine treatment increased survival rate, decreased plasma aspartate transaminase and alanine aminotransferase activity, increased superoxide dismutase and glutathione peroxidase and decreased malondialdehyde, tumor necrosis factor- and myeloperoxidase activities in mice with LPS/D-GalN-induced liver failure. Furthermore, Oxymatrine activated nuclear factor erythroid 2-related factor (Nrf) 2 and heme oxygenase (HO)-1 protein expression, and suppressed Toll like receptor (TLR)4, myeloid differentiation primary response 88 and nuclear factor-κB protein expression in mice LPS/D-GalN mice. Overall, the present study suggests that oxymatrine effectively attenuates LPS/D-GalN-induced acute liver failure by oxidative damage via activation of Nrf2/HO-1 and modulation of TLR4-dependent inflammatory signaling pathways.

Introduction

Acute liver failure (ALF) is a type of severe liver disease which frequently occurs in patients with no medical history of liver disease, characterized by sudden apoptosis of liver cells over a short period, rapid deterioration of liver function and complications including hepatic encephalopathy and coagulation disorders (1). Due to high incidence, rapid development, severe illness, poor prognosis and high mortality rate, acute liver failure seriously endangers the life of patients and has become a challenge to clinicians (2). ALF exhibits a complex pathogenesis, and is a complicated pathophysiological process involving numerous factors. Endotoxemia and special liver toxic substances are important factors that lead to the incidence and development of liver failure (1). It is believed that lipopolysaccharide (LPS)/D-galactosamine (D-GalN) may lead to the injury of liver cells in mice by various mechanisms including liver cell apoptosis, generation of free radicals and lipid peroxidation (3). Research regarding the role of hepatocyte apoptosis and oxidative stress in the pathogenesis of ALF is of primary concern (4). It has previously been demonstrated that hepatic tissues of patients with ALF generate increased reactive oxygen species (ROS), resulting in an oxidation-reduction imbalance and thereby oxidative stress injury, which results in the apoptosis of liver cells by a mitochondrial pathway. Therefore, antioxidants and anti-apoptotic therapeutics are necessary in the treatment of ALF (5). Excluding liver transplantation, there is no effective therapeutic strategy in the treatment of ALF (6). Therefore, early therapeutic intervention in ALF has high research value and clinical significance.

A previous study suggested that transcription factor nuclear factor erythroid 2-related factor (Nrf) 2 is a key factor in the regulation of numerous antioxidants, which may maintain the balance of oxidation and reduction, inhibit apoptosis and protect against inflammation (7). The expression of Nrf2 is decreased under normal circumstances, very few stable Nrf2 units translocate into the nucleus and bind to antioxidant response elements (ARE) (8). However, the balance is disturbed under oxidative stress; Nrf2 escapes the Keap1-mediated ubiquitin degradation pathway and rapidly
translocates into the nucleus, binds to ARE to initiate the transcription of phase II detoxifying enzymes and various antioxidant enzyme genes downstream of ARE, thus resisting external harmful stimuli. Target genes in Nrf2 include heme oxygenase (HO-1), superoxide dismutase (SOD), dependent coenzyme/II oxidoreductase-1 and glutamylcysteine synthase. Deficiency or activation disorders of Nrf2 may increase the sensitivity of stimuli, leading to cell dysfunction and apoptosis, prolonged inflammatory repair and other pathological alterations (7,9).

Intestinal endotoxemia is important in liver failure. The pathophysiological process of endotoxin-induced liver injury involves a variety of signaling molecules and signal transduction pathways, of which the LPS/Toll like receptor (TLR)4 signal transduction pathway exhibits a predominant role (10). TLR4/nuclear factor (NF)-κB are classical inflammatory signals, and high expression of TLR4/NF-κB is associated with liver injury. It has been indicated that therapeutic intervention of the expression of TLR4 and NF-κB may improve liver function to a certain extent.

Oxymatrine (Fig. 1), additionally termed matrine, is an alkaloid with a tetracyclicquinolizidine structure extracted from Sophora alopecuroides L. (11), which belongs to the Ningxia herbal medicine Sophora. With anti-inflammatory, hepatoprotective and anti-neoplastic functions, Oxymatrine is effective in the treatment of cardiovascular disease (12). As a drug with various pharmacological effects, Oxymatrine has been applied to the treatment of hepatitis B and liver fibrosis, in addition to preventing chronic kidney disease development into renal interstitial fibrosis in patients, with low rates of adverse reaction (11,13). Therefore, the specific purpose of the present study was to investigate the protective effects of oxymatrine against LPS/D-GalN-induced acute liver failure and potential mechanisms in the mouse model.

Materials and methods

Animals and experimental protocol. Male, C57BL/6 mice (weight, 20-22 g; age, 6 weeks old) were obtained from the Center of Experimental Animals of Chongqing University (Chongqing, China) and were housed under standard conditions (temperature, 22±2˚C; humidity, 55±5%, 12-h light/dark cycle) with free access to food and water. All animal experiments carried out in the present study were approved by the Care and Use of Laboratory Animals of Fuling Center Hospital of Chongqing (Chongqing, China). Mice were randomly assigned to normal, ALF model or Oxymatrine groups. In the ALF model and Oxymatrine groups, mice were administrated 4 mg/kg LPS and 600 mg/kg D-GalN (intraperitoneal injection) for 24 h. Then, mice of the Oxymatrine group were treated with 120 mg/kg/day oxymatrine for 4 weeks. Mice in the normal and ALF model groups were treated with normal saline.

Blood clinical analyses. Following treatment with Oxymatrine, blood samples were collected and centrifuged at 2,000 x g for 10 min at 4˚C. The serum was then collected and used to measure aspartate transaminase (AST) and alanine aminotransferase (ALT) levels using ELISA kits (C010-2 and C009-2 respectively; Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Analysis of oxidative stress and inflammation. Following treatment with Oxymatrine, the tissue samples were homogenized in lysis buffer (Beyotime Institute of Biotechnology, Jiangsu, China) and used to analyze superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), malondialdehyde (MDA), tumor necrosis factor (TNF)-α and myeloperoxidase (MPO) activities using ELISA kits (A001-1-1, A005, A003-1, H052 and A044 respectively; Nanjing, Jiancheng Bioengineering Institute).

Western blot analysis. Following treatment with Oxymatrine, the tissues samples were homogenized in radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology), and the protein concentrations were determined using a BCA protein assay reagent (Beyotime Institute of Biotechnology). A total of 50-60 µg proteins were separated on 10% SDS-PAGE gel and transferred onto polyvinylidene membranes (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Following blocking with 5% skim milk for 1 h at 37°C, the membranes were incubated with primary antibodies against Nrf2 (1:500; cat. no. sc-81342), HO-1 (1:500; cat. no. sc-136256) TLR4...
(1:300; cat. no. sc-293072), myeloid differentiation primary response 88 (MyD88; sc-11356; 1:300), NF-κB (1:300; cat. no. sc-136970) and GAPDH (1:300; cat. no. sc-59540) all obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA) at 4˚C overnight. Following washing with TBST 3 times, the membranes were probed with a horseradish peroxidase-conjugated secondary antibody (1:5,000; Cell Signaling Technology, Inc., Danvers, MA USA) at room temperature for 2 h. Protein was developed with the ECL Plus Western Blotting Detection system (GE Healthcare Life Sciences, Little Chalfont, UK) and analyzed using Image Lab version 3.0 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis. Data are presented as the mean ± standard deviation using SPSS version 19.0 (IBM Corp., Armonk, NY, USA). Comparison among groups was determined by one-way analysis of variance followed by the Tukey post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

**Oxymatrine increases survival rate in ALF mice.** In LPS/D-GalN -induced ALF mice, survival rate was 40% which was a decreased value compared with normal control group. Treatment with Oxymatrine significantly increased survival rate of LPS/D-GalN-induced ALF mice, compared with the LPS/D-GalN-induced ALF model group (Fig. 2).

**Oxymatrine decreases plasma AST and ALT serum levels in ALF mice.** Conversely, AST and ALT serum levels of ALF model mice were increased compared with normal control group. Treatment with Oxymatrine significantly reduced AST and ALT serum levels in ALF mice, compared with ALF model group (Fig. 3).

**Oxymatrine exhibits varied effects on SOD, GSH-Px and MDA activities in ALF mice.** It was observed that there was a significant decrease in SOD and GSH-Px, and an increase in MDA activities in the ALF model group, compared with...
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normal control group. Pre-treatment with Oxymatrine significantly increased SOD and GSH-Px activities and decreased MDA activities in ALF mice, compared with the ALF model group (Fig. 4).

Oxymatrine activates Nrf2 and HO-1 protein expression in ALF mice. To explore the potential effects of Oxymatrine on ALF mice, Nrf2 and HO-1 protein expression was measured using western blot analysis. The results of the western blot analysis demonstrated that Nrf2 and HO-1 protein expression of ALF mice was downregulated in ALF mice, compared with normal control group. Oxymatrine significantly increased Nrf2 and HO-1 protein expression in ALF mice, compared with ALF model group (Fig. 5).

Oxymatrine decreases plasma TNF-α and MPO levels in ALF mice. The results demonstrated that TNF-α and MPO levels of the ALF model group were upregulated, compared with normal control group. Treatment with Oxymatrine significantly decreased TNF-α and MPO levels in ALF mice, compared with ALF model group (Fig. 6).

Oxymatrine suppresses TLR4, MyD88 and NF-κB protein expression in ALF mice. The present study next sought to investigate the role of TLR4, MyD88 and NF-κB. Protein expression was measured using Western blot analysis. As presented in Fig. 7, TLR4, MyD88 and NF-κB protein expression in ALF mice was increased, compared with normal control group. Oxymatrine significantly suppressed TLR4, MyD88 and NF-κB protein expression in ALF mice, compared with ALF model group (Fig. 7).

Discussion

The incidence of liver disease is high in China due to hepatitis B and C viral infections. According to the report of the World Health Organization, ~2 billion people are infected with hepatitis B worldwide and 350 million of these cases...
are chronic infections (3). In addition, ~1 million fatal cases of liver cirrhosis, liver failure and hepatocellular carcinoma are induced by hepatitis B every year (14). Hepatic cirrhosis accounts for 10-20% of all liver diseases based on pathological features and progression of disease (15). Liver failure may be divided into four categories, including acute liver failure, sub-acute liver failure, acute-on-chronic liver failure and chronic liver failure, each of which account for ~0.25-5% of advanced liver diseases, however the mortality rate may be up to 60-80% (2,15). Chronic or acute/subacute liver failure frequently occurs in China, accounting for >90% of all liver diseases (16). The present study firstly observed that Oxymatrine effectively increased the survival rate and reduced AST and ALT activities in LPS/D-GalN-induced ALF mice. Li et al (17) reported that the effects of oxymatrine prevents against arsenic trioxide-induced liver injury.

The body has developed a set of complex systems in response to the damage that may result from oxidative stress, one of which is ARE (18). ARE is a cis-acting element located upstream of phase II detoxifying enzymes and antioxidant protein/enzymes genes (19). It has previously been demonstrated that Nrf2 is the activator of ARE and the key transcription factor which regulates the oxidative stress response, the sensor for toxic substances and exogenous oxidative stress, and is closely associated with incidence and development of inflammation, respiratory system disease, malignant tumors, precancerous lesions and cardiovascular diseases (8). Under normal circumstances, Nrf2 is in an inactive state via binding to cytoplasmic specific receptor Keap-1 in the form of a heterologous dipolymer (19). It is activated by oxidative stress, uncycles from Keap1 and translocates into the nucleus to bind to ARE. The activated ARE then mediates target gene transcription, to increase the resistance of cells to oxidative stress. SOD, HO-1 and various other Nrf2 target genes are expressed in the liver. HO-1, additionally termed heat shock protein 32, is an endogenous antioxidant enzyme which is of current research interest (20). It has previously been demonstrated that HO-1 and metabolic products of heme may protect against oxidative stress, and the targeted activation of HO-1 may prevent injury to the liver (9,21). The results of the present study demonstrated that Oxymatrine significantly increased SOD and GSH-Px activities and decreased MDA activities in ALF mice through activating Nrf2/HO-1 expression. Jiang et al (22) demonstrated that Oxymatrine ameliorates renal ischemia-reperfusion injury via the Nrf2/HO-1 pathway.

TLR4 is one of the natural immune recognition receptors which is primarily used to recognize LPS and mediate transmembrane signal transduction. LPS activates Kupffer cells (KC) via a TLR-mediated signal transduction pathway, with a mechanism as follows: LPS and TLR4 interact with each other to transfer LPS into TLR4/lymphocyte antigen 96 (MD2) by LPS binding protein (LBP) and cluster of differentiation 14, and then LPS binds to TLR4/MD2 to induce the accumulation of TLR4, leading to the activation of intracellular MyD88 dependent pathway, and thereby promoting the expression and activation of NF-κB in cells, so as to stimulate cells to produce a variety of inflammatory mediators including TNF, IL-1, IL-6, nitric oxide, leukotrienes and thus resulting in the inflammatory reaction (23-25). This aids in the body's removal of endotoxins, however an excessive inflammatory reaction results in liver cell apoptosis and necrosis (26). It was demonstrated that Oxymatrine significantly suppressed TNF-α and MPO activities via the TLR4/MyD88/Myeloid differentiation primary response 88-dependent inflammatory signaling pathways. Fan et al (27) reported that Oxymatrine protects rat brains against focal ischemia and downregulates TLR4, TLR2, MyD88 and NF-κB.

In conclusion, oxymatrine effectively attenuated LPS/D-GalN-induced ALF through oxidative damage, by activation of Nrf2/HO-1 and suppression of TLR4-dependent inflammatory signaling pathways. Identification of the mechanism...
underlying these effects of Oxymatrine may aid in the development of a novel therapeutic strategy and subsequent clinical application in the treatment of ALF in the future.

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References