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Hepatoprotective effect of cryptotanshinone from *Salvia miltiorrhiza* in D-galactosamine/lipopolysaccharide-induced fulminant hepatic failure

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ABSTRACT

Cryptotanshinone from *Salvia miltiorrhiza Bunge* was investigated for hepatoprotective effects in D-galactosamine (GalN)/lipopolysaccharide (LPS)-induced fulminant hepatic failure. Cryptotanshinone (20 or 40 mg/kg) was orally administered 12 and 1 h prior to GalN (700 mg/kg)/LPS (10 μg/kg) injection. The increased mortality and TNF-α levels by GalN/LPS were declined by cryptotanshinone pretreatment. In addition, cryptotanshinone attenuated GalN/LPS-induced apoptosis, characterized by the blockade of caspase-3, -8, and -9 activation, as well as the release of cytochrome c from the mitochondria. In addition, cryptotanshinone significantly suppressed JNK, ERK and p38 phosphorylation induced by GalN/LPS, and phosphorylation of TAK1 as well. Furthermore, cryptotanshinone significantly inhibited the activation of NF-κB and suppressed the production of proinflammatory cytokines. These findings suggested that hepatoprotective effect of cryptotanshinone is likely associated with its anti-apoptotic activity and the down-regulation of MAPKs and NF-κB associated at least in part with suppressing TAK1 phosphorylation.

Introduction

Fulminant hepatic failure (FHF) is a life-threatening clinical syndrome and characterized by coagulopathy, jaundice and multisystem organ failure and a very high mortality, even though there is still no available therapy except liver transplantation limited by the chronic shortage of donor livers (Van Thiel et al. 2002).

D-Galactosamine (GalN) and lipopolysaccharide (LPS)-induced hepatitis in mice is a commonly used test model with endotoxemic shock and fulminant hepatic failure, which is similar to fulminant hepatic failure in clinic (Hishinuma et al. 1990). In the GalN/LPS model, LPS, as the bacterial cell wall component is used to initiate the inflammatory response. Because rodents are less sensitive to LPS exposure than humans, LPS is combined with the amino sugar GalN to sensitize the animals. In response to the infection in mammals LPS is detected by immune cells such as monocytes, macrophages and hepatic Kupffer cells (Liaskou et al. 2012). The combined exposure to GalN/LPS activates Kupffer cells to produce tumor necrosis factor (TNF)-α, consequently results in hepatocyte apoptosis, in the early stages of the LPS-induced liver injury in GalN-sensitized mice (Jaeschke et al. 1998). Then in the later stages of liver injury neutrophils transmigrate and attack hepatocytes, and eventually cause massive hepatocyte necrosis and multiorgan failure (Ramaiah and Jaeschke 2007).

Mitogen-activated protein kinases (MAPKs) family including p38 kinase, c-Jun N-terminal kinase (JNK) and extracellular signal regulated kinase (ERK) serve to regulate diverse cellular responses to extracellular stimuli, and modulate various cellular activities including gene expression, mitosis, differentiation and cell survival/apoptosis. With regard to the regulation of MAPKs, it is well known that nuclear factor (NF)-κB is an important transcriptional factor involved in the expression of TNF-α and interleukin (IL)-1 (Aggarwal 2004). In unstimulated cells, NF-κB is sequestered in an inactive form in the cytoplasm bound to inhibitory IκB protein. Stimulation leads to the rapid phosphorylation, ubiquitination, and degradation of IκB, which frees NF-κB to translocate to the nucleus and activate the transcription of proinflammatory genes (Karin

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; ERK, extracellular signal regulated kinase; FHF, fulminant hepatic failure; GalN, D-galactosamine; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; IL-1, interleukin-1; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinases; NF-κB, nuclear factor-κB; TAK1, TGF-β-activated kinase 1; TNF-α, tumor necrosis factor-α.

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and Ben-Neriah 2000). Therefore, an insight into the regulation of signaling pathways by MAPKs and NF-κB is indispensable for developing a treatment based on their inhibition.

Despite an increasing need for agents to protect the liver from damage, much less modern medicine is reliable regarding how to protect liver from diverse risks. Therefore, intense attention has been devoted to natural sources for the prevention and treatment of various liver diseases. Components from natural products used in folk herbs are expected to be therapeutically effective and have much lower toxicity when clinically used. Recently, we screened a numbers of natural substances, which are used traditionally for liver diseases (Lian et al. 2010b; Nan et al. 2004; Wan et al. 2010). Especially, we have reported that, in murine macrophage 264.7, cryptotanshinone (CTN) effectively inhibited LPS-triggered TLR4 signaling and NF-κB downstream pathways (Li et al. 2011). Cryptotanshinone is a major active lipid-soluble constituent of *Salvia miltiorrhiza Bunge* (reputed "Danshen") (Zhong et al. 2009), which is commonly used in the Traditional Chinese Medicine system. It was reported that *Salvia m*. ameliorates cirrhosis and portal hypertension, and also protects against doxorubicin-induced hepatic toxicity (Wagner and Ulrich-Merzenich 2013 and references therein). Cryptotanshinone possesses anti-cancer, anti-inflammatory and anti-oxidative activities (Lee et al. 2009; Li et al. 2011; Tang et al. 2011). In previous research, we also found that CTN significantly increased the survival rate against LPS challenge in GalN-sensitized mice. Additionally Park et al. (2009) have reported that cryptotanshinone-containing purified extract of *Salvia m.* protects hepatocytes from GalN-induced liver toxicity *in vitro*. Those together with previous *in vitro* results (Li et al. 2011) intrigued us to further investigate whether cryptotanshinone could attenuate GalN/LPS-induced fulminant liver failure in mice.

Materials and methods

Experimental animals and protocol

Male C57BL/6 mice (20–22 g) were obtained from Animal Division of Jilin University (Jilin, China) and housed in the animal facilities at the Pharmacy College of Yanbian University for at least 1 week before the experiment. All animal experiments were in accordance with the criteria of the "Guide for the Care and Use of Laboratory Animals" published by the USA National Institutes of Health (1996). Animals were treated humanely and with regard for alleviation of pain and distress. The animal experimental protocol was approved by Animal Research Committee of the University.

Cryptotanshinone (CTN; National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China; purity ≥99% by HPLC) or silymarin was freshly prepared for animal experiments and administered at a constant volume of 10 ml/kg body weight by oral gavage. The mice were randomly assigned to 6 groups; two groups of them (normal and control groups) received saline orally and the other groups received CTN (20 or 40 mg/kg) or silymarin (100 mg/kg) at 12 h and 1 h before GalN/LPS treatment. The last group of mice was only administrated with CTN (40 mg/kg) alone. All animals (except for the normal and CTN alone) were injected intraperitoneally with GalN (700 mg/kg body weight; Sigma Chemical Co., St. Louis, MO, USA)/LPS (10 μg/kg body weight; *Escherichia coli* 055:B5; Sigma Chemical Co.). Animals were anesthetized for blood sampling at 1 or 6 h after administration of GalN/LPS (Lian et al. 2010a). Blood was removed by cardiac puncture, and the serum was used for blood biochemistry. Mice were then euthanized and removed the livers. A portion of liver was preserved in formalin for histological sections. The remaining livers were snap frozen in liquid nitrogen and stored at −80 °C for additional analyses.

Determination of lethality

CTN was administered at a dose of 20 or 40 mg/kg to mice 12 h and 1 h before D-GalN/LPS injection. Control mice were given vehicle. The survival rate of mice was monitored for 24 h after D-GalN/LPS injection.

Blood biochemistry and TNF-α ELISA assay

Serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were detected at 6 h after GalN/LPS administration using an Autodry Chemistry Analyzer (SPOTCHEM™ SP4410, Arkray, Kyoto, Japan). Serum TNF-α was determined at 1 h after GalN/LPS injection by Quantikine® Mouse TNF-α Immunoassay kit (R&D, Minneapolis, MN, USA) according to the manufacturer's protocol.

DNA fragmentation analysis

Genomic DNA was extracted according to instructions supplied by the manufacturer of DNA Ladder kit (Beyotime, Jiangsu, China) according to the manufacturer's protocol and electrophoresed in 1.5% agarose gel at 100 V for 0.5 h and stained with 0.1 μg/ml ethidium bromide.

Histological analysis

At 6 h after GalN/LPS injection, liver tissues were removed from a portion of left lobe, fixed immediately in 10% neutral buffered formalin, embedded in paraffin and cut serially into 4 μm sections. The hematoxylin and eosin-stained sections were evaluated by light microscopy.

Immunoblotting

Preparation of total protein extracts from mouse liver was performed as previously described (Lian et al. 2010a). The isolation of cytosolic and nuclear fractions was performed using the NE-PER™ Nuclear and Cytoplasmic Extraction kit (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer's instructions. Forty micrograms of protein was loaded per lane on 10–12% polyacrylamide gels for electrophoresis. Proteins were electrophoretically transferred to polyvinylidenefluoride (PVDF) membranes. The PVDF membranes were blocked with 5% skim milk in PBSTween-20 (0.1%, v/v) for 1 h and then incubated with antibodies against mouse JNK, p-JNK, p-ERK, ERK, p38, p-P38, TAK1, p-TAK1 (Cell Signaling Technology, Beverly, MA, USA), caspase-3, caspase-8, caspase-9, NF-κB P65, p-NF-κB P65, IκB-α, p-IκB-α (Santa Cruz Biotechnology, CA, USA), α-tubulin (Sigma–Aldrich, St. Louis, MO, USA) at 4 °C overnight. Horseradish peroxidase conjugated anti-rabbit or anti-mouse IgG (Santa Cruz Biotechnology) was used as the secondary antibody. Immunoreactive proteins were visualized by the BeyoECL plus kit (Beyotime, Jiangsu, China). Band density was quantitatively analyzed using Quantity One software (Bio-Rad, USA). Each immunoreactive band from three independent experiments was digitized and normalized to α-tubulin levels.

Extraction of total RNA and reverse transcription

Total RNA was prepared from mice livers by use of the Beyozol reagent according to the manufacturer's protocol (Beyotime, Jiangsu, China). The cDNA was reverse transcribed from 1 μg of total RNA per 25 μl RT reaction with Oligo (dT) 15 primer and the AMV Reverse Transcriptase. RT-PCR was performed using primers specific for the mouse IL-1α, IL-1β and for the mouse housekeeping gene glyceraldehydes-3-phosphate dehydrogenase