International Immunopharmacology 15 (2013) 30–37

Contents lists available at SciVerse ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Protective effects of probiotic *Lactobacillus casei* Zhang against endotoxin- and D-galactosamine-induced liver injury in rats *via* anti-oxidative and anti-inflammatory capacities

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ARTICLE INFO

Article history:
Received 14 September 2012
Received in revised form 23 October 2012
Accepted 26 October 2012
Available online 9 November 2012

Keywords:
Lactobacillus casei Zhang
Lipopolysaccharide
D-Galactosamine
Liver injury
TNF-α
TLR4

ABSTRACT

Lactobacillus casei Zhang (LcZ) has been recently isolated from the traditional Mongolian beverage koumiss and has a set of favorable probiotic properties, including aciduricity, bile resistance and ability to colonize the gastrointestinal tract. We have previously reported the anti-oxidative properties of LcZ in the hyperlipidemic rats. In this study, the hepatoprotective effects of LcZ against lipopolysaccharide (LPS) and D-galactosamine (D-GalN)-induced liver injury were investigated. We found that pretreatment with LcZ significantly improved survival of rats challenged with LPS/D-GalN. In addition, pretreatment with LcZ significantly decreased alanine transaminase (ALT) and aspartate aminotransferase (AST) levels in LPS/D-GalN-challenged rats, which were accompanied by diminished liver injuries, reduced malondialdehyde (MDA) content and increased superoxide dismutase (SOD) activity in liver homogenates. Pretreatment with LcZ also markedly reduced LPS/D-GalN-induced production of hepatic nitric oxide (NO), activation of inducible nitric oxide synthase (iNOS) and expression of tumor necrosis factor-α (TNF-α). Furthermore, hepatic toll-like receptor 4 (TLR4) mRNA and protein levels, the phosphorylation of I-κB and translocation of nuclear factor κB (NF-κB) were significantly down-regulated by pretreatment with LcZ. These results suggest that pretreatment with LcZ protects against LPS/D-GalN-induced liver injury in rats *via* its anti-oxidative and anti-inflammatory capacities. The hepatoprotective effects of LcZ are associated with an inhibition of TLR4 expression and TLR4 signaling.

1. Introduction

Fulminant hepatic failure (FHF) is a dramatic and devastating clinical syndrome with poor prognosis and high mortality. Causes such as viruses, drugs, toxins, alcohol, metabolic diseases, or chronic autoimmune hepatitis can lead to the onset of FHF [1]. Liver transplantation is the only proven effective therapy for FHF. However, this possibility is limited due to the shortage of live donors and the rapidity of progression of FHF [2]. Effective prophylactic or therapeutic interventions are urgently needed to improve prognosis of FHF patients.

The combination of D-galactosamine (D-GalN) and lipopolysaccharide (LPS) is used frequently as an ideal animal model for FHF because it closely mimics the cascade of events leading to clinical hepatitis caused by endotoxemia and sepsis [3–6]. Animal model studies demonstrate that the innate immune system overreacts in the presence of LPS in the systemic circulation, leading to overproduction of pro-inflammatory mediators during the process of FHF. It is also known that TNF-α is one of the major mediators which contributes to hepatocyte apoptosis and organ failure [7–9]. Overproduction of reactive oxidative species (ROS) also plays an etiological role in the process of FHF induced by LPS/D-GalN [10–12]. Consequently, interventions in the production of pro-inflammatory mediators and oxidative substances are potential strategies for the prevention or therapy of FHF.

The use of probiotics is one such strategy for preventing and treating FHF. Probiotics, by definition, are live microorganisms which confer a health benefit on the host when administered in adequate amounts [13]. Studies reported that probiotics are effective in the prevention and treatment of specific pathogen infections in the gut [14–16]. Lactobacilli have been reported to be able to significantly inhibit LPS-induced TNF-α and prostaglandin E2 (PGE2) secretion *in vitro* and protect against DSS-induced ulcerative colitis (UC) in mice *via* anti-inflammation and immunomodulatory activities [17]. Due to the

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http://dx.doi.org/10.1016/j.intimp.2012.10.026

strong relationship between the gut and liver, the use of probiotics to prevent liver injury is receiving increasing attention. Probiotics have proved to be beneficial in treating chronic liver diseases resulting from alcohol consumption, viral infection and metabolic disorders [18–20]. Recent data showed that oral administration of *Lactobacillus*, *Bifidobacterium* and blueberry reduced liver injury induced by endotoxin and D-GalN [4]. In addition, *Lactobacillus plantarum* co-supplemented with L-arginine protected against endotoxin-induced liver injury [21]. However, the precise molecular mechanisms of probiotics still need to be elucidated.

Lactobacillus casei Zhang (LcZ) which has been recently isolated from koumiss (a traditional Mongolian beverage), exhibits favorable probiotic properties, including aciduricity, bile resistance and ability to colonize the gastrointestinal tracts [22]. Our previous experiment showed that oral administration of LcZ increased the production of IgA and IFN-γ in mice [23]. Furthermore, LcZ has been reported to have potentially anti-oxidative effects in hyperlipidemic rats [24]. We hypothesize that LcZ can be beneficial for treating acute liver injury. In this study, we evaluated the hepatoprotective effects of LcZ on LPS/D-GalN-induced liver injury in rats and examined the underlying mechanisms.

2. Materials and methods

2.1. Materials

LPS (*Escherichia coli* serotype O55:B5) and D-GalN were purchased from Sigma (St Louis, MO, USA). MDA and SOD kits were purchased from Jiancheng Institute of Biotechnology (Nanjing, China). Dexamethasone was obtained from Zhejiang Conba Pharmaceutical Co., Ltd (Hangzhou, China). Rabbit anti-TLR4 antibody was obtained from Research Santa Cruz (Santa Cruz, CA). Polyclonal Abs against actin, nucleoporin p62, monoclonal Abs for phospho-IκB-α and NF-κB p65 were purchased from Cell Signaling Technology (Beverly, CA).

2.2. Lactobacillus strain and growth conditions

L. casei Zhang is a novel strain of lactic acid bacteria (LAB) isolated from koumiss, the probiotic properties of which were described previously [22]. The strain was grown in de Man, Rogosa, and Sharpe (MRS) broth (Hopebio Co., Qingdao, China) as previously reported [23]. Cell pellets were harvested at 5000×*g* for 5 min, washed twice with PBS, and lyophilized. Lyophilized powder was suspended in physiological saline and adjusted to 1×10^9 CFU/ml for oral administration to rats.

2.3. Animals

Male Wistar rats, weighting 120–140 g of 5–6 weeks old, were purchased from Vital River Lab Animal Technology Co., Ltd., Beijing, China [certification no.: SCXK (Jing) 2007–0009]. The animals were kept in wire cages at 20–22 °C and 55 ± 10% relative humidity, under a 12-h light/dark cycle. Animals were fed with a standard laboratory diet and given water *ad libitum*. Animal experiments began after one week of adaptation. The procedures for animal experiments were approved by the Animal Research Committee of Inner Mongolia Agricultural University and performed in accordance with the NIH and Inner Mongolia Agricultural University guidelines for the Care and Use of Laboratory animals.

2.4. Experimental protocol

Forty-eight male Wistar rats were randomly divided into the following four groups (n = 12 per group): health control group (CTRL), LPS and D-GalN induced-liver injury group (L/G), *L. casei* Zhang group (L/G + LCZ), and dexamethasone group (L/G + DXM). The CTRL group, L/G group and L/G + DXM group received oral gavage with saline, and the L/G + LCZ group were gavaged with 1 ml of LcZ (1×10^9 CFU/ml) for 30 days prior to exposure to LPS and D-GalN. Rats were then injected intraperitoneally (*i.p.*) with 50 μg/kg LPS and 300 mg/kg D-GalN. L/G + DXM rats were first *i.p.* injected with 10 mg/kg dexamethasone and then challenged with similar doses of LPS and D-GalN. The CTRL rats were injected with saline. All the rats were sacrificed 16 h after LPS/D-GalN challenge when they were still alive. Blood was collected and plasma was prepared by centrifugation at 12,000×*g* for 20 min at 4 °C. A 1×1 cm piece was taken from the right lobe of the liver and fixed in 10% formalin for histological analysis. The remainder of liver was stored in liquid nitrogen. Long-term (14 days) survival was assessed using an additional 62 male Wistar rats divided into the following four groups: the CTRL group (n = 16), the L/G group (n = 16), the L/G + LCZ group (n = 20), and the L/G + DXM group (n = 10).

2.5. Biochemical assays

The levels of ALT and AST in plasma were measured by an Olympus 2700 biochemistry analyzer using ALT and AST kits according to the manufacturer's instructions (Leadman, Beijing, China).

2.6. Measurement of MDA, SOD, NO, and iNOS levels

Liver tissues were homogenized (100 mg/ml) in cold physiological saline, and then centrifuged at 12,000×*g* (4 °C) for 10 min and supernatants were collected. The protein concentration in the supernatants was measured by BCA protein assay (Pierce) according to manufacturer's instructions. The concentration of NO in the liver homogenates was detected with Griess reagent (Sigma Aldrich, St. Louis, MO, USA) as described previously [25]. The concentration of MDA, the activities of iNOS and SOD were measured by the corresponding kits according to the manufacturer's instructions (Jiancheng Institute of Biotechnology, Nanjing, China). The values of NO, iNOS, MDA and SOD were normalized by the total protein concentration of the same sample.

2.7. RNA extraction and real-time PCR assay

Total RNA was extracted by Trizol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). The quality of RNA was verified by evaluating the optical density at 260 nm (OD 260) and at 280 nm (OD 280). One microgram total RNA was reversely transcripted to cDNA using the PrimeScript RT-PCR kit (Takara, Dalian, China). Real-time PCR analysis for TNF-α and TLR4 gene expression were performed on ABI 7300 (Applied Biosystems) with the SYBR Real Time-PCR kit (Takara, Dalian, China). The following primers were used to amplify TNF-α cDNA: sense, 5′-TCAGTTCCATGGCCCAGAC-3′, and antisense, 5′-GTTGTCTTTGAGATCCATGCCATT-3′; TLR4 cDNA: sense, 5′-CTCACAACTTCAGTGGCTGATTTA-3′, and antisense, 5′-TGTCT CCACAGCCACCAGATTC-3′; GAPDH cDNA: sense, 5′-GCAAGTTCAACGG CACAG-3′, antisense, 5′-GCCAGTAGACTCCACGACAT-3′. The real-time PCR parameters were initiated by 5 min at 95 °C, followed by 40 cycles of 5 s at 95 °C and 1 min at 60 °C. The relative quantities of target transcripts were normalized against the data of house-keeping gene, GAPDH.

2.8. Histological analysis and immunohistochemistry staining

Livers were collected 16 h after the induction of acute liver injury in rats, and then fixed in 10% paraformaldehyde (PFA)/PBS for 24 h. The fixed livers were then embedded in paraffin. Liver sections (5 μm) were processed for staining with hematoxylin and eosin, and then assessed under a light microscope in a blind fashion. For immunohistochemistry staining, liver sections (5 μm) were treated with