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Protective effect of linarin against D-galactosamine and lipopolysaccharide-induced fulminant hepatic failure

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ABSTRACT

Linarin was isolated from *Chrysanthemum indicum* L. Fulminant hepatic failure is a serious clinical syndrome that results in massive inflammation and hepatocyte death. Apoptosis is an important cellular pathological process in D-galactosamine (GalN)/lipopolysaccharide (LPS)-induced liver injury, and regulation of liver apoptosis might be an effective therapeutic method for fulminant hepatic failure. This study examined the cytoprotective mechanisms of linarin against GalN/LPS-induced hepatic failure. Mice were given an oral administration of linarin (12.5, 25 and 50 mg/kg) 1 h before receiving GalN (800 mg/kg)/LPS (40 μg/kg). Linarin treatment reversed the lethality induced by GalN/LPS. After 6 h of GalN/LPS injection, the serum levels of alanine aminotransferase, aspartate aminotransferase, tumor necrosis factor (TNF)-α, interleukin-6 and interferon-γ were significantly elevated. GalN/LPS increased toll-like receptor 4 and interleukin-1 receptor-associated kinase protein expression. These increases were attenuated by linarin. Linarin attenuated the increased expression of Fas-associated death domain and caspase-8 induced by GalN/LPS, reduced the cytosolic release of cytochrome *c* and caspase-3 cleavage induced by GalN/LPS, and reduced the pro-apoptotic Bim phosphorylation induced by GalN/LPS. However, linarin increased the level of anti-apoptotic Bcl-xL and phosphorylation of STAT3. Our results suggest that linarin alleviates GalN/LPS-induced liver injury by suppressing TNF-α-mediated apoptotic pathways.

1. Introduction

Fulminant hepatic failure is a potentially devastating syndrome that accompanies hepatic encephalopathy, severe coagulopathy, jaundice and hydroperitoneum, and is associated with high mortality clinically (Lee, 1994). Administration of a subtoxic dose of D-galactosamine (GalN) together with lipopolysaccharide (LPS) has often been used in an animal model of fulminant hepatic failure (Galanos et al., 1979; Hishinuma et al., 1990). Increased intestinal permeability and impaired macrophage clearance following GalN/LPS injection results in systemic inflammation characterized by increased levels of proinflammatory cytokines (Leithead et al., 2009). During development of fulminant hepatic failure, large numbers of apoptotic hepatocytes have been considered as one of major etiology (Hatano, 2007; Kuhla et al., 2008). Hepatocellular apoptosis is not only an essential process that mediates liver failure but is also a trigger for activation of inflammatory leukocytes and Kupffer cells, which further aggravates the injury (Eipel et al., 2007).

Tumor necrosis factor (TNF)-α is the most potent contributor to various models of liver injury, and TNF-α level correlates with the extent of tissue damage (Hishinuma et al., 1990; Colletti et al., 1990). Conventionally, TNF-α is considered as a first-wave cytokine that leads to a burst in downstream proinflammatory cytokine release. Furthermore, TNF-α acts as a strong inducer of neutrophil migration and macrophage activation, and induces massive hepatocyte necrosis during fulminant hepatic failure. In addition, TNF-α that is produced in response to GalN/LPS injection participates in the initiation of the extrinsic apoptotic pathway, and its signal transduction mechanisms have been characterized recently (Kim et al., 2010; Nagaki and Moriwaki, 2008). As the importance of TNF-α-dependent molecular mechanisms

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; DISC, death-inducing signaling complex; FADD, Fas-associated death domain; GalN, D-galactosamine; H&E, hematoxylin and eosin; IFN, interferon; IL, interleukin; IRAK, interleukin-1 receptor-associated kinase; JNK, Jun-N-terminal kinase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; STAT, signal transducer and activator of transcription; TLR, toll-like receptor; TNF, tumor necrosis factor; TUNEL, Terminal deoxynucleotidyl transferase dUTP nick end labeling

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underlying hepatocyte apoptosis in fulminant hepatic failure has become recognized, therapeutic approaches to regulate these signaling molecules are currently being investigated.

Chrysanthemum indicum L. has been widely used as a tea in Korea and China traditionally, and also as an herbal medicine for alleviating vertigo, hypertensive symptoms and several inflammatory diseases such as colitis and stomatitis (Kato et al., 1987). *C. indicum* suppressed auricle edema induced by dimethylbenzene in mice, and showed anti-inflammatory properties by suppressing NF-κB and mitogen-activated protein kinase (MAPK) activity in LPS-treated RAW 264.7 macrophages (Cheng et al., 2005; Cheon et al., 2009). Linarin is a natural occurring flavanol glycoside derived from *C. indicum* that exerts remarkable analgesic and anti-inflammatory activities (Martinez-Vazquez et al., 1998). Recently, the anti-apoptotic effect of linarin was shown in an amyloid-β-induced PC12 cell death model where it induced the anti-apoptotic protein Bcl-2 through the PI3K/Akt pathways (Lou et al., 2011). However, the effect of linarin on liver, particularly on *in vivo* models of hepatotoxicity, has not been documented.

In this study, we investigated the protective mechanisms of linarin against GalN/LPS-induced liver injury, with a particular focus on apoptotic signaling pathways.

2. Materials and methods

2.1. Plant materials

The flowers of *C. indicum* were collected in September 2011 at Jeju Island, Korea, and identified by Prof. Je-Hyun Lee, College of Oriental Medicine, Dongguk University. A voucher specimen is deposited in the College of Pharmacy, Chungnam National University (12A1001).

2.2. Isolation and identification of linarin

The dried flowers of *C. indicum* (8.0 kg) were extracted with methanol (MeOH) three times at room temperature. The MeOH extract was concentrated to dryness under reduced pressure, and the residue was suspended in H_2O. The resulting solution was partitioned with dichloromethane (CH_2Cl_2) and ethyl acetate (EtOAc) consecutively. The EtOAc fraction (30 g) was subjected to silica gel (Kiesel gel 60, 230–400 mesh; Merck, Whitehouse Station, NJ, USA) column chromatography using a mixture of CH_2Cl_2: MeOH with an increasing amount of MeOH (1:0–0:1) to give 11 subfractions (EA-A–EA-K). Subfraction EA-F was rechromatographed over a silica gel column (EtOAc:MeOH:H_2O = 100:10:7) and also on a RP-C18 (LiChroprep® RP-18, 40–63 μm; Merck) column with 50% MeOH–H_2O as eluents to obtain linarin (2.0 g). The structure of linarin was determined by spectroscopic analysis. The ^{1}H NMR (700 MHz) and ^{13}C NMR (176 MHz) spectra were recorded on a NMR spectrometer (Avance III 700; Bruker, Billerica, MA, USA) using DMSO-d_6 as a solvent. The ESI-MS spectrum was obtained on an Agilent 1100 LC/MSD trap classic (Agilent, Santa Clara, CA, USA).

2.3. Treatment of animals

Male ICR mice weighing 24–26 g were fasted for 18 h, but given access to water *ad libitum*. All animals received care in compliance with the Principles of Laboratory Animal Care formulated by the National Institute of Health (NIH Publication no.86-23, revised 1985) and the guidelines of the Sungkyunkwan University Animal Care Committee. Mice (except for control) were injected intraperitoneally with GalN (800 mg/kg; Sigma-Aldrich, St Louis, MO, USA) and LPS (40 μg/kg *Escherichia coli* 026: B6; Sigma-Aldrich) dissolved in phosphate-buffered saline. In the linarin-treated group, mice were administered 12.5, 25 and 50 mg/kg of linarin (dissolved in 10% Tween 80 in saline, vehicle) orally 1 h before the GalN/LPS treatment, while other groups received an equivalent volume of vehicle. Animals were randomly assigned to the following groups (each group, $n = 8-10$): (a) vehicle-treated control group, (b) 50 mg/kg linarin-treated control group, (c) vehicle-treated GalN/LPS group, (d) 12.5 mg/kg linarin-treated GalN/LPS group, (e) 25 mg/kg linarin-treated GalN/LPS group, and (f) 50 mg/kg linarin-treated GalN/LPS group. The dose of linarin was selected based on previous report (Han et al., 2002) and our preliminary study. The survival analysis of mice was monitored for 24 h after GalN/LPS injection. Mice were anesthetized with ketamine/xylazine and then killed by decapitation at 6 h after GalN/LPS injection. Blood and liver samples were collected for further assessment.

2.4. Serum aminotransferase activities

Serum alanine aminotransferase (ALT) activity and aspartate aminotransferase (AST) were determined by standard spectrophotometric procedures using the ChemiLab ALT assay kit (IVDLab Co., Uiwang, Korea).

2.5. Histological analysis

After 6 h of GalN/LPS injection, liver tissues were removed from a portion of the left lobe. Tissue samples were sectioned and fixed immediately in 10% neutral-buffered formalin. Then, the sample was embedded in paraffin and sliced into 5 μm sections. The hematoxylin and eosin (H&E)-stained sections were evaluated using an optical microscope (Olympus Optical, Tokyo, Japan). Apoptotic cells were detected using Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining with a commercially available kit (*in situ* Apoptosis Detection Kit; Takara, Shiga, Japan).

2.6. Serum TNF-α, interleukin (IL)-6 and interferon (IFN)-γ levels

Serum levels of TNF-α, IL-6 and IFN-γ were quantified at 6 h after GalN/LPS injection using commercial mouse enzyme-linked immunosorbent assay (ELISA) kits (BD Bioscience, San Diego, CA, USA) according to the method reported by Kang et al. (2011).

2.7. Preparation of protein extracts and western blot immunoassay

Fresh liver tissue was isolated and homogenized in PRO-PREP (Intron Biotechnology, Seongnam, Korea) for whole protein samples and in NE-PER® (Thermo Fisher Scientific Inc., Rockford, IL, USA) for extraction of nuclear and cytosolic protein samples, according to manufacturer's instructions. Protein concentrations were determined using the BCA Protein Assay kit (Thermo Fisher Scientific Inc.). Whole protein samples of 20 μg were used to determine the content of toll-like receptor 4 (TLR4), interleukin-1 receptor-associated kinase (IRAK), Fas-associated death domain (FADD), caspase-3, caspase-8, Bim, Bcl-xL, signal transducer and activator of transcription3 (STAT3), phosphorylated STAT3 (p-STAT3). Cytosolic protein samples of 20 μg were used to determine the content of cytosolic cytochrome *c*. Protein samples were separated by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE) and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA) using the Semi-Dry Trans-Blot Cell (Bio-Rad Laboratories, Hercules, CA, USA). After transfer, the membranes were washed with 0.1% Tween-20 in tris-buffered saline (TBS/T) and blocked for 1 h at room temperature with 5% (w/v) skim milk powder in TBS/T. Blots were then incubated overnight at 4 °C with the primary

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