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Protective effects of morin on lipopolysaccharide/D-galactosamine-induced acute liver injury by inhibiting TLR4/NF-κB and activating Nrf2/HO-1 signaling pathways

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ABSTRACT

Morin, a bioactive flavonoid extracted from the bark of *Moraceae* plants and many medicinal herbs, has anti-inflammatory and antioxidative effects. In this research, we explored the protective effects of morin against lipopolysaccharide (LPS) and D-galactosamine (D-GalN) induced acute liver injury in mice. Mice were given an intraperitoneal injection of morin before LPS and D-GalN treatment and the HepG2 cells were only given morin to investigate its effects. The results showed that morin markedly inhibited the production of serum alanine transaminase (ALT), aspartate aminotransferase (AST), interleukin-6 (IL-6), tumor necrosis factor (TNF-α) and hepatic TNF-α, IL-6, and myeloperoxidase (MPO) induced by LPS/D-GalN. In order to evaluate morin effect in the future, we investigated the expression of nuclear factor E2 related factor 2 (Nrf2), nuclear factor-kappaB (NF-κB), toll like receptor 4 (TLR4) on liver injury. Taken together, these results suggested that morin could exert the anti-inflammatory and anti-oxidative effects against LPS/D-GalN-induced acute liver injury by activating Nrf2 signal pathways and inhibiting NF-κB activation.

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1. Introduction

The occurrence of liver injury is often accompanied by endotoxemia and sepsis and can cause high mortality in animals and human beings due to the lack of effective drugs [1,2]. Therefore, finding an effective drug to treat acute liver injury is in desperate need. LPS and D-GalN-induced acute liver injury in mice model is widely used in human liver damage [3,4]. The mice model has been recognized as a potential treatment of drug research method [5]. D-GalN is a kind of hepatotoxic agent and can cause hepatotoxicity in liver mainly through inhibiting hepatocyte RNA and protein synthesis [6]. LPS is a kind of endotoxin that could activate inflammatory cytokines, which can bring about liver tissue injury [7,8].

Nuclear factor E2 related factor 2 is an important regulatory factor that regulates cellular defense against oxidative stress. It can protect against oxidative damage triggered by liver injury and inflammation by regulating the expression of antioxidant proteins. Nuclear factor κB, a nuclear transcription factor, plays a great role in the regulation of inflammation. The activation of NF-κB is considered to respond to the oxidative stress. Recent study revealed that LPS/D-GalN was capable of activating NF-κB and Nrf2 signaling [9]. However, the molecular basis of morin mitigating the LPS/D-GalN-induced liver damage by induction of Nrf2 remains elusive. This led us to reason whether morin can activate Nrf2 and inhibit NF-κB signaling, which exert protective effect against LPS/D-GalN-mediated oxidant damage. If the above hypothesis is feasible, our results will show that morin may be a potential drug target to mitigate liver damage via inhibition of NF-κB and up-regulation of Nrf2 signal pathways.

Morin (20, 3, 40, 5, 7-pentahydroxyflavone), a kind of flavonol, is extracted from herbal medicine which has anti-oxidant [10], anti-hyperglycemic [11] and liver-protective effects [12]. Previous studies showed that morin could inhibit the Fyn kinase, an enzyme linked to Nrf2 degradation [13,14] and influence LPS-BV2 cells by suppressing NF-κB activity and activating HO-1 induction [15]. In addition, morin was also found to inhibit LPS-induced acute lung injury in mice [16]. However, the effects of morin on LPS/D-GalN-induced liver injury remain unclear.

In this study, we evaluated whether morin played a positive role in protecting liver tissue against LPS/D-GalN-induced acute liver injury. We demonstrated, for the first time, that morin could show functional and morphological protection against LPS/D-GalN-induced liver injury by alleviating inflammatory and oxidative stress.

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2. Materials and methods

2.1. Materials

Morin (purity ≥98%) was purchased from Chengdu Reference Products (Chengdu, China). Dimethylsulfoxide (DMSO), LPS (*Escherichia coli* lipopolysaccharide, 055:B5) were purchased from Sigma-Aldrich (St. Louis, MO, USA). CCK8 was purchased from Beyotime Biotechnology (Beijing. China). Fetal bovine serum (FBS), Dulbecco's Modified Eagle's Medium (DMEM), penicillin and streptomycin were purchased from Invitrogen-Gibco (Grand Island, NY). We purchased D-galactosamine hydrochloride from Aladdin Industrial Corporation (Shanghai, China). Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) detection kits were provided by Jiancheng Bioengineering Institute of Nanjing (Nanjing, Jiangsu, China). Mouse TNF-α, and IL-6 enzyme-linked immunosorbent assay (ELISA) kits were provided by BioLegend (CA, USA), and Rabbit Ab Phosphor-NF-κB, Rabbit Ab phosphor-IκB and IκB were purchased from Abcam (USA). Anti-HO-1 and anti-Nrf2 monoclonal antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Anti-Lamin B, anti-TLR4 monoclonal antibodies were purchased from Proteintech Group Inc. (Boston, MA, USA), and HRP-conjugated goat anti-rabbit and goat anti-mouse antibodies were provided by GE Healthcare (Buckinghamshire, UK). All other chemicals were of reagent grade.

2.2. Animals

C57/BL6 mice (male, 6–8 weeks old, weighing approximately 18 to 22 g each) were purchased from Liaoning Changsheng Biotechnology (Liaoning, China). These mice were given adequate food and water ad libitum and housed in clean cages for 2–3 d. The laboratory temperature was 24 ± 1 °C and relative humidity was 40%–80%. All studies were performed in accordance with the National Institutes of Health guide for the Care and Use of Laboratory Animals published by the USA National Institutes of Health.

2.3. In vitro study

2.3.1. Cell culture and treatment

The HepG2 human hepatoma cell line was purchased from the China Cell Line Bank (Beijing, China). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated Fetal bovine serum, 3 mM Glutamine, antibiotics (100 U/ml penicillin and 100 U/ml streptomycin) at 37 °C and 5% CO_2 conduction. All cell experiments were incubated in the presence or absence of various concentrations of morin for 12 h followed by natural culture.

2.3.2. CCK8 assay for cell viability

HepG2 cells were plated at a density of 4×10^5 cells/ml onto 96-well plates and incubated in 37 °C and 5% CO_2 incubator for 12 h (100 μl/well) after trypsin treated. Then the cell culture medium was discarded, and the cells were treated with 100 μl morin of distinctive concentrations (0–128 μg/ml). After 12 h, 10 μl CCK8 was added to each well, and the cells were incubated for an extra 3 h at 37 °C with 5% CO_2. The optical density was measured at 450 nm on a microplate reader (TECAN, Austria).

2.4. In vivo study

2.4.1. LPS/D-GalN-induced acute liver injury mode

The C57/BL6 mice were randomly divided into six groups: the control group, LPS/D-GalN-stimulated group (intraperitoneal injection with 600 mg/kg D-GalN and 10 μg/kg LPS) and morin (25, 50, and 100 mg/kg) groups which were treated with morin 1 h before LPS/D-GalN stimulation. The negative control was only given morin (100 mg/kg) treatment. Firstly, mice were given intraperitoneal injection (i.p.) of different concentrations of morin. Then PBS was given in the control group. One hour later, the mice were given LPS/D-GalN treatment. In addition, others mice were given morin (25, 50, and 100 mg/kg) treatment. Mice blood and liver tissues were collected subsequently for future research.

2.4.2. MPO (myeloperoxidase) assay

MPO is a marker of neutrophil activation, oxidative stress and oxidative tissue damage. MPO levels were detected 3 h after LPS/D-GalN injection. Liver tissues were homogenized for further research through using MPO ELISA kit (Jiancheng Company, Nanjing, China). MPO levels were determined by measuring the absorbance under 450 nm using a spectrophotometer.

2.4.3. Cytokine assays

The C57/BL6 mice were randomly divided into six groups and were treated as described before. In the end, the blood and liver tissues were collected for measuring TNF-α and IL-6 by using ELISA kits (BioLegend) according to the production directions.

2.4.4. Histopathology evaluation of the liver tissues

Livers were collected 3 h after LPS/D-GalN treatment and tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, sliced and stained with hematoxylin and eosin (H&E). The pathological changes of liver tissues were observed with a light microscope.

2.4.5. Analysis of liver enzymes

Blood were collected from all mice to detect plasma enzyme of ALT and AST changes. The ALT and AST levels marked the degree of Hepatocyte damage. Serum was collected to detect ALT and AST levels with test kits purchased from Jiancheng Bioengineering Institute of Nanjing according to the instructions.

2.5. Western blot

HepG2 cells were trypsinized and plated onto 6-well plate. The density of cells was 4×10^5 cells/ml. The cells were incubated in 37 °C and 5% CO_2 for 12 h. afterwards, morin in different concentrations (8, 16, and 32 μg/ml) were added into every plate. After 8 h, the cellular proteins were collected. Liver tissues were collected 3 h after LPS/D-GalN injection. Nuclear and cytoplasmic proteins of the liver tissues and cells were extracted from livers by using Nuclear and cytoplasmic protein Extraction Kit (Beyotime). The protein concentrations were assayed by using BCA protein assay kit (Thermo, USA) following the manufacturer's instructions. 30 μg of sample proteins were regularly collected, followed by being fractionated on 12% polyacrylamide-SDS gel and transferred to polyvinylidene fluoride (PVDF) membrane. The membranes were blocked in 5% skim milk on the table for 2 h at room temperature and incubated with primary antibody (1:1000) at 4 °C overnight. The membranes were washed by TBST four times and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h (1:5000). Then the membranes were washed by TBST four times and the immunoreactive proteins were detected by chemiluminescence (ECL) Western blotting detection kit (Thermo, USA).

2.6. Statistical analysis

All values were expressed as mean ± SEM. Differences between mean values of normally distributed data were assessed by two-tailed Student's *t*-test. Statistical significance was accepted at $P < 0.05$ or $P < 0.01$.

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