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Original article

# Ligustrazine disrupts lipopolysaccharide-activated NLRP3 inflammasome pathway associated with inhibition of Toll-like receptor 4 in hepatocytes

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ABSTRACT

Intestine microbial products may translocate into the liver via portal vein and trigger or exacerbate hepatocyte inflammatory responses during liver injury. The NLRP3 inflammasome pathway plays a key role in regulation of inflammatory cytokines in response to bacterial products. The present study was aimed to investigate the effects of ligustrazine, a natural alkaloid compound, on the NLRP3 inflammasome pathway activation and interleukin-1β (IL-1β) generation in hepatocytes. We cultured human LO2 hepatocytes and treated them with lipopolysaccharide (LPS), a membrane component of Gram-negative bacteria, for mimicking hepatic exposure to microbial products *in vitro*. The results demonstrated that LPS upregulated NLRP3 and cleaved-caspase-1, and promoted the expression and secretion of IL-1β in LO2 cells. Ligustrazine was found to reduce NLRP3 and cleaved-caspase-1, prevented IL-1β cleavage, and decreased IL-1β secretion into extracellular environment. Further examinations showed that LPS upregulated the expression of Toll-like receptor 4 (TLR4), but ligustrazine repressed TLR4 expression in LPS-treated hepatocytes. Moreover, pharmacological inhibition of TLR4 by its specific inhibitor TAK-242 downregulated NLRP3 and cleaved-caspase-1, and combination treatment with TAK-242 and ligustrazine led to more significant inhibitory effects on the NLRP3 pathway. TAK-242 also reduced cleaved-IL-1β, and this reducing effect was enhanced by ligustrazine. Collectively, the current results revealed that ligustrazine interrupted LPS-activated NLRP3 inflammasome signaling and reduced generation of IL-1β in hepatocytes, which was associated with inhibition of TLR4. This study uncovered a novel mechanism for ligustrazine as a potential hepatoprotective agent.



## 1. Introduction

Liver injury can be caused by a variety of factors. The close interplay between the liver and gut seems to play an important role in induction and furthering the progression of liver injury, because more than 70% of the hepatic blood supply derives from the portal vein, the direct venous outflow of the intestine [1]. A key role in the maintenance of gut-liver axis health has been attributed to intestinal bacteria. Although the amount of bacterial products reaching the liver is minuscule under normal circumstances, increased bacterial translocation occurs in chronic liver injury and may result in hepatic inflammation [2]. Microbial products such as lipopolysaccharide (LPS) are potent inducers of inflammatory responses. Actions of LPS are mediated through Toll-like receptor 4 (TLR4), one of pattern recognition receptors that recognize microbial components and trigger innate immune defenses [3]. In the liver, TLR4 is expressed in many different cell types including hepatocytes, Kupffer cells, and hepatic stellate cells. It is increasingly recognized that the liver is exposed to LPS from the intestinal microbiota, even in early stages of liver disease, suggest that LPS/TLR4 signaling acts as an important link between hepatic inflammation and injury [4].

*Abbreviations:* DMEM, Dulbecco's modified eagle medium; DMSO, dimethyl-sulfoxide; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; IL-1β, interleukin-1β; LPS, lipopolysaccharide; NASH, non-alcoholic steatohepatitis; NLR, NOD-like receptor; NLRP3, NACHT, LRR, and PYD domains-containing protein 3; NOD, nucleotide binding oligomerization domain; PBS, phosphate buffered saline; TLR4, Toll-like receptor 4.

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Inflammasome refers to large multiprotein complexes composed by nucleotide binding oligomerization domain (NOD)-like receptor (NLR) family [5]. Inflammasome can be activated by multiple types of tissue damage or pathogen-associated signatures and result in the autocatalytic cleavage of caspase-1, ultimately leading to the processing and thus secretion of pro-inflammatory cytokines, most importantly interleukin-1β (IL-1β) and IL-18 [5]. To date, there are four known NLRs that participate in the formation of inflammasome complexes: NLRP1, NLRP3, NLRC4 and AIM2 [5]. NLRP3 (NACHT, LRR, and PYD domains-containing protein 3) is the most intensively characterized member of the inflammasome family, and its activation requires two steps. Cell priming with an NF-κB activator, such as LPS, is the first step leading to upregulation of NLRP3 expression, while the second signal includes a variety of activators [6]. Increasing evidence has indicated that NLRP3 inflammasome activation is component of the liver pathophysiology [7]. For example, increased mRNA expression of NLRP3 inflammasome components was found in human livers of non-alcoholic steatohepatitis patients [8]. In a mouse model of alcoholic fatty liver, NLRP3 and pro-caspase-1 were upregulated in the liver [9]. Furthermore, acetaminophen-caused liver damage was attenuated in mice lacking components of NLRP3 inflammasome, suggesting a role for NLRP3 inflammasome in drug-induced liver injury [10]. However, the NLRP3 inflammasome in hepatocytes in the gut microbiota-related liver injury has been rarely investigated. We previously demonstrated that the natural compound ligustrazine has potent activity against hepatic fibrosis by inhibiting stellate cell activation [11–13]. It was also reported that ligustrazine had hepatoprotective effect on acute econazole-induced liver injury [14]. The present study was aimed to examine the effects of ligustrazine on the NLRP3 inflammasome pathway in hepatocytes upon LPS challenge.

## 2. Materials and methods

### 2.1. Reagents and antibodies

LPS from *Escherichia coli* 026:B6 was provided by Sigma (St Louis, MO, USA). It was dissolved in phosphate buffered saline (PBS) for experiments. Ligustrazine was purchased from Sigma (St Louis, MO, USA). TAK-242 was obtained from Shanghai Chembest Research Laboratories Limited (Shanghai, China). These two compounds were dissolved in dimethylsulfoxide (DMSO) for experiments. The following primary antibodies were used in this study: NLRP3 (Novus Biologicals, Littleton, CO, USA); cleaved-caspase-1 (Millipore, Burlington, MA, USA); cleaved-IL-1β and β-actin (Cell Signaling Technology, Danvers, MA, USA); and TLR4 (Santa Cruz Technology, Santa Cruz, CA, USA).

### 2.2. Cell culture

Human hepatocytes LO2 cells were purchased from the Cell Bank of Chinese Academy of Sciences (Shanghai, China) and was cultured in Dulbecco's modified eagle medium (DMEM; Invitrogen, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Wisent Biotechnology Co., Ltd., Nanjing, China), 100 U/ml penicillin and 100 mg/ml streptomycin, and grown in a 95% air and 5% $CO_2$ humidified atmosphere at 37 °C.

### 2.3. Western blot analyses

Whole cell protein extracts were prepared from treated cells. The protein levels were determined using a BCA assay kit (Pierce, USA). Proteins (50 μg/well) were separated by SDS-polyacrylamide gel, transferred to a PVDF membrane (Millipore, Burlington, MA, USA), blocked with 5% skim milk in Tris-buffered saline containing 0.1% Tween 20. Target proteins were detected by corresponding primary antibodies, and subsequently by horseradish peroxidase-conjugated secondary antibodies. Protein bands were visualized using chemiluminescence reagent (Millipore, Burlington, MA, USA) by Bio-Rad Universal Hood II DOC Electrophoresis Imaging Cabinet. Equivalent loading was confirmed using an antibody against β-actin. The levels of target protein bands were densitometrically determined using Image Lab Software 3.0. The variation in the density of bands was expressed as fold changes compared to the control in the blot after normalization to β-actin. Presented blots are representative of three independent experiments.

### 2.4. Enzyme-linked immunosorbent assay (ELISA)

The levels of IL-1β in LO2 cell culture supernatant were determined with an ELISA kit (Nanjing Jiancheng Bioengineering Institute) according to the protocol. Briefly, cells were seeded in 6-well plates and cultured in DMEM with 10% FBS for 24 h. Cells were treated with vehicle, LPS and/or ligustrazine at indicated concentrations for 24 h. Subsequently, samples of 100 μl were added to each well of the 96-well plates coated with antibody, followed by incubation at room temperature for 2 h. Working detector solution

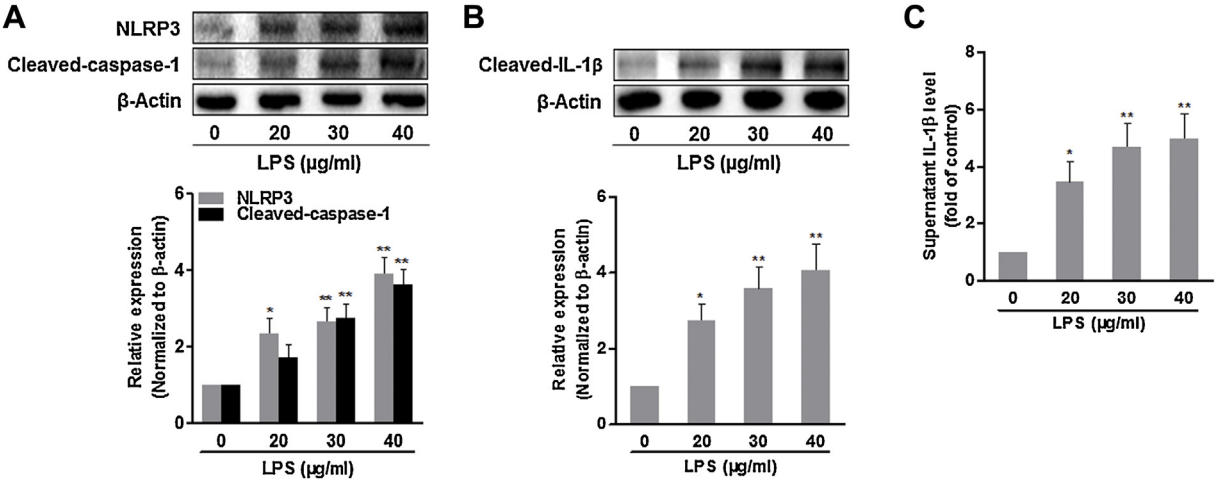


**Fig. 1.** LPS activates the NLRP3 inflammasome pathway in hepatocytes. LO2 cells were treated with vehicle PBS and LPS at indicated concentrations for 24 h. Western blot analyses of NLPR3 and cleaved-caspase-1 (A), and cleaved-IL-1β (B) with densitometry. Significance: $^{*}P < 0.05$ versus vehicle, $^{**}P < 0.01$ versus vehicle. (C) IL-1β levels in culture supernatant were examined by ELISA. Significance: $^{*}P < 0.05$ versus vehicle, $^{**}P < 0.01$ versus vehicle.