

Original Research Article

Antioxidant and anti-inflammatory effect of ligustilide on sepsis-induced acute kidney injury via TLR4/NF- κ B and Nrf2/HO-1 signaling

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Abstract

Purpose: To investigate the protective effect of ligustilide on sepsis-induced acute kidney injury (AKI) and the signaling pathways involved.

Methods: Sepsis-induced AKI was established by cecal ligation and puncture (CLP) in mice. Histopathological renal damage was examined using hematoxylin and eosin (H & E) staining while creatinine and cytokines were measured using commercial kits. Protein levels were determined by Western blotting.

Results: Vacuoles, dilations, degeneration, and necrosis were observed in CLP mouse kidneys, but these alterations were countered by 20 mg/kg of ligustilide. Serum creatinine, blood urea nitrogen (BUN), tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , and IL-6 were significantly increased in CLP mice compared with control. Furthermore, the serum levels of these indicators in serum were lowered by ligustilide ($p < 0.01$). The expression levels of Toll-like receptor 4 (TLR4) and phosphorylated nuclear factor (NF)- κ B in CLP mice were also downregulated by ligustilide. Malondialdehyde (MDA) and myeloperoxidase (MPO) levels increased in CLP mice, but were attenuated by ligustilide ($p < 0.01$). Superoxide dismutase (SOD) and glutathione (GSH) levels decreased in CLP mice but were increased by ligustilide ($p < 0.01$). Increased expression of Nrf2 and heme oxygenase-1 (HO-1) were observed in CLP mice, and were further enhanced by ligustilide.

Conclusion: Ligustilide exerts antioxidant and anti-inflammatory effects on sepsis-induced AKI via TLR4/NF- κ B and Nrf2/HO-1 signaling pathways.

Keywords: Ligustilide, Sepsis, Acute kidney injury, TLR4/NF- κ B signaling pathway, Nrf2/HO-1 signaling pathway

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INTRODUCTION

Sepsis is a systemic disorder characterized by acute organ dysfunction and high mortality [1]. Over the past decade, various treatments have

been applied to improve the outcomes of septic patients, and mortality has decreased from 37% to 30% [2]. An estimated 40 – 50 % cases of acute kidney injury (AKI) are caused by sepsis, which increases the risk of death by 6- to 8-fold,

as well as the risk of chronic kidney disease [3]. The pathology of sepsis-induced AKI is affected by complex factors including inflammation, oxidative stress, coagulation disturbances, and endothelial dysfunction, which contributes to histological renal damage such as tubular vacuolization, dilation, and necrosis [4].

Sepsis-induced AKI is highly associated with inflammatory factors (tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , and IL-6) [5]. During sepsis, renal epithelial and dendritic cells are activated by pathogens through pattern-recognition receptors including nucleotide oligomerization domain-like receptors, C-type lectin receptors, and Toll-like receptors (TLRs) that initiate the release of inflammatory mediators [6]. Oxidative stress is also a crucial contributor to sepsis-induced AKI [4]. Reactive oxygen species (ROS) production is increased by bacteria and promotes apical epithelial tubular cell vacuolization, which results in the apoptosis or necrosis of tubular cells, indicating that oxidative stress is closely related to the dysfunction of tubular [4]. Thus, suppressing inflammation and oxidative stress may prevent sepsis-induced AKI.

Ligustilide (3-butenyl-4,5-dihydro-1(3H)-isobenzofuranone) is a bioactive phthalide derivative isolated from *Angelica* and *Chuanxiong* [7]. The pharmacological effects of ligustilide are wide-ranging and include neuroprotection, anti-inflammation, and anti-cancer [8]. In human umbilical vein endothelial cells, Ligustilide can prevent endothelial inflammation via activation of the Nrf2/heme oxygenase-1 (HO-1) pathway [8]. In RAW 264.7 macrophages, it was reported that ligustilide suppressed lipopolysaccharide-mediated inducible nitric oxide synthase expression through inactivation of nuclear factor (NF)- κ B signaling pathway and ROS production [9]. However, there are no reports considering the effects of ligustilide on sepsis-induced AKI. This study aimed to investigate the protective effects of ligustilide on sepsis-induced AKI and identify the signaling pathways involved, which may provide new insights into the clinical treatment of sepsis-induced AKI.

EXPERIMENTAL

Animal model

Male C57BL/6 mice (6-8 weeks, 18-20 g) were purchased from Guangdong Medical Laboratory Animal Center (Guangdong Province, China) and kept in a controlled environment (23 ± 2 °C/ $55 \pm 15\%$ humidity) under a 12-h

light/12-h dark cycle. All experiment were approved by our hospital (approval no. 2019-007-025) and followed the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (no. 85-23, 1996) [10]. Sepsis-induced AKI was induced through cecal ligation and puncture (CLP) in the previous study [11]. In brief, a midline incision (1-cm) was made on the anterior abdomen in mice, and the cecum was ligated 12 mm from its tip. The cecum was punctured by a needle (22-gauge), and the abdomen was closed. For sham surgery, the operation was performed without ligation and puncture.

Mice were grouped (n = 8 in each group): Sham group, Sham mice were treated with normal saline (tail vein injection); Sham + Lig group, Sham mice were treated with 20 mg/kg of ligustilide (Sigma-Aldrich, USA; dissolved in normal saline; 1 mg/ μ L; tail vein injection); CLP group, CLP mice were treated with normal saline; CLP + Lig group, CLP mice were immediately treated with 20 mg/kg of ligustilide. Mice were sacrificed by 100 mg/kg of pentobarbital sodium 12 h after operation. Blood was collected from the heart into 1.5-mL tubes and centrifuged (3,500 rpm; 5 minutes), and the supernatant (serum) was collected. Renal tissues and serum were stored at -80 °C.

Histopathological examination

Kidney tissues were fixed, embedded, cut into sections (5- μ m thickness), and stained with hematoxylin and eosin (H&E) and safranin O (Sigma-Aldrich, USA). The sections were finally photographed under a microscope (Nikon, Japan) at 200 \times magnification.

Measurement of serum creatinine, BUN, MDA, SOD, GSH, MPO, and cytokines

After CLP or sham operation, creatinine and blood urea nitrogen (BUN) levels in serum and malondialdehyde (MDA), superoxide dismutase (SOD), glutathione (GSH), and myeloperoxidase (MPO) levels in renal tissues were measured by commercial kits (Abcam, UK). Cytokines, (TNF- α , IL-1 β , and IL-6) in serum and renal tissues were quantified using commercial enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, USA).

Western blotting

Cell lysates were extracted in radioimmunoprecipitation lysis buffer (Beyotime, China). 5 μ g of total proteins were loaded in sodium dodecyl sulfate-polyacrylamide gels,

separated, and transferred to polyvinylidene fluoride membranes that were blocked with 5% milk. The membranes were then incubated with primary antibodies at 4 °C overnight and then incubated with corresponding secondary antibodies for 2 h at room temperature. Protein band signals were detected using enhanced chemiluminescent detection reagents (Sigma-Aldrich, USA). The primary antibodies used were TLR4 (ab13867, 1:1000 dilution), p-NF-κB (Cell Signaling Technology [CST]#3031, 1:1000 dilution), NF-κB (CST#3031, 1:2000 dilution), Nrf2 (ab137550, 1:1500 dilution), and GAPDH (ab9485, 1:5000 dilution).

Statistical analysis

All results were presented as mean ± SEM and analyzed using SPSS 19.0 (IBM, USA). Student's *t*-tests and one-way analyses of variance were used to analyze differences between two groups or among multiple groups. *P* < 0.05 was considered statistically significant.

RESULTS

Ligustilide decreased CLP-induced AKI in mice

Serum creatinine was higher in CLP mice than that in Sham group; however, this induction was attenuated by 20 mg/kg of ligustilide. (Figure 1 A). BUN level in blood serum was also higher in CLP mice than that in Sham mice, which was decreased by 20 mg/kg of ligustilide (Figure 1 B). Numerous vacuoles, dilations, widespread degeneration, and necrosis were observed in renal tissues from CLP mice, which was attenuated by 20 mg/kg of ligustilide (Figure 1 C). Ligustilide had no obvious effect on serum creatinine and BUN levels or kidney tissue morphology in normal mice (Figure 1).

Ligustilide prevented CLP-induced inflammation in mice

TNF-α, IL-1β, and IL-6 levels in blood serum were higher in CLP mice than that in Sham mice, but these levels were reduced by 20 mg/kg of ligustilide (Figure 2 A). TNF-α, IL-1β, and IL-6 levels in renal tissues were also higher in CLP mice than that in Sham mice, and reduced by 20 mg/kg of ligustilide (Figure 2 B).

Ligustilide reduced CLP-induced oxidative stress in mice

MDA and MPO levels in renal tissues were higher in CLP mice than that in Sham mice, which were reduced by 20 mg/kg of ligustilide

(Figure 3 A and D). SOD and GSH levels in renal tissues were lower in CLP mice than that in Sham mice, which were increased by 20 mg/kg of ligustilide (Figure 3 B and C).

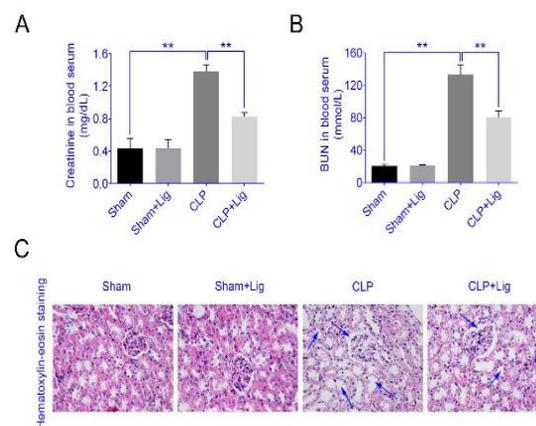


Figure 1: Ligustilide decreased CLP-induced AKI in mice. Ligustilide inhibited the enhancements in serum creatinine (A) and serum BUN level (B) in CLP mice; (C) Ligustilide attenuated renal damage in CLP mice; ***p* < 0.01 vs. Sham or CLP. Sham, sham surgery; Lig: 20 mg/kg ligustilide; CLP: cecal ligation and puncture

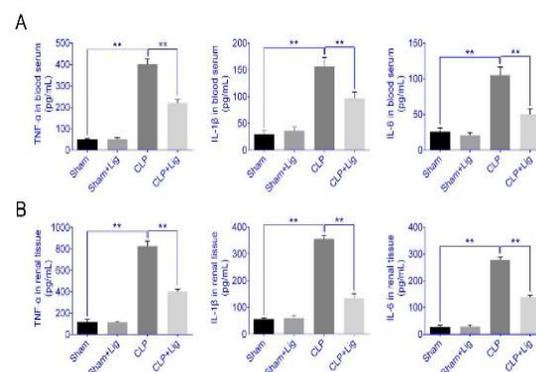


Figure 2: Ligustilide prevented CLP-induced inflammation in mice. (A) Ligustilide inhibited the enhancement in (A) serum and (B) renal tissue levels of TNF-α, IL-1β, and IL-6 levels in CLP mice; ***p* < 0.01 vs. Sham or CLP

Ligustilide inactivated TLR4/NF-κB signaling and activated Nrf2/HO-1 signaling

Relative protein expression was determined using western blotting. The results showed that the levels of TLR4 and phosphorylation of NF-κB were up-regulated in CLP mice, but these changes were attenuated in CLP mice treated with 20 mg/kg ligustilide (Figure 4). Increased Nrf2 and HO-1 expression were observed in CLP mice, and ligustilide treatment further increased the levels of these proteins (Figure 4).

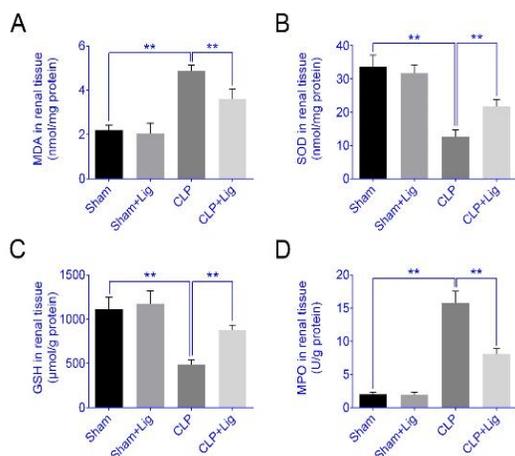


Figure 3: Ligustilide reduced CLP-induced oxidative stress in renal tissue of CLP mice. MDA levels (A), SOD levels (B), (C) GSH levels, and (D) MPO in renal tissues of mice were detected by ELISA kits; ** $p < 0.01$ vs. Sham or CLP

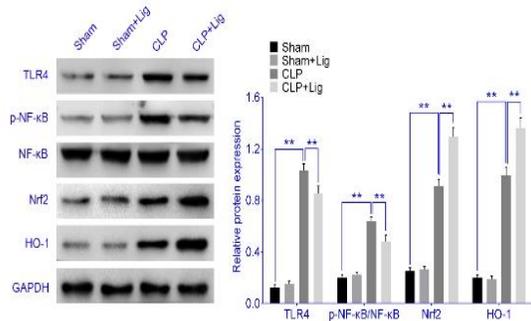


Figure 4: Ligustilide inhibited TLR4/NF-κB signaling and activated Nrf2/HO-1 signaling in CLP mice; ** $p < 0.01$ vs. Sham or CLP

DISCUSSION

The pharmacological effects of ligustilide are wide-ranging and include neuroprotection, anti-inflammation, and anti-cancer [8]. This is the first study to explore the efficacy of ligustilide in treating sepsis-induced AKI. During early sepsis, stimulation of macrophages leads to the release of TNF- α , IL-1 β , and IL-6, thereby causing a systematic inflammatory response [5]. In this study, ligustilide caused the reduced production of these pro-inflammatory cytokines, suggesting that it has an inhibitory effect on sepsis. Moreover, ligustilide reduced the serum levels of creatinine and BUN and inhibited renal tubule vacuoles, dilations, degeneration, and necrosis, indicating that ligustilide protected against sepsis-induced AKI.

During sepsis, bacterial endotoxin can activate TLR4 and induce the activation of NF-κB

signaling, which phosphorylate NF-κB p65 subunit and translocate from the cytoplasm into nucleus. Afterwards, p65 regulates target gene expression to produce and release TNF- α , IL-1 β , and IL-6 [12]. Here, ligustilide downregulated the expression levels of TLR4 and phosphorylated NF-κB, implying that the anti-inflammatory roles of ligustilide were mediated via inactivation of TLR4/NF-κB signaling in sepsis-induced AKI. MPO is a member of the heme peroxidase family linked to activation of inflammation and increased oxidative stress [13]. The reduced SOD activity and increased MDA level indicated increased oxidative stress [14]. GSH is an endogenous antioxidant that helps protect other antioxidants from inactivation [15].

Our results showed that ligustilide downregulated expression levels of TLR4 and phosphorylated NF-κB, which were associated with decreased levels of MDA and MPO and increased levels of SOD and GSH. These findings indicated that the anti-oxidant effects of ligustilide were mediated by inactivation of TLR4/NF-κB signaling in sepsis-induced AKI.

Nrf2 is well-known as a transcription factor sensitive to redox, belonging to the CNC essential leucine zipper protein family, and it regulates resistance to oxidative stress [16]. In normal physiological settings, Nrf2 is located in cytoplasm in a complex with Keap1 [17]. With increased oxidative stress, phosphorylated Nrf2 can dissociate from the Nrf2/Keap1 complex and translocate to the nucleus to exert regulatory effects on the expression of downstream genes including HO-1, GSH peroxidase, and other antioxidant enzymes [17]. We demonstrated that ligustilide enhanced Nrf2/HO-1 signaling in CLP mice, suggesting that the anti-oxidant functions of ligustilide were at least partially, mediated by further activation of Nrf2/HO-1 signaling. The Nrf2/HO-1 signaling pathway has been verified has anti-inflammatory effects on bacterial endotoxin-induced inflammation [18]. These findings suggest that ligustilide exert anti-inflammatory functions by activation of the Nrf2/HO-1 signaling pathway, which was consistent with previous conclusions [8].

CONCLUSION

The findings of this study demonstrate that ligustilide exerts antioxidant and anti-inflammatory effects on sepsis-induced AKI by inhibiting TLR4/NF-κB signaling and activating Nrf2/HO-1 pathway. Thus, ligustilide is a potential treatment of sepsis-induced AKI, but its clinical use needs further investigations.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Xiumin Yang and Chencai Qiao designed the study and supervised the data collection, Chao Zheng analyzed and interpreted the data. Qingjun Deng prepared the manuscript for publication and reviewed the drafts. All authors have read and approved the final manuscript.

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