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Original Article

# Overexpression of CISD2 alleviates septic acute kidney injury via activating Sonic Hedgehog signaling pathway

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**Abstract**

Patients with sepsis are often complicated by acute kidney injury (AKI), which greatly increases mortality. In this study, our purpose was to explore the expression and function of CDGSH iron sulfur domain 2 (CISD2) in septic AKI and the underlying molecular mechanism. Western blot and quantitative real-time polymerase chain reaction (RT-PCR) were employed to detect protein and mRNA levels in cells. The inflammation level of cells was evaluated by detecting the content of inflammatory factors (TNF-α, IL-1β, IL-6). Apoptosis of cells was evaluated by Caspase-3 activity assay, flow cytometry and terminal deoxynucleotidyl transferase-mediated dUTP Nick-End Labeling (TUNEL) staining. CISD2 was down-regulated in HK-2 cells treated with lipopolysaccharide (LPS). LPS treatment increased the level of inflammatory factors, the activity of Caspase-3, and the rate of apoptosis in HK-2 cells. However, overexpression of CISD2 significantly suppressed these effects. Moreover, overexpression of CISD2 activated the Sonic Hedgehog (SHH) signaling pathway. The use of cyclopamine (Cyc), an SHH signaling pathway inhibitor, eliminated the effect of overexpressing CISD2, that is, inhibiting LPS-induced inflammation and apoptosis of HK-2 cells. LPS treatment down-regulated CISD2 in HK-2 cells, and overexpression of CISD2 could inhibit LPS-induced inflammation and apoptosis of HK-2 cells by activating the SHH signaling pathway.

**Keywords:** Sepsis, Acute kidney injury, CISD2, Lipopolysaccharide, Inflammation, apoptosis, Sonic Hedgehog signaling pathway

## 1. Introduction

Sepsis is a common fatal disease that can cause the host to be unable to reactively control the active infection of microorganisms and their products that invade the body, causing systemic inflammatory response syndrome, which further develops into septic shock and multiple organ dysfunction syndrome (MODS) [1]. Sepsis is the main cause of death in critically ill patients in the intensive care unit (ICU). About 20% to 25% of severe sepsis patients will die, and the mortality rate is increasing [2]. Sepsis usually causes acute kidney injury (AKI) and is the main cause of AKI. More than 30% of AKI patients are caused by sepsis [3]. AKI is very common in critically ill patients, and the case fatality rate is twice that of similar patients without AKI. 50% of patients with severe AKI cannot recover and eventually progress to chronic kidney disease [4]. Therefore, how to prevent the occurrence and development of AKI, one of the most common complications of sepsis, has become one of the most serious medical problems in contemporary medicine.

In recent years, great progress has been made in the study of the cellular and molecular etiological mechanisms of AKI, and there is a new understanding of the pathogenesis of AKI. It was originally believed that AKI was associated with shock caused by surgery, heart failure, sepsis, and hypovolemia. Therefore, it was believed that AKI was caused by decreased renal blood flow. However, the clinical treatment of AKI has little effect, indicating that ischemia may not be the only factor leading to AKI. More and more studies have shown that inflammation, immune response, disturbance of renal tubule and glomerular microvascular blood flow may be involved [5,6]. These studies have focused on the damage of renal tubular epithelial cells.

CDGSH iron sulfur domain 2 (CISD2) is an evolutionarily highly conserved gene. CISD2 contains a transmembrane domain, a CDGSH domain and a conserved amino acid sequence bound by an iron ion [7]. The CISD2 gene is located on human chromosome 4 and the encoded protein is mainly located in the outer mitochondrial membrane, which plays a role in regulating the integrity of mitochondria in mammals [8]. CISD2 has been reported to be widely involved in the development and progression of tumors, including proliferation, apoptosis, and autophagy

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[9-11]. Moreover, CISD2 has been shown to be involved in the regulation of cellular aging [12-14]. In addition, Lin et al. [15] demonstrated that curcumin could inhibit LPS-induced inflammation and apoptosis of neuronal cells in a CISD2-dependent manner. However, the role of CISD2 in septic AKI is not known.

In this article, we tried to describe the role of CISD2 in septic AKI. We used LPS to construct an *in vitro* model of septic AKI, and found that CISD2 expression decreased in LPS-treated HK-2 cells. And overexpression of CISD2 could inhibit LPS-induced inflammation and apoptosis of HK-2 cells by activating the SHH signaling pathway. Our findings provide a potential therapeutic target for septic AKI.

## 2. Materials and methods

### 2.1. Cell culture and transfection

The HK-2 cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). HK-2 cells were cultured in complete medium containing dulbecco's modified eagle medium / F-12 (DMEM / F-12) (Gibco, Rockville, MD, USA), 10% fetal bovine serum (FBS, Gibco, Rockville, MD, USA) and 1% penicillin/streptomycin (Gibco, Rockville, MD, USA) and incubated in a cell incubator containing 5% $CO_2$ at 37℃. HK-2 cells were treated with 500 ng/ml LPS to establish an in vitro model of septic AKI.

The CISD2 overexpression plasmid was constructed by Shanghai Genechem Co., LTD (Shanghai, China) and transfected into HK-2 cells with Lipofectamine™ 3000 (Invitrogen, Carlsbad, CA, USA) according to the instructions. Cells were divided into 4 groups: control group, LPS treatment group, LPS+vector group, and LPS+CISD2 group. In order to study the role of the SHH pathway, the SHH pathway inhibitor cyclopamine (Cyc, MedChemExpress, Shanghai, China) was used to inhibit the activity of the SHH pathway. Cells were divided into 2 groups: LPS+CISD2 group, LPS+CISD2+Cyc group.

### 2.2. Western blot

Total protein of HK-2 cells was extracted using radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China) and protein concentration was measured using the bicinchoninic acid (BCA) method (Beyotime, Shanghai, China). 5 ×sodium dodecyl sulphate (SDS) protein loading buffer (Beyotime, Shanghai, China) was added to the protein sample before the protein sample was boiled. The protein was separated in sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel at a voltage of 120 V, and then transferred to polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The membrane was then incubated in 5% skim milk to block non-specific antigens. After that, the membrane was incubated with primary antibodies (CISD2, Abcam, Cambridge, MA, USA, Rabbit, 1:1000; SHH, Abcam, Cambridge, MA, USA, Rabbit, 1:1000; Gli1, Abcam, Cambridge, MA, USA, Rabbit, 1:1000; GAPDH, Abcam, Cambridge, MA, USA, Rabbit, 1:1000). Then the membrane was incubated with secondary antibody (Abcam, Cambridge, MA, USA, Rabbit, 1:5000). Blots were developed with Clarity Western ECL Substrate (Bio-Rad, Shanghai, China).

### 2.3. Quantitative real-time polymerase chain reaction (RT-PCR) analysis

HK-2 cells were seeded in a 24-well plate and 0.5 mL of TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was employed to lyse the cells. Then 0.1 ml of chloroform was added. The mixture was shaken vigorously for 30 seconds and then allowed to stand for 5 minutes, followed by centrifugation at 4℃ with a centrifugal force of 12,000 g for 15 minutes. The upper aqueous phase was collected and transferred into a new Eppendorf (EP) tube, and isopropyl alcohol was added and mixed fully. After the mixture was allowed to stand for 10 minutes, it was centrifuged at 12,000 g for 10 minutes at 4℃. Subsequently, the supernatant was discarded, 75% ethanol was added and then the RNA was precipitated by centrifugation at 7500 g at 4 ℃ for 10 minutes. Finally, 20 μL of diethyl pyrocarbonate (DEPC)-treated Water (Beyotime, Shanghai, China) was used to dissolve the RNA. The purity and concentration of the total RNA was detected using NanoDrop 2000C Ultramicrospectrophotometer. HiScript® III RT SuperMix for qPCR (Vazyme, Nanjing, China) was utilized to perform reverse transcription of mRNA. AceQ Universal SYBR qPCR Master Mix (Vazyme, Nanjing, China) was used to perform RT-PCR. GAPDH was the internal control. All the primers were listed in Table 1.

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

The HK-2 cell supernatant was collected. The contents of inflammatory cytokines (TNF-α, IL-1β, IL-6) in the supernatant of HK-2 cells were detected using corresponding ELISA detection kits (Beyotime, Shanghai, China).

### 2.5. Caspase-3 activity

Caspase-3 activity of HK-2 cells was detected using Caspase-3 activity detection kit (Beyotime, Shanghai, China).

### 2.6. Flow Cytometry

HK-2 cells were collected using Trypsin Solution without EDTA (ethylenediaminetetraacetic acid) (Beyotime, Shanghai, China). Then the cells were washed using phosphate buffered saline (PBS). After that, the cells were resuspended in 100 μL of Binding Buffer, and then 5 μL of Annexin V-FITc (KeyGen, Shanghai, China) and PI

**Table 1.** Real time PCR primers.

| Gene name | Forward (5'>3') | Reverse (5'>3') |
|---|---|---|
| CISD2 | GCAAGGTAGCCAAGAAGTGC | CCCAGTCCCT GAAAGCATTA |
| SHH | GATGAGGAAAACACGGGAGC | CTGCTCGACCCTCATAGTGT |
| Gli1 | TCTGTGATGGGCAATGGTCT | TCTGGGGTGGGATCAGGATA |
| GAPDH | ACAACTTTGGTATCGTGGAAGG | GCCATCACGCCACAGTTTC |

RT-PCR, quantitative real time polymerase chain reaction.

(KeyGen, Shanghai, China) were both added. Then another 400 μl of Binding Buffer was added. The apoptosis rate was analyzed by flow cytometry.

### 2.7. Terminal deoxynucleotidyl transferase-mediated dUTP Nick-End Labeling (TUNEL) staining

HK-2 cells were first fixed using 4% paraformaldehyde and then 0.1% Triton X-100 was used to increase cell permeability. After that, the cells were incubated with One Step TUNEL Apoptosis Assay Kit (Beyotime, Shanghai, China) at 37 ℃ for 1 hour. Then 4',6-diamidino-2-phenylindole (DAPI) (Beyotime, Shanghai, China) was used to stain the nucleus. The TUNEL-positive cells were observed using a fluorescence microscope.

### 2.8. Statistical analysis

Measurement data were expressed as $\chi \pm s$, and the measurement data were tested for normality. Differences between two groups were analyzed by using the Student's t-test. Comparison between multiple groups was done using One-way ANOVA test followed by Post Hoc Test (Least Significant Difference). $P<0.05$ indicated a significant difference.

## 3. Results

### 3.1. CISD2 was down-regulated in LPS-treated HK-2 cells

We used LPS to treat HK-2 cells, thus establishing a cell model of septic AKI. Then, through Western blot and RT-PCR, we detected the protein and mRNA levels of CISD2 and found that compared with the control group, the expression of CISD2 in the LPS-treated group decreased significantly (Figure 1A and 1B).

### 3.2. Up-regulation of CISD2 inhibited LPS-induced inflammation and apoptosis of HK-2 cells

When the CISD2 overexpression plasmid was transfected into HK-2 cells, the expression of CISD2 increased dramatically (Figure 2A). Compared with the control group, the levels of inflammatory cytokines (TNF-α, IL-1β, IL-6) in the LPS group were notably increased, and overexpression of CISD2 suppressed the levels of these inflammatory cytokines (Figure 2B~2D). Moreover, LPS treatment increased the activity of Caspase-3 in HK-2 cells and also increased the apoptosis rate and the percentage of TUNEL-positive cells. However, increasing CISD2 clearly reversed these effects (Figure 2E~2G).

### 3.3. Up-regulation of CISD2 activated the SHH signaling pathway

By Western blot, we detected the expression of SHH and Gli1 in HK-2 cells. Compared with the control group, the expressions of SHH and Gli1 in the LPS group were markedly reduced, suggesting that LPS treatment inhibited the SHH pathway. After up-regulating CISD2, the SHH signaling pathway was activated, which was manifested by the increased expression of SHH and Gli1 (Figure 3A). We also tested the levels of SHH mRNA and Gli1 mRNA, and the results were consistent with the above (Figure 3B and 3C).

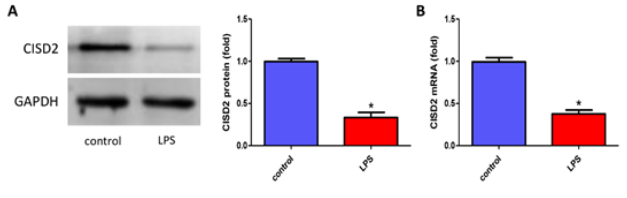


**Fig. 1.** CISD2 was down-regulated in LPS-treated HK-2 cells. (A) Western blot showed the expression of CISD2 ("*" $P<0.05$ vs. control, n=3). (B) CISD2 mRNA expression was detected by RT-PCR ("*" $P<0.05$ vs. control, n = 3).

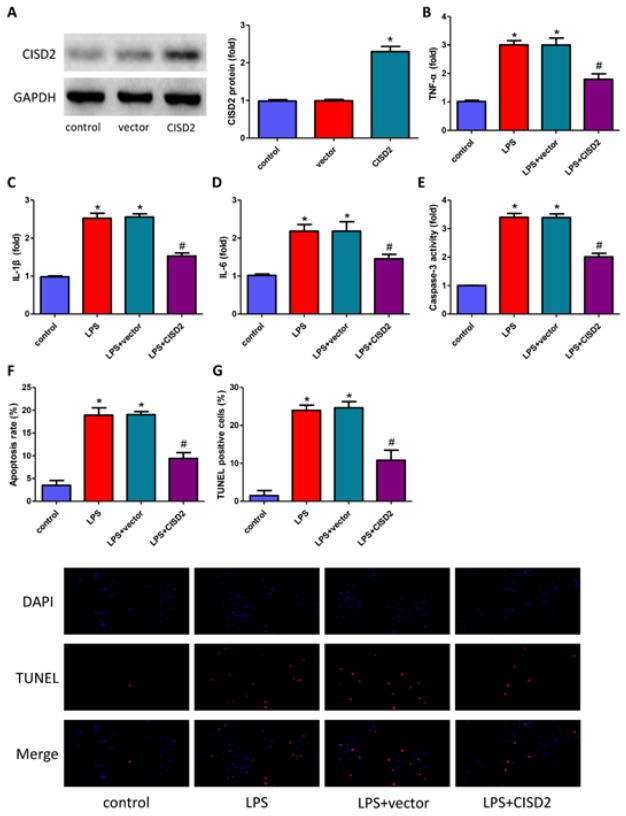


**Fig. 2.** Up-regulation of CISD2 inhibited LPS-induced inflammation and apoptosis of HK-2 cells. (A) CISD2 expression was detected by Western blot ("*" $P<0.05$ vs. vector, n = 3). The contents of TNF-α (B) and IL-1β (C) and IL-6 (D) in supernatant of HK-2 cells ("*" $P<0.05$ vs. control, "#" $P<0.05$ vs. LPS+vector, n=3). (E) The Caspase-3 activity in HK-2 cells ("*" $P<0.05$ vs. control, "#" $P<0.05$ vs. LPS+vector, n=3). (F) Apoptosis rate was detected by flow cytometry ("*" $P<0.05$ vs. control, "#" $P<0.05$ vs. LPS+vector, n=3). (G) Results of TUNEL staining in each group (magnification: 200×) ("*" $P<0.05$ vs. control, "#" $P<0.05$ vs. LPS+vector, n=3).

### 3.4. CISD2 inhibited LPS-induced inflammation and apoptosis of HK-2 cells via activating SHH signaling pathway

We used the SHH pathway inhibitor cyclopamine (Cyc) to inhibit the activation of the SHH pathway. The inhibition of SHH pathway obviously eliminated the effect of CISD2 overexpression to inhibit LPS-induced inflammation and apoptosis of HK-2 cells. Compared with the LPS+CISD2 group, not only the level of inflammatory cytokines increased remarkably, but also the activity of Caspase-3 and the rate of apoptosis increased markedly in the LPS+CISD2+Cyc group (Figure 4A~4F). These indicated that the inhibition of LPS-induced inflammation and apoptosis of HK-2 cells by CISD2 was dependent on the SHH pathway.

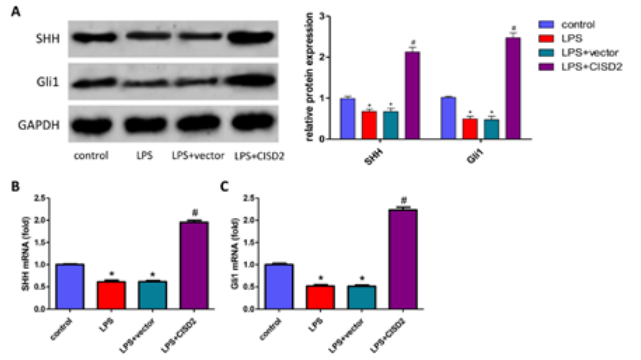


**Fig. 3.** Up-regulation of CISD2 activated the SHH signaling pathway. (A) The expression of SHH and Gli1 was detected by Western blot ("*" P<0.05 vs. control, "#" P<0.05 vs. LPS+vector, n=3). The expression of SHH mRNA (B) and Gli1 mRNA (C) was detected by RT-PCR ("*" P<0.05 vs. control, "#" P<0.05 vs. LPS+vector, n=3).

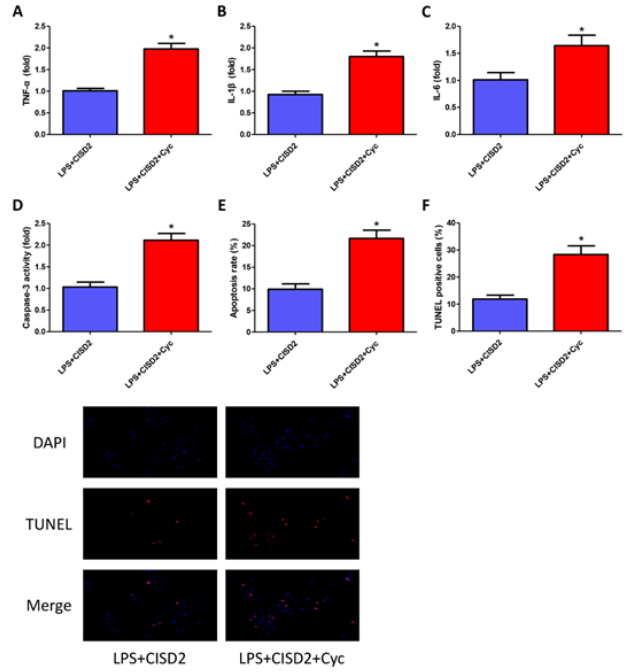


**Fig. 4.** CISD2 inhibited LPS-induced inflammation and apoptosis of HK-2 cells *via* activating SHH signaling pathway. The contents of TNF-α (A) and IL-1β (B) and IL-6 (C) in supernatant of HK-2 cells ("*" P<0.05 vs. LPS+CISD2, n=3). (D) The Caspase-3 activity in HK-2 cells ("*" P<0.05 vs. LPS+CISD2, n=3). (E) Apoptosis rate was detected by flow cytometry ("*" P<0.05 vs. LPS+CISD2, n=3). (F) Results of TUNEL staining in each group (magnification: 200×) ("*" P<0.05 vs. LPS+CISD2, n=3).

## 4. Discussion

Sepsis can easily lead to AKI and is closely related to the prognosis of critically ill patients [16]. AKI associated with sepsis is one of the most common organ dysfunctions in critically ill patients. There is currently no reliable drug for prevention and treatment and the prognosis is poor.

Inflammation and renal tubular epithelial cell apoptosis are both mechanisms of acute renal injury in sepsis. Under the action of endotoxin or endotoxin-like substances released by bacteria, the body's neutrophils, monocytes, and vascular endothelial cells undergo a complex immune network reaction, and release a large number of endogenous inflammatory mediators (including IL-1, IL-6, TNF-α, PAF, prostaglandin, etc.) into the blood circulation, causing damage to multiple organs including the kidney. The TLR4/NF-κB pathway has been shown to be involved in the process of renal inflammatory response, and inhibition of TLR4/NF-κB-mediated inflammatory response has a protective effect on LPS-induced AKI [17,18]. Apoptosis is through a series of gene activation, expression and regulation to maintain the stable programmed death of the cell environment. After receiving pathological stimulation, the disorder of apoptosis leads to excessive cell death and organ dysfunction. According to previous views, acute tubular necrosis (ATN) caused by renal ischemia and inflammatory factors plays a leading role in septic AKI. However, recent studies have shown that apoptosis is an important mechanism for the development of AKI caused by sepsis [19]. Previous studies have found that apoptosis related to Fas and Caspase signaling pathways mediates extensive death of renal tubular epithelial cells in septic AKI [20]. These mitochondrial-dependent apoptosis pathways start with the generation of oxidative stress and then promote the Bax and Bcl-2 protein complex to enter the mitochondria, resulting in increased mitochondrial permeability, release of cytochrome C, activation of Caspase-3, and initiation of apoptosis pathways [21,22].

SHH signaling pathway is one of the most conservative signaling pathways in biological evolution. It is involved in multiple processes of embryonic development in vertebrates and invertebrates and is crucial for the development of nervous system and the formation and construction of organs. In the classical SHH signaling pathway, the SHH protein binds to a Patched receptor by autocrine or paracrine, removing the inhibition of the Smoothed receptor and enabling Gli1, Gli2 and Gli3 to enter the nucleus and initiate the expression of a series of target genes that control the growth, survival and differentiation of cells [23]. Administration of exogenous recombinant SHH protein can significantly improve neurobehavioral score, reduce cerebral infarction area, and promote angiogenesis of peripheral tissues of ischemia and colonization of neural stem cells in rats with cerebral ischemia-reperfusion injury [24]. Activating the SHH pathway could also inhibit LPS-induced lung inflammation and reduce acute lung injury[25].

In this study, we found for the first time that the expression of CISD2 decreased in LPS-induced AKI. Moreover, LPS obviously induced inflammation and apoptosis of renal tubular epithelial cells, and inhibited the activity of SHH signaling pathway. Overexpression of CISD2 can greatly inhibit LPS-induced inflammation and apoptosis, and activate the SHH pathway. However, blocking the SHH pathway eliminated the protective role of CISD2 in septic AKI.

## 5. Conclusion

CISD2 was down-regulated in septic AKI, and up-regulation of CISD2 could inhibit LPS-induced inflammation and apoptosis of HK-2 cells by activating the SHH signaling pathway.

## Conflict of Interests

The author has no conflicts with any step of the article preparation.

## Consent for publications

The author read and approved the final manuscript for

publication.

**Ethics approval and consent to participate**
No human or animals were used in the present research.

**Informed Consent**
The authors declare not used any patients in this research.

**Availability of data and material**
The data that support the findings of this study are available from the corresponding author upon reasonable request.

**Authors' contributions**
Wenjun Wu and Jia Zhang designed the study and performed the experiments, Jia Zhang and Fei Chen collected the data, Hongzhuan Song and Xiujie Zhou analyzed the data, Wenjun Wu prepared the manuscript. All authors read and approved the final manuscript.

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