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Signa Vitae

ORIGINAL RESEARCH

Overexpression of *HIC1* plays a protective effect on renal cell injury caused by lipopolysaccharide by inhibiting IL-6/STAT3 pathway

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Abstract

Sepsis is a life-threatening condition that can even occur due to an infection. Systemic inflammatory response syndrome acts a pivotal role in acute kidney injury (AKI). Although great advancements have been achieved for treating sepsis-induced AKI, its prognosis and pathophysiology remain unclear. In order to gain insights into the relevant role of *hypermethylated in cancer 1* (*HIC1*) in AKI, a cellular model of AKI caused by sepsis was performed in lipopolysaccharide (LPS)-treated human kidney 2 (HK-2) cell line. The overexpression vector pcDNA3.1-*HIC1* was transfected into HK-2 cell line to examine the effects of *HIC1* on LPS-treated HK-2 cell line. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR), western blot and enzyme-linked immunosorbent assay (ELISA) assays were performed to examine the alterations in the expression levels of *HIC1*, cell apoptosis, or inflammation-related biomarkers. The apoptotic rate of HK-2 cell line was measured by flow cytometry. This study suggested that LPS treatment downregulated *HIC1* and inhibited HK-2 cell viability, whereas *HIC1* overexpressing reversed these effects. Importantly, *HIC1* has a protective effect on LPS-induced cellular apoptosis and inflammatory response. Moreover, overexpression of *HIC1* suppressed the LPS-induced activation of IL-6/STAT3 signaling pathway in HK-2 cell line. *HIC1* protects HK-2 cell line against LPS-induced damage, which was partly through the inhibition of IL-6/STAT3 signaling pathways.

Keywords

Sepsis; Renal cell injury; HIC1 cell line; IL-6/STAT3; LPS

1. Introduction

Sepsis is a disease caused by an infection, usually with a severe systemic inflammatory response, resulting in various organ dysfunction, and its mortality rate is high, especially in elderly [1, 2]. Acute kidney injury (AKI) is a serious and lethal complication of the sepsis process, represented by insufficient blood filtration, imbalance of water and ions, and damaged urine production [3, 4]. Numerous studies have shown that systemic inflammatory response syndrome act a pivotal role in AKI [5, 6]. The inflammatory factors such interleukin 6 (IL-6), inducible nitric oxide synthase (iNOS), interleukin 1 beta (IL-1β), and tumor necrosis factor α (TNF-α) were reported as crucial immune response factors which occur and damage the kidney [7]. Recent researches have demonstrated that the cellular apoptosis, inflammatory response, and the expression levels of oxidative stress in renal tubular cells may be associated with the pathological process of AKI [8, 9]. Although great advancements have been achieved for treating sepsis-induced AKI, its prognosis and pathophysiology remain unclear.

Hypermethylated in Cancer 1 (*HIC1*) is a tumor suppressor gene located at 17p13.3 [10], which could regulate target genes, including fibroblast growth factor binding protein 1, cyclin D1, cyclin dependent kinase inhibitor 1C and p21, which were involved in the occurrence and progression of various tumors [11–13]. A recent study has indicated that *HIC1* reduced cell invasion and metastasis by suppressing the IL-6/STAT3 signaling pathway in human pancreatic cancer [14]. Another study has also reported that *HIC1* regulated high glucose-induced reactive oxygen species (ROS) accumulation in renal tubular epithelial cells through epigenetic inhibition of silent information regulator 1 (SIRT1) transcription [15]. However, there are rare reports considering the role of *HIC1* in AKI caused by sepsis.

The signal transducers and activators of transcription (STAT) family proteins can regulate principal cellular progression, including cell proliferation, differentiation, inflammatory response, angiogenesis, and metastasis [16]. The STAT signal transduction pathway can be activated in response to many protein ligands, including cytokines, growth factors, and interferons [17, 18]. In particular, STAT3 acts as

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a transcription factor for oncogenes and mediates oncogenic transformation [19]. Excessive inflammatory response and cytokine storm cause serious damage to multiple organs. IL-6, TNF-α, IL-1β are important inflammatory factors that are released when the immune response occurs and cause damage to the kidney. IL-6 is a key cytokine for immune response and tumorigenesis [20], and IL-6 and its main factor STAT3 are also well-known as tumor-promoting factors in numerous cancers [21]. A previous study reported that adiponectin could attenuated the expression of inflammatory factors TNF-α and IL-6 through the IL-6/STAT3 signaling pathway, accordingly suppressing lung injury caused by sepsis [22]. Furthermore, STAT3 is one of the proteins associated with *HIC1*, which can inhibit the activity of related molecules and regulate the expression of target gene mediated by STAT3 and involved in the cellular biological functions [23]. Thus, this study investigated the role of *HIC1* in regulating IL-6/STAT3 signaling pathway, which thus participates in sepsis-induced renal cell injury.

2. Methods

2.1 Cell culture

Human renal proximal tubular epithelial cell line (HK-2) was purchased from American Type Culture Collection (Manassas, WV, USA) and maintained in DMEM/Ham's F12 growth medium (Gibco, Waltham, MA, USA) containing fetal bovine serum (10%, Gibco) and penicillin/streptomycin (1%). HK-2 cell line was maintained (37 °C, 5 % Carbon dioxide, CO_2) and pre-transfected with pcDNA3.1 or pcDNA3.1 *HIC1* at 24–48 hours before treatment of LPS (5 μg/mL).

2.2 qPCR analysis

RNA was obtained from HK-2 cell line using TRIzol method (Invitrogen, Carlsbad, CA, USA). The Prime-Script RT reagent kit (Tiangen, Beijing, China) was used to synthesize cDNA. QRT-PCR was performed on an ABI 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA) and detected by using SYBR Green PCR Master Mix (TaKaRa, Dalian, China).

The primers were as follow: *HIC1*: Forward (F): 5'-GAGTTGGGGAAGAGATGTGGAG-3'; reverse ®' 5'-CCAACCCCAAATACTCCTAAACA'-3', TNF-α: F' 5'-AGGACACCATGAGCACTGA'-3'; R' 5'-CCGATCACTCCAAAGTGCA'-3', IL-1β: F' 5'-CTCTCTCCTTTCAGGGCCA'-3'; R' 5'-GCGGTTGCTCATCAGAATG'-3', IL-6: F' 5'-ATGAACTCCTTCTCCACAAG'-3'; R' 5'-CTACATTTGCCGAAGAGCCCTCAGGCTGGACT'-3', GAPDH: F' 5'-TCTTTTGCGT CGCCAGCCGA'-3'; R' 5'-TGACCAGGCG CCCAATACGA'-3'.

2.3 Western blot

HK-2 cell line was collected using RIPA buffer and centrifuged (13,000 g, 4 °C, 15 minutes). Protein samples (20 μg) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gel (8%) and then transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). The primary antibodies against *HIC1* (1:2000, Santa Cruz Biotechnology, Inc. (SCB), Dallas, TX, USA), β-actin (1:10,000, SCB), BCL2 Associated X (Bax) (1:2000, Abcam, Cambridge, MA, USA), Cleaved Caspase 3 (1:2000, SCB), STAT3 (1:2000, Cell Signaling Technology (CST), Beverly, MA, USA), phosphorylation of Signal Transducer And Activator Of Transcription 3 (p-STAT3) (1:1000, CST), IL-6 (1:2000, Abcam), RAR-related orphan receptor gamma (RORγt) (1:2000, SCB), Suppressor Of Cytokine Signaling 3 (SOCS3) (1:2000, CST) and B-cell lymphoma 2 (Bcl-2) (1:2000, Abcam) overnight (4 °C). Horseradish peroxidase-labeled secondary antibody was used to incubate the membrane (37 °C, 1 hour). The proteins on the membrane were visualized by the enhanced chemiluminescence (Sigma-Aldrich, St. Louis, MO, USA). β-actin was used as the internal control.

2.4 MTT assay

After pre-transfected with pcDNA3.1 or pcDNA3.1 *HIC1* at 24–48 hours before treatment of LPS (1 μg/mL), HK-2 cell line was seeded (5 $\times$ 10^4/well, 96-well plate, 24 hours), and incubated (10 μL, 5 mg/mL, MTT; Sigma, St. Louis, MO, USA). After incubation (4 hours, 37 °C), solubilized the formazan crystals in DMSO. The microplate reader was used to detect the absorbance (570 nm).

2.5 Enzyme-linked immunosorbent assay (ELISA)

The expression levels of TNF-α, IL-6, and IL-1β in the supernatant from treated HK-2 cell line were measured using ELISA (R&D Systems, Minneapolis, MN, USA).

2.6 Flow cytometry

The flow cytometry Annexin V-FITC/PI apoptosis detection kit was used to determine the apoptosis in HK-2 cell line. Cells were harvested and resuspended at a concentration of 10^6 cells/mL. FITC-Annexin V and PI were added and kept on ice (in the dark, 15 minutes). The ratio of apoptotic cells was detected by flow cytometry.

2.7 Statistics

All experiments were obtained in triplicate and analyzed using GraphPad Prism Software 6.0 (GraphPad Software, La Jolla, CA, USA). All data were presented as mean $\pm$ standard error of the mean (SEM). One-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc test was used for comparison. A statistically significant difference was defined as a $p < 0.05$.

3. Results

3.1 Overexpression of HIC1 promotes the viability of LPS treated HK-2 cell line

LPS, a widely conceded stimulator of cellular apoptosis and inflammatory response, was used to treated HK-2 cell

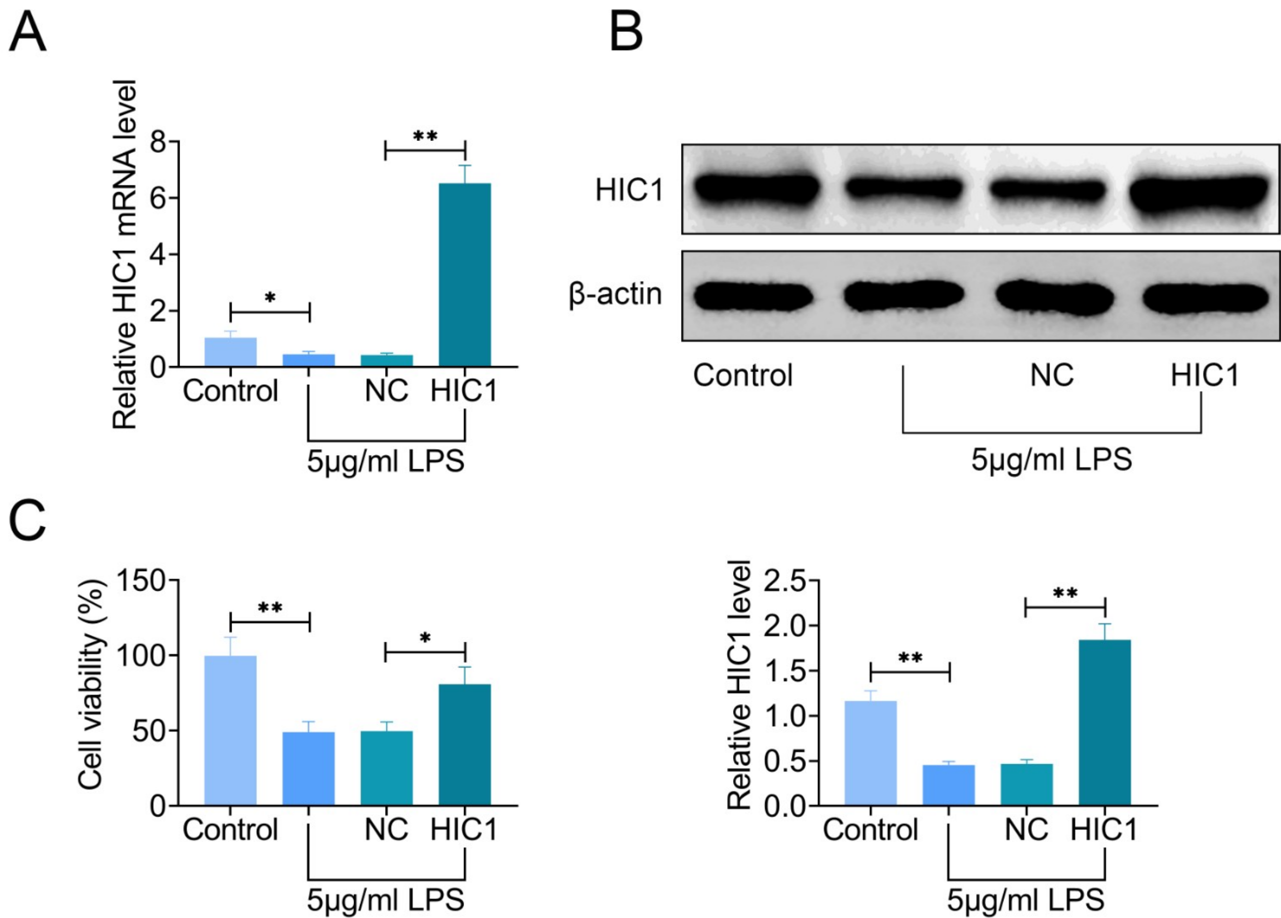

FIGURE 1. Effects of *HIC1* on the LPS-induced viability in HK-2 cell line, HK-2 cell line was transfected with control vector (pcDNA3.1) (NC) or *HIC1* overexpression vector, followed by LPS treatment. (A) *HIC1* expression was determined by qPCR. (B) Western blot analysis and quantitative data of *HIC1* expression in LPS-treated HK-2 cell line. (C) Cell viability was estimated using MTT assay * $p < 0.05$ and ** $p < 0.01$ *vs.* control or LPS + HIC, data are expressed as mean ± SEM.

line to establish a cellular model of AKI caused by sepsis. QPCR and western blot assays showed that *HIC1* expression was remarkably suppressed in HK-2 cell line upon LPS treatment (Fig. 1A,B). HK-2 cell line that transfected with *HIC1* overexpressing vector remarkably increased the *HIC1* expression levels compared with pcDNA3.1 transfection (NC group) (Fig. 1A,B). In addition, the viability of LPS treated HK-2 cell line was significantly raised after transfection of *HIC1* overexpressing vector (Fig. 1C). These data indicated that LPS downregulated *HIC1* expression and reduced the viability in HK-2 cell line, and *HIC1* overexpressing vector could reverse the effects induced by LPS.

3.2 Overexpression of HIC1 suppressed LPS-induced apoptosis in HK-2 cell line

The flow cytometry was performed to identify the regulatory effect of *HIC1* overexpression on cellular apoptosis. The number of apoptosis-positive cells was remarkably increased in the LPS group as compared to the control group, while decreased by *HIC1* overexpression. Similar findings were detected by western blot, revealed by determining the expression levels of apoptosis-related biomarkers in HK-2 cell line (Fig. 2A). As presented in Fig. 2B, the expression levels of Bax and Caspase 3 in LPS treated HK-2 cell line were markedly increased compared with the control group, while decreased after transfected with *HIC1* overexpressing vector. Moreover, LPS treatment induced a reduction in the Bcl-2 expression, which was enhanced by transfection with *HIC1* overexpressing vector. These results implied that *HIC1* might be involved in the pathophysiological processes in LPS-induced cellular apoptosis.

3.3 LPS-induced inflammation in HK-2 cell line was suppressed by overexpression of HIC1

QPCR assay indicated that the mRNA expression levels of proinflammatory cytokines (IL-6, TNF-α, and IL-1β) in HK-2 cell line were significantly increased by LPS treatment (Fig. 3A), while transfection of *HIC1* overexpression vector attenuated LPS-induced proinflammatory cytokine gene expressions in HK-2 cell line. In addition, ELISA assay verified that the protein expression levels of proinflammatory cytokines were increased by LPS treatment. Transfection of *HIC1* overexpression vector in LPS-treated HK-2 cell line

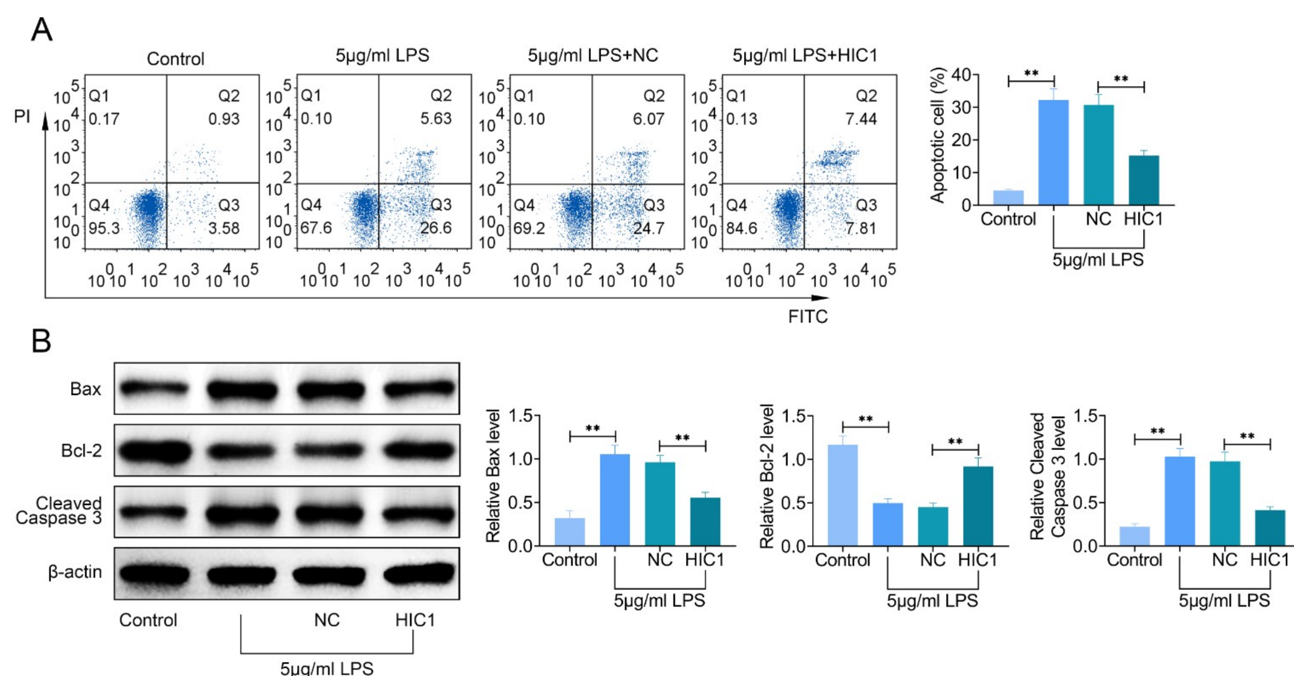

FIGURE 2. Overexpression of *HIC1* suppressed apoptosis in HK-2 cell line induced by LPS. (A) The apoptotic rate of HK-2 cell line was measured by flow cytometry. (B) The protein expressions of Bax, Cleaved Caspase 3, and Bcl-2 were examined by Western blot analysis. * $p < 0.05$ and ** $p < 0.01$ *vs.* control or LPS + HIC. Data are expressed as mean ± SEM.

obviously reversed these results (Fig. 3B). Together, these data supported a role of *HIC1* in mediating the regulatory role of LPS in inflammatory response.

3.4 Overexpression of HIC1 suppressed LPS-induced activation of IL-6/STAT3 signaling pathway in HK-2 cell line

IL-6/STAT3 signaling pathway has been reported closely related to the enhanced inflammatory response, further study was explored its involvement in the regulation of *HIC1* in LPS-treated HK-2 cell line. Western blot analysis revealed that LPS activated the protein expressions of STAT3, p-STAT3, IL-6, and RORγt. Conversely, the expression level of SOCS3, related to the function of anti-inflammatory, was significantly decreased in LPS-treated HK-2 cell line. Transfection of *HIC1* overexpression vector could reverse the simulative effect of LPS on IL-6/STAT3 signaling pathway (Fig. 4). These results highlighted that *HIC1* exerted a protective influence on LPS-induced renal cell injury by suppressing the IL-6/STAT3 signaling pathway.

4. Discussion

AKI is one of common complications of sepsis and associated with renal inflammation, whose main characteristics are acute renal dysfunction. Multiple evidences have indicated that AKI is precipitated by unique and complex mechanisms, including abnormal kidney damage biomarkers, such as insulin-like growth factor-binding protein 7, interleukin-18 and L-type fatty acid binding protein [24]. LPS is a classic TLR4 agonist commonly used to induce sepsis in an animal model, which can cause a direct and consequential inflammatory response, thus stimulating the activation of the innate immune system in sepsis. The molecular mechanism of *HIC1* as a tumor suppressor is to suppress tumor development by regulating the interactions between its target genes or proteins [25]. Previous studies have reported that *HIC1* was constantly epigenetically silenced or deleted in prevalent human cancers [10, 26]. Despite extensive investigations and the determination that *HIC1* expression is detectable in a wide range of cells, there are no studies on the molecular basis of *HIC1* inhibition of sepsis-induced AKI progression. Here, the present study initially found that *HIC1* expression was notably downregulated after LPS treatment and the overexpression of *HIC1* significantly improved the viability in LPS-treated HK-2 cell line. Previous reports have shown that overexpression of *HIC1* was related to tumor proliferation, migration, apoptosis and invasion [25, 27]. In the present study, its anti-apoptotic effect was demonstrated through two different kinds of experiments. The results from flow cytometry assay indicated that the number of apoptosis-positive cell was significantly increased in the LPS treatment group, while overexpression of *HIC1* reversed these results. Moreover, the results from western blot demonstrated similar results. Though a recent research has reported that hypermethylation of *HIC1* promoter and overexpression of *HIC1* protein appear in some cancer cells [28], this is the first report showing that *HIC1* overexpression was correlated with LPS-induced acute renal cell dysfunction. LPS induces mitochondrial dysfunction and inflammation in multiple cell types and is frequently used to develop renal or intestine injury models. LPS mediates cellular apoptogenic signals via the release of cytochrome C and alterations in the mitochondrial membrane potential [29].

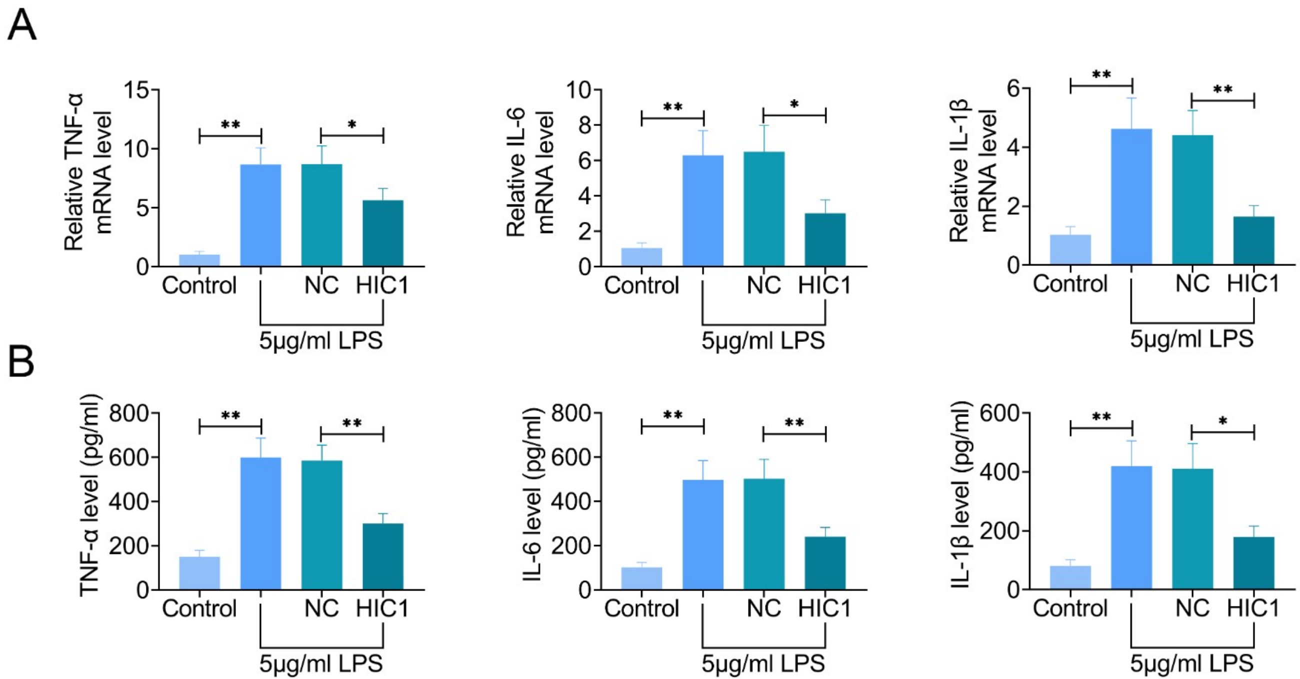

FIGURE 3. Overexpression of *HIC1* attenuated LPS-induced inflammation in HK-2 cell line. (A) QPCR results and (B) ELISA results of TNF-α, IL-6, and IL-1β expressions in LPS-treated HK-2 cell line. * $p < 0.05$ and ** $p < 0.01$ *vs.* control or LPS + HIC. Data are expressed as mean ± SEM.

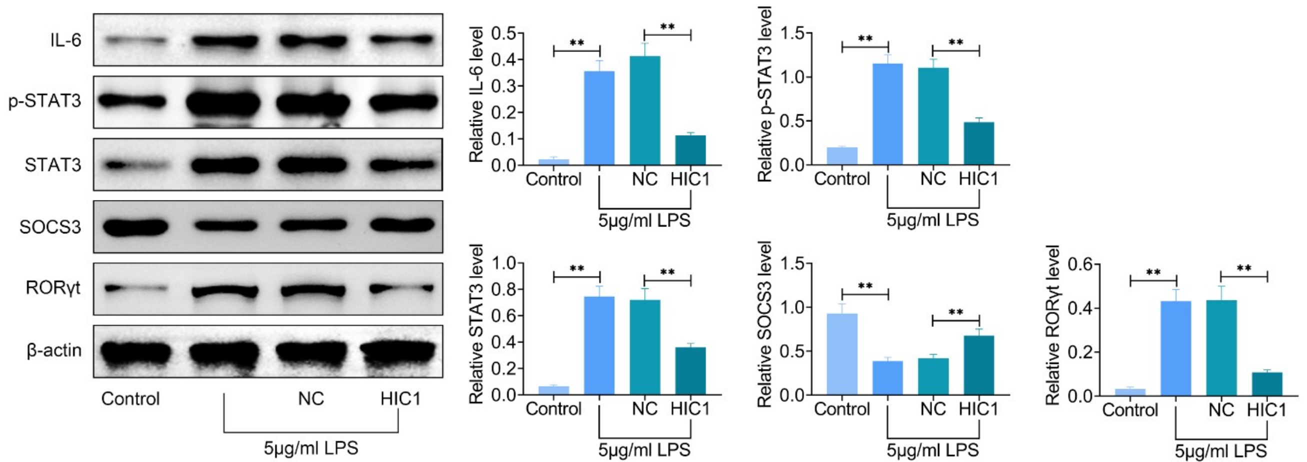

FIGURE 4. *HIC1* regulated LPS-induced injury in HK-2 cell line via regulating IL-6/STAT3 signaling pathway. Western blot analysis and quantitative data for STAT3, p-STAT3, IL-6, RORγt and SOCS3 expression in LPS-treated HK-2 cell line. * $p < 0.05$ and ** $p < 0.01$ *vs.* control or LPS + HIC. Data are expressed as mean ± SEM.

In addition, LPS could be recognized by Toll-like receptors and NF-κB signaling pathway, a critical pathway of all the signal transduction pathways mediated by LPS, indicating that LPS may be an essential target to enhance the pro-inflammatory response and cell injury [30, 31]. Indeed, this study suggested that the protein expression levels of proinflammatory cytokines (TNF-α, IL-6, and IL-1β) were increased by LPS treatment. Conversely, *HIC1* overexpression markedly inhibited the LPS-induced inflammatory response in HK-2 cell line.

Numerous studies have reported that IL-6 and its major effector STAT3 are pro-tumorigenic agents in many cancers and are critical in managing essential cellular progressions, such as cell proliferation, differentiation, inflammation, angiogenesis, and metastasis [32, 33]. The activation of STAT3 was involved in the progression of oncogenic transformation and tumor formation. Recent studies have indicated that netrin-1 protected kidney function is dependent on the inhibition of IL6/STAT3 pathway [34]. Moreover, the suppression of renal fibrosis and pentraxin 3 induced STAT3 activation is mediated by IL-6 [35]. Previous studies reported that STAT3 is one of

the *HIC1*-interacting proteins and *HIC1* also suppressed the STAT3-mediated reporter activity [36]. STAT3 is regularly triggered in a wide range of human cancers, and hypermethylation of *HIC1* promoter in most cancers may increase the STAT3 activity and accordingly promote oncogenesis [27]. In non-small cell lung cancer, *HIC1* was found to be a suppressor of IL-6, which could in turn inhibit *HIC1* expression [37, 38]. Promoting *HIC1* expression or *HIC1*-STAT3 interaction may provide a potential therapeutic strategy in antagonizing STAT3-associated human cancers. From this result, it can be concluded that the expressions of *HIC1* and IL-6 could be reciprocally regulated by each other. However, previous studies have focused on its regulatory relationship in cancer research, and there has not been reported the mechanism of IL-6 expression inhibited by *HIC1* in renal cell injury. This is the first study showing that *HIC1* could inhibit the LPS-induced expressions of STAT3, p-STAT3, IL-6, and RORγt in HK-2 cell line. A limitation of this study is that only study on the cell-based level, the animal study will be the direction of the future.

5. Conclusion

In summary, these results suggested that overexpression of *HIC1* could promote viability and suppress apoptosis and inflammatory response in the LPS-treated HK-2 cell line. Furthermore, the protective effect of *HIC1* overexpression was partly generated through the inhibition of IL-6/STAT3 signaling pathways. *HIC1* and IL-6/STAT3 pathway are essential to elucidate the mechanism of renal cell injury, and these findings may help to identify the promising targets for clinical sepsis therapy.

AUTHOR CONTRIBUTIONS

XY, JFG—designed the study, supervised the data collection; XSC—analyzed the data, interpreted the data; YJY—prepared the manuscript for publication and reviewed the draft of the manuscript, all authors have read and approved the manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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