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*Original Article*

# CXCL16 is activated by p-JNK and is involved in $H_2O_2$-induced HK-2 cell injury via p-ERK signaling

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**Abstract:** Acute kidney injury (AKI) leads to an abrupt deterioration of renal function. CXC chemokine ligand 16 (CXCL16) is a CXC-soluble chemokine and a cell surface scavenger receptor that is involved in tissue injury and inflammation. It is induced in AKI patients, but the molecular mechanism remains unclear. In this study, we have worked to determine the function of CXCL16 and to investigate the involvement of ERK and JNK signaling in AKI. We used $H_2O_2$ and recombinant human CXCL16 protein (rh CXCL16) to treat the human renal tubular epithelial cell line, HK-2, *in vitro*. The present results indicate that $H_2O_2$ inhibits proliferation, induces apoptosis, and up-regulates expression of CXCL16 and the CXCL16 receptor, CXCR6, in HK-2 cells. Furthermore, $H_2O_2$-induced proliferation inhibition, apoptosis, and inflammation in HK-2 cells were ameliorated by NAC (N-acetyl cysteine, the ROS scavenger) and SP600125 (the JNK inhibitor). The expression levels of CXCL16 and CXCR6 were also positively correlated with the phosphorylation level of JNK (p-JNK). Additionally, rh CXCL16 treatment led to effects like those of $H_2O_2$ treatment in HK-2 cells, with symptoms being effectively improved by CXCR6 siRNA and the ERK inhibitor (PD98059). In addition, rh CXCL16-induced HK-2 cell apoptosis, inflammation, and collagen deposition were lessened by CXCR6 siRNA and PD98059 treatment. In summary, the present results have indicated that CXCL16 is involved in $H_2O_2$-induced HK-2 cell injury, that CXCL16 is activated by p-JNK, and that the regulatory function of CXCL16 is involved in the phosphorylation of ERK.

**Keywords:** Acute kidney injury, CXCL16, CXCR6, JNK, ERK

## Introduction

Acute kidney injury (AKI) is defined as an acute renal failure and is characterized by a sudden decline of renal function, leading to dysregulation of acid-base status, electrolytes, and fluid balance [1]. AKI can be caused by the introduction of many factors such as cisplatin, ischemia-reperfusion, sulphasalazine, and fructose, and mechanistic studies have revealed that the abnormal increase of reactive oxygen species (ROS) is the primary cause of renal injury [2-5]. Mitochondria are the producers of most cellular energy, and mitochondrial dysfunction is closely associated with an increase in cellular ROS levels [6]. Oxidative stress acts as the cause or consequence of mitochondrial dysfunction, which leads to nuclear DNA damage, cell death, and tubular cell injury [6-8]. Mitogen-activated protein kinases (MAPKs, including ERK, JNK, and p38 MAPK) are crucial mediators that are activated in cells to regulate survival, cell proliferation, and apoptosis of cells that are exposed to oxidative stress. Suppressing the ROS-mediated JNK/ERK/p38 pathway is, therefore, the critical target for the treatment of renal injury [4, 8].

CXC chemokine ligand 16 (CXCL16) is a member of the CXC chemokine family. It is a soluble chemokine and cell surface-bound molecule that only binds to its unique receptor, CXCR6 [9, 10]. Previous reports have shown that CXCL16 aggravates chronic hypertensive renal injury and cisplatin-induced AKI by regulating inflammation and apoptosis [11, 12]. Further, Overexpression of CXCL16 is closely related to the pathogenesis of ischemia-reperfusion-induced renal injury [10]. CXCL16 CXCL16 exists, not only in a transmembrane form as an

adhesion molecule but also as a soluble chemokine that mediates infiltration of circulating cells into injury sites [13, 14].

Although CXCL16 has been established as having a critical role in renal injury, the initial molecular events involved in this role remain elusive [15]. In the present study, we have assessed the expression of peripheral blood CXCL16 in AKI patients. Further, different concentrations of $H_2O_2$ were used to treat the human renal tubular epithelial cell line, HK-2, *in vitro* to mimic ROS-induced AKI and to further study the resulting function of CXCL16 and the involvement of the JNK and ERK signal pathway.

## Materials and methods

### *Chemicals and reagents*

Human CXCL16, TNF-α, IL-6 and TGF-β ELISA kits were obtained from Cell Signaling Technology Inc. (Beverly, MA, USA). A CCK-8 assay kit was purchased from Signalway Antibody (SAB, USA). An Annexin V-FITC cell apoptosis detection kit was purchased from Beyotime Biotech (Shanghai, China). Primary antibodies of CXCL16 and CXCR6 were obtained from Abcam Biotech (Cambridge, MA, USA); primary antibodies of Bcl2 and Bax were obtained from Santa Cruz (CA, USA); primary antibodies of p-ERK1/2, ERK1/2, collagen type (COL)-I, COL-III, matrix metalloproteinase 2 (MMP2), MMP9, and GAPDH were obtained from Cell Signaling Technology Inc. (Beverly, MA, USA). Trizol reagent was purchased from Invitrogen (Thermo Fisher, MA, USA). Recombinant Human CXCL16 protein was obtained from Abcam Biotech (Abcam, Cambridge, UK).

### *Experimental design*

*Patients:* All patients suffering from AKI received cardiac bypass surgery. Informed consent was obtained from all of the study participants. Our experimental procedures were up to the standards of medical ethics formulated by the 1975 Helsinki Declaration (revised in 2005) and were approved of by the Experimentation Ethics Committee of our hospital.

### *Measurement of CXCL16 concentration in peripheral blood*

The peripheral blood concentrations of CXCL16 in healthy controls (n = 20) and AKI patients (n = 20) were determined using a human CXCL16 ELISA kit according to the manufacturer's instructions.

### *Effects of $H_2O_2$ stimulation on HK-2 cells*

The HK-2 cells were obtained from SIBCB (Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, Shanghai, China). Cells were cultured in DMEM (Dulbecco's modified Eagle's medium; GBICO, USA) with 10% FBS (fetal bovine serum; GBICO) and penicillin (100 U/mL) (5% $CO_2$ at 37°C). The third generation of HK-2 cells was used in follow-up experiments.

HK-2 cells were cultured with different concentrations of $H_2O_2$ (0, 200, or 400 μM). The proliferation of HK-2 cells was determined by a CCK8 assay kit at 0, 24, 48 and 72 h after treatment. In addition, the amount of CXCL16, TNF-α, IL-6, and TGF-β in the supernatant, as well as mRNA and protein levels of CXCL16 and CXCR6 in HK-2 cells, were tested at the end of the experiment.

### *The involvement of JNK signaling in $H_2O_2$-mediated HK-2 cell injury*

HK-2 cells were divided into four groups: normal control group, $H_2O_2$ (400 μM) treatment group, $H_2O_2$ (400 μM) + NAC (N-acetyl cysteine, the ROS scavenger [16]) treatment group, and $H_2O_2$ (400 μM) + SP600125 (the JNK inhibitor [17]) treatment group. The last two groups had added inhibitors, with NAC at a concentration of 5 mM being added to the $H_2O_2$ + NAC treatment group, and with SP600125 at a concentration of 25 μM was added to the $H_2O_2$ + SP600125 treatment group. Cells in all groups were incubated at 37°C for 2 h. Then, $H_2O_2$ (400 μM) was added to the three treatment groups, and cells were cultured for 24 h. The apoptosis rate; the amount of CXCL16, IL-6, TNF-α and TGF-β in the supernatant; and the mRNA expression and protein levels of CXCL16 and CXCR6 were tested at the end of the experiment.

### *Effects of different concentrations of CXCL16 on HK-2 cells*

HK-2 cells were cultured with different concentrations (50, 100, or 200 μg/L) of recombinant human CXCL16 protein dissolved in dimethyl sulfoxide (DMSO). The culture conditions for the negative control group were the same, but with an equal amount of solvent replacing the

**Table 1.** CXCR6 (NM_006564.1) RNAi sequence and primers

| RNAi sequence and primers | Sequence (5'-3') |
|---|---|
| RNAi sequence (842-860 bp) | GCAGCACACACTGGGAATA |
| CXCR6-Forward | CCGGGCAGCACACACTGGGAATACTCGAGTATTCCCAGTGTGTGCTGCTTTT |
| CXCR6-Reverse | AATTAAAAAGCAGCACACACTGGGAATACTCGAGTATTCCCAGTGTGTGCTGC |

Underlined sequences: restriction sites.

**Table 2.** Primers used for qRT-PCR

| Gene name/number | Sequence (5'-3') | Description |
|---|---|---|
| CXCL16 (NM_001100812.1) | CCCGCCATCGGTTCAGTTC | Forward |
| CXCL16 | CCCCGAGTAAGCATGTCCAC | Reverse |
| CXCR6 (NM_006564.1) | GACTATGGGTTCAGCAGTTTCA | Forward |
| CXCR6 | GGCTCTGCAACTTATGGTAGAAG | Reverse |
| GAPDH (NM_001256799.1) | CACCCACTCCTCCACCTTTG | Forward |
| GAPDH | CCACCACCCTGTTGCTGTAG | Reverse |

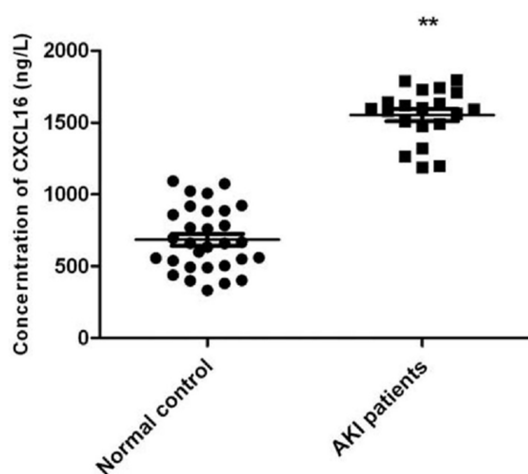


**Figure 1.** The CXCL16 level in peripheral blood of AKI patients (n = 20). **P < 0.01 vs. healthy controls (n = 20).

recombinant protein solution. The proliferation of HK-2 cells was tested at 0, 24, 48 and 72 h. Moreover, the apoptosis rate of HK-2 cells; the protein levels of CXCL16, CXCR6, Bcl2, Bax, and ERK1/2; and the phosphorylation of ERK1/2 (p-ERK1/2) were tested at 72 h after the experiment.

*Functional study of CXCL16 in HK-2 cells*

The CXCR6 siRNA lentivirus vectors (sequence and primers shown in **Table 1**) were constructed by JRDun Biotech (Shanghai, China). Viral supernatants were diluted in culture medium to the required concentration and added to the exponential-phase monolayer of HK-2 cells. HK-2 cells were divided into four groups: the empty plasmid control (EPC) group, CXCL16 (200 μg/L) + EPC group, CXCL16 (200 μg/L) + CXCR6 siRNA group, and CXCL16 (200 μg/L) + EPC + PD98059 (the ERK inhibitor [17]) group. The viral supernatants were added to the HK-2 cells. Cells in all groups were cultured at 37°C for 24 h, and PD98059 (10 μM) was added to the last group at 22 h. Then, the recombinant human CXCL16 protein was added, and cells were cultured for another 24 h. The apoptosis rate of HK-2 cells, as well as the protein levels of COL-I, COL-III, MMP2, MMP9, Bcl2, Bax, ERK1/2, and p-ERK1/2, were determined at the end of the experiment.

*Experimental methods*

*CCK8 assay:* The cultured HK-2 cells were harvested at 0, 24, 48 and 72 h. The proliferation of HK-2 cells was tested using a CCK8 assay kit according to the manufacturer's instructions. The absorbance of HK-2 cells in each group was measured at a wavelength of 450 nm using a microplate reader (Thermo Fisher, MA, USA).

*Apoptosis assay*

The cultured HK-2 cells were harvested and incubated with FITC-labelled annexin-V and propidium iodide (PI) at 25°C for 20 min according to the manufacturer's instructions. Subsequently, the intensity of annexin-V or PI fluorescence was analyzed by FACScan (Becton-Dickinson, San Diego, CA, USA). For each sample, 10,000 cells were tested.

*ELISA*

The cultured HK-2 cells were harvested at the end of the experiment, and the concentrations of CXCL16, TNF-α, IL-6 and TGF-β in the super-

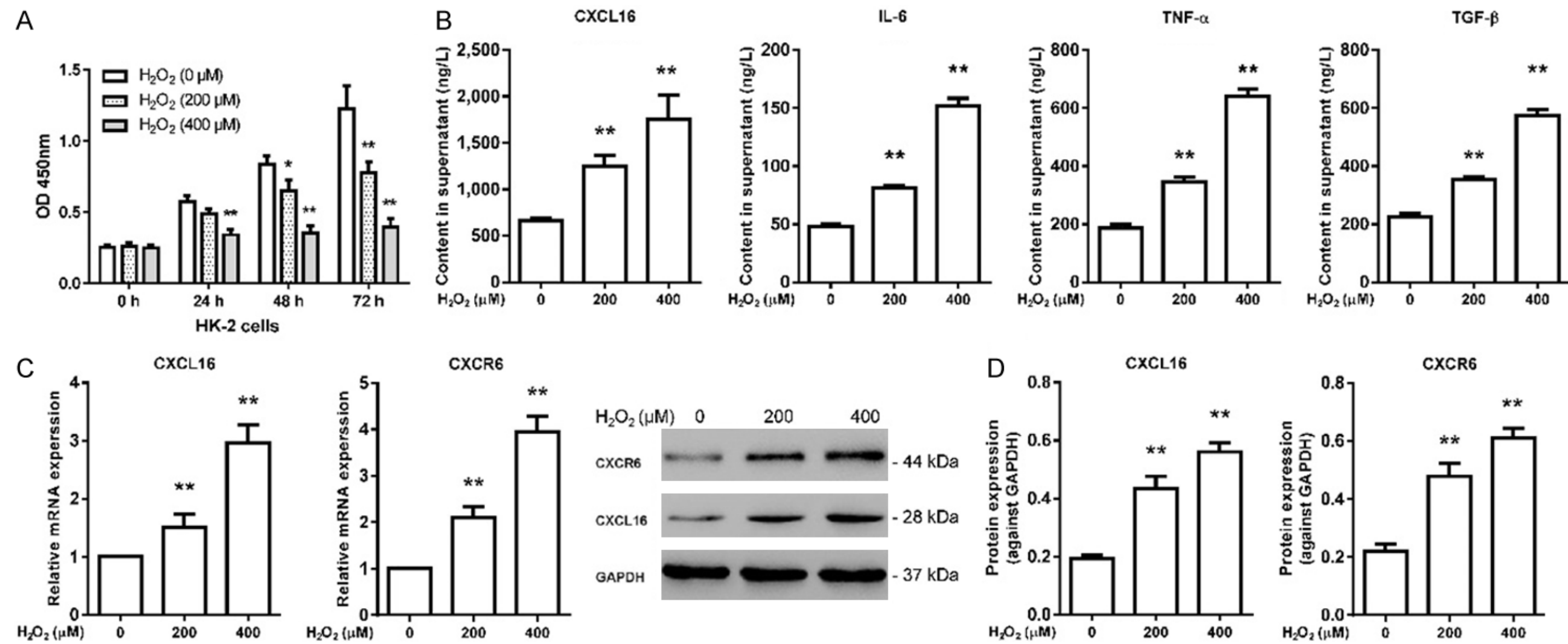


**Figure 2.** Effects of different concentrations of $H_2O_2$ treatment on HK-2 cells. A: $H_2O_2$ treatment inhibited the proliferation of HK-2 cells. B: $H_2O_2$ treatment resulted in higher concentrations of CXCL16, IL-6, TNF-α, and TGF-β in the supernatant. C: $H_2O_2$ treatment increased the protein levels of CXCL16 and CXCR6. D: $H_2O_2$ treatment increased mRNA and protein expression levels of CXCL16 and CXCR6. *$P < 0.05$, **$P < 0.01$ vs. $H_2O_2$ (0 μM) treatment group (n = 3).

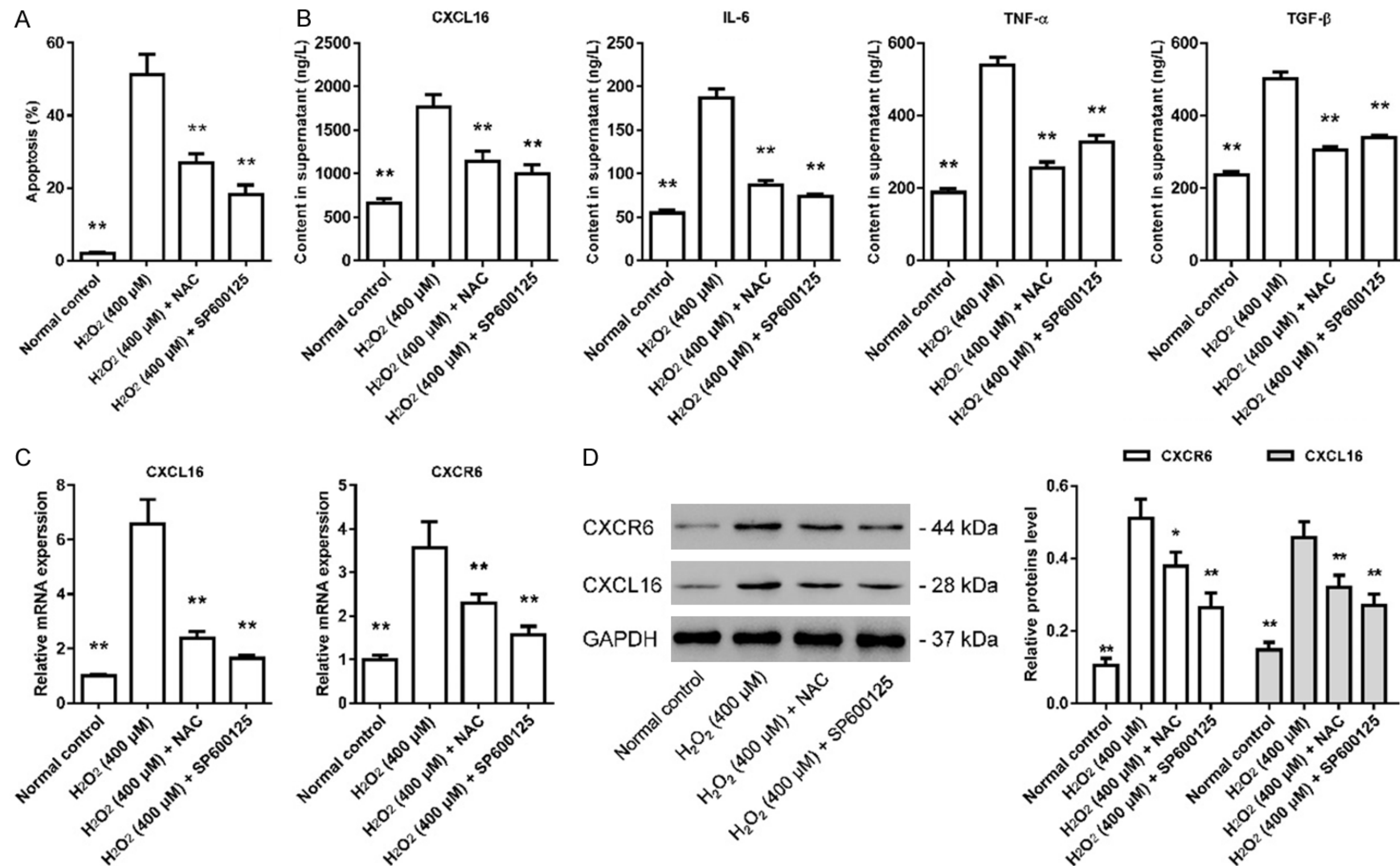


**Figure 3.** The involvement of JNK signaling in $H_2O_2$-mediated HK-2 cell injury. A: $H_2O_2$-induced HK-2 cell apoptosis was lessened by treatment with the ROS scavenger, NAC, and the JNK inhibitor, SP600125. B: $H_2O_2$-induced CXCL16, IL-6, TNF-α, and TGF-β contents in the supernatant were lessened by NAC and SP600125 treatment. C: The mRNA expression levels of CXCL16 and CXCR6 were increased by $H_2O_2$ treatment but were decreased by NAC and SP600125 treatment. D: The protein levels of CXCL16 and CXCR6 were increased by $H_2O_2$ treatment but were down-regulated by NAC and SP600125 treatment. *$P < 0.05$, **$P < 0.01$ vs. $H_2O_2$ (400 μM) treatment group (n = 3).

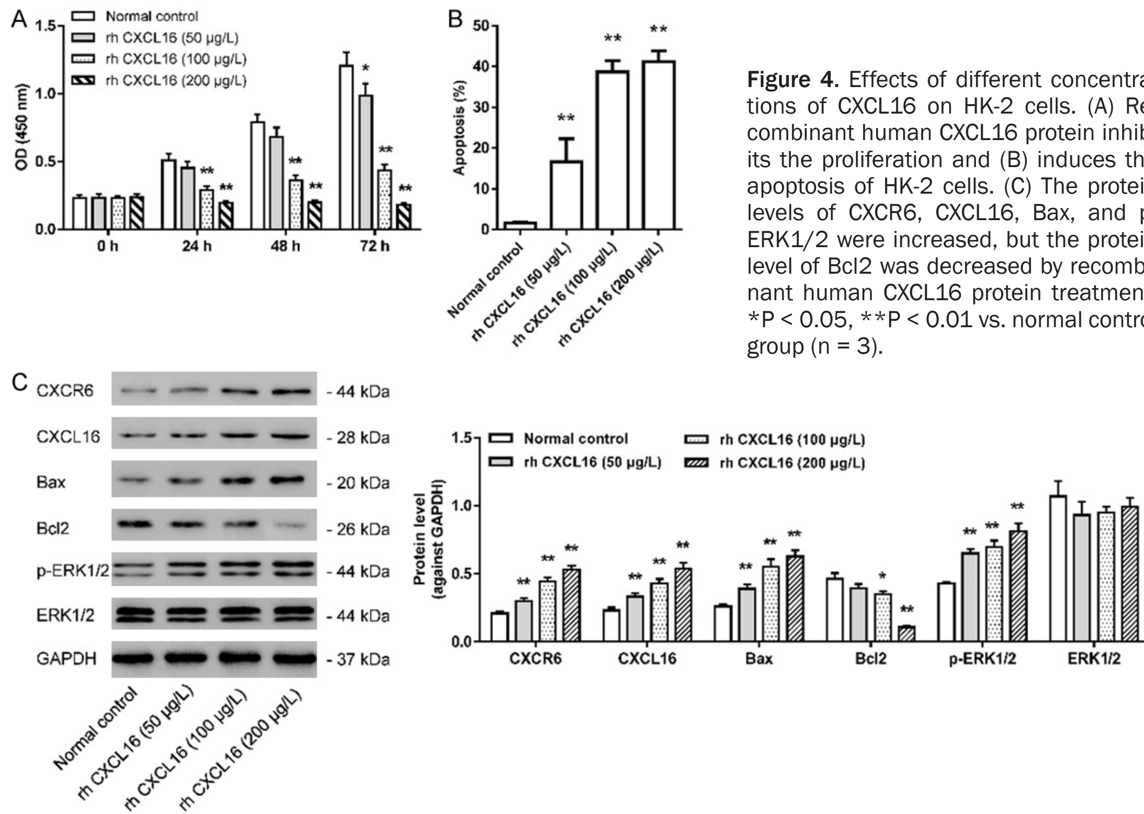


**Figure 4.** Effects of different concentrations of CXCL16 on HK-2 cells. (A) Recombinant human CXCL16 protein inhibits the proliferation and (B) induces the apoptosis of HK-2 cells. (C) The protein levels of CXCR6, CXCL16, Bax, and p-ERK1/2 were increased, but the protein level of Bcl2 was decreased by recombinant human CXCL16 protein treatment. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. normal control group (n = 3).

natant were measured by an ELISA assay kit with a microplate reader according to the manufacturer's instructions.

*RT-qPCR*

Relative mRNA expression was determined by RT-qPCR. Total mRNA was isolated using Trizol according to the manufacturer's instructions. Two μg of total RNA for each sample was reverse transcribed into cDNA using the First Strand cDNA Synthesis kit (Thermo, MA, USA) and a quantitative real-time PCR machine (ABI-7300, USA).

Primers used for RT-qPCR are shown in **Table 2**. Relative mRNA expressions were evaluated using a $2^{-\Delta\Delta Ct}$ relative quantitative analysis against GAPDH.

*Western blot*

The total protein of each sample was extracted, and the protein content was tested using a bicinchoninic protein assay kit. Then, the protein (30 μg) was separated with a 10% SDS-PAGE gel and transferred onto a PVDF membrane. Membranes were blocked with 5% skim milk at 25°C for 1 h. Resulting membranes were incubated with the appropriate primary antibody: CXCR6 (1:1,000, Abcam), CXCL16 (1:500, Abcam), Bcl2 (1:400, Santa Cruz), Bax (1:400, Santa Cruz), p-ERK1/2 (1:1,000, CST), ERK1/2 (1:1,000, CST), COL-I (1:1,000, CST), COL-III (1:1,000, CST), MMP2 (1:1,000, CST), MMP9 (1:1,000, CST), or GAPDH (1:2,000, CST). Following this, the membranes were incubated with secondary antibody for 1 h.

Finally, the protein bands were tested with an ECL-detecting kit (Beyotime Biotech, Shanghai, China). Blots against GAPDH served as a loading control.

*Statistical analysis*

Data are expressed as Mean ± SD. The Student's t-test was used for analysis between two groups, and a one-way analysis of variance (ANOVA) test was used for analysis of three or more groups. The analysis was done using SPSS 20.0 software (SPSS Inc., USA). A $P$ value of less than 0.05 was considered to be significant.

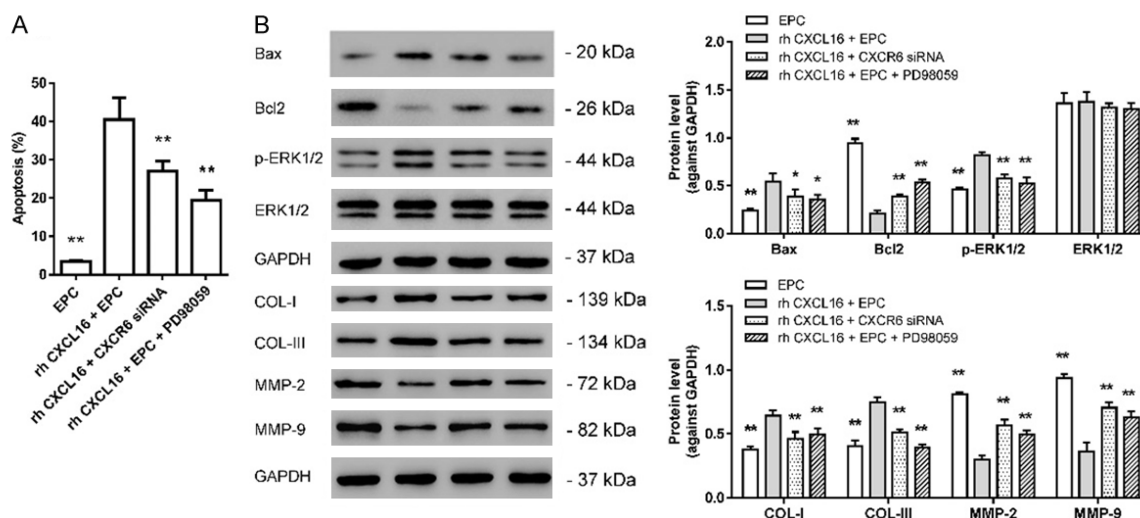


**Figure 5.** The involvement of CXCL16 in HK-2 cell injury. A: Recombinant human (rh) CXCL16 protein (200 μg/L)-induced HK-2 cell apoptosis was lessened by PD98059 (10 μM) and CXCR6 siRNA treatment. B: The protein levels of Bax, p-ERK1/2, COL-I, and COL-III were up-regulated, and the protein levels of Bcl2, MMP2, and MMP9 were down-regulated by recombinant human CXCL16 protein treatment. These same effects were weakened by PD98059 and CXCR6 shRNA treatment. EPC: empty plasmid control. $^*P < 0.05$, $^{**}P < 0.01$ vs. rh CXCL16 + EPC group (n = 3).

## Results

### *Expression of CXCL16 was increased in AKI patients*

The concentration of CXCL16 in peripheral blood was tested for using an ELISA kit. As is shown in **Figure 1**, the CXCL16 level increased significantly in AKI patients compared with that of those in the normal control group.

### *$H_2O_2$ treatment increased CXCL16 expression and inflammation in HK-2 cells*

Different concentrations of $H_2O_2$ were used to treat HK-2 cells *in vitro* to mimic ROS-induced AKI. The results indicated that the proliferation of HK-2 cells was inhibited by $H_2O_2$ in a time- and dose-dependent manner (**Figure 2A**). Further, the concentration of CXCL16 and inflammatory cytokines IL-6, TNF-α, and TGF-β were increased in the supernatant following $H_2O_2$ stimulation (**Figure 2B**). Moreover, the mRNA and protein expression levels of CXCL16 and CXCR6 were increased by $H_2O_2$ treatment in HK-2 cells (**Figure 2C** and **2D**).

### *JNK signaling is involved in $H_2O_2$-induced HK-2 cell injury*

The involvement of JNK signaling in ROS-mediated HK-2 cell injury was further assessed. As is shown in **Figure 3**, the apoptosis rate (**Figure 3A**); the amount of CXCL16, IL-6, TNF-α, and TGF-β in the supernatant (**Figure 3B**); and the mRNA (**Figure 3C**) and protein expression levels of CXCL16 and CXCR6 were up-regulated following $H_2O_2$ treatment in HK-2 cells. These effects, however, were weakened by the ROS scavenger, NAC, and the JNK inhibitor, SP600125. Additionally, the expression levels of CXCL16 and CXCR6 were positively regulated by p-JNK. These results suggest that $H_2O_2$-induced inflammation and apoptosis in HK-2 cells is dependent on the phosphorylation of JNK signaling.

### *Effects of different concentrations of CXCL16 on HK-2 cells*

We further investigated the effects of recombinant human CXCL16 protein (rh CXCL16) on HK-2 cells. The results indicate that the proliferation of HK-2 cells was inhibited by recombinant human CXCL16 protein in a time- and dose-dependent manner (**Figure 4A**) and that the apoptosis rate was increased (**Figure 4B**). Moreover, the protein expression levels of CXCL16, CXCR6, Bax, and p-ERK1/2 were up-regulated by recombinant human CXCL16 protein in a dose-dependent manner. The protein level of Bcl2, however, showed an opposite trend. The protein level of ERK1/2 remained unchanged (**Figure 4C**).

*CXCL16 is involved in HK-2 cell injury via CXCR6 and p-ERK signaling*

The apoptosis of HK-2 cells (**Figure 5A**) and the protein levels of Bax, p-ERK, COL-I, and COL-III were increased by rh CXCL16, but this effect was ameliorated by CXCR6 siRNA and PD98059 (ERK inhibitor) treatment. On the other hand, the protein levels of Bcl2 and the ECM components decomposers MMP2 and MMP9 [18] showed the opposite trend. The protein level of ERK1/2 remained unchanged (**Figure 5B**).

## Discussion

AKI can be caused by many factors, all of which lead to the abrupt deterioration of renal function [10]. Previous studies have described multiple molecular mechanisms that are involved in AKI. The primary phase of calcium overload and energy deficit, and the secondary phase of inflammation and oxidative stress were described as the main causes of renal ischemia-reperfusion (RIR)-induced AKI [19]. Further, the pathogenesis of AKI is related to multiple stresses such as energy depletion, hypoxia, endoplasmic reticulum (ER) stress, oxidant injury, genotoxic stress, inflammation, and other damaging insults that induce autophagy [20-22]. In addition, the injury of endothelial cells and tubular epithelial cells present in the microvasculature is associated with an extension phase of AKI [23]. Oxidative stress leading to the activation of the apoptotic pathway and overproduction of ROS results in the increase of apoptosis of tubular epithelial cells via the PI3K/Akt/eNOS pathway [24]. In the multiple molecular mechanisms involved in AKI, ROS-mediated activation of MAPK signaling plays a critical role. Excessive autophagy during the process of cisplatin-induced AKI was closely related to overactivation of p38 and ERK signaling [25]. In the present study, the phosphorylation of JNK was activated by $H_2O_2$ in HK-2 cells.

CXCL16 is a CXC-soluble chemokine, and it is known as an adhesion molecule and cell surface scavenger receptor. Previous reports have described CXCL16's involvement in tissue injury and inflammation [26-28]. Acetaminophen (APAP) induces hepatotoxicity via activation of JNK signaling in mice, and CXCL16 deficiency attenuates tissue injury and inflammation [29]. In addition, genetic silencing of CXCL16 inhibits inflammation and apoptosis in cisplatin-induced AKI [11]. Moreover, soluble CXCL16 levels were increased following gene knockdown of the inflammatory factors IL-6 and CXCL16 in HepG2 cells. The gene knockdown was done using CXCL16 siRNA and resulted in decreased ROS production, lipid accumulation, and ECM excretion [30]. In the present study, the expression of CXCL16 was up-regulated by $H_2O_2$ in HK-2 cells but was weakened by the suppression of ROS-mediated activation of JNK signaling. Meanwhile, recombinant human CXCL16 protein induced collagen deposition and suppression of collagen degradation, both of which were reversed upon introduction of CXCR6 siRNA and the ERK inhibitor PD98059. These results have indicated that the expression of CXCL16 is positively regulated by p-JNK.

Moreover, recombinant human CXCL16 protein further induced apoptosis and collagen deposition while inhibiting proliferation of HK-2 cells via the activation of ERK, the down-regulation of apoptosis-related Bcl-2, and the up-regulation of Bax [31]. Blocked ERK signal and CXCR6 siRNA, however, could effectively relieve this symptom. The effectiveness of this blocking mechanism has suggested that JNK/ERK signal transduction is mediated by CXCL16/CXCR6 in ROS-induced HK-2 cell injury.

In summary, the present results suggest that CXCL16 is involved in $H_2O_2$-induced HK-2 cell injury. CXCL16 was up-regulated by ROS-mediated activation of JNK signaling. Further, the regulatory function of CXCL16 was related to the phosphorylation of ERK.

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## Disclosure of conflict of interest

None.

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