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CXC195 suppresses proliferation and inflammatory response in LPS-induced human hepatocellular carcinoma cells via regulating TLR4-MyD88-TAK1-mediated NF-κB and MAPK pathway

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ABSTRACT

CXC195 showed strong protective effects in neuronal apoptosis by exerting its antioxidant activity. However, the anti-cancer effects of CXC195 is still with limited acquaintance. Here, we investigated the role of CXC195 in lipopolysaccharide (LPS)-induced human hepatocellular carcinoma (HCC) cells lines (HepG2) and the possible signaling pathways. CXC195 exhibited significant anti-proliferative effect and induced cell cycle arrest in LPS-induced HepG2 cells. In addition, CXC195 suppressed the release of pro-inflammatory mediators in LPS-induced HepG2 cells, including TNF-α, iNOS, IL-1β, IL-6, CC chemokine ligand (CCL)-2, CCL-22 and epidermal growth factor receptor (EGFR). Moreover, CXC195 inhibited the expressions and interactions of TLR4, MyD88 and TAK1, NF-κB translocation to nucleus and its DNA binding activity, phosphorylation of ERK1/2, p38 and JNK. Our results suggested that treatment with CXC195 could attenuate the TLR4-mediated proliferation and inflammatory response in LPS-induced HepG2 cells, thus might be beneficial for the treatment of HCC.

1. Introduction

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related deaths worldwide. Approximately 80% of HCC patients develop in fibrotic or cirrhotic livers, which occur in response to chronic liver injury caused by persistent inflammation [1,2]. Treatment options for HCC include resection, liver transplantation, percutaneous ablation and chemoembolisation. However, the prognoses of HCC patients, especially those with high staging or chemoresistant tumors, are still unsatisfactory [3].

Chronic inflammation plays a critical role in carcinogenesis in various organs such as the lung, colon and liver [4]. Toll-like receptor 4 (TLR4), the receptor for lipopolysaccharide (LPS), is an important mediator of the host inflammatory response to infection and plays a role in the development and progression of various human cancers, including HCC [5–7]. LPS induces the interaction of TLR4 with adaptor molecule MyD88 and TAK1, which activates downstream MAPK and NF-κB signaling pathways and subsequently causes inflammatory mediators production [25,26]. Therefore, identifying endogenous molecules of TLR4-mediated inflammatory reaction in HCC and providing effective pharmacological intervention may probably offer possibilities to develop effective therapies in human HCC.

Tetramethylpyrazine (TMP) is the most important extract from the traditional Chinese herb *Chuanxiong*, which has been widely used in China for the treatment of various human cancers, including lung cancer [8], osteosarcoma [9], gliomas [10,11], ovarian carcinoma [12], breast cancer [13], gastric cancer [14] and HCC [15]. CXC195, a TMP analogue, was synthesized through replacing the methyl group of TMP with the (4,4′-difluoro)biphenylmethyl-1-piperazine methyl group of flunarizine and showed the strongest protective effects on H_2O_2-induced human umbilical vein endothelial cells through inhibition of the mitochondria- and caspase3- dependent pathway [16,17]. Moreover, CXC195 had a neuroprotective effect in transient focal ischemia, it is most likely due to its antioxidant activity and anti-apoptotic effect by inhibiting NADPH Oxidase and iNOS expression [18] and regulating PI3K/Akt/GSK3β pathway [19]. However, there is no report about the anti-cancer effect of CXC195 and its mechanism [12]. Despite evidence

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indicating the anti-cancer effect of TMP, there is a lack of data describing the anti-tumor activity of its analogue CXC195 on HCC. We therefore investigate the roles and explore the underlying mechanisms of CXC195 in LPS-induced HepG2 cells.

2. Materials and methods

2.1. Cell lines and culture

Human hepatocarcinoma HepG2 cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) containing 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 10 μg/ml streptomycin, at 37 °C and 5% CO_2. LPS was purchased from Sigma–Aldrich chemical. CXC195 (Fig. 1A) was synthesized by 2-chloromethyl-3,5,6-trimethylpyrazine hydrochloride directly reacting with the 4,4′-difluorobenzhydrylpiperazine. Its purity (>98%) was determined by high-performance liquid chromatography. The tested compounds and positive control were dissolved in DMSO.

2.2. Cell viability assay

Cell viability was assessed using the MTT assay. Spent medium was removed and 10 μl MTT solution (5 mg/ml) was added to 100 μl of respective growth medium without phenol red, and plates were incubated at 37 °C for 4 h in a humidified 5% CO_2 atmosphere. Then, formazan crystals formed by mitochondrial reduction of MTT were solubilized in DMSO (100 μl/well) and absorbance was read at 540 nm using a microplate reader (BioRad, Hercules, CA, USA). Percent inhibition of cytotoxicity was calculated as a fraction of control (with DMSO) and expressed as percentage of cell viability.

2.3. Determination of apoptotic HepG2 cells

Double staining for Annexin V-FITC and propidium iodide (PI) was performed to estimate the apoptotic rate of HepG2 cells. Briefly, HepG2 cells were cultured 48 h after treatment with LPS (10 ng/ml) in the presence or absence of 100 μM CXC195. Subsequently, HepG2 cells were trypsinized and washed twice with PBS, centrifuged at 800 rpm for 5 min. Then, 1×10^6 cells were suspended in binding buffer and double-stained with Annexin V-FITC and PI for 30 min at room temperature. After that, the fluorescence of each sample was quantitatively analyzed by FACS calibur flow cytometer and CellQuest software. The results were interpreted as follows: PI positive and Annexin V-FITC-positive stained cells were considered in apoptosis.

2.4. Measurement of the cell cycle

HepG2 cells were cultured 48 h after treatment with LPS (10 ng/ml) in the presence or absence of 100 μM CXC195. The cells were then treated with 20 μg/ml RNase A, followed by 25 μg/ml PI. The population of cells at each stage of the cell cycle was determined by examining the intensity of PI fluorescence with a flow cytometer using an argon laser and a 570 nm bandpass filter (FAC-Sort, Becton Dickinson).

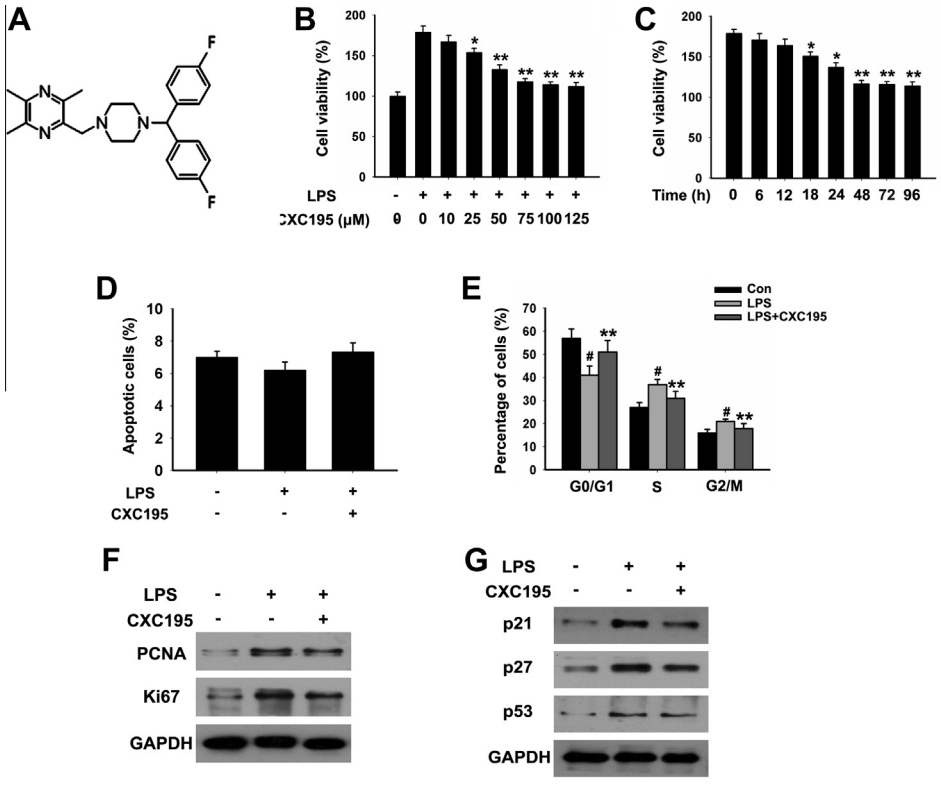

Fig. 1. Effects of CXC195 on the proliferation of LPS-induced HepG2 cells. (A) Chemical structures of CXC195. HepG2 cells were cultured 48 h after treatment with LPS (10 ng/ml) in the presence or absence of CXC195. Cell viability was assayed by the MTT method. (B) Effect of different doses of CXC195 on the cell viability of LPS-induced HepG2 cells. The control group set at 100%. (C) Effect of 100 μM CXC195 on the cell viability of LPS-induced HepG2 cells in a time-dependent manner. LPS induced HepG2 cells treated with 150 μM CXC195 for 0 h set at 100%. (D) Effect of 100 μM CXC195 on the apoptotic rate of LPS-induced HepG2 cells. Cells were analyzed by flow cytometry after staining with Annexin-V and PI. (E) Cell cycles were examined by flow cytometry. (F) PCNA and Ki67 proteins were detected by western blot analyses. (G) p21, p27 and p53 proteins were detected by western blot analyses. GAPDH was used to confirm equal protein loading. All data are presented as means ± SD ($n = 6$, $^{\#}P < 0.01$, significantly different from the control group; $^{*}P < 0.05$, $^{**}P < 0.01$ significantly different from the LPS-induced group).

2.5. Measurement of inflammatory mediators

Media was collected and centrifuged 48 h after treatment with LPS (10 ng/ml) in the presence or absence of CXC195. IL-6, IL-8, CCL-2, CCL-22, EGFR, HGF, TGFβ1 and TGFβ2 were measured by specific ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's protocol.

2.6. NF-κB assay

Nuclear extracts from treated HepG2 cells were prepared using the Nuclear Extract Kit (Active Motif, Carlsbad, CA). NF-κB activity was measured by a NF-κB p65 assay kit (Active Motif) according to the manufacturer's protocol.

2.7. Immunoprecipitation (IP)

To examine protein–protein interactions, HepG2 cells cultured were pretreated with 100 μM CXC195 for 48 h and then exposed to LPS for 90 min. The cells were lysed in 1 ml buffer consisting of 50 mM Tris HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 10 mM NaF, 1 mM Na_3VO_4, 10 g/ml leupeptin, 10 g/ml aprotinin and 20 mM PMSF after harvesting. Aliquots of the cellular lysates (containing 500 μg proteins) were incubated with proper primary anti-TLR4 and anti-MyD88 antibodies with rocking overnight at 4 °C. The immune complexes were allowed to bind to 40 μl of Recombinant Protein G Agarose beads (Invitrogen, USA) at 4 °C for 2 h, and the beads were washed three times with lysis buffer. The washed beads were resuspended in electrophoresis sample buffer and boiled for 10 min. After centrifugation, the supernatants were obtained as immunoprecipitates for western blot analysis.

2.8. Western blot analysis

Protein samples from the HepG2 cells extracts were separated by 8% or 10% SDS–PAGE and transferred to a nitrocellulose membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK). The membrane was blocked with 5% skim milk and incubated with primary antibodies, which were purchased from these companies: Santa Cruz (PCNA, Ki67, p21, p27, p53, TNF-α, iNOS, COX-2, IL-1β, GAPDH), Cell Signaling (p38, ERK, JNK, phospho-p38, phospho-ERK, phospho-JNK, IκB, phospho-IκB, TLR4, MyD88, TAK1 and phospho-TAK1). After washing with TBST, HRP-conjugated secondary antibodies (goat anti-rabbit IgG, Amersham Pharmacia Biotech; donkey anti-goat IgG, Santa Cruz Biotechnology) were applied. The blots were developed using ECL Western Blotting Detection Reagents (Amersham Pharmacia Biotech). Densitometry analysis of bands was performed with the Image Master™ 2D Elite software, version 3.1 (Amersham Pharmacia Biotech).

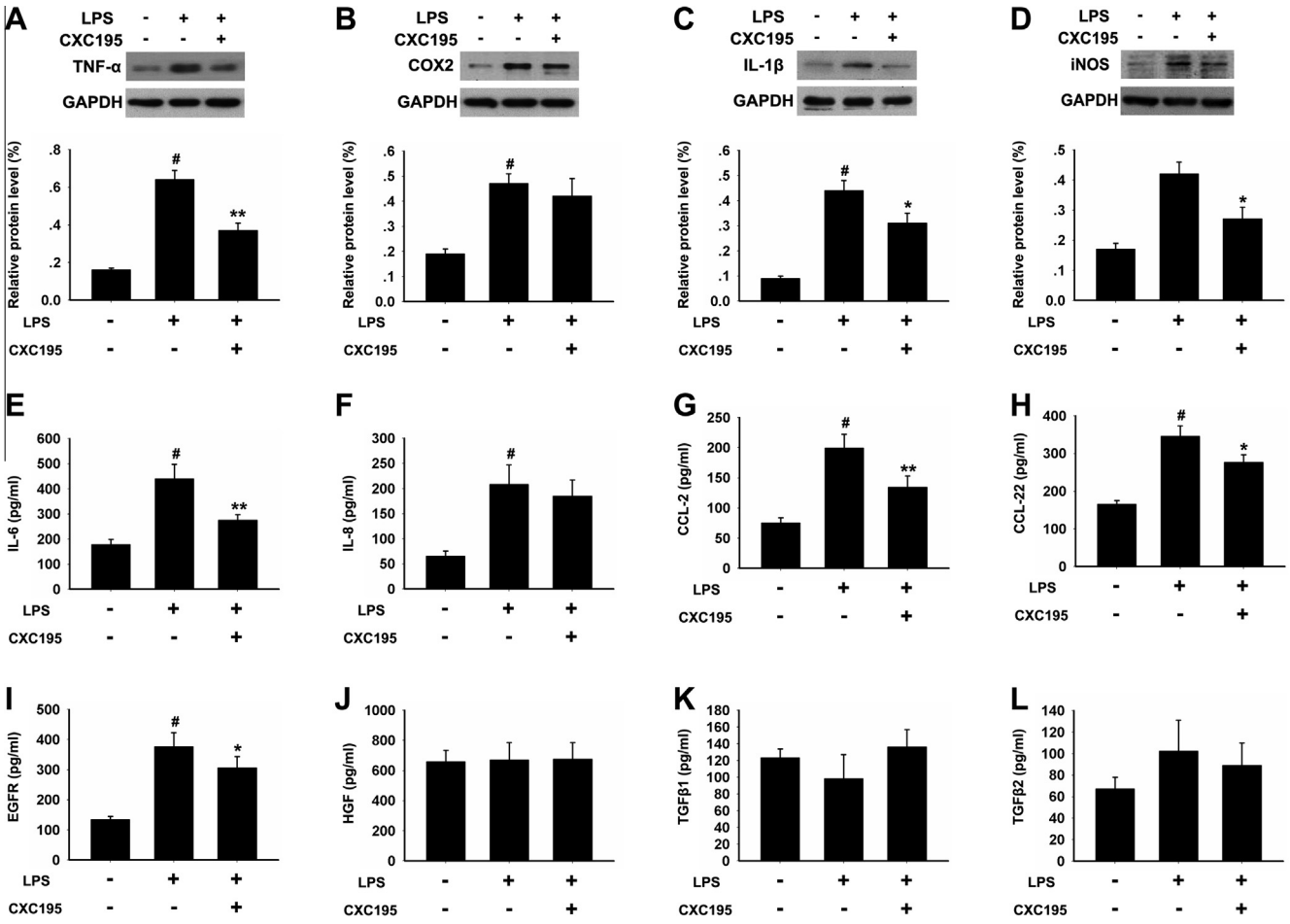

Fig. 2. Effects of CXC195 on the expressions of key inflammatory mediators in LPS-induced HepG2 cells. HepG2 cells were cultured 48 h after treatment with LPS (10 ng/ml) in the presence or absence of 100 μM CXC195. (A–D) TNF-α, iNOS, COX-2 and IL-1β proteins were detected by western blot analyses. GAPDH was used to confirm equal protein loading. (E–L) IL-6, IL-8, CCL-2, CCL-22, EGFR, HGF, TGFβ1 and TGFβ2 were measured by specific ELISA kit. All data are presented as means ± SD ($n = 6$, $^{\#}P < 0.01$, significantly different from the control group; $^{*}P < 0.05$, $^{**}P < 0.01$ significantly different from the LPS-induced group).

2.9. Statistical analysis

Statistical calculations of the data were performed using an unpaired Student's *t*-test and ANOVA analysis. Statistical significance was at $P < 0.05$.

3. Results

3.1. Effects of CXC195 on the proliferation of LPS-induced HepG2 cells

We first investigated the anti-proliferative effect of CXC195 on LPS-induced HepG2 cells at various concentrations (10, 25, 50, 75, 100 and 125 μM) and time points (6, 12, 18, 24, 48, 72, 96 h) by the MTT assay. As illustrated in Fig. 1B, cell viability induced by LPS in HepG2 cells was down-regulated gradually when treated with 25 and 50 μM CXC195 and maintained at lowest levels when pre-treated with 75–125 μM CXC195 for 48 h. Moreover, 100 μM CXC195 induced strong down-regulation of cell viability induced by LPS in a time-dependent manner, with minimum activation of cell viability detected at 48–96 h (Fig. 1B). Therefore, 100 μM CXC195 treated for 48 h was chosen for most of the subsequent experiments.

Annexin V/PI binding was used to evaluate the effect of CXC195 on the type of cell death. As shown in Fig. 1D, there were no apparent changes in apoptotic HepG2 cells among LPS or/and CXC195 treatments. Cell cycle analysis showed that LPS promoted cell cycle progression in HepG2 cells (decreased cells in G0/G1 phase and increased cells in S and G2/M phase) (Fig. 1E), which was partly abolished after 100 μM CXC195 treated for 48 h. We also found that CXC195 significantly inhibited expressions of cell proliferative markers (PCNA and Ki67; Fig. 1F) and elevated expressions of tumor suppressor genes (p21, p27 and p53; Fig. 1G) in LPS-induced HepG2 cells. These results indicated that CXC195 could inhibited the proliferation of LPS-induced HepG2 cells.

3.2. Effect of CXC195 on the expressions of key inflammatory mediators in LPS-induced HepG2 cells

In order to confirm that LPS-induced inflammatory mediators release could be inhibited by CXC195, we tested the TNF-α, COX-2, IL-1β and iNOS expression levels, which we employed as biological markers for inflammation. The western blot results showed that the expressions of inflammatory mediators were increased in LPS-induced HepG2 cells. Conversely, most of these markers were decreased after 100 μM CXC195 treated for 48 h (Fig. 2A, C and D). However, CXC195 treatment did not inhibit the protein expression of COX-2 in LPS-induced HepG2 cells (Fig. 2B). Moreover, Our ELISA results indicated that the expression levels of IL-6 (Fig. 2E), IL-8 (Fig. 2F), CCL-2 (Fig. 2G), CCL-22 (Fig. 2H) and EGFR (Fig. 2I) that were abundant in cells at sites of inflammation were significantly increased in LPS-induced HepG2 cells. LPS treatment did not induce the expressions of HGF (Fig. 2J), TGFβ1 (Fig. 2K) and TGFβ2 (Fig. 2L) in HepG2 cells. CXC195 treatment reduced the expressions of IL-6 (Fig. 2E), CCL-2 (Fig. 2G), CCL-22 (Fig. 2H) and EGFR (Fig. 2I) in LPS-induced HepG2 cells. However, CXC195 treatment did not inhibit the expressions of IL-8 (Fig. 2F), HGF (Fig. 2J), TGFβ1 (Fig. 2K) and TGFβ2 (Fig. 2L) in LPS-induced HepG2 cells. These findings suggested that CXC195 could inhibit the activation of the inflammatory cascade in LPS-induced HepG2 cells.

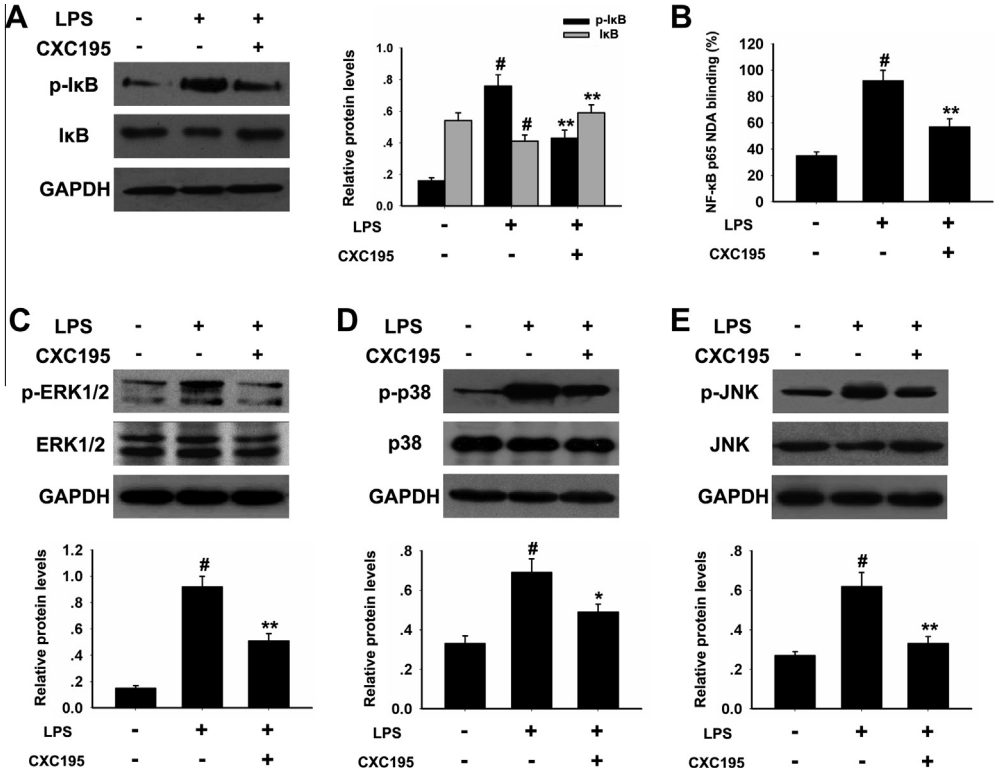

Fig. 3. Effects of CXC195 on the activation of NF-κB and MAPK pathway in LPS-induced HepG2 cells. After pre-treated with 100 μM CXC195 for 48 h, HepG2 cells were treated with LPS (10 ng/ml) for 90 min. (A) Protein levels of IκB and p-IκB were evaluated by western blot analysis. GAPDH was used to ensure equal loading. Densitometric analysis of effects of CXC195 on expressions of IκB and p-IκB. (B) Nuclear extracts were prepared by using a nuclear extract kit. NF-κB activity was measured using an ELISA kit. Effect of CXC195 on the protein expressions of p-JNK (C), p-ERK (D) and p-p38 (E) were examined by western blot analysis. Densitometric analysis of effects of CXC195 on expressions of p-JNK, p-ERK and p-p38. Data were shown as mean ± SEM ($n = 6$, $^{\#}P < 0.01$, significantly different from the control group; $^{*}P < 0.05$, $^{**}P < 0.01$ significantly different from the LPS-induced group).

3.3. Effect of CXC195 on activation of NF-κB and MAPK pathway in LPS-induced HepG2 cells

NF-κB is an important nuclear transcription factor, which initiates transcription of genes associated with inflammation in HepG2 cells. NF-κB is inactivated in the cytosol by binding to IκB, and becomes active through translocation to the nucleus preceded by LPS-induced proteolytic degradation of IκB. As shown in Fig. 3A, IκB was phosphorylated and degraded 90 min after LPS treatment. Pre-treatment of HepG2 cells with 100 μM CXC195 for 48 h decreased to phosphorylation of IκB in response to LPS, indicating that the subsequent NF-κB inactivation induced by CXC195. Moreover, the effects of CXC195 on NF-κB activity were investigated using an NF-κB ELISA kit analysis. As shown in Fig. 3B, LPS significantly enhanced the DNA binding activity of nuclear NF-κB p65 in HepG2 cells. The increase in NF-κB activity was significantly decreased by pre-treating HepG2 cells with 100 μM CXC195 for 48 h.

To further investigate the signaling pathways involved in the mechanism in LPS-induced HepG2 cells, we assessed activation of the MAPK pathway (ERK1/2, p38, and JNK) in HepG2 cells. As shown in Fig. 3C–E, LPS treated for 90 min strongly induced phosphorylation of ERK1/2, p38, and JNK. Pre-treatment with 100 μM CXC195 for 48 h reduced expressions of p-JNK, p-ERK1/2 and p-p38 in LPS-induced HepG2 cells. These results revealed that CXC195 inhibited the activation of NF-κB and MAPK pathways, which might be involved in the inhibition of proliferation and inflammatory mediators release in LPS-induced HepG2 cells.

3.4. Effect of CXC195 on TLR4 expression and its interactions with MyD88 or TAK1 in LPS-induced DRG neurons

TLR4 is a key regulators involved in regulating the LPS-induced inflammatory mediators expression through the activation of NF-κB and MAPK pathway. HepG2 cells cultured were pre-treated with 100 μM CXC195 for 48 h and then exposed to LPS for 90 min. As shown in Fig. 4A, pre-treatment with CXC195 reduced the protein expression of TLR4, which was induced by LPS in HepG2 cells.

The interaction of TLR4 with adaptor molecule MyD88 and TAK1 is critical for TLR4 to activate downstream signaling pathways and induce inflammatory response, so the effect of CXC195 on interactions of TLR4 with its adaptor molecules was investigated. As shown in Fig. 4B, LPS stimulation of HepG2 cells for 90 min caused an increase in MyD88 and p-TAK1 expressions. 100 μM CXC195 pre-treated for 48 h exhibited a statistically inhibitory effect on the increased expressions of MyD88 and p-TAK1. Moreover, as shown in Fig. 4C, the formation of TLR4/MyD88 complex significantly increased after LPS stimulation. Pre-treated with CXC195 showed a reduction in the intensity of the MyD88 band co-immunoprecipitated using anti-TLR4 antibody compared to LPS induced group. Furthermore, the increased ligand association of TLR4 with TAK1 was detected after stimulation with LPS. CXC195 significantly attenuated the LPS-stimulated formation of TLR4/TAK1 complex. Likewise, the reverse co-immunoprecipitation of TLR4 using the MyD88 antibody was also diminished in HepG2 cells pre-treated with CXC195 compared to LPS induced group

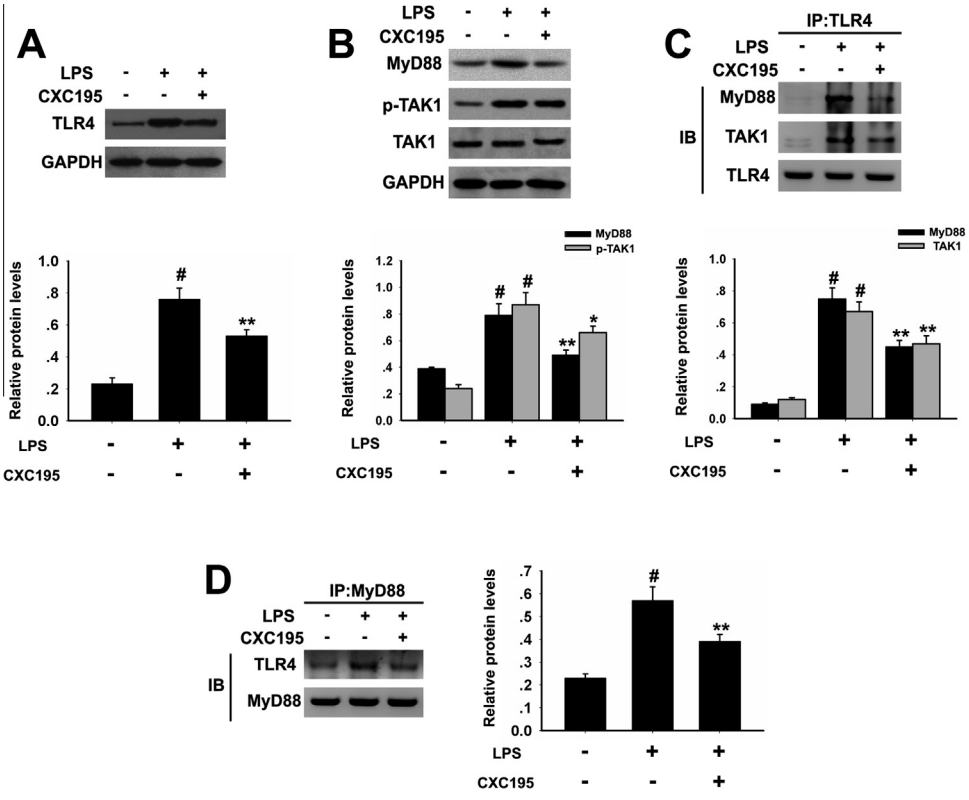

Fig. 4. Effects of CXC195 on TLR4 expression and its interactions with MyD88 or TAK1 in LPS-induced HepG2 cells. After pre-treated with 100 μM CXC195 for 48 h, HepG2 cells were treated with LPS (10 ng/ml) for 90 min. (A) Protein level of TLR4 was evaluated by western blot analysis. Densitometric analysis of effects of CXC195 on expressions of TLR4. (B) Protein levels of MyD88 and p-TAK1 were evaluated by western blot analysis. Densitometric analysis of effects of CXC195 on expressions of MyD88 and p-TAK1. (C) The complexes of TLR4/MyD88 and TLR4/TAK1 were precipitated by antibody against TLR4 first and then analyzed by western blot analysis for MyD88 and TAK1. The protein levels of TLR4, MyD88 and TAK1 in whole cell lysate were analyzed by western blot analysis. Densitometric analysis of effects of CXC195 on interactions of TLR4 with MyD88 and TAK1. (D) The complexes of TLR4/MyD88 were precipitated by antibody against MyD88 first and then analyzed by western blot analysis for TLR4. The protein levels of TLR4 and MyD88 in whole cell lysate were analyzed by western blot analysis. Densitometric analysis of effects of CXC195 on interactions of MyD88 with TLR4. Data were shown as mean ± SEM. GAPDH was used to ensure equal loading ($n = 6$, $^{\#}P < 0.01$, significantly different from the control group; $^{*}P < 0.05$, $^{**}P < 0.01$ significantly different from the LPS-induced group).

(Fig. 2G and H). These data suggested that CXC195 could inhibit activation of TLR4-MyD88-TAK1 pathway, which might contribute to its effects against proliferation and inflammatory mediators release in LPS-induced HepG2 cells.

4. Discussion

Chronic inflammation of the liver is a well-recognized risk factor for carcinogenesis [4], 80% of all cases of HCC being associated with cirrhosis or fibrosis, which are mainly characterized by persistent cycles of liver injury, inflammation, and compensatory hepatocyte proliferation [2]. Inflammation depends largely on gene expression and shares key regulators. The activation of MAPK pathway, such as ERK1/2, p38 and JNK, plays a critical role in progression of HCC [20,21]. In our present study, LPS induced the phosphorylation of ERK1/2, p38 and JNK in HepG2 cells, which was reduced by pre-treated with 100 μM CXC195 for 48 h. NF-κB is another pro-inflammatory transcription factor that controls transcription of genes involved in cell proliferation and cell survival. Constitutive expression of NF-κB is an emerging hallmark of cancer. In fact, constitutive NF-κB activation is generally associated with cancer proliferation, survival, chemoresistance and progression of HCC [22–24]. Thus, NF-κB emerges as an important target for developing adjuvant cancer treatment. Our present study showed that CXC195 significantly suppressing NF-κB DNA binding activity and reduces degradation of IκB and thereby inhibits NF-κB pathway in LPS-induced HepG2 cells.

TLR4 is a member of the Toll-like receptor family of pattern recognition receptors that specifically mediates signaling by LPS. Classically, TLR4 recognizes the microbial lipids in homodimer format, and thus activates various intracellular signaling pathways, such as NF-κB and MAPK pathway. TLR4 have now been identified in HCC and may play a role in progression of HCC [7]. LPS-induced activation of TLR4 signaling promoted HCC cells survival and proliferation, which was associated with regulation on the activation of NF-κB and MAPK pathways [7]. Animal models in vitro showed that inactivating TLR4 had no effect on HCC tumor incidence, but significantly reduced tumor number and size. By contrast, continuous administration of low doses of LPS increased tumor number and size [5]. Therefore, targeting TLR4 provides a promising intervention strategy to reduce the expression of inflammatory mediators induced by these pathologies. As mentioned above, we found that the protein level of TLR4 were up-regulated after LPS-induction, the pre-treatment with CXC195 reduced the expression of TLR4 in LPS-induced HepG2 cells, which might be a reason for its anti-inflammatory effects.

Activation of TLR4 signaling at the plasma membrane by LPS stimulated NF-κB and MAPK pathways through the formation of MyD88-IRAK-TRAF6-TAK1 signaling complex [25]. TLR4 is unique among the TLRs because of its ability to combine MYD88 adapters, which results in activation of NF-κB and consequently production of pro-inflammatory cytokines [26]. TAK1 was recruited to TLR4 after stimulation with LPS, after that it dissociated from the receptor presumably to bifurcate the signal into NF-κB and MAPK pathways. Therefore, MyD88 and TAK1 serve as the key TLR4 adaptor protein, linking the receptors to downstream kinases. In the present study, CXC195 decreased the protein level of MyD88 and p-TAK1. Moreover, CXC195 also inhibited the formation of the complexes of TLR4 with MyD88 and TAK1, which indicated that CXC195 could disturb the association of TLR4 with its adaptors (MyD88 and TAK1), leading to inactivation of TLR4 in LPS-induced HepG2 cells.

In conclusion, our study firstly confirmed anti-proliferative and anti-inflammatory effects of CXC195 on LPS-induced HepG2 cells through inhibiting the activation of TLR4-MyD88-TAK1 mediated NF-κB and MAPK signaling pathway. Further studies are required to explore anti-cancer of CXC195 in the animal model of HCC.

Conflict of interest

The authors declare no conflict of interest.

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