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Ulinastatin suppresses lipopolysaccharide induced neuroinflammation through the downregulation of nuclear factor-κB in SD rat hippocampal astrocyte

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ABSTRACT

Astrocyte activation plays a pivotal role in neuroinflammation, which contributes to neuronal damage, so the inhibition of astrocyte activation may alleviate the progression of neurodegeneration. Recent studies have proved that urinary trypsin inhibitor ulinastatin could inhibit NF-kB activation. In our study, the inhibitory effects of ulinastatin on the production of pro-inflammatory mediators were investigated in lipopolysaccharide (LPS)-reduced primary astrocyte. Our results showed that ulinastatin significantly inhibited LPS-induced astrogliosis, which is measured by MTT and BrdU. Ulinastatin decreased the production of pro-inflammatory cytokines, such as TNF-α, IL-6, IL-1β, it significantly decreased both the mRNA and the protein levels of these pro-inflammatory cytokines and also increased the protein levels of IκB-α binded to NF-κB, which blocked NF-κB translocation to the nucleus and prevented its activity. Our results suggest that ulinastatin is able to inhibit neuroinflammation by interfering with NF-κB signaling. The study provides direct evidence of potential therapy methods of ulinastatin for the treatment of neuroinflammatory diseases.

1. Introduction

Neuroinflammation is characterized by the activation of microglia and astrocyte, which has been implicated in the pathogenesis of neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease and multiple sclerosis [1,2]. Astrocytes are the major glial cell within the central nervous system (CNS) and have a number of important physiological properties related to CNS homeostasis [3]. Astrocytes participate in all essential CNS functions, including blood flow regulation, energy metabolism, ion and water homeostasis, immune defence, neurotransmission, and adult neurogenesis [4]. Astrocytes respond to all forms of CNS insults through a process referred to as reactive astrogliosis, which has become a pathological hallmark of CNS structural lesions. Increasing evidence points towards the potential of reactive. Astrogliosis to play either primary or contributing roles in CNS disorders via loss of normal astrocyte functions or gain [5]. Activated astrocyte can both secrete and respond to a number of important cytokines affecting the cellular state of both surrounding cells such as microglia and neurons as well as astrocytes themselves. For example, cytokines such as IL-1β, TNF-α, IL-6 and transforming growth factor-β1 (TGF-β1) can act to up- or down-regulate other pro- and anti-inflammatory genes including nitric oxide synthase-2 (NOS-2) and cyclooxygenase-2 (COX-2) [6–8]. The transcription factor, nuclear factor kappa B (NF-κB), has been shown to control inflammatory responses in microglial cells. Activation of NF-κB is triggered by phosphorylation and subsequent degradation of inhibitor of κB (IκB). This process subsequently leads to translocation of the free NF-κB to the nucleus where it promotes the expression of proinflammatory genes such as the proinflammatory [9].Urinary trypsin inhibitor (UTI) is one of the Kunitz-type protease inhibitors found in urine [10].With its anti-protease and anti-inflammatory effects, UTI has been widely used as a drug for patients with acute inflammatory disorders, such as disseminated intravascular pancreatitis, shock, and coagulation [11]. Earlier, we showed that Ulinastatin produces *in vivo* anti-inflammatory effects [12]. Although the anti-inflammatory effects of Ulinastatin in the periphery have been documented, its effects on cells in the CNS, specifically the hippocampal astrocyte *in vitro*, are not known.

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In the present study, we sought to investigated the effects of UTI on lipopolysaccharide (LPS)-stimulated pro-inflammatory responses in astrocyte and the signaling mechanism by which Ulinastatin modulates the pro-inflammatory response.

2. Material and methods

2.1. Chemicals and reagents

2.1.1. Cell cultures

Sprague–Dawley rats were obtained from the Experimental Animal Center of Capital medical University (Beijing, China).All the experimental procedures rats in this study were carried out according to the National Institutes of Health Guide for Care and the use of Laboratory Animals and were approved by the Bioethics Committee of Capital Medical University. Primary astrocyte of hippocampus were cultured from postnatal day 4 Sprague–Dawley rats as previously described [13]. Briefly, by removing the meninges and the blood vessels, hippocampus was collected with ice-cold calcium/magnesium free HBSS at pH 7.4.The tissue was then minced and trypsinized (trypsin-EDTA 0.25%) for 5 min 37 °C. 5 min later the solution was neutralized with DMEM/F12 medium containing 10% fetal bovine serum (FBS) and penicillin-streptomycin solution, then the cells were filtered through a mesh bag. After centrifuging at 1000 rpm for 5 min, the tissues were resuspended in DMEM/F12 containing 10% FBS, 100 U/ml penicillin and 100 mg/ml streptomycin at 37 °C in the humidified atmosphere of 5% CO_2 and 95% air. The cultures were refreshed with DMEM/F12 medium containing 10% fetal bovine serum (FBS) and penicillin-streptomycin solution twice a week. 7–8 days later, astrocytes were separated from microglia and oligodendrocyte by shaking for 18 h (200 rpm 37°). Isolated astrocytes were cultured on 6-well plates at a density of 2×10^5 cells/well, and cultures with >95% purity of astrocyte (identified by GFAP antibody) were used. The cells can be used for drug treatment 2 days later.

2.1.2. Cell viability assay

Cell viability was evaluated by the tetrazolium salt 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT, Sigma–Aldrich) assay. Briefly, cells were seeded in 96-well plates at the concentration of 1×10^5 cells/well. After 24 h incubation, cells were preadministrated with various concentrations of UTI (400 U/ml) for 1 h, then treated with or without LPS (1 μg/mL) for 24 h followed by the incubation of MTT solution (0.5 mg/mL) to each well for 4 h.200 μL DMSO was added to each well, the solubilized formazan products were performed at a test wavelength of 570 nm. Each experiment was performed in triplicate.

2.1.3. BrdU incorporation assay

BrdU staining was conducted using a 5-Bromo-2′-deoxy-uridine Labeling and Detection Kit I (Roche) according to the manufacture's instructions. BrdU from the Kit I was added to the culture wells for 24 h incubation, prior to fixation with 70% ethanol in 50 Mm glycine (PH 2.0). After washing with PBS three times, the wells were incubated with anti-BrdU-FLUOS antibody and DAPI for 30 min at 37 °C. Images were collected using a fluorescent microscope.

2.1.4. Immunofluorescence

Immunofluorescence was performed as previous described [14]. To determine level of GFAP, we performed immunofluorescence assay in the cultured astrocyte. The cells were incubated to primary rabbit polyclonal antibody for GFAP (1/1000, Millipore Inc. Cambridge, MA, USA) overnight at 4 °C. After being washed with ice-cold PBS followed by treatment with an anti -mouse secondary antibody labeled with Alexa Flour 488 (1/100 dilution, Molecular Probes, Inc, Eugene, OR, USA) for 2 h at room temperature, immunofluorescence images were acquired with a fluorescence pictures taken on a Leica TCS SP2 scanning confocal microscope.

2.1.5. Real-time PCR assay

Total RNA was extracted by Trizol (Invitrogen, Paisley, UK) from induced cell cultures. The ABI Primer Express software had been used to design the PCR primers. The primer sequences were TNF-α:GGGCAGGTCTACTTTGGAGTCATTG (F), GGGCTCTGAGGAGTAGACGATAAAG(R);IL-1β:CCCAACTGGTACATCAGCACCTCTC(F),CTATGTCCCGACCATTGCTG(R);IL-6:GATTGTATGAACAGCGATGATGC(F),-AGAAACGGAACTCCAGAAGACC(R);GAPDH:TGGAGTCTACTGGCGTCTT(F),TGTCATATTTCTCGTGGTTCA(R).We quantified the PCR amplifications using SYBR Green PCR Master Mix and the relative mRNA expression by cycle time(Ct) values, normalized with GAPDH gene expression.

2.1.6. Western blot analysis

Protein samples extracted from astrocyte were separated by 8 or 10% SDS-PAGE and transferred to polyvinylidene difluoride membrane (Amersham Pharmacia Biotech, UK). The membrane was incubated with 5% non-fat milk in Tris-buffered saline with Tween (TBS-T) for 2 h, probed with primary antibody against IL-1β, TNF-α, IL-6,NF-kB (Abcam, Cambridge, MA, UAS) at 4 °C overnight then incubated with the horseradish peroxidase-conjugated secondary antibodies for 2 h at room temperature. Immunoreactive bands were detected with an enhanced ECL (GE).

2.1.7. Enzyme linked immunosorbent assay for proinflammatory cytokine measurement

Cells (1×10^6) were seeded in 24-wells plates, the supernatants were collected for measurement of TNF-α, IL-1βand IL-6 using the enzyme-linked immunosorbent assay (ELISA) kits from R&D Systems.

2.1.8. Statistical analysis

Statistical analyses were performed using SPSS 13.0. Data are presented as mean ± S.E.M. The difference between means was determined by one-way ANOVA followed by a Student–Newman–Keuls test for multiple comparisons. A probability value of $P < 0.05$ was taken to be statistically significant.

3. Results

3.1. UTI antagonized astrocyte overactivation and proliferation caused by LPS

The first step astrocyte respond to external stimuli is presented as astrocyte activation excessive activation as astrogliosis. To investigate the effect of ulinastatin on LPS-induced astrocyte activation, we measure the expression of GFAP by western blot. In our study, as showed in Fig. 1A control cells expressed weak band ,however, it expressed at high level following stimulation with 1.0 μg/ml LPS for 24 h, shown as 4.98 expression fold compared to the control group (**P < 0.01, Fig. 1B). Pretreatment of astrocyte with 400 U/ml, 800 U/ml for 1 h prior to LPS stimulation led to a corresponding reduction in GFAP expression, as showed in Fig. 1A, the protein band was weakened, while proved by the statistical results there is no significant difference versus LPS group ($P > 0.05$ vs. LPS group, Fig. 1B), the GFAP expression was still maintained at a high level. However, 1600 U/ml ulinastatin could significantly reduce the expression of GFAP ($^{@}P < 0.05$ vs. LPS group, Fig. 1B). Meanwhile, we noticed that even 1600 U/ml ulinastatin didn't recover the GFAP expression to normal level ($^{\#\#}P < 0.01$ for LPS/400 U/ml group and $^{\#}P < 0.05$ for LPS/800 U/ml and LPS/1600 U/ml

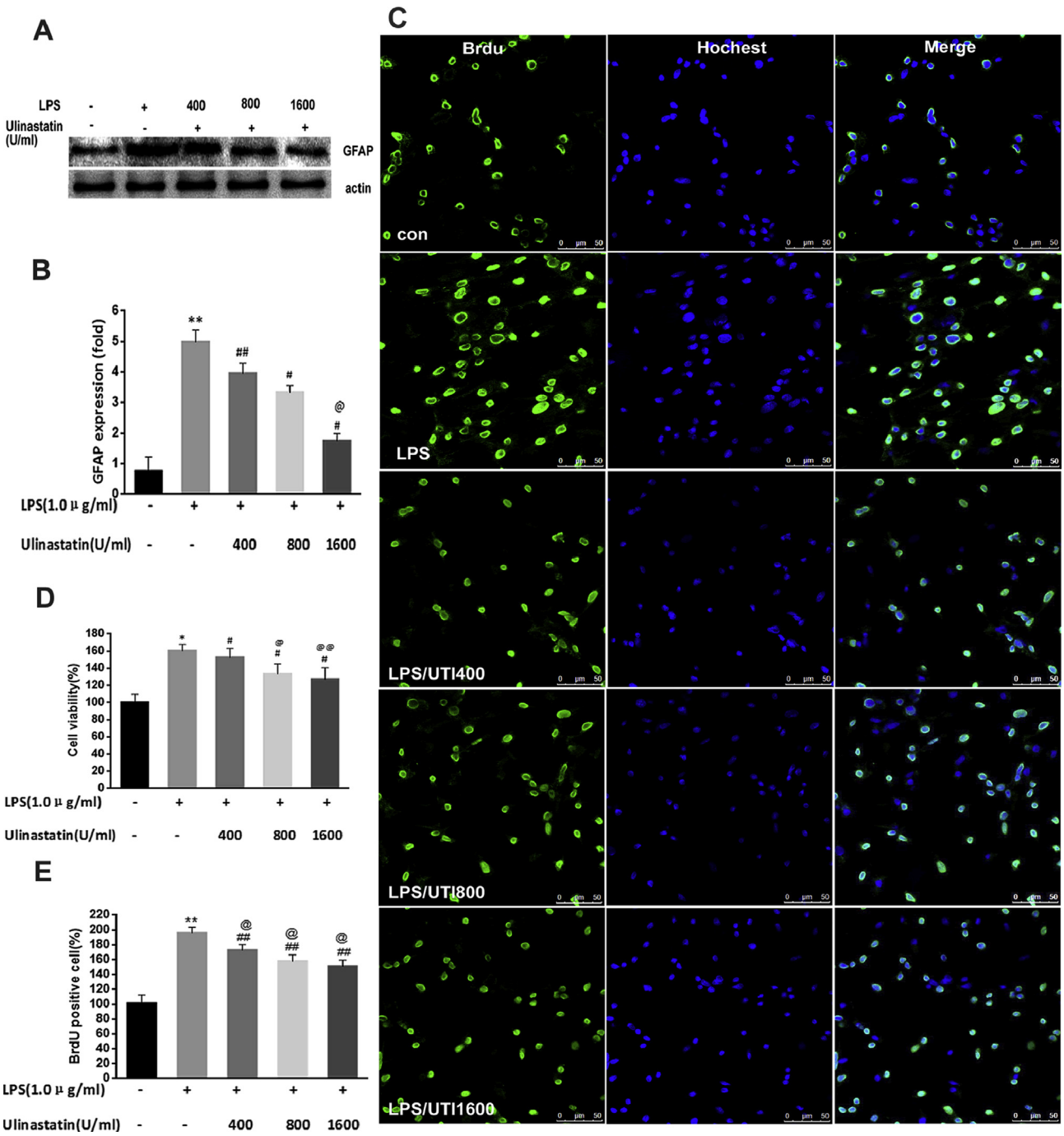

Fig. 1. Ulinastatin inhibited GFAP overexpression and proliferation in astrocytes increased by LPS. A. Representative western blot showing the expression of GFAP. B. Quantification of the expression of GFAP. Astrocytes in the control group were incubated in standard culture medium without ulinastatin and LPS. Astrocytes in LPS, LPS + UTI400 U/ml, LPS + UTI800 U/ml, LPS + UTI1600 U/ml, respectively. Results are mean ± S.E.M (n = 5). $^{**}P < 0.01$ versus control group, $^{@}P < 0.05$, $^{@@}P < 0.01$ versus LPS group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus control group. C. The immunofluorescence of BrdU positive (green), Co-staining with DAPI (blue) allowed the identification of nuclear. D. The viability was examined by MTT assay. E. Data of BrdU positive cells were presented as percentage of the control group. Astrocytes in the control group were incubated in standard culture medium without UTI and LPS. Astrocytes in LPS, LPS/UTI400 U/ ml, LPS/UTI800 U/ml, LPS/UTI1600 U/ml were treated with LPS only, LPS + UTI400 U/ml, LPS + UTI800 U/ml, LPS + UTI1600 U/ml, respectively. Results are mean ± S.E.M of five different independent experiments. $^{**}P < 0.01$ versus control group, $^{@}P < 0.05$, $^{@@}P < 0.01$versus LPS group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus control group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

group vs. control group, Fig. 1B).This results indicate that ulinastatin has an inhibitory effect on astrocyte activation, but just partly antagonized astrocyte overactivation induced by LPS, there might be other regulatory molecules involved in LPS-induced astrocyte inflammation.

To investigate the effect of ulinastatin on LPS-induced astrocyte *in vitro*, we firstly tested whether ulinastatin affected cell growth. MTT assay displayed treatment with LPS (1.0 μg/ml) induced 52.33% increase in cell proliferation ($^{**}P < 0.01$ vs. control group, Fig. 1D). 400 U/ml UTI reduced the proliferation rate to 48.17%, without

statistical difference compared to LPS group ($P < 0.05$, Fig. 1D). With the increase of UTI concentration, 800 U/ml and 1600 U/ml further reduced the cell proliferation to 33.83% and 22.50%, respectively, with significant statistical difference compared to LPS group ($^{@}P < 0.05$ for LPS/UTI800 U/ml group and $^{@@}P < 0.01$ for LPS/UTI1600 U/ml group, Fig. 1D). At the same time, we noticed even when UTI concentration reached to 1600 U/ml, significant difference was still remained versus control group ($^{\#\#}P < 0.01$ for LPS/UTI1600 U/ml group, Fig. 1D).

We used bromodeoxy-uridine (BrdU) staining to monitor astrocyte proliferation, which can be incorporated into newborn proliferating cells. As showed in Fig. 1C, the ratio of Hochest/GFAP in the merged images was calculated as the incorporation rate of BrdU. In the present study, it was demonstrated that there was a significantly increased in the percentage of BrdU positive cells, ($^{**}P < 0.01$, Fig. 1C,E). LPS/400, 800, 1600 U/ml treatment also significantly reduced the percentage of BrdU-positive cells ($^{@}P < 0.05$), However, UTI could partly reverse astrocyte over-proliferation induced by LPS ($^{\#\#}P < 0.01$ *vs.* control group). All the above findings were demonstrated that UTI inhibited astrocyte activation and proliferation induced by LPS in a concentration dependence.

3.2. Ulinastatin inhibits the expression of cytokines in LPS-stimulated primary rat astrocyte

It had demonstrated that UTI inhibited TNF-α induced by LPS and significantly reduced TNF-α expression in a dose-dependent manner in rat lung tissue [15]. To elucidate the potential effect of UTI on proinflammatory cytokine in LPS-stimulated primary rat astrocyte, in this study, we measured the expression of IL-1β, TNF-α, IL-6 by Immunofluorescence western blot, ELISA and RT-PCR, as showed in Fig. 2. The secretion level of IL-1β, TNF-α, IL-6 were significantly increased following exposure of astrocyte to LPS 1.0 μg/ml for 24 h ($^{*}P < 0.01$ *vs.* control group,). The secretion level of IL-6 and IL-1β from astrocyte were significantly reduced by UTI 400 U/ml ($^{@}P < 0.01$ and $^{@}P < 0.05$ *vs.* LPS group, respectively), while had no significant effect on TNF-α($P > 0.05$ *vs.* LPS group). All proinflammatory cytokines by astrocyte were significantly decreased by UTI 800 U/ml ($^{@@}P < 0.01$ *vs.* LPS group). These results indicated that UTI can effectively down-regulate the expression of IL-1β, TNF-α, IL-6 of LPS-stimulated primary astrocyte.

3.3. Inhibition of LPS-induced NF-kB activation by ulinastatin

NF-kB has been demonstrated to play an essential role in the LPS-induced expression of both pro-inflammatory cytokines. We performed Immunofluorescence, western blot and ELISA to investigate whether ulinastatin can inhibit NF-kB activation in LPS-stimulated primary astrocyte. As showed in Fig. 3, ulinastatin led to expression of NF-κB significantly decreased in concentration dependence.

In our study, we determined the protein levels of IκB-α by western blot. Because the activation of NF-kB was depended on the IκB-α degradation in cytoplasm, the level of IκB-α in LPS-induced astrocytes was significantly decreased (Fig. 3G LPS group vs.

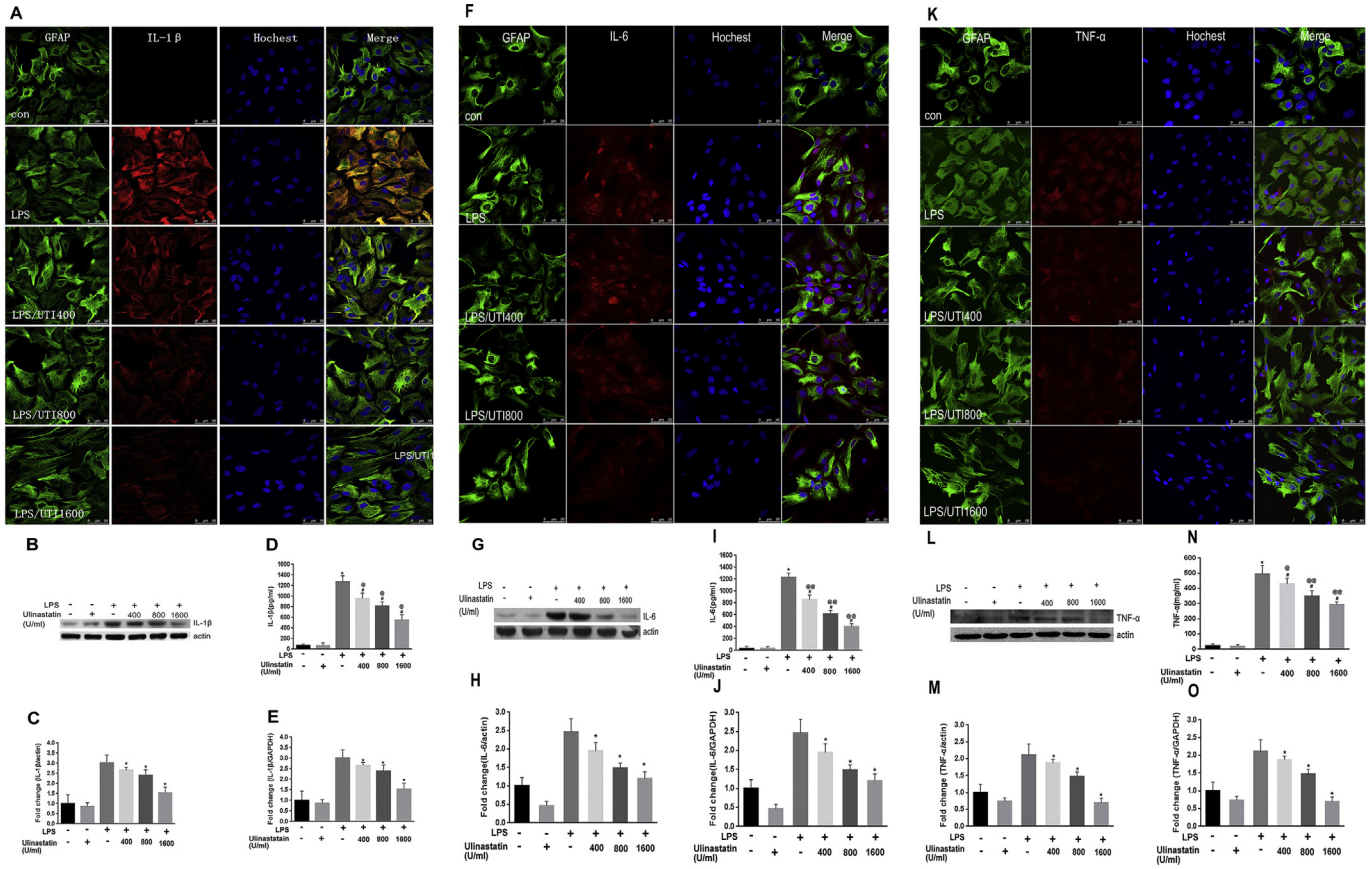

Fig. 2. Ulinastatin inhibits pro-inflammatory cytokines in LPS-induced primary astrocyte. A, F, K. The immunofluorescence of GFAP (green), Hochest (blue), IL-1β, IL-6, TNF-α(red) and merge. B, G, L. Western blot of IL-1β,IL-6,TNF-α. C, H, M. Quantification of the expression of IL-1β,IL-6,TNF-α. D, I, N. ELISA for IL-1β, IL-6, TNF-α. E, J, O. The mRNA levels of IL-1β, IL-6, TNF-α. The data are presented as the means ± S.E.M (n = 5) for three independent experiments. Results are mean ± S.E.M of five different independent experiments. $^{**}P < 0.01$ versus control group; $^{@}P < 0.05$, $^{@@}P < 0.01$versus LPS group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus control group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

control group:0.37 ± 0.08, $^{*}P < 0.05$). Pretreatment with ulinastatin before primary astrocyte exposed to LPS resulted in significant increase in the cytoplasm level of IκB-α (Fig. 3F LPS + ulinastatin group *vs.* LPS group: 1.08 ± 0.13, $^{**}P < 0.01$), while the protein level of phosphorylated IκB-α in nucleus was significantly reduced (Fig. 3G LPS + ulinastatin group vs. LPS group: 1.27 ± 0.08 vs. 2.00 ± 0.11, $^{**}P < 0.01$ or $^{*}P < 0.05$). In order to investigate whether or not ulinastatin can inhibit the NF-kB activity, we compared results by ulinastatin with PDTC, which is a specific inhibitor of NF-κB. The results in Fig. 3 indicated that ulinastatin (1600 U/ml) alone had no effect on the IκB-α degradation and phosphorylation in cytoplasm of primary astrocyte (Fig. 3F. 0.99 ± 0.08, Fig. 3G. 0.97 ± 0.09). However, it can prevent LPS-induced degradation and phosphorylation of IκB-α in cytoplasm (Fig. 3F. 1.17 ± 0.07, $^{**}P < 0.01$, Fig. 3G. 1.20 ± 0.05, $^{**}P < 0.01$). Ulinastatin inhibited NF-κB activity as PDTC (300 mM) in similar degree (Fig. 3A–G).All above results have demonstrated that ulinastatin can effectively inhibit LPS-induced NF-κB activation by preventing IκB-α degradation and phosphorylation in primary astrocytes *in vitro*.

3.4. The effects of ulinastatin on LPS-induced pro-inflammatory mediators in different time course

In order to supply clinical clue for the time to preadministrate ulinastatin, in this study, exposure of cells to 1.0 μg/ml LPS significantly increased the secretion of IL-1β, TNF-α, IL-6 at 8 h, 16 h,24 h ($^{***}P < 0.001$ vs. control group), we pretreated astrocyte with ulinastatin at the concentration of 800 U/ml induced by LPS 1 μg/ml for 8 h, 16 h, 24 h. We measure the expression of IL-1β, TNF-α, IL-6 by Immunofluorescence, western blot, RT-PCR as showed in Fig. 4, cytokines decreased significantly ($^{***}P < 0.001$). These suggested that UTI negatively regulates the expression of IL-1β, TNF-α, IL-6 in LPS-stimulated primary astrocyte and better to preadministrate as early as possible. It has been demonstrated that neutrophil protease release, excessive inflammatory responses, reduce the release of oxygen free radicals and consumption of superoxide dismutase can be inhibited following by early administration of UTI [16].

4. Discussion

There is increasingly evident that neuroinflammatory mechanisms are implicated in the pathogenesis and progression of neurodegenerative disorders like AD. What's more, some evidence has demonstrated microglia and astrocytes were involved in sustained inflammatory responses in animal models of neurodegeneration [17]. From this point of view, it is very important to prevent the occurrence of neuroinflammation through the development of therapeutic strategies. During screening anti-inflammatory drug, ulinastatin attracted our attention by its anti-inflammatory characteristics and broad application in clinical practice. In this study, we focused on the effect of ulinastatin against neuroinflammation of astrocytes induced by LPS from hippocampus. To the best of our knowledge, the present study was possibly first to demonstrate that ulinastatin can inhibit the neuroinflammatory activation of astrocyte in hippocampus induced by LPS *in vitro*. In LPS-activated primary astrocyte *in vitro*, ulinastatin can significantly decrease the activation of astrocyte, astrogliosis and inflammatory cytokines, such as IL-1β, IL-6, TNF-α production, and the RT-PCR analysis showed that ulinastatin markedly suppressed the gene expression of IL-1β, IL-6, TNF-α. The anti-inflammatory properties of ulinastatin are

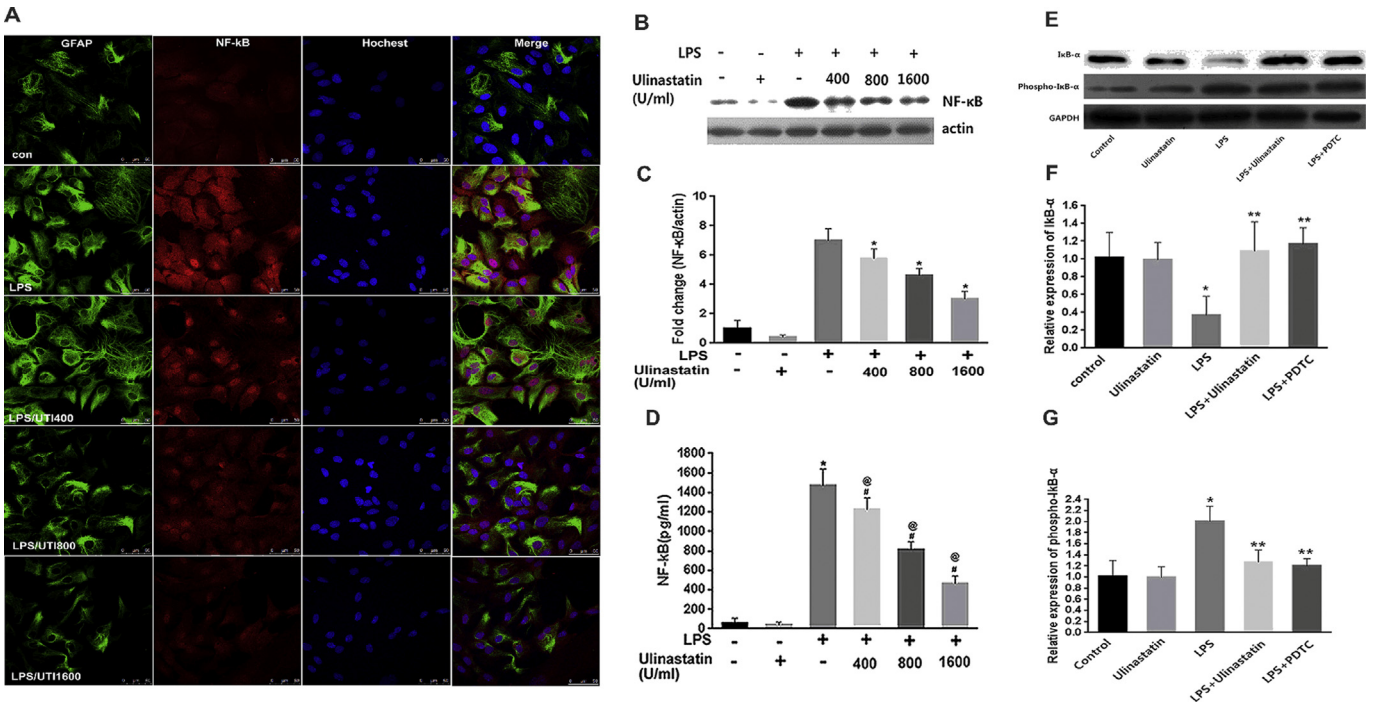

Fig. 3. Ulinastatin inhibits NF-κB p-65 activity in LPS-induced primary astrocyte. A. The immunofluorescence of GFAP (green), Hochest (blue), NF-κB (red) and merge. B. Western blot of NF-κB p-65. C. Quantification of the expression of NF-κB p-65. D. ELISA for NF-κB p-65. The DNA binding activity of NF-kB p-65 was evaluated by western blotting in astrocytes, preadministrated with ulinastatin at the concentration of 400 U/ml, 800 U/ml, 1600 U/ml for 1 h incubation with LPS (1 μg/ml). E. After treatment with ulinastatin (1600 U/ml) or PDTC (a specific inhibitor of NF-kB, 300 mM) for 1 h, primary SD rat astrocytes were stimulated with LPS (1 μg/ml) for 24 h. F,G. The LPS-induced degradation and phosphorylation of IκB-α were analyzed by western blot analysis. Data are presented as the means ± S.E.M from five separate experiments. $^{**}P < 0.01$ versus control group, $^{@}P < 0.05$, $^{@@}P < 0.01$ versus LPS group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus control group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

mediated by the interruption of NF-κB signaling pathway in astrocyte.

Astrocytes nearly constitute the half of the cells in the human brain and almost all CNS disease were substantially involved in astrocytes [18]. Astrocytes are responsible for a wide variety of complex and essential functions in the healthy CNS, including primary roles in synaptic transmission and information processing by neural circuit functions [5]. Expression of glial fibrillary acid protein (GFAP) can be recognized as a prototypical marker for immunohistochemical identification of astrocytes. It was expressed lower in physiological, steady state, but get enhanced expression in activated state [19]. Reactive astrogliosis is used widely as a pathological hallmark of diseased CNS tissue, there is various up-regulation of expression of GFAP and other genes in mild or

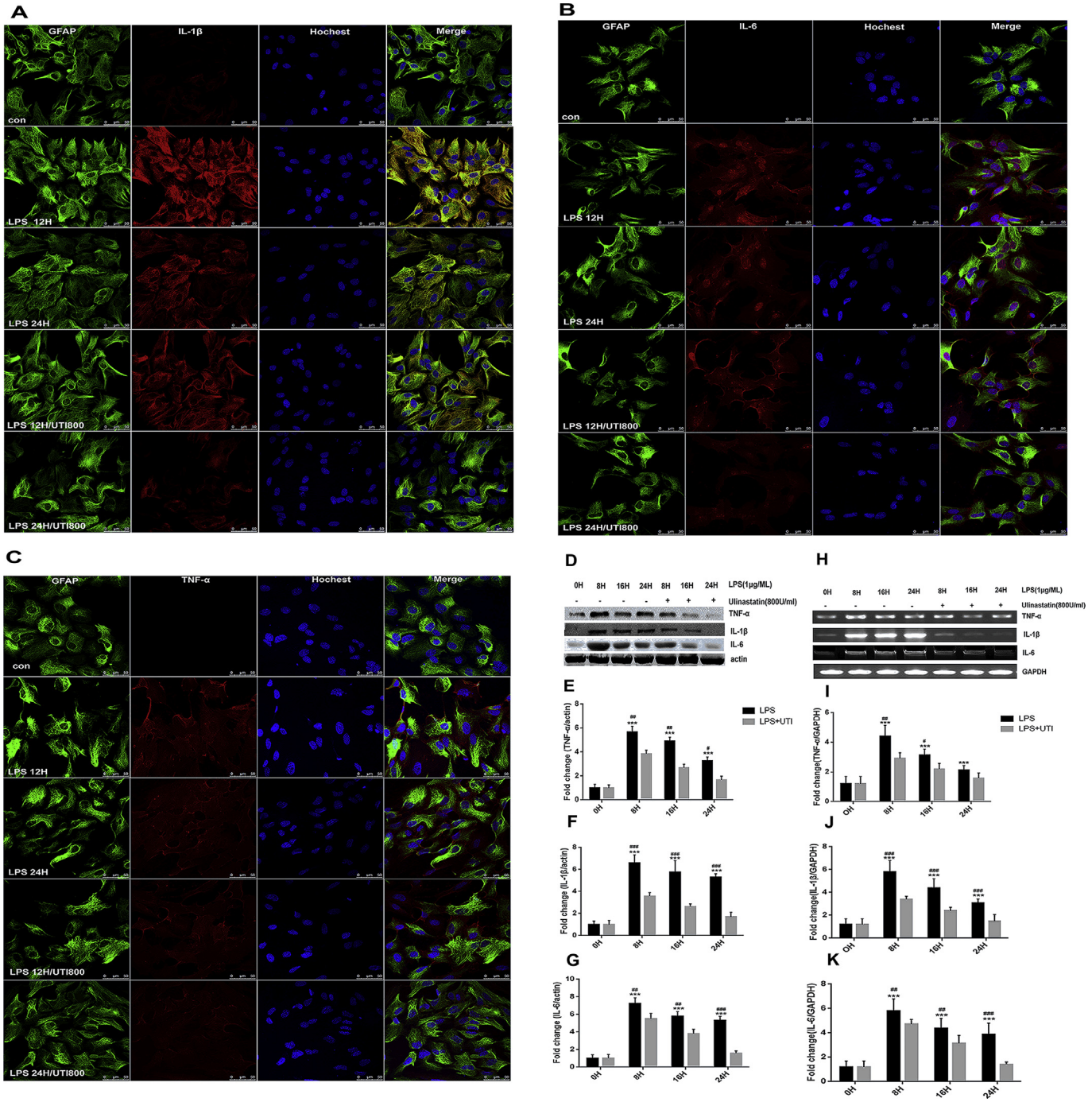

Fig. 4. The effects of ulinastatin on LPS-induced pro-inflammatory mediators in different time course. A, B, C. immunofluorescence of IL-1β, IL-6, TNF-α in SD astrocyte treated with or without LPS 1 μg/ml treated for 12 h or 24 h in the concentration of 800 U/ml UTI. D–G. mRNA expression levels of TNF-α, IL-1β , IL-6 in SD astrocyte treated with LPS for 6, 12, or 24 h treated in the presence or absence of UTI were established by RT-PCR. H–K. Protein expression levels of TNF-α, IL-1β, IL-6 in SD astrocyte treated with LPS for 6, 12, or 24 h treated in the presence or absence of UTI were established by western blot. Data are presented as the means ± S.E.M from five separate experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* Control. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs* LPS group.

moderate reactive astrogliosis [20]. Therefore, antagonism of astrogliosis and pro-inflammatory cytokines from astrocyte could block neuroinflammation following LPS stimuli. In our study, GFAP expression was significantly increase (Fig. 1A) in the presence of LPS (1 μg/ml).Our experiments did not detect the exact role of GFAP in LPS-induced inflammation, but it was sufficient to demonstrate that LPS (1 μg/ml) can induce GFAP-rapidly-overactivated ($^{**}P < 0.01$ vs. control group, Fig. 1B). UTI at the concentration of 1600 U/ml can significantly reduce the expression of GFAP ($^{**}P < 0.01$ *vs.* LPS, Fig. 1B). While another evidence for astrocyte overactivation is excessive proliferation, which was detected by two methods of MTT assay and BrdU incorporation assay in our experiments as showed in Fig. 1C–E, UTI significantly decreased the proliferation of astrocyte.

A large body of experimental studies had indicated that reactive astrocytes can exert both pro- and anti-inflammatory regulatory functions *in vivo* in a context dependent manner, which is regulated by specific molecular signaling pathways [21,22]. Activated astrocyte produce a wide range of proinflammatory mediators including IL-6, TNF-a, IL-1β, ROS, and NO [23], while TNF-α and IL-1β are the most important mediators, and they are known to be secreted during the early phase of inflammatory disease [24]. NF-κB is considered as the ubiquitous and essential transcription factor for the expression of many inflammation-related genes, including IL-6, TNF-α, IL-1β, COX-2 and iNOS [25]. UTI inhibit activation in several cell types, UTI suppresses lipopolysaccharide-induced prostaglandin E2 synthesis and nitric oxide production through the downregulation of NF-κB in BV2 mouse microglial cells [26]. UTI inhibited growth and apoptosis of breast carcinoma by activating NF-κB signaling, which showed the inhibitory effect of ulinastatin on NF-κB signal transduction and suppress the proliferation and induce the apoptosis of human breast cancer cells [27]. UTI attenuates seawater-induced acute lung injury by influencing the activities of nuclear factor-κB and its related inflammatory mediators [28]. Due to the anti-inflammatory effect, the properties of UTI in LPS-induced astrocyte was investigated, in our study, we confirm that the signaling pathways of NF-κB activated by LPS and inhibited by UTI, as showed in Fig. 3, LPS-mediated proinflammatory response, the successfully reduced the production of pro-inflammatory cytokines such as IL-6, TNF-α, IL-1β proved that UTI inhibited LPS-activated inflammation in a concentration-dependent manner.

NF-κB heterodimer consists of p50 and p65 subunits. The activity of NF-κB is regulated by its subcellular localization, in resting cells, NF-κB is sequestered in the cytoplasm by the IκB family, including IκB-a and IκB-b [29]. The activation of the IκB proteins can be induced by a variety of stimulation, such as LPS and pro-inflammatory cytokines, which results in the phosphorylation of the IκB proteins by a complex of IκB kinases (IKK). Phosphorylation-IκB proteins are rapidly degraded by the proteasome, which allows NF-κB to be released from IκB and translocate to the nucleus where it can initiate transcription by binding to numerous specific gene promoter elements [30]. In the present study, we proved that UTI increase the IκB-α, the corresponding expression of phospho-IκB-α decreased. Therefore, increased expression of IκB-α enhanced the binding to NF-κB, which further blocked NF-κB translocation to the nucleus and prevented its activity.

In conclusion, the present findings indicated that UTI can prevent neuroinflammation through the inhibition of astrocyte activation and the production of proinflammatory cytokines IL-6, IL-1β, TNF-α and iNOS *in vitro*. These neuroprotective effects might be closely associated with NF-κB pathway. The study extends the anti-inflammatory effects of UTI, implicating that UTI may be a promising candidate for the treatment of neuroinflammatory diseases that are characterized by excessive astrocyte activation and proliferation.

Conflict of interest

The authors have no conflict of interest.

Acknowledgment

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Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.01.155.

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