Original Paper

Ketamine Inhibits Lipopolysaccharide-Induced Astrocytes Activation by Suppressing TLR4/NF- κ B Pathway

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Key Words

Ketamine • Astrocyte • Inflammation • TLR4 • NF- κ B • GFAP • Cytokine

Abstract

Background/Aims: Ketamine has been reported to exert anti-inflammatory effects on astrocytes stimulated by lipopolysaccharide (LPS) *in vitro* and *in vivo*. However, the mechanism has not been elicited clearly. The aim of this study was to investigate the effects of ketamine on TLR4 expression and NF- κ B-p65 phosphorylation, as well as the production of proinflammatory cytokines in LPS challenged astrocytes. **Methods:** Astrocytes were stimulated with LPS (1 μ g/ml) in the absence and presence of various concentrations of ketamine (10, 100, 1000 μ M). The concentrations of IL-1 β , IL-6 and TNF- α were measured by ELISA, the expression of glial fibrillary acidic protein (GFAP) in astrocytes was detected by immunofluorescence staining, the level of phosphorylated NF- κ B p65 and the expression of TLR4 were detected by western blotting. **Results:** LPS increased TLR4 expression and the phosphorylation of NF- κ B p65 subunit as well as GFAP expression and the production of IL-1 β , IL-6 and TNF- α in cultured astrocytes. Ketamine (100 and 1000 μ M) reduced the expression of GFAP and the production of these proinflammatory cytokines, inhibited the expression of TLR4 and attenuated the phosphorylation of NF- κ B p65 in astrocytes challenged by LPS. **Conclusion:** The inhibitory effects of ketamine on LPS-induced astrocytes activation and inflammation response may be mediated by suppressing NF- κ B activation through reducing the expression of TLR4.

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Introduction

In the central nervous system (CNS), the interaction among various cell types, neurons, astrocytes, microglia and oligodendrocytes, is important in the immune and inflammatory responses. Among these cells, astrocytes are known to play a pivotal role in CNS immunity

as immunocompetent cells by secreting cytokines and inflammatory mediators [1, 2]. Activation of astrocytes leads to an inflammatory response during disease, infection, trauma, and ischemia [3], producing diverse proinflammatory cytokines such as IL-1 β , TNF- α and IL-6 [4]. A sustained inflammatory response present in acute and chronic brain disorders may be a first step in the development of several neurodegenerative diseases [5, 6]. Therefore, the regulation of astrocyte inflammatory response may be essential in pathological processes in the central nervous system.

Ketamine, an intravenous anesthesia inducing agent, has been showed anti-inflammatory actions in various immune cells, such as macrophages and peripheral leucocytes, stimulated with lipopolysaccharide (LPS) *in vitro* and *in vivo* [7-9]. Recent *in vitro* and *vivo* studies demonstrated that ketamine inhibited astrocyte activation and blunted LPS-induced astrocyte TNF- α production [10-12]. However, the detailed mechanisms underlying the antiinflammatory effects of ketamine in astrocytes stimulated by LPS have not been clearly revealed.

Toll-like receptor 4 (TLR4) is a member of the IL-1R/TLR superfamily that is required for LPS responsiveness and is involved in the host defense against Gram-negative bacteria [13]. According to recent findings TLR4 is expressed in primary astrocytes, providing a mechanistic link between bacterial challenge and immune responses in the inflammatory CNS disorders [14]. After ligand binding, TLRs/IL-1Rs dimerize and undergo the conformational change required for the recruitment of downstream signaling molecules and stimulation of downstream kinases, including activation of the transcriptional factors NF- κ B [15]. The activation of NF- κ B leads to the induction of genes encoding proinflammatory cytokines such as IL-1 β , IL-6 and TNF- α [16].

The present study addressed the question whether ketamine could attenuate LPS-induced astrocyte inflammatory response through inhibiting TLR4 expression and NF- κ B activation, reducing proinflammatory cytokines production in astrocyte. To this end, we investigated the effects of ketamine on LPS-induced production of IL-1 β , IL-6 and TNF- α and relative mechanisms in primary cultures of rat astrocytes *in vitro*.

Materials and Methods

Primary culture of mixed glial cells

Astrocytes were prepared from whole spinal cord of 1-day-old SD rats using the procedure described as the following literature [17] with some modifications. The meninges and blood vessels were carefully removed, and the tissue was minced with a mesh bag (200 μ m) and trypsinized (trypsin-EDTA 2.5%) for 10 min. After centrifuging for 10 min at 1000 rpm, the tissues were resuspended in DMEM-F-12 containing FBS 10%, penicillin 100 U ml⁻¹ and streptomycin 100 mg ml⁻¹. Cells were plated on 75 cm² culture flasks and kept in DMEM-F-12 supplemented with 10% FBS and the antibiotics in a humidified 5% carbon dioxide atmosphere at 37°C. The medium was changed every 3 days after shaking the flasks to remove neuron and other glial cells.

Secondary culture of astrocytes

Mixed glials cultured for 12 or 13 days in 75 cm² flasks were shaken at 150 r.p.m. for 18h at 37°C on a gyratory shaker. The remaining source cultures were dissociated using trypsin and then collected by centrifuging (1000 r.p.m. for 5 min). The cells were seeded onto 24-well culture plates at 2 $\times$ 10⁵ cells cm⁻² and cultured for 24 h before being used as astrocytes.

Immunofluorescence for GFAP

The astrocytes were fixed with 4% paraformaldehyde for 30 min and rinsed three times with PBS. Non-specific binding was blocked with 10% Goat serum for 1h at room temperature. Subsequently, the astrocytes were incubated with glial fibrillary acidic protein (GFAP) antibody (BOSTER) in the blocking buffer for 24 h at 4°C. After washing three times with PBS, the cells were incubated with the secondary antibody for 2 h at room temperature and rinsed. The preparations were mounted with glycerin and examined using an

Fig. 1. GFAP staining of secondary cultures of astrocytes. More than 95% of the purified cells were GFAP-positive identified by immunofluorescence staining. Scale bar = 50 μ m.

inverted microscope equipped with fluorescence optics (OLYMPUS, Japan).

Treatment of astrocytes

Cells were divided into five groups treated with medium (control), LPS ($1.0 \mu\text{g ml}^{-1}$), different concentrations of ketamine (10, 100 and $1000 \mu\text{M}$) and LPS ($1.0 \mu\text{g ml}^{-1}$) respectively. Ketamine was administered 15 min before LPS treatment. After a 24 h LPS treatment, the cultured cells and supernatant were collected respectively. The supernatant was used to detect the level of proinflammatory cytokines by ELISA. The collected cells were used to detect the expression of TLR4 and the activation of NF- κ B by western blotting.

ELISA for IL-1 β , IL-6 and TNF- α

IL-1 β , IL-6 and TNF- α levels in supernatant were measured by enzyme-linked immunoadsorbent assay (ELISA) according to the protocols of the kits. Briefly, Cellfree supernatant was added to each well of a monoclonal rabbit anti-rat IL-1 β (or IL-6, or TNF- α) antibody coated micro titre plates (ELISA plates) for 12 h at 4°C . Unbound material was washed off and a biotinylated monoclonal rabbit anti-rat IL-1 β (or IL-6, or TNF- α) antibody was used for 45 min. Bound antibody was detected by addition of avidin-peroxidase for 30 min followed by incubation of the ABTS substrate solution. Absorbance was measured 20 min after addition of substrate. A standard curve was constructed using various dilutions of IL-1 β (or IL-6, or TNF- α) standard preparation in the kit. The contents of IL-1 β , IL-6 and TNF- α in the supernatant were determined by extrapolation of absorbance to the standard curve.

Western Blot Analysis for TLR4 and NF- κ B

After drug treatment, cell lysates were prepared in ice-cold lysis buffer. Protein concentrations were quantified using a bicinchonic acid protein assay kit (Beyotime). The protein sample ($100\mu\text{g/well}$) was separated on a sodium dodecyl sulfate-polyacrylamide gel and electrophoretically transferred onto a nitrocellulose membrane in Tris-glycine transfer buffer. After blocking, TLR4, NF- κ Bp-65 and phosphorylation NF- κ Bp-65 (p-p-65) were immunodetected using different rabbit polyclonal antibodies. Cellular β -actin protein was immunodetected as the internal standard.

Statistics analysis

Data were presented as Mean $\pm$ SD. SPSS-13.0 was used for data analysis. Differences among groups were determined by one way analysis of variance (ANOVA), followed by the Student-Newman-Keuls post hoc test. Significance was defined as $p < 0.05$.

Results

Identification of astrocytes

Glial fibrillary acidic protein (GFAP) is a consensually accepted specific marker of astrocytes. In the present study, the immunofluorescence technique was used to identify

Fig. 2. Effects of ketamine on proinflammatory cytokines production in LPS activated astrocytes. Astrocytes were treated with ketamine (10, 100, and 1000 μ M) for 15 minutes, followed by the addition of LPS (1 μ g ml^{-1}) for 24 hours. The PBS group added an isovolumetric PBS buffer for 24 hours. Cell-free supernatants were assayed for IL-1 β (A), IL-6 (B) and TNF- α (C) levels by ELISA. Data are presented as the Mean $\pm$ SD (n=3). **P<0.01, compared to PBS group; ## P<0.01, compared to LPS group.

Fig. 3. Effect of ketamine on GFAP expression in astrocytes stimulated by LPS. The expression intensity of GFAP in astrocytes was examined by immunofluorescence. A: Astrocytes treated with medium (control). B: Astrocytes treated with LPS (1.0 μ g ml^{-1}). The cells were hypertrophied and showed thicker GFAP-positive staining. C: Astrocytes treated with ketamine (1000 μ M) followed by treatment with LPS (1.0 μ g ml^{-1}). After pretreatment with ketamine, the activation of astrocytes by LPS was markedly attenuated.

astrocytes by detecting GFAP expression in cultured cells. As shown in Fig. 1, the cells were uniformly labeled by green fluorescence, which indicated that the cells utilized in the present study were astrocytes.

Levels of IL-1 β , IL-6 and TNF- α by ELISA

As shown in Fig. 2, the levels of IL-1 β , IL-6 and TNF- α in the cell culture supernatant were markedly increased after stimulation by LPS compared to PBS control group. Ketamine (100 and 1000 μ M) pretreatment for 15 min significantly inhibited the production of IL-1 β , IL-6 and TNF- α from astrocytes stimulated by LPS (1 μ g ml^{-1}). In contrast, ketamine at the concentration of 10 μ M did not appreciably affect release of these proinflammatory cytokines from LPS-activated astrocytes.

Fig. 4. TLR4 expression in astrocytes after LPS treatment with or without the presence of ketamine. LPS significantly enhanced TLR4 expression. Ketamine at a concentration of 100 and 1000 μ M suppressed TLR4 expression after LPS challenge. At 10 μ M ketamine did not reduce the expression of TLR4. ** $p < 0.01$, vs PBS group; # $p < 0.05$, vs LPS group.

Fig. 5. Total NF- κ B p-65 expression in astrocytes after LPS treatment with or without the presence of ketamine. Ketamine or LPS had no notable effects on the total NF- κ B p-65 expression.

Effect of ketamine on GFAP expression in astrocytes stimulated by LPS

The expression intensity of GFAP in astrocytes was examined by immunofluorescence. After LPS stimulation, the size of astrocytes was increased and the expression of GFAP was markedly enhanced. Ketamine significantly inhibited increased cell body volume and elevated expression of GFAP in astrocytes challenged by LPS (Fig. 3).

Western Blot Analysis for TLR4

Subsequently, in order to clarify the mechanism through which ketamine inhibits astrocyte activation, we further investigated the expression of TLR4 in astrocytes by western blot analysis. In this study TLR4 expression in astrocytes was markedly upregulated by LPS stimulation. Ketamine at the concentration of 100 and 1000 μ M significantly suppressed TLR4 expression challenged by LPS. In contrast, 10 μ M ketamine did not reduce LPS induced expression of TLR4 (Fig. 4).

Western Blot Analysis for NF- κ B

Both total and phosphorylated NF- κ B p-65 in astrocytes were also detected by western blot analysis. As shown in Fig. 5, there was no significant difference among all the experiment groups for total NF- κ B p-65 expression in astrocytes. However, the phosphorylated NF- κ B p-65 in astrocytes was markedly upregulated by LPS stimulation. Ketamine at the concentration of 100 and 1000 μ M significantly attenuated LPS induced phosphorylation of NF- κ B p-65 in astrocytes. In contrast, 10 μ M ketamine did not inhibit the expression of phosphorylated NF- κ B p-65 challenged by LPS. (Fig. 6)

Fig. 6. Phosphorylated NF- κ B p-65 expression in astrocytes after LPS treatment with or without the presence of ketamine. LPS significantly stimulated NF- κ B p-65 phosphorylation. At 10 μ M ketamine did not significantly reduce the phosphorylation of NF- κ B p-65, ketamine at the concentration of 100 and 1000 μ M markedly suppressed NF- κ B p-65 phosphorylation following challenge by LPS. ** $p < 0.01$, vs PBS group; # $p < 0.05$, ## $p < 0.01$, vs LPS group.

Discussion

In this study we investigated the effect of ketamine on inflammatory responses of primary culture of rat astrocytes stimulated with LPS. We found that ketamine blunted the activation of astrocytes by LPS and reduced LPS-stimulated production of IL-1 β , IL-6 and TNF- α in astrocytes. Ketamine was also found to inhibit LPS-induced TLR4 expression and NF- κ B activation in astrocytes. Significant effects on astrocytes were observed at ketamine concentrations of 100 and 1000 μ M in media supplemented with serum. A previous study demonstrated that plasma levels of ketamine varied from 9000 to 25800 ng/ml or 37.8-108.4 μ M in patients 1 minute after intravenous ketamine injection at a dose of 2.0-2.2 mg/kg [18]. Thus, a ketamine concentration of 100 μ M is observed *in vivo*. Our results suggest that clinically relevant or higher concentrations of ketamine may modulate some of the inflammatory responses of astrocytes stimulated by LPS *in vitro* by suppressing TLR4/NF- κ B pathway, assuming that protein binding is comparable in the serum-supplemented media and plasma.

Astrocytes, the most abundant glial cell type in central nervous system, provide metabolic and trophic support for neurons and modulate synaptic transmission and plasticity [19, 20]. They have a major role in regulation of the extracellular ionic environment and protect neurons from oxidative stress and excitotoxicity [21, 22]. In some instance, excessive activation of astrocytes and sustained production of proinflammatory cytokines will lead to acute or chronic brain disorders [23]. Therefore, the regulation of astrocyte inflammatory response may be important in the maintenance of normal central nervous system function.

Astrocytes become activated in response to various stimuli, from subtle changes in their microenvironment to massive tissue damage. This process, known as reactive astrogliosis, far from being an all-or-none event, is a finely graded and non-homogeneous response that varies according to the type, severity, time and duration of the insult [24]. Histopathologically, reactive astrocytes undergo hypertrophy and increase the expression of GFAP [24]. Reactive astrocytes release a wide array of mediators, including pro-inflammatory cytokines [24]. The present study shows that ketamine markedly inhibited hypertrophy of astrocytes and enhanced expression of GFAP in astrocytes induced by LPS. In addition, the production of IL-1 β , IL-6 and TNF- α in astrocytes stimulated by LPS was also significantly suppressed. The observations suggest that ketamine attenuates the activation and the excessive inflammatory responses of astrocytes by LPS and that ketamine thus protects against inflammation of the central nervous system. This anti-inflammatory action of ketamine found in our study was consistent with the previous reports [10].

Although the present and previous studies have shown the antiinflammatory actions of ketamine on astrocytes challenged by LPS [10], the exact mechanisms responsible for these actions are incompletely understood. Toll-like receptor 4 (TLR4) is a transmembrane receptor protein with extracellular leucine-rich repeated domains and a cytoplasmic

signaling domain. TLR4 is involved in immune responses, especially in the activation of innate immunity, but it also triggers adaptive immunity [25-27]. LPS, a major component of the outer membrane of gram-negative bacteria, is a well known exogenous ligand for TLR4 [28]. LPS binds to lipopolysaccharide-binding protein and is transferred to CD14 [29, 30]. TLR4 is a CD-14 associated transmembrane signal transducer, which is necessary for the LPS-induced cellular response [31-33]. Recent studies have suggested that the association of TLR4 with myeloid differentiation factor 88 (MyD88) may induce the activation of IL-1R associated kinase and TNF receptor-associated factor [34], which triggers inflammatory cascade reactions.

Nuclear factor kappa B (NF- κ B) is an important nuclear transcription factor. NF- κ B heterodimer consists of p50 and p65 (Rel A) subunits. It plays a pivotal role in immune and inflammatory responses through the regulation of the expression of several proteins, including pro-inflammatory cytokines, chemokines, and adhesion molecules. Uncontrolled activation of the NF- κ B pathway is involved in the pathogenesis of many acute and chronic inflammatory diseases [35]. In its inactive state, the NF- κ B dimer is present in the cytosol, where it is bound to an inhibitory protein, I- κ B. Activation of NF- κ B by several stimuli, including LPS, induces the release and degradation of the inhibitory protein I- κ B from the dimeric complex, followed by phosphorylation of NF- κ B p65 and translocation to the nucleus [36]. In the nucleus, NF- κ B is bound to corresponding sites to regulate transcription of many proinflammatory genes [37, 38].

TLR4-mediated signaling pathways mainly stimulate the activation of nuclear factor kappa B (NF- κ B) [39]. This important nuclear transcription factor regulates many pro-inflammatory genes, such as cytokines, chemokines, cyclooxygenase-2 (COX-2), matrix metalloproteinase-9 (MMP-9), and inflammatory mediators involved in the pathogenesis of many central nervous system (CNS) diseases [37, 38]. TLR4-mediated NF- κ B signaling plays a vital role in the initiation of neuropathic pain, cerebral ischemic injury, traumatic brain injury and other inflammatory or autoimmune CNS diseases [40, 41].

In the present study, we used LPS to induce an inflammatory response of astrocytes. The results showed that LPS elevated the expression of TLR4 and activated NF- κ B in astrocytes, a finding in agreement with previous studies [42, 43]. Here, we also show that ketamine treatment could reduce LPS-induced TLR4 expression and NF- κ B phosphorylation in astrocytes. Since TLR4 is an essential upstream sensor for LPS and may mediate the NF- κ B activation, and NF- κ B activation can increase the production of IL-1 β , IL-6 and TNF- α , it is possible that ketamine decreases the production of proinflammatory cytokines IL-1 β , IL-6 and TNF- α by suppressing the activation of TLR4/NF- κ B signaling in LPS challenged astrocytes. Our results suggest that suppression of TLR4/NF- κ B signaling may be the probable mechanism through which ketamine attenuated LPS induced inflammatory responses in astrocytes.

In conclusion, our findings indicate that ketamine blunts the activation of astrocytes by LPS and reduces the production of proinflammatory cytokines IL-1 β , IL-6 and TNF- α by interfering with TLR4/NF- κ B signaling. These properties of ketamine may contribute to its anti-inflammatory effects in the central nervous system.

Acknowledgements

This work was supported by National Natural Science Foundation of China (81171013, 81070889, 81001431 and 81171041), Key Subject of Colleges and Universities Natural Science Foundation of Jiangsu Province (10KJA320052), Natural Science Foundation of Jiangsu Higher Education Institutions of China (11KJB310014), Xuzhou Scientific and Technological Project (xzzd1052) and A Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions. We declare that there is no conflict of interest that would prejudice its impartiality.

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