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Original Article

Dexmedetomidine inhibits lipopolysaccharide-induced inflammatory response in hippocampal astrocytes *in vitro*

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Abstract: Neuroinflammation mediated by astrocytes has been implicated in neurodegenerative diseases. Meanwhile, dexmedetomidine (DEX) has potent anti-inflammatory properties. The present study aimed to assess the effects of DEX on proinflammatory mediator production and release in astrocytes after lipopolysaccharide (LPS) induction. Cultured astrocytes, derived from hippocampi of 4-day-old rats, were treated with DEX at 0.1, 1, 10, and 100 μM, respectively, in the presence or absence of LPS (1 μg/ml). Then, mRNA and protein levels of the proinflammatory cytokines TNF-α, IL-6 and IL-1β were measured. The protein levels of IκB-α were also assessed. DEX at 0.1 μM or 1 μM did not affect the production of proinflammatory mediators. However, higher DEX levels (10 and 100 μM) significantly decreased the amounts of TNF-α, IL-6, and IL-1β, both at the mRNA and protein levels, and increased the protein levels of IκB-α. These findings indicate that DEX inhibits neuroinflammation by interfering with NF-κB signaling, and may constitute a potential therapeutic agent for protecting patients from neuroinflammation associated diseases.

Keywords: Dexmedetomidine, astrocytes, NF-κB, lipopolysaccharide

Introduction

Astrocytes are responsible for diverse functions in the central nervous system (CNS), and play pivotal roles in maintaining the physiological functions of neurons. However, when overactivated under pathological conditions, astrocytes become a center of inflammatory processes. Consequently, they release and respond to a number of important cytokines, including IL-1β, TNF-α, IL-6, and nuclear factor kappa B (NF-κB), which in turn affect microglia and neurons, as well as astrocytes themselves. Ultimately, the normal physiological interaction between glia and neurons may be impaired. Such effects finally damage neuronal function and cause clinically detectable cognitive changes [1-3]. Therefore, suppression of excessive astrocyte activation may constitute a potential therapeutic mechanism to alleviate the progression of neurological diseases which are closely associated with neuroinflammation [1, 4]. Dexmedetomidine, a hypnotic drug with high selectivity for the α_2-adrenergic receptor, has been used as a sedative or an anesthetic adjuvant. Its advantages include reduced respiratory suppression, high quality of sedation, anti-agitation features [5, 6], anti-delirium [7], and anesthetic and analgesic-sparing effects [8, 9]. DEX has potential anti-inflammatory properties [10], as well as potent cyto- or organo-protective features. For example, DEX attenuates ischemia-reperfusion induced kidney injury [11] and even protects against remote lung injury induced by renal ischemia-reperfusion in mice [12, 13]. DEX also reduces the mortality rate and dampens the inflammatory response during endotoxemia [14].

In present study, we assessed the effects of DEX on the production of proinflammatory mediators in primary hippocampal astrocytes induced by LPS, and explored the cell signaling mechanisms by which DEX modulates proinflammatory responses.

Material and methods

Chemicals and reagents

Cell culture: Timed pregnant Sprague Dawley rats were obtained from the Experimental Animal Center of Capital medical University (Beijing, China). All research protocols were approved by the Bioethics Committee of Capital Medical University. Primary astrocytes of hippocampus were prepared as previously described [15]. Briefly, hippocampi of 4-day-old Sprague Dawley rats were harvested with ice-cold calcium/magnesium free HBSS at pH 7.4. The tissues was minced and trypsinized (trypsin-EDTA 0.25%) for 5 min at 37°C; trypsin neutralization was performed with DMEM/F12 medium (Gibco, Grand Island, NY) containing 10% fetal bovine serum (FBS) (Gibco) and the penicillin-streptomycin solution (Gibco). Finally, cells were filtered through a mesh (40 μm). After centrifugation 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 a 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 twice a week. After 7-8 days, astrocytes were separated from microglia and oligodendrocytes by shaking for 18 h (200 rpm 37°C). The isolated astrocytes were cultured in 6-well plates at a density of 2×10^5 cells/well; the cultures were>95% purity of astrocytes as verified by immunocytochemistry.

Experimental grouping

Purified astrocytes were randomly divided into seven groups of 5 wells. In the control group, cells were cultured with serum-free culture medium for 24 h. In the nLPS group, cells were treated with DEX (Jiangsu Hengrui Medicine Co., Ltd.) at the final concentration of 100 μM for 1 h, and cultured with serum-free culture medium for 24 h. In the LPS group, LPS was added for 24 h. In the DEX0.1, DEX1, DEX10 and DEX100 groups, the astrocytes were treated with DEX at concentrations of 0.1, 1, 10, and 100 μM, respectively, followed by LPS (1 μg/ml) for 24 h.

Cell viability assay

[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT, Sigma-Aldrich, St. Louis, MO, USA) was used to evaluate cell viability, according to the manufacturer's instructions. Briefly, cell viability was reflected by the formation of blue formazan metabolized from colorless MTT by mitochondrial dehydrogenases, which are active only in live cells. Astrocytes were seeded into 96-well plates at a density of 1×10^5 cells/well for 24 h. Astrocytes were then treated with various concentrations of DEX for 1 h, and with or without LPS treatment (1 μg/ml) for 24 h. After treatment, MTT solution (0.5 mg/ml) was added to each well for 4 h. After removal of the cell culture medium, DMSO (200 μl) was added per well to dissolve the formazan crystals before absorbance measurement at 570 nm on a microplate reader (Model, Bio-Rad). Each group was measured in triplicate.

Real-time PCR

Total RNA was extracted from astrocytes with TRIzol reagent (Invitrogen, Paisley, UK) according to the manufacturer's instructions. The ABI Primer Express software was used to design the polymerase chain reaction (PCR) primers used in this study: NF-κB, ACGATCTGTTTCCCCTCATC (F) and TGCTTCTCTCCCCAGGAATA (R); TNF-α, GGGCAGGTCTACTTTGGAGTCATTG (F) and GGGCTCTGAGGAGTAGACGATAAAG (R); IL-1β, CCCAACTGGTACATCAGCACCTCTC (F) and CTATGTCCCGACCATTGCTG (R); IL-6, GATTGTATGAACAGCGATGATGC (F) and AGAAACGGAACTCCAGAAGACC (R); GAPDH, TGGAGTCTACTGGCGTCTT (F) and TGTCATATTTCTCGTGGTTCA (R). PCR was carried out on a Real-Time PCR System (ABI 7500, Applied Biosystems, USA), and relative mRNA expression levels were assessed by cycle threshold (Ct) values, normalized to GAPDH.

Western blot

Protein samples (30 μg) extracted from astrocytes were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride membranes (Amersham Pharmacia Biotech, UK). The membranes were incubated with 5% non-fat milk or fetal bovine serum (FBS) in Tris-buffered saline with Tween (TBS-T) for 2 h at room temperature, to block nonspecific binding. After washing, the membranes were incubated with primary antibodies against NF-κB, phospho-IκBα, or total IκBα (Abcam, Cambridge, MA, UAS) at 4°C overnight, and subsequently with horseradish peroxidase-

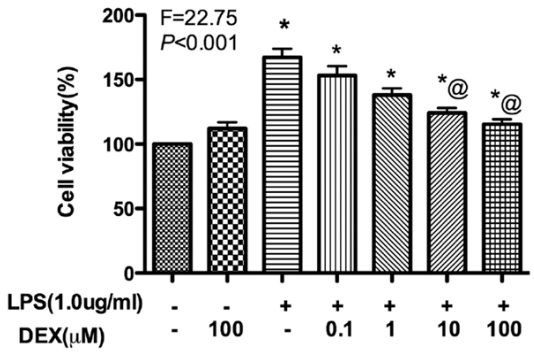

Figure 1. Effect of DEX on cell viability in primary astrocytes. Data are mean ± SEM (n = 5) from three independent experiments. *P<0.001 versus control group; @P<0.05, versus LPS group.

conjugated secondary antibodies for 2 h at room temperature. Immunoreactive bands were detected with an enhanced ECL kit (GE); imaging was performed on Image Quant™ LAS500 imager (GE) using the IQ LAS500 control software™.

Enzyme linked immunosorbent assay for proinflammatory cytokine measurements

Cells (1×10^6) were seeded in 24-wells plates, and culture supernatants after treatment (with or without LPS and Dex) were collected for the measurement of TNF-α, IL-1β and IL-6 by enzyme-linked immunosorbent assay (ELISA) using kits from R&D Systems (Minneapolis, U.S.A.). The concentrations of IL6, IL-1β, and TNF-α were calculated based on standard curves generated with recombinant cytokines provided in the respective ELISA kits.

Statistical analysis

Statistical analyses were performed with SPSS 20.0. Data are mean ± SEM, and were assessed by one-way ANOVA followed by a Student-Newman-Keuls test for multiple comparisons. $P<0.05$ was considered statistically significant. All experiments were repeated independently at least three times.

Results

DEX is not cytotoxic, and inhibits LPS induced astrocyte over-activation and proliferation

To assess the effects of DEX on astrocytes, cell viability/growth was first evaluated. The viability of astrocytes was not significantly changed by DEX treatment at various concentrations (0.1-100 μM) compared to the control group ($P>0.05$). Astrocyte activation is the first step of the response to external stimuli, and excessive activation is regarded as astrogliosis [16]. Compared with the control group, LPS at 1.0 μg/ml, a dose that induces proinflammatory responses, caused excessive astrocyte activation and increased cell viability; pretreatment with 10 and 100 μM DEX for 1 h dramatically inhibited LPS induced excessive astrocyte activation ($P<0.05$) (**Figure 1**).

DEX inhibits cytokine expression in LPS-stimulated primary rat astrocytes

Proinflammatory cytokines are soluble mediators of inter- and intracellular communications, and increase during inflammatory responses. In the present study, we measured the expression levels of IL-1β, TNF-α, IL-6 by Immunofluorescence, ELISA and RT-PCR (**Figure 2**). As expected, LPS significantly increased the release of the above cytokines. Astrocytes pretreated with DEX (10 or 100 μM) for 1 h before exposure to LPS (1.0 μg/ml) for 24 h, showed significantly attenuated release of the proinflammatory cytokines IL-1β, TNF-α, and IL-6 compared with the LPS-alone treatment group. Pretreatment with DEX (0.1 and 1 μM) did not significantly inhibit LPS-induced cytokine production compared with the LPS-alone-treatment group (**Figure 2**).

DEX inhibits LPS induced NF-κB activation

NF-κB plays an essential role in LPS-induced expression of pro-inflammatory cytokines. In this study, we performed immunofluorescence, Western blot and RT-PCR to assess whether DEX inhibits NF-κB activation in LPS-stimulated primary astrocytes. As shown in **Figure 3**, DEX significantly decreased NF-κB expression in a concentration dependent manner. We also measured the protein levels of IκB-α in the cytoplasm as well as phosphorylated IκB-α in the nucleus by Western blot. Because NF-κB activation depends on IκB-α degradation in the cytoplasm, IκB-α amounts in LPS-induced astrocytes were significantly decreased (**Figure 3F**, $P<0.05$). IκB-α expression in the cytoplasm of primary astrocytes was increased significantly after pretreatment with DEX 10 or 100 μM before exposure to LPS (**Figure 3F**, $P<0.01$). Meanwhile, the protein levels of phosphorylat-

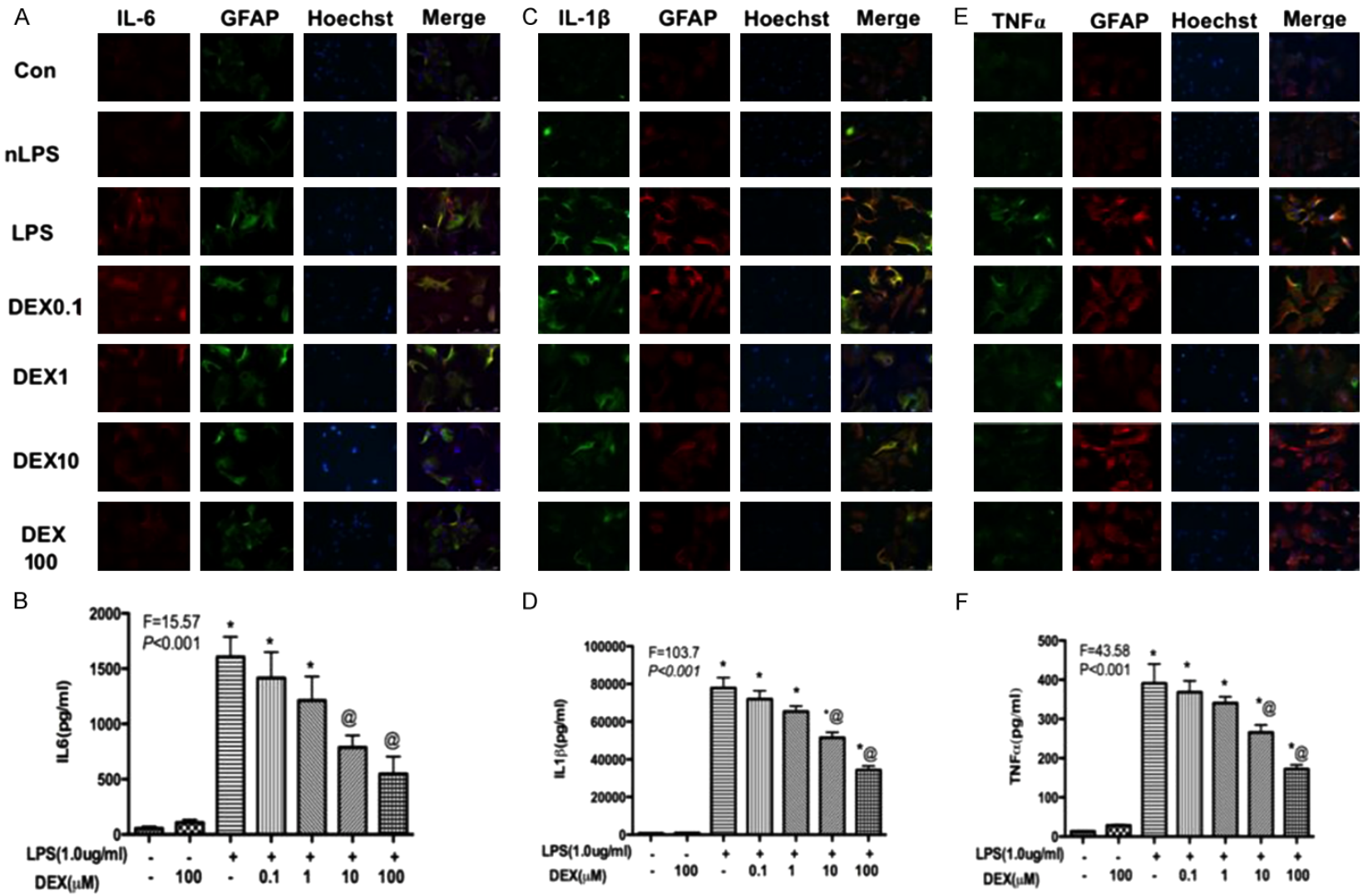

Figure 2. Dexmedetomidine inhibits pro-inflammatory cytokines in LPS-induced primary astrocytes. A. Immunofluorescent staining of IL-6 (red), GFAP (green), Hoechst (blue), and merge. B. ELISA detection of IL-6. Data are mean ± SEM (n = 5) from three independent experiments. *P<0.001 versus control group; @P<0.05, versus LPS group. C. Immunofluorescent staining of IL-1β (green), GFAP (red), Hoechst (blue), and merge. D. ELISA detection of IL-1β. Data are mean ± SEM (n = 5) from three independent experiments. *P<0.001 versus control group; @P<0.05, versus LPS group. E. Immunofluorescent staining of TNF-α (green), GFAP (red), Hoechst (blue), and merge. F. ELISA detection of TNF-α. Data are mean ± SEM (n = 5) from three independent experiments. *P<0.001 versus control group; @ P<0.05, versus LPS group.

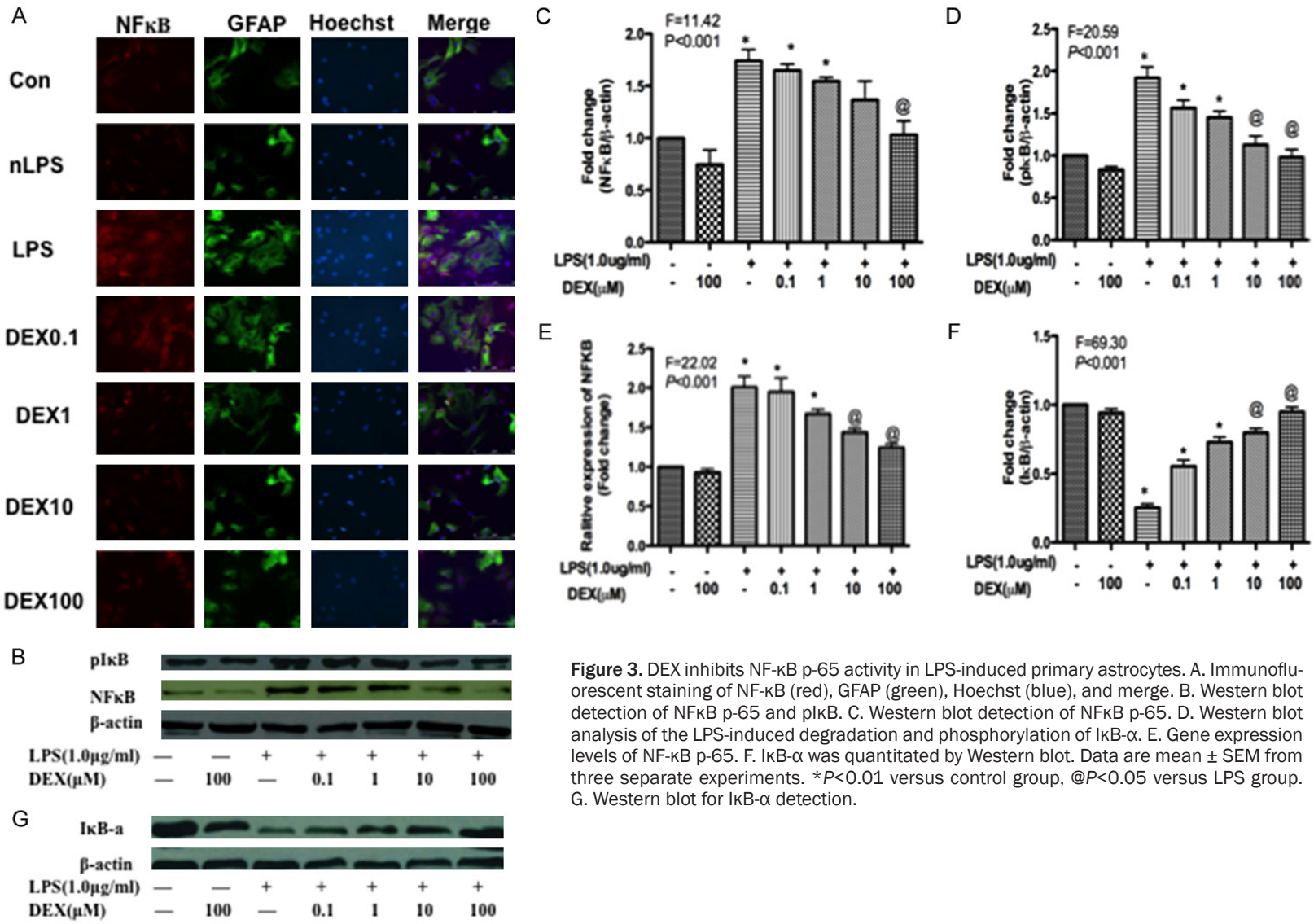

Figure 3. DEX inhibits NF-κB p-65 activity in LPS-induced primary astrocytes. A. Immunofluorescent staining of NF-κB (red), GFAP (green), Hoechst (blue), and merge. B. Western blot detection of NFκB p-65 and pIκB. C. Western blot detection of NFκB p-65. D. Western blot analysis of the LPS-induced degradation and phosphorylation of IκB-α. E. Gene expression levels of NF-κB p-65. F. IκB-α was quantitated by Western blot. Data are mean ± SEM from three separate experiments. **P*<0.01 versus control group, @*P*<0.05 versus LPS group. G. Western blot for IκB-α detection.

ed IκB-α in the nucleus were significantly reduced (**Figure 3D**; $P<0.05$ or $P<0.01$).

Discussion

This study showed that DEX remarkably suppressed the increased amounts of NF-κB, IL-1β, IL-6 and TNF-α in astrocytes after LPS induction, both at the gene and protein levels. These anti-inflammatory properties of DEX are likely through the modulation of NF-κB signaling.

In the human brain, astrocytes account for about half of all cells and are substantially involved in almost all CNS diseases [17]. As they appear to play a dominant role in the inflammatory process, astrocytes are considered a central element in neurological diseases. Astrocytes are responsible for a wide variety of complex and essential functions in the healthy CNS, with primary roles in synaptic transmission and information processing by neural circuit functions [18, 19]. Neuroinflammation is a common feature of multiple neuro-degenerative diseases in onset and progression. It was proposed that delirium after surgery is associated with neuroinflammation induced by astrocyte activation [20, 21]. Suppression of astrocyte activation may therefore help alleviate postoperative delirium. To this end, exploring new drugs or interventions, which prevent astroglia activation, may have clinical significance.

LPS, a bacterial membrane component, is a potent astrocyte activator and an inducer of brain inflammation associated with pro-inflammatory cytokines in many experimental models *in vivo* and *in vitro* [22, 23]. A large body of experimental studies have indicated that reactive astrocytes exert both pro- and anti-inflammatory regulatory functions *in vivo*, which are controlled by specific molecular signaling pathways [24, 25]. Activated astrocytes produce a wide range of proinflammatory mediators, including IL-6, TNF-α, IL-1β and beyond. However, TNF-α and IL-1β are the most important mediators, and secreted during the early phase of inflammatory disease [26]. A recent study by Cuiying Xie, et al [27] showed that medium and high concentrations of DEX post-treatment could inhibit the expression and release of inflammatory factors. In the present study, however, we found that DEX may attenuate the inflammatory response by inhibiting the NF-κB pathway. NF-κB is considered a common and essential transcription factor for the expression of many inflammation-related genes, including IL-6, TNF-α, and IL-1β [28]. The NF-κB heterodimer consists of the p50 and p65 subunits. NF-κB activity is regulated by its subcellular localization. In resting cells, NF-κB is sequestered in the cytoplasm by the IκB family of proteins, including IκB-α and IκB-b [29]. IκB proteins can be induced by a variety of stimuli, such as LPS and proinflammatory cytokines, which results in the phosphorylation of IκB proteins by a complex of IκB kinases (IKKs). Phosphorylated-IκB proteins are rapidly degraded by the proteasome, allowing NF-κB to be released from IκB and translocated to the nucleus where it can initiate transcription by binding to numerous specific gene promoter elements [30]. In the present study, DEX increased IκB-α levels, and decreased the amounts of phospho-IκB-α. Therefore, increased expression of IκB-α enhanced binding to NF-κB, which further blocked NF-κB translocation to the nucleus and prevented its activity. It was recently reported [31] that IκB-α is a critical mediator of NFκB activity in astrocytes. Compared with astrocytes, neurons exhibit negligible NFκB activity. Therefore, suppressing astrocyte activation would play a critical role for the astrocyte IκB-α/NFκB loop in neuronal homeostasis through a novel neuron-glia signaling pathway. Due to anti-inflammatory properties, DEX inhibits NF-κB signaling pathways in LPS stimulated astrocytes, successfully reducing the production of proinflammatory cytokines such as IL-6, TNF-α, and IL-1β. Ya-juan Zhu, et al [32] also found that pretreatment with DEX could inhibit NF-κB signaling and neuroinflammation in hippocampal astrocytes after tibial fractures in rats.

A preliminary study showed an association between microglia and astrocyte activation in the human brain and delirium in elderly patients, adding to the accumulating evidences that inflammatory mechanisms are involved in delirium [30]. Interestingly, IL-1β can inhibit acetylcholine release and cholinergic-dependent memory function [20, 33]. Therefore, it could be reasonably postulated that the anti-delirium of DEX reported recently [7] might be due to its anti-inflammatory effects although other beneficial features, e.g. improving sleep quality [34], cannot be ruled out.

In conclusion, the present findings indicate that DEX is a potent suppressor of LPS-induced inflammation in activated astrocytes, and decreases the production of proinflammatory cytokines such as IL-6, IL-1β, and TNF-α. These favorable effects are closely associated with NF-κB pathway modulation in astroglia by DEX. Therefore, DEX may be a potent therapeutic agent for preventing or treating neurological disorders closely associated with neuroinflammation triggered by excessive activation of astrocytes.

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Disclosure of conflict of interest

None.

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