

Dexmedetomidine Inhibits Nlrp3 Inflammasome Priming in BV-2 microglia through the NF- κ B Pathway and the ROS-Nlrp3-IL-1 β Signaling Axis

Jiawei Xie (✉ 760664654@qq.com)

Jinan University <https://orcid.org/0000-0001-8423-7678>

Li Chen

Jinan University

Yuling Luo

Sun Yat-Sen University

Jianling Li

Jinan University First Affiliated Hospital

Xianxue Wang

First People's Hospital of Changde City

Jieke Tang

Jinan University First Affiliated Hospital

He Tian

Jinan University First Affiliated Hospital

Shuqing Liang

Jinan University

Biao Xi

The First Affiliated Hospital of Bengbu Medical College

Yalan Li

Jinan University <https://orcid.org/0000-0002-3495-4721>

Research Article

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Abstract

In the brain, the NOD-like receptor family pyrin domain containing 3 (Nlrp3) inflammasome is mostly expressed in microglia and is considered to be the primary cause of perioperative neurocognitive dysfunction (PND). Dexmedetomidine (Dex), a novel kind of clinical anesthetic with anti-inflammatory properties, has been shown to be effective in preventing PND in surgical patients. However, the mechanism of its anti-neuroinflammatory activity is still quite unclear. We examined the impact of Dex administration on Nlrp3 priming in activated BV-2 cells in this research. To investigate the mechanism by which Dex impacts Nlrp3 priming, we employed the inhibitors pyrrolidine dithiocarbamate (PDTC) and N-acetyl-L-cysteine (NAC) to block the NF- κ B p65 and the reactive oxygen species (ROS)-Nlrp3-interleukin (IL)-1 β signaling axis, respectively. The results showed that Dex substantially decreased the expression of Nlrp3 and p65 and significantly inhibited the levels of the inflammatory factors IL-1 β and tumor necrosis factor (TNF)- α in BV-2 cells stimulated with lipopolysaccharide (LPS). Additionally, when the NF- κ B pathway was inhibited by PDTC, Dex could aggravate the downregulation of Nlrp3 and IL-1 β in BV-2 cells. What is more, Dex negatively regulated the expression of Nlrp3 and IL-1 β in activated BV-2 cells when NAC was added. These results showed that Dex inhibited Nlrp3 priming in LPS-induced BV-2 cells, presumably via blocking the NF- κ B pathway and the ROS-Nlrp3-IL-1 β signaling axis.

Introduction

Perioperative neurocognitive dysfunction (PND) refers to changes in cognitive function before or after surgery, and the clinical manifestations include abnormalities in learning, memory, language, thinking, spirit and emotion [1]. Microglia, which are resident macrophages in the central nervous system (CNS), can not only continuously monitor the brain microenvironment but also regulate various cellular responses [2]. There is evidence that surgery may activate microglia and enhance proinflammatory factor expression in the brain [3, 4]. In the CNS, uncontrolled microglial activation and the accompanying inflammation are thought to lead to the development of PND [5, 6]. Microglia contribute to PND by producing proinflammatory cytokines including interleukin-1 (IL-1) and interleukin-6 (IL-6), as well as tumor necrosis factor- α (TNF- α) [7]. The upregulation of these proinflammatory factors predisposes individuals to cognitive impairment [8, 9]. Therefore, understanding how to control microglial activation in the brain after surgery still requires further research.

In PND, microglia expressed NOD-like receptor family pyrin domain containing 3 (Nlrp3), and other components of the Nlrp3 inflammasome were elevated and activated [10]. The most researched class of NOD-like receptors is Nlrp3 (NLRs). NLRs are pattern-recognition receptors (PRRs) that can detect the presence of certain microbial components known as pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) [11]. Through proximity-induced self-cleavage, Nlrp3 activation activates caspase-1, resulting in the maturation of proinflammatory factors such as interleukin-1 β (IL-1 β) and interleukin-18 (IL-18) [12]. By modulating neuroinflammation, hippocampal IL-1 β contributes significantly to surgically caused cognitive impairment. Pretreatment with IL-1 receptor antagonists or knockdown of IL-1 receptors in mice were effective in alleviating cognitive dysfunction

induced by surgical trauma [13]. Recent research has shown that isoflurane-induced cognitive impairment in elderly mice is associated with elevated Nlrp3 levels in the hippocampus, and that this damage may be repaired by inhibiting the Nlrp3-caspase-1-IL-1 β pathway [10]. The mitochondrial reactive oxygen species (mtROS)/Nlrp3 inflammasome/IL-1 β signaling pathway may provide an attractive target for disrupting the pathophysiology of PND [14].

Dexmedetomidine (Dex), a highly selective agonist of the α_2 receptor, is commonly used to give mild analgesia and sedation to surgical patients undergoing general anesthesia. A few preexisting meta-analyses have reported that Dex reduced postoperative delirium among patients who underwent cardiac and noncardiac surgery [15, 16]. Dex inhibits microglia-mediated release of TNF α , interleukin 1 β , and other factors that are essential for the proinflammatory cascade, and IL-1 β and TNF α are related to the development of PND [17]. Furthermore, Dex inhibited the Nlrp3 inflammasome in different animal models, such as lung injury [18], acute kidney injury [19] and brain injury [20], which suggests that Dex may have a protective effect on PND patients. The mechanism by which Dex suppresses the Nlrp3 inflammasome in microglia, on the other hand, remains unknown. As a result, we investigated the underlying mechanisms by which Dex suppresses the Nlrp3 inflammasome via upstream regulatory molecules involved in Nlrp3 priming.

Materials And Methods

Cell culture and treatments

FuHeng Biology (FuHeng Biology, Shanghai, China) provided the BV-2 microglial cell line in mice. At 37 °C and 5% CO₂ in humidified air, BV-2 microglia were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% sodium pyruvate. At least 12 hours prior to treatment with or without the test reagents lipopolysaccharide (LPS), Dex, pyrrolidine dithiocarbamate (PDTC), and N-acetyl-L-cysteine (NAC), BV-2 cells were inoculated at a density of 4×10^6 cells/well in 6-well plates.

To determine cytotoxicity, BV-2 cells were seeded at a density of 1×10^4 cells per well in 96-well plates and treated with various concentrations of LPS (0, 10, 50, 100, 200, 400, 800 ng/L) (Sigma-Aldrich, St. Louis, MO, USA) for various times (6, 12, 24 h) or with various concentrations of Dex (0, 0.1, 1, 5, 10, 20 μ M) for 24 h. To test if Dex has anti-inflammatory activity against BV-2 cells, the cells were treated with 10 μ M Dex and then subjected to 100 ng/mL LPS. PBS-treated cells served as the control group to ensure experimental rigor.

Cell viability

Cell viability was determined using a CCK-8 test kit (Beyotime Institute of Biotechnology, Nanjing, China). For treatment, BV-2 cells were seeded into each well of a 96-well microtiter plate, followed by the addition of 10 μ L of CCK-8 solution and incubation at 37 °C for 4 hours. The absorbance of each well was then

determined at 490 nm (OD490) using a microplate reader (BioTek Instruments, Vermont, USA). Three separate tests were conducted three times each.

ELISA

The cytokines IL-1 β and TNF- α were determined in cell culture supernatants using an ELISA kit (R&D Systems, Minneapolis, Minnesota, USA) in accordance with the manufacturer's instructions.

Total RNA extraction and real-time reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Trizol reagent was used to extract total RNA from BV-2 cells (Life, Massachusetts, USA). To synthesis cDNA, we utilized a reverse transcriptase kit and an SYBR Premix Ex Taq kit (Takara, Tokyo, Japan) in a CFX96 Touch real-time PCR system (Bio-Rad, California, USA). The expression of β -actin served as a control to standardize the quantity of cDNA in the various samples. The comparable Ct method was used to assess real-time qPCR products according to the manufacturer's recommendations. The primer sequences for qRT-PCR were listed in Table 1.

Table1. Sequences of the primers used in the experiments

qRT-PCR Primers	Sequences
Mouse Nlrp3	Forward 5'-GAAGAAGAGTGGATGGGTTTG-3'
	Reverse 5'-CTGCGTGTAGCGACTGTTGAG-3'
Mouse Caspase-1	Forward 5'-GACAAGGCACGGGACCTATGT-3'
	Reverse 5'-CAGTCAGTCCTGGAAATGTGC-3'
Mouse IL-1 β	Forward: 5'-GTGTCTTTCCCGTGGACCTT-3'
	Reverse: 5'-CGTTGCTTGGTTCTCCTTG-3'
Mouse β -actin	Forward: 5'-GGGAATGGGTCAGAAGGACT-3'
	Reverse: 5'-TTT GAT GTC ACG CAC GAT TT-3'

Western blotting

Total proteins were isolated from BV-2 cells according to the manufacturer's procedure using protein extraction kits (Sigma, St Louis, MO, USA). BCA protein assays (Beyotime Institute of Biotechnology,

Nanjing, China) were used to measure the protein content in the lysate supernatant. For electrophoresis, equal quantities of protein (20 µg) each well were loaded onto 12 percent sodium dodecyl sulfate-polyacrylamide gels and then transferred to polyvinylidene difluoride membranes (PVDF; Millipore, Bedford, MA, USA). After blocking nonspecific binding sites on the membrane for 1 hour at room temperature with 5% nonfat milk in TBS containing 0.1% Tween-20 (TBS-T), the membranes were incubated overnight at 4 °C with the following primary antibodies: Nlrp3 (1:1000, ab214185, Abcam, Shanghai, China), Caspase-1 (1:800, ab138483, Abcam, USA), p65 (1:1000, #8242, CST, MA, USA) and GAPDH (1:1000, #5174, CST, MA, USA). Following three washes with TBS-T, the membranes were incubated for 1 hour at room temperature with horseradish peroxidase-labeled secondary antibodies (1:2000, CST, MA, USA). Luminescent liquid was subsequently added, and photographs were taken with a gel imager. Finally, we used a chemiluminescence (ECL) system to visualize the relative expression level of the proteins and used ImageJ software to analyze the intensities of the bands, with GAPDH as an internal control.

ROS analysis

We measured ROS fluorescence intensities using a ROS staining kit (Beyotime Institute of Biotechnology, Nanjing, China) according to the manufacturer's procedure. Briefly, cells were seeded in a 6-well plate and then washed three times with PBS after drug treatment. The cells were then incubated for 20 minutes at 37 °C in the dark with 2 mol/L DCFH-DA probe. After washing with PBS, the nuclei were counterstained with Hoechst 33342 (Sigma, St Louis, MO, USA). A fluorescent microscope was used to determine the fluorescence intensity of BV-2 cells.

Results

BV-2 cell viability in the presence or absence of LPS or Dex

The CCK-8 method was used to determine the viability of BV-2 cells exposed to a range of LPS concentrations (0–800 ng/mL) over a range of time periods (6 h, 24 h, or 48 h). When activated BV-2 cells were subjected to doses higher than or equivalent to 100 ng/mL LPS for 24 hours, viability reduced significantly ($p < 0.05$, Fig. 1a). To evaluate the impact of Dex on BV-2 cell viability, we treated the cells with different doses of Dex (0, 0.1, 1, 5, 10, or 20 µM). The CCK-8 test revealed no change in viability between BV-2 cells treated with various Dex doses ($p > 0.05$, Fig. 1b).

Dex suppresses the inflammatory reactions produced by LPS in BV-2 cells

To evaluate the anti-inflammatory effect of Dex, the mRNA expression of Nlrp3, Caspase-1, interleukin-1 β and the protein expression of Nlrp3, Caspase-1, and p65 were measured. In addition, the levels of IL-1 β

and TNF- α were determined in cell culture supernatants. The RT-qPCR results showed that Dex decreased Nlrp3 (Fig. 2a) and IL-1 (Fig. 2c) expression in LPS-induced BV-2 cells but did not affect caspase-1 expression (Fig. 2b). Western blot analysis revealed that LPS treatment enhanced Nlrp3 and p65 protein expression in BV-2 cells. However, Nlrp3 and p65 protein expression levels were decreased by 10 μ M Dex in comparison to the LPS group (Fig. 2d). Furthermore, Caspase-1 protein expression was not enhanced in LPS-activated BV-2 cells, and 10 μ M Dex had no effect on Caspase-1 expression in comparison to the LPS group (Fig. 2d). The ELISA results showed that Dex inhibited the expression of IL-1 β and TNF- α in BV-2 cells stimulated by LPS (Fig. 2h and i).

Dex suppresses the activation of NF- κ B by LPS in BV-2 cells

To determine whether Dex's inhibitory impact on the Nlrp3 inflammasome was due to the NF- κ B signaling pathway, we employed the NF- κ B inhibitor PDTC to disrupt the NF- κ B pathway. As shown in Fig. 3, PDTC was efficient at inhibiting NF- κ B p65 protein production as well as the inflammatory mediators IL-1 β and TNF- α . This finding suggested that PDTC successfully blocked the NF- κ B pathway. Moreover, when the NF- κ B pathway was inhibited, the LPS+PDTC+Dex group demonstrated a reduction in Nlrp3 and the inflammatory mediators IL-1 β and TNF- α when compared to the LPS+PDTC group. These results indicated that Dex acted on the Nlrp3 inflammasome through other pathways than the NF- κ B pathway.

Dex's anti-inflammatory actions on LPS-induced BV-2 cells are mediated via the ROS-Nlrp3-IL-1 β pathway

The impact of Dex on the signaling molecule ROS upstream of the Nlrp3 inflammasome was confirmed. To assess Dex's impact on the ROS-NLRP3-IL-1 β axis, the ROS-specific inhibitor NAC was employed as a positive control (Fig. 4a and b). The findings indicated that NAC substantially decreased the amount of reactive oxygen species (ROS) in LPS-activated BV-2 cells, as well as the expression of the downstream protein Nlrp3, as shown by the ROS assay and Western blotting (Fig. 4c-f). Dex had a similar impact to NAC on LPS-activated BV-2 cells. This finding suggested that Dex may have anti-inflammatory effects through the ROS-Nlrp3-IL-1 β pathway.

Discussion

The current research established conclusively that Dex has anti-inflammatory properties in LPS-activated microglia. Dex inhibited the generation of TNF- α and IL-1 β by LPS-activated BV-2 cells, as shown in our research. These results suggested that Dex exerted a significant anti-inflammatory impact, which may contribute to the reduction of postoperative tissue damage. Neuroinflammation may be generated in the brain when systemic inflammation is caused by surgery on peripheral tissues and organs [21, 22]. A large number of studies have indicated that neuroinflammation, which is characterized by microglial activation, plays a vital role in PND [23]. Since Dex has anti-inflammatory effects, this property of Dex may mediate

the reduction in PND. Additionally, previous research indicates that Dex may help prevent PND by decreasing the expression of the Nlrp3 inflammasome [24]. However, the exact mechanism is still unclear. The current study contributes to a better understanding of how Dex works as an anti-inflammatory.

Dex has been shown in a growing number of studies to inhibit the expression of the Nlrp3 inflammasome in a variety of disease models. It is currently recognized that microglial activation plays a vital role in PND [25]. Therefore, we adopted the widely used experimental model of BV-2 microglial activation by LPS [26, 27]. According to a previous paper, we treated BV-2 cells with 0.1–20 μ M Dex to examine cytotoxicity and found that Dex treatment did not induce cytotoxicity. In addition, we found that treatment with 10 μ M Dex inhibited LPS-induced Nlrp3 and p65 activation in BV-2 cells. These results were consistent with those of previous study. LPS has been shown to cause proinflammatory and cytotoxic reactions in microglia [28]. Hu's research [29] shows that Nlrp3 inflammasome activation requires the participation of LPS and ATP. Moreover, LPS is involved in the initiation of Nlrp3 and does not cause an increase in caspase-1. We utilized LPS alone as a stimulant for BV-2 cells in this research and found enhanced expression of Nlrp3 and upstream signaling components. The LPS-induced BV-2 cell model was applied to measure Dex's mechanism of action in the Nlrp3 inflammasome's upstream signaling pathway.

Recent research revealed that NF- κ B, an upstream factor of the Nlrp3 inflammasome, plays a key regulatory function in inflammasome activation [30]. NF- κ B p65 is the main component of the NF- κ B heterodimer, which is phosphorylated on Ser536 site in response to LPS and translocates to the nucleus to induce Nlrp3 and IL-1 β production [31]. We obtained results similar with earlier research following LPS stimulation of BV-2 cells in the current study. Furthermore, we discovered that Dex inhibited NF- κ B p65 and Nlrp3 protein expression. Yao et al demonstrated that Dex mitigated LPS-induced acute kidney injury (AKI) by inhibiting oxidative stress injury and NLRP3 inflammasome activation through the NF- κ B pathway [32]. The NF- κ B inhibitor PDTC can effectively block NF- κ B in microglia, which has been well confirmed [33]. Interestingly, after blocking NF- κ B p65 with PDTC in LPS-activated BV-2 cells, Dex could still reduce Nlrp3 and IL-1 β protein expression. No previous study has shown a similar result. These data suggest that Dex can affect Nlrp3 expression through other upstream factors.

In terms of upstream factors affecting Nlrp3 expression, the ROS-Nlrp3-IL-1 signaling axis is critical. Mitochondrial dysfunction generates ROS, which trigger Nlrp3 oligomerization through the ROS-Nlrp3-IL-1 β signaling axis, thus acting upstream of Nlrp3 activation [34]. NAC, an inhibitor of ROS, is commonly used to suppress the production of ROS and the Nlrp3 inflammasome. To clarify the effect of Dex on upstream Nlrp3 factors, we used NAC to block ROS in BV-2 cells treated with LPS. Our results showed that NAC substantially inhibited ROS generation and Nlrp3 inflammasome activation in BV-2 cells treated with LPS. These findings corroborated earlier research [35]. Additionally, we found that Dex had a similar impact to NAC on LPS-activated BV-2 cells. Dex substantially reduced the formation of LPS-induced ROS in BV-2 cells, suggesting that ROS suppression may be a process by which Dex suppresses Nlrp3 activation.

In summary, our study demonstrated that Dex inhibits Nlrp3 activation in LPS-induced BV-2 microglia through the NF- κ B signaling pathway and the ROS-Nlrp3-IL-1 β signaling axis (Fig. 5). When NF- κ B p65 is inhibited, Dex can still suppress inflammatory mediators by reducing ROS and Nlrp3 protein expression. Dex blocks the ROS signaling pathway, which was consistent with the effects observed when we blocked the ROS signaling pathway using the specific ROS inhibitor NAC. Dex may contribute to anti-neuroinflammation through its inhibitory effect on the Nlrp3 inflammasome's upstream signaling pathway. In conclusion, our data reveal a possible mechanism by which Dex reduces the expression of inflammatory mediators.

Declarations

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Conflicts of Interest The authors declare that the publishing of this article does not involve any conflict of interest.

Availability of Data and Material The original data will be provided if required.

Code Availability Not applicable.

Authors' contributions Jiawei Xie, Li Chen, and Yalan Li conceived the research, designed and conducted the experiments, and wrote the manuscript. Yuling Luo, Jianling Li, and Xianxue Wang contributed to the data presentation by supplying BV-2 cells, conducting qPCR testing, and assisting with data analysis. Jieke Tang, He Tian, Shuqing Liang, and Biao Xi revised the scientific content of the manuscript.

Ethics approval This study used the BV-2 mouse microglial cell line commercially available, thereby not requiring ethics.

Consent to participate Not applicable.

Consent for publication Not applicable.

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Figures

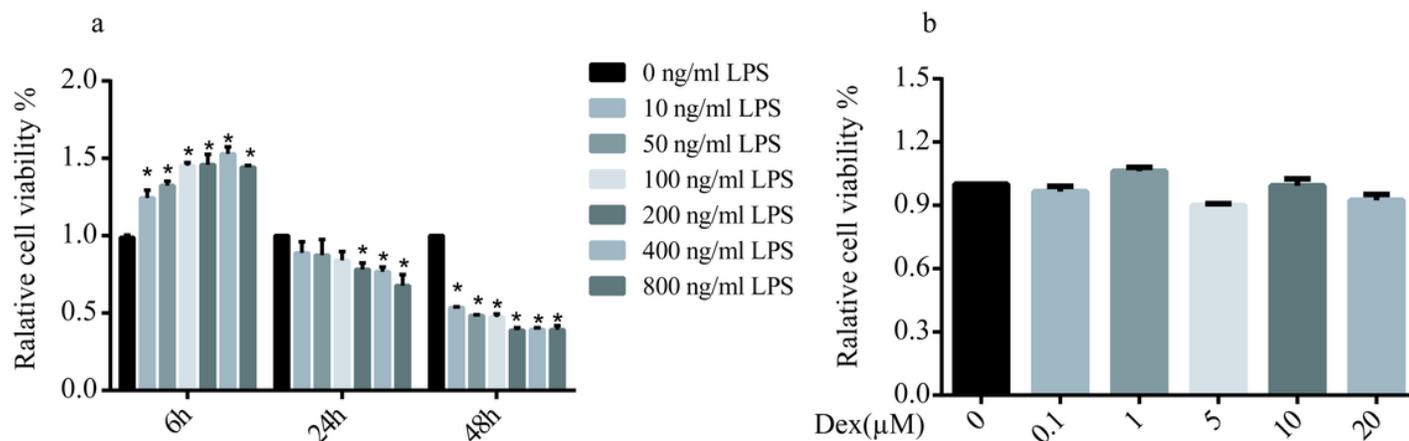


Figure 1

The viability of BV-2 cells in the presence or absence of LPS or Dex. (a): The vitality of BV-2 cells treated to various doses (0–800 ng/mL) of LPS over various time periods (6, 24, or 48 h) was determined. (b): The viability of BV-2 cells treated to various doses of Dex (0–20 μ M) over 24 hours. The results of three separate trials are given as the means standard error of the mean ($n = 3$). * $p < 0.05$ versus the control group. Dex, dexmedetomidine; LPS, lipopolysaccharide

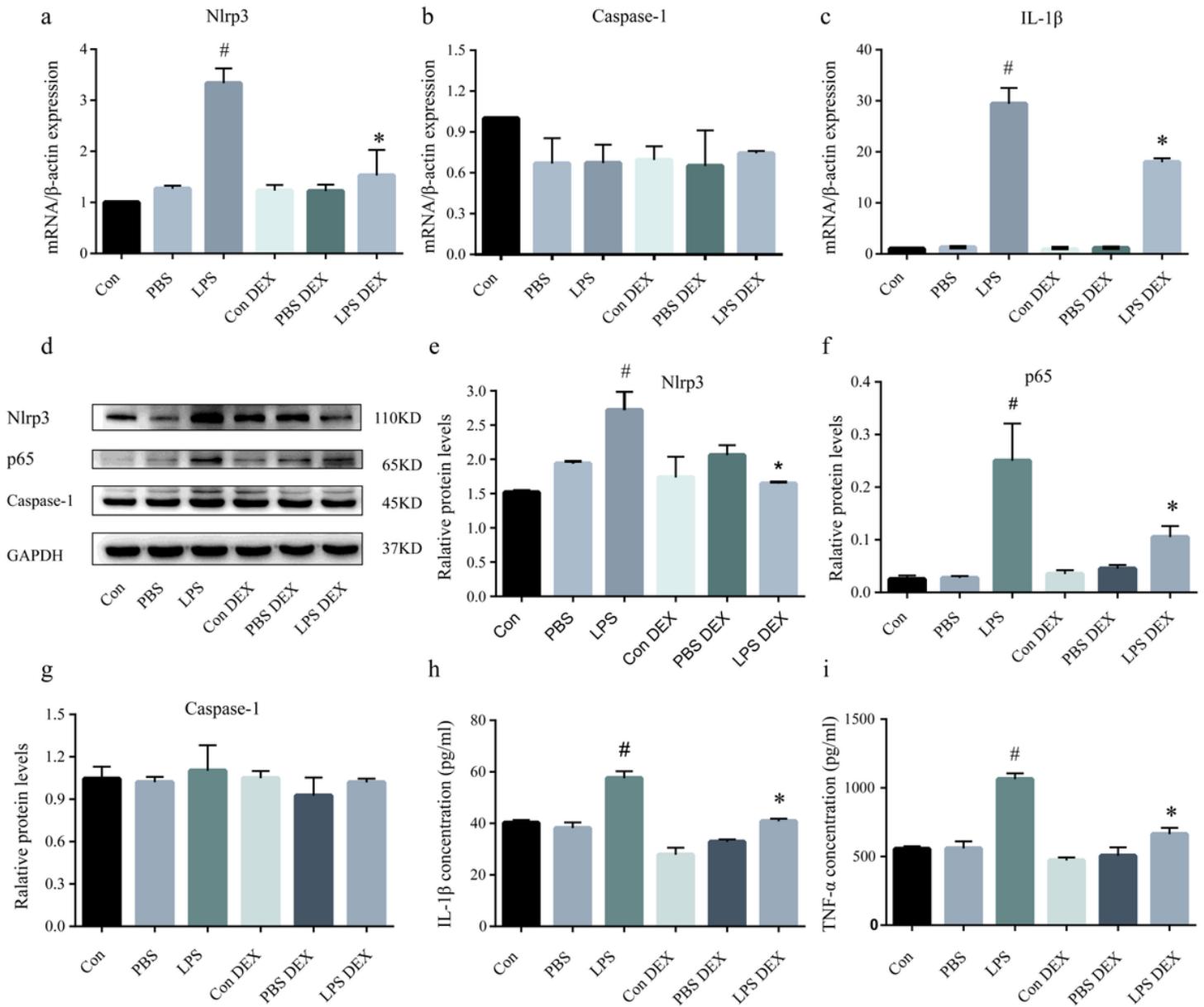


Figure 2

Dex inhibits the LPS-induced inflammatory responses in BV-2 cells. 100 ng/mL LPS and 10 μ M Dex were used alone or in combination to treat BV-2 cells. RT-qPCR was used to measure the mRNA levels of Nlrp3 (a), Caspase-1 (b), and IL-1 β (c). Western blotting was used to measure Nlrp3, p65, and Caspase-1 protein levels (d–g) in BV-2 cells, and ELISA was performed to measure IL-1 β (h) and TNF- α (i) levels in cell culture supernatants. # p < 0.05 vs. the control. * p < 0.05 vs. the LPS group. Dex, dexmedetomidine; LPS, lipopolysaccharide; PBS, phosphate buffer saline

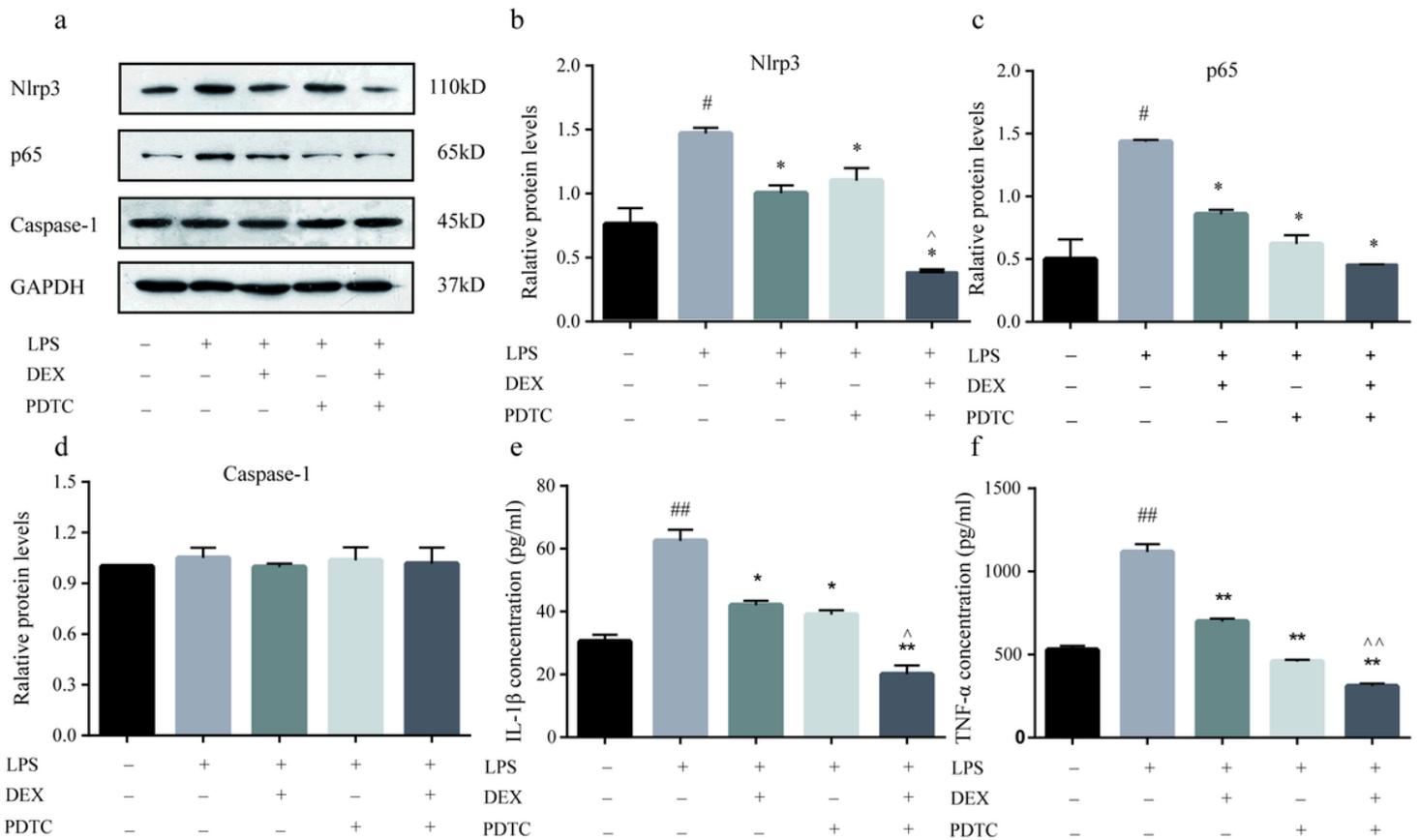


Figure 3

Other pathways than NF-κB are involved in the anti-inflammatory effect of Dex on LPS-induced BV-2 cells. LPS, PDTC, and DEX were used to treat BV-2 cells, either alone or in combination. Western blotting was used to determine the amounts of Nlrp3, p65, and caspase-1 protein in BV-2 cells (a–d), and ELISA was used to determine the levels of IL-1β (e) and TNF-α (f) in cell culture supernatants. # $p < 0.05$, ## $p < 0.01$ vs. the control. * $p < 0.05$, ** $p < 0.01$ vs. the LPS group. ^ $p < 0.05$, ^^ $p < 0.01$ vs. the LPS+PDTC group. Dex, dexmedetomidine; ELISA, enzyme-linked immunosorbent assay; IL, interleukin; LPS, lipopolysaccharide; PDTC, pyrrolidine dithiocarbamate; TNF, tumor necrosis factor

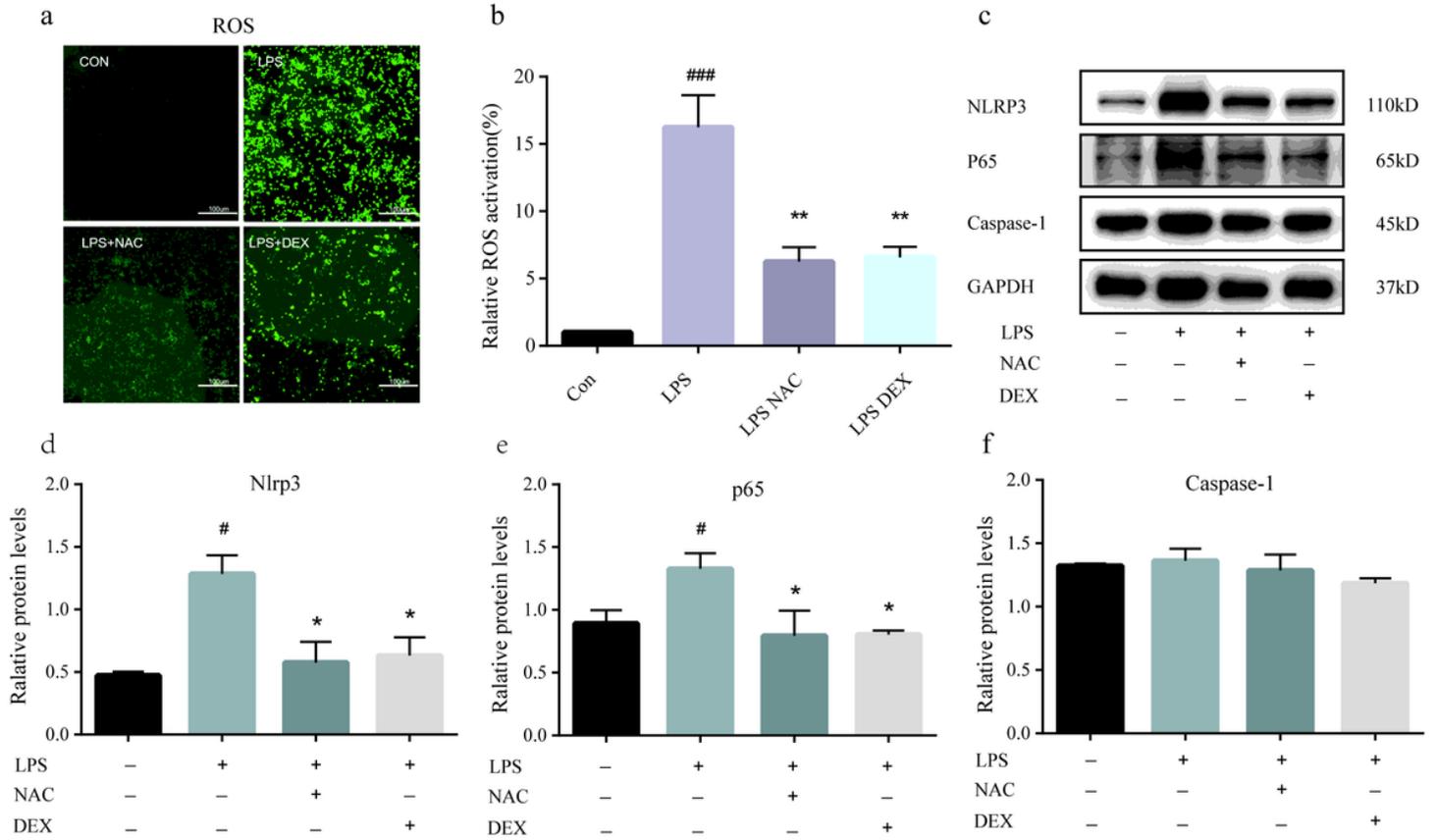


Figure 4

Dex's anti-inflammatory actions on LPS-induced BV-2 cells are mediated via the ROS-Nlrp3-IL-1 β pathway. (a) and (b): Dex protects BV-2 cells against oxidative stress caused by LPS. For 24 hours, BV-2 cells were exposed to LPS and treated with Dex. Intracellular ROS was measured using DCFH-DA. Scale bar = 100 μ m. (c–f): Western blot examination of the levels of Nlrp3, p65, and caspase-1 proteins in BV-2 cells. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. the control. * $p < 0.05$, ** $p < 0.01$ vs. the LPS group. DCFH-DA, xxx; Dex, dexmedetomidine; IL, interleukin; LPS, lipopolysaccharide; NAC, N-acetyl-L-cysteine; TNF, tumor necrosis factor; ROS, reactive oxygen species

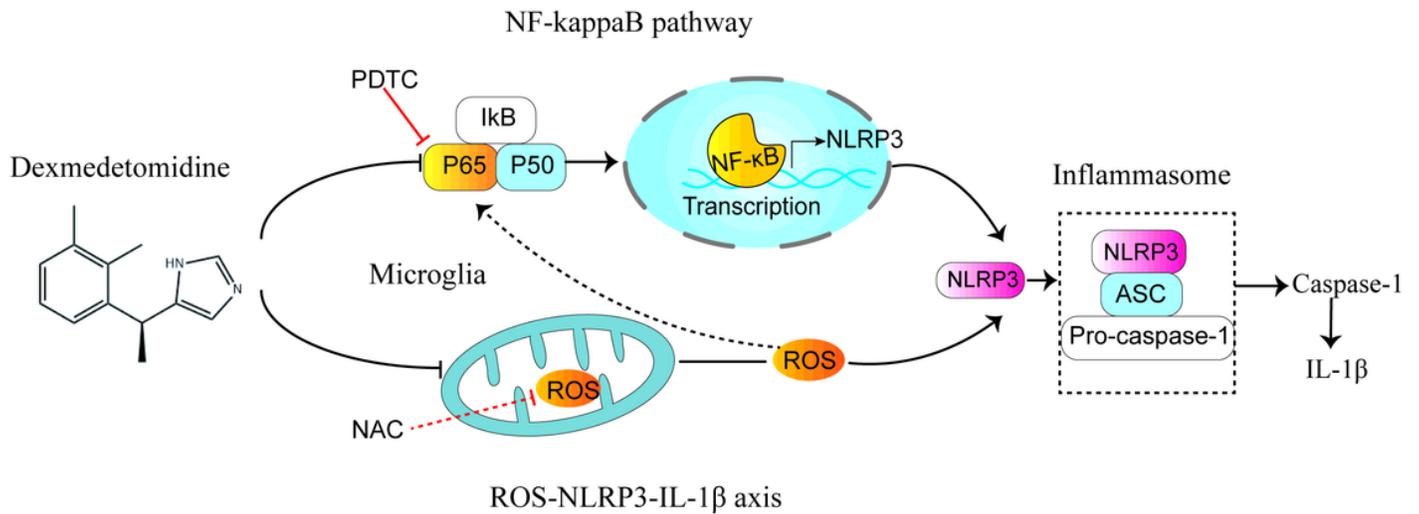


Figure 5

Schematic diagram of the mechanism of the anti-inflammatory effect of Dex. Microglia transform to the M1 phenotype under LPS stimulation. Then, the NF- κ B signaling pathway and the ROS-Nlrp3-IL-1 β signaling axis are activated. This activation leads to the massive induction of the downstream signaling factor Nlrp3. Dex can reverse the activation of these two signaling pathways and inhibit the initiation of Nlrp3. ASC, Apoptosis-associated speck-like protein containing a CARD; IL, interleukin; LPS, lipopolysaccharide; NAC, N-acetyl-L-cysteine; PDTC, pyrrolidine dithiocarbamate; PND, perioperative neurocognitive disorder; ROS, reactive oxygen species; TNF, tumor necrosis factor