Oxycodone ameliorates the inflammatory response induced by lipopolysaccharide in primary microglia

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Background: Activation of microglia participates in a wide range of pathophysiological processes in the central nervous system. Some studies reported that oxycodone (6-deoxy-7,8-dehydro-14-hydroxy-3-O-methyl-6oxomorphine) could inhibit the overactivation of glial cells in rats’ spinal cords. In the present study, we observed the effect of oxycodone on inflammatory molecules and pathway in lipopolysaccharide (LPS)-stimulated primary microglia in rats.

Materials and methods: Neonatal rats’ primary microglia were exposed to various concentrations (25, 50, 100 ng/mL) of oxycodone for 1 h after LPS stimulation for 24 h. The levels of pro-inflammatory mediators, IL-1β, TNF-α, and TGF-β1/smads2/3 signaling pathway were measured. The activation situation of microglia and the expression of TβR1 were observed by immunofluorescence.

Results: Oxycodone at 25 ng/mL did not change the levels of proinflammatory molecules and TGF-β1/smads2/3 signaling pathway in primary microglia, which was increased by LPS. Oxycodone at 50 and 100 ng/mL could significantly suppress LPS-induced production of TNF-α and IL-1β and the expression of TGF-β1/mRNA, IL-1β/mRNA, and TGF-β1/smads2/3 signaling pathway.

Conclusion: These findings indicate that oxycodone, at relatively high clinically relevant concentration, can inhibit inflammatory response in LPS-induced primary microglia. The detailed mechanism needs to be investigated in future.

Keywords: oxycodone, inflammatory, microglia

Introduction

Microglia, innate immune cells of the central nervous system (CNS), are considered to be important participants in the natural immunity and adaptive immunity of the CNS.¹

Activation of microglia by injury or external stimulus is widely perceived as contributing to a series of inflammatory signaling pathways and produces various proinflammatory cytokines, involved in a broad range of pathophysiological conditions, including neurodegeneration, neuropathic pain, and traumatic brain injury.²,³ Therefore, suppression of these inflammatory molecules, such as IL-1β and TNF-α, may be a potential and effective therapeutic method to mitigate the progression of previously mentioned neuroinflammatory conditions.

Oxycodone (6-deoxy-7,8-dehydro-14-hydroxy-3-O-methyl-6oxomorphine), μ and κ opioid receptor agonist, is widely used for the treatment of a large variety of painful disorders.⁴ Previous studies have shown that controlled-release oxycodone improved pain management after uterine artery embolization for symptomatic fibroids, and relieved neuropathic pain in diabetic patients.⁵,⁶ And in an animal model, oxycodone was
reported to suppress the overactivation of glial cells and the subsequent release of pro-inflammatory cytokines. Besides, dextromethorphan could potentiate the effects of oxycodone.7 However, little is known about the effect of oxycodone on the inflammatory response in microglia in vitro.

Some studies and experimental models have confirmed that lipopolysaccharide (LPS) can induce inflammatory response and promote the release of proinflammatory cytokines in microglia.8,9 So, in this study, we attempt to investigate whether oxycodone could reduce the production of proinflammatory molecules and regulate the signaling pathway associated with inflammatory response. This study may provide several novel directions in understanding the analgesic and anti-inflammatory effect of oxycodone in patients and animal models.

Materials and methods

Cell culture

Male neonatal Sprague–Dawley (SD) rats were purchased from the center for disease control and prevention in Hubei Province. All related animal protocols were carried out according to the US National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Experimental Animal Center Review Board of Wuhan University (Wuhan, People’s Republic of China).

Microglial cultures were prepared as previously described.8,9 In brief, using 75% alcohol to disinfect skin, healthy 1-day old SD rats were decapitated and the brain tissue removed on an ultraclean bench. Then the brain tissue was placed in a sterile petri dish containing precooled PBS (Genom, Hangzhou, People’s Republic of China) solution, cleaned twice. The meninges and cerebellum were removed (Genom, Hangzhou, People’s Republic of China) solution, was placed in a sterile petri dish containing precooled PBS tissue removed on an ultraclean bench. Then the brain tissue described.8,9 In brief, using 75% alcohol to disinfect skin, the Experimental Animal Center Review Board of Wuhan Province. All related animal protocols were carried out from the center for disease control and prevention in Hubei

Experimental protocol

Microglial cells were randomly divided into the following five groups: 1) control group (the microglia in the untreated group with no LPS [from Escherichia coli; Sigma-Aldrich Co., St. Louis, MO, USA] and oxycodone [Mundipharma, Beijing, People’s Republic of China]); 2) LPS group (the microglia were treated with 1 μg/mL LPS); and 3) O25, O50, and O100 (the cells were treated with 25, 50, 100 ng/mL oxycodone, respectively, for 1 h after the treatment with 1 μg/mL for 24 h).

Cell viability assay

After the microglia were treated with different reagents, we used Cell Counting Kit-8 (CCK-8) assay to assess the effect of LPS and/or oxycodone on cells’ viability according to the manufacturer’s instructions (Beyotime, Shanghai, People’s Republic of China). Microglia were plated into 96-well plates at a density of 1×10^4 cells per well and cultured at 37°C with 5% CO₂ for 24 h and then washed. Microglia were treated with different concentrations of oxycodone and/or LPS, then CCK-8 reagent (10 μL) was added to each well. Four hours later, the absorbance for all wells was measured at 450 nm using a microplate reader (model: DR-200Bs, Diatek, Wuxi, China).

RT-PCR

According to the manufacturer’s instructions, the total RNA was isolated from the primary microglia with Trizol Reagent (Thermo Fisher Scientific). Total RNA from these samples was reverse-transcribed into cDNA using PrimeScript™ RT Reagent Kit with gDNA Eraser (TaKaRa, Kyoto, Japan). After reverse-transcription, the RT-PCR was performed using SYBR Green PCR Master Mix (TaKaRa). PCR primers were as follows (forward and reverse): TGF-β1: 5′-GTGGCGTAACAGGGAGACG-3′ and 5′-AGGTGTGGAGGACCTTTCCAG-3′; TNF-α: 5′-CAC- CAGCTTTCTCTTGACTG-3′, and 5′-GCTACGGGGCTTGACTCG-3′; IL-1β: 5′-GTGGCTACTACCTATGT CTTCG-3′ and 5′-CCACTTGGCTCTTCTTTCG-3′; GAPDH: 5′-CGCTACATCAATGGGGTG-3′ and 5′-TTGGTGCAGAAATCAGGGAG-3′. PCR amplification conditions: 40 cycles of denaturation at 95°C for 15 s, annealing at 56°C for 20 s, and extension at 72°C for 45 s. The relative expression levels were calculated by the 2−ΔΔCt method. The mRNA levels of the target gene were normalized to the levels of GAPDH. GAPDH was used as the endogenous “housekeeping” control gene for these analyses.
Cytokine assay
The presence of TNF-α, IL-1β, and TGF-β1 in the culture supernatant was determined using an ELISA kit (R&D Systems, Inc., Minneapolis, MN, USA). The results were expressed in pg/mL.

Western blot
The protein extraction and Western blot were performed as described previously. Cultured cells were collected and cell lysates were prepared in the lysis buffer (Beyotime). The cell lysates were × g for 5 min at 4°C to collect the supernatants. Then the total protein concentrations in the supernatants were measured by BCA protein assay kit (Aspen, Wuhan, People’s Republic of China). Protein samples (30 μg) were subjected to electrophoresis on 10% SDS-polyacrylamide gel (Aspen, Wuhan, China) and transferred to PVDF membranes (EMD Millipore, Billerica, MA, USA). After blocking with 5% non-fat milk for 1 h at room temperature, the membranes were probed at 4°C overnight with the following primary antibodies: 1:600 for p-Smad2 (Cell Signaling, MA, USA), 1:600 for p-Smad3 (Cell Signaling), and 1:5000 for GAPDH (Cell Signaling).

Secondary antibody (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) was used at a 1:10000 dilution for 30 min at room temperature. Finally, the immunoreactions were detected by enhanced chemiluminescence (Aspen) and visualized by Alpha Ease FC software for image analysis.

Immunofluorescence
The microglia from each group were seeded onto cover slips and fixed in 4% formaldehyde for 30 min at room temperature before cells permeabilized for 20 min in PBS containing 0.5% Triton X-100. The cells were incubated with a rabbit monoclonal antibody against CD11b (1:200, Abcam, Cambridge, UK) and TβR1 (1:100, Abcam) respectively at 4°C overnight. Next, after washing, cover slips were incubated for at least 1 h with TRITC-labeled goat anti-rabbit IgG or anti-mouse IgG (Aspen). Then cover slips were washed three times with blocking buffer and cells were dyed nucleus with DAPI (Aspen) for 15 min. Finally, the fluorescence signals were analyzed using a fluorescence microscope (Olympus Corporation, Tokyo, Japan).

Statistical analysis
The data were expressed as the mean ± standard error of the mean. Statistical significance between groups was analyzed by using a one-way ANOVA with Tukey’s multiple comparison test. A value of \( P < 0.05 \) was accepted as statistically significant. All statistical analyses were performed using SPSS 11.0 software.

Results
No significant change in the cell viability treated with oxycodone and/or LPS
As shown in Figure 1, cytotoxic effects of oxycodone and/or LPS were tested by measuring the viability of microglial cells by using the CCK-8 assay. Compared with serum-free DMEM-treated group, the viability of microglia was not significantly changed by treatment with oxycodone and/or LPS \( (F=2.113, P=0.102) \).

Oxycodone reduced the pro-inflammatory cytokines' production and downregulated the expression of related mRNAs
Utilizing ELISA, we evaluated the effect of oxycodone on the release of LPS-induced cytokines, including TNF-α and IL-1β, in culture supernatants. As shown in Figure 2A and 2C, compared to control group, LPS could induce the inflammatory response in primary microglia and increase the production of TNF-α \( (P<0.01) \) and IL-1β \( (P<0.01) \). Treatment with oxycodone at 25 ng/mL had no effect on LPS-induced production of TNF-α and IL-1β \( (P=0.105) \). Treatment with oxycodone at 50 and 100 ng/mL could significantly suppress the levels of TNF-α and IL-1β in primary microglia \( (TNF-\alpha: P=0.034 \text{ and } IL-1\beta: P=0.0234) \).
To clarify the underlying mechanism responsible for the associated effect of oxycodone on cytokines in microglia, we subsequently detected the cytokines’ mRNA expression using qRT-PCR. In line with the results acquired from cytokine production, LPS could increase the mRNA expression levels of TNF-α (P < 0.01) and IL-1β (P < 0.01) in primary microglia (Figure 2B and 2D).

Oxycodone at 50 and 100 ng/mL could significantly suppress the mRNA levels of TNF-α and IL-1β in primary microglia (TNF-α: P=0.040 and IL-1β: P=0.032).

Oxycodone downregulated the LPS-stimulated inflammatory TGF-β1/smad2/3 signaling pathway in primary microglia

Activated microglia leads to increased production of TGF-β1 cytokines which have anti-inflammatory actions. To investigate the effect of oxycodone on TGF-β1 and smad2/3 expression in LPS-induced primary microglia, we examined the expression of TGF-β1 mRNA, the concentration of TGF-β1, and the levels of its downstream mediators, including p-Smad2 and p-Smad3, by qRT-PCR, ELISA, and Western blot, respectively.

As shown in Figure 3, expression of TGF-β1 mRNA and the concentration of TGF-β1 were clearly increased in LPS-stimulated primary microglia, as well as its downstream mediators, p-Smad2 and p-Smad3 (TGF-β1 mRNA: P<0.01; TGF-β1: P<0.01; p-Smad2 and p-Smad3: P<0.01).

After treatment with oxycodone at 50 or 100 ng/mL, oxycodone reduced the production of TGF-β1 and downregulated the expression of TGF-β1 mRNA in LPS-stimulated microglia (TGF-β1 mRNA: P=0.041 and TGF-β1: P=0.037). Likewise, Western blot showed that p-Smad2 and p-Smad3 were inhibited by oxycodone at 50 or 100 ng/mL in LPS-induced microglia (p-Smad2: P<0.01 and p-Smad3: P<0.01).
CD11b and TβR1 expression in cultured microglia

To indicate the activation of microglia in each group, we tested the expression of microglial marker CD11b. A one-way ANOVA for the data reported in Figure 4 identified significant differences in CD11b expression (Figure 4; \( F = 99.816, P < 0.01 \)) among the groups. LPS could induce the activation of primary microglia and the expression of CD11b was clearly increased compared with control group (\( P < 0.01 \)); compared with LPS group, oxycodone at 50 or 100 ng/mL suppressed the expression of CD11b (\( P = 0.023 \) and \( P < 0.01 \), respectively).

To further investigate the expression of TGF-β1 in primary microglia, we analyzed surface expression of TβR1 on LPS-stimulated primary microglia. A one-way ANOVA for the data reported in Figure 5 identified significant differences in TβR1 expression (Figure 5; \( F = 41.242, P < 0.01 \)) among the groups. LPS enhanced the surface expression of TβR1 on primary microglia (\( P < 0.01 \)) and treatment with oxycodone at 50 or 100 ng/mL resulted in TβR1 expression on the surface of primary microglia (\( P = 0.018 \) and \( P < 0.01 \), respectively). Collectively, oxycodone downregulated the LPS-stimulated inflammatory TGF-β1/smad2/3 signaling pathway in primary microglia (Figure 5).

Discussion

Our study’s main findings are as follows: first, LPS at 1 μg/mL can successfully induce activation of primary microglia and the activated microglia leads to a series of cytokine release. The TGF-β1/smad2/3 signaling pathway, which is a novel factor in cell cycle and associated with cell growth, apoptosis, and inflammatory response, is also upregulated in primary microglia induced by LPS. A low concentration of oxycodone (25 ng/mL) exerts no effect on the regulation of inflammatory molecules and pathways in activated primary microglia. Conversely, median and high concentrations of oxycodone (50 and 100 ng/mL) are capable of inhibiting the pro-inflammatory cytokines, including IL-1β, and TNF-α and the expression of mRNA. For TGF-β1/smad2/3 signaling pathway, median and high concentrations of oxycodone...
also exhibited the ability to inhibit TGF-β1 protein, its down-  
stream mediators pSmad2/3, and the surface expression of  
TβR1 on activated primary microglia.

Based on a previous study, we found that LPS at 1 μg/mL  
can both activate primary microglia and have no effect on its  
cell viability. So, in vitro, we prepared primary microglia  
from neonatal rats’ brain tissue to establish the inflammatory  
response model induced by LPS at 1 μg/mL. The clinically  
relevant plasma concentration range of oxycodone is 5–50  
ng/mL. Therefore, we consider 25–50 ng/mL as the  
range of clinically relevant concentrations achievable during  
oxycodone analgesia. Although oxycodone at 100 ng/mL  
is much higher than the clinical concentration, we could  
also observe its effects of cytotoxicity and inflammatory  
response on microglial cells and provide reference for opioid  
accumulation or abuse.

Exaggerated neuroinflammatory responses in the brain  
appear fundamental to neuroinflammatory-associated CNS  
disorders. Altered inflammatory potential in the brain is  
due, in part, to changes in microglia, the predominant innate  
immune cells of the brain and the major CNS source of  
inflammatory cytokines. Activated microglia play a pleio-  
tropic and crucial role in the CNS pathophysiology process,  
for instance neurodegenerative disorder, traumatic brain  
injury, and neuropathic pain. In the early stage, activated  
microglia participate in maintaining tissue homeostasis, sens- 
ing the environment, searching for tissue damage or invading  
microorganisms, which play a positive and beneficial role  
in pathological processes. Nonetheless, when persistently  
activated, microglia ramp up a mass of pro- and anti-  
inflammatory cytokines and apoptotic cascades, which lead  
to excessive and unnecessary neuron loss and CNS injury.  
In this regard, our data demonstrated that activated microglia  
could increase both pro-inflammatory molecules, IL-1β, and  
TNF-α, and anti-inflammatory cytokine, TGF-β1. Both the  
increase of pro- and anti-inflammatory cytokines maintain  
a balance in the inflammatory response microenvironment.  
Meanwhile, our data also indicated that relatively high con-  
centrations of oxycodone (50 and 100 ng/mL) attenuated the  
pro-inflammatory cytokines, including IL-1β and TNF-α,
Oxycodone ameliorates the inflammatory response induced by lipopolysaccharide

Figure 5 Effects of oxycodone on the expression of TβR1 in each culture medium. Notes: Immunofluorescent staining was used to observe the expression of TβR1 in each group, which represented the expression situation of TGF-β1. Oxycodone at 50 and 100 ng/mL alleviated upregulation of TβR1 in microglia. Original magnification ×100, scale bar: 50 μm. Data are mean ± standard error of the mean. n=3, *P<0.01 vs control group; **P<0.01 vs LPS group; #P<0.05 vs LPS group.

Abbreviation: LPS, lipopolysaccharide.

and the expression of mRNA. Thus, oxycodone possesses a special advantage that other opioid drugs do not.

Oxycodone has been used in a clinical setting since 1917, and is increasingly used worldwide to treat acute and chronic pain in two types of dosage forms: oral administration and intravenous administration. Of note, although there are some limitations to the epidural administration of oxycodone, it could provide much higher cerebral spinal fluid concentrations, possibly better analgesic efficacy, and decrease the total dose of rescue fentanyl. Unlike other μ-opioid analgesics, oxycodone provides not only potent analgesia but also has less side effects, such as low tolerance, respiratory depression, and pruritus. And recently, several studies indicated that oxycodone may have the property of relieving inflammatory response, in addition to its antinociceptive effects. In an in vivo model, Yang et al showed that oxycodone can ameliorate the spinal nerve injury-induced activation of glial cells (astrocytes and microglia) and plasma levels of proinflammatory cytokines (IL-6, IL-1β, and TNF-α) in rats, and co-administration of dextromethorphan potentiated this effect. Moreover, Yang et al also reported that oxycodone exerts protective effects on LPS-induced acute lung injury in rats and decreases the production of inflammatory cytokines, including IL-1β, TNF-α, and HMGB-1. Consistent with these results, our study found that oxycodone at 50 and 100 ng/mL attenuated upregulation of several inflammatory molecules in primary microglia induced by LPS. Furthermore, using immunofluorescence, we found that oxycodone can also decrease surface expression of TβR1 on LPS-stimulated primary microglia, suggesting that oxycodone not only inhibits pro-inflammatory cytokines but also regulates anti-inflammatory cytokines in activated microglia. However, Kokki et al showed that oxycodone (0.5–2 mM) has potential cytotoxicity in human neuroblastoma and mouse motoneuronal cells. These results seem to be in conflict with our present study. Some factors could explain this inconsistent phenomenon. First, our study utilized the microglial cells of neonatal rats rather than human neuroblastoma and mouse motoneuronal cells. Second, the time of oxycodone treatment was just 1 h rather than 24 h or 48 h as in Kokki’s study. Due to the differences in exposure time and types of cultured cell lines, oxycodone may exhibit different degrees of cytotoxicity. For TGF-β1, current evidence has focused on its multiple roles, including modulating inflammation and neuronal survival in neurodegenerative diseases and CNS injury. Chen et al demonstrated that TGF-β1 could suppress glial and T cell-mediated neuroinflammation and thereby alleviate Alzheimer's disease-related neurodegeneration; furthermore, Hu et al reported that over-activated microglia surrounding neuritic plaques could recruit more neurotoxic microglia and lead to further neuroinflammation. They utilized TGF-β1 to suppress the chemotactic migration of BV-2 microglia toward Aβ plaques, and its potential mechanism may be involved in SMAD signaling and down-regulation of CCL5. In addition, TGF-β1/smad2/3 signaling has also been shown to be a “double edged sword” in several pain models. On the one hand, in chronic constriction injury-induced neuropathic rats, Chen et al showed that suppressing p38 and ERK activity affects TGF-β1-induced analgesia during neuropathy, suggesting that it may be a therapeutic target for neuropathic pain. However, in chronic pancreatitis, Zhu et al found that TGFβ1 enhanced neuronal excitability and increased the concentration of intracellular calcium. Furthermore, intrathecal injection of TGFβ1 produced abdominal hyperalgesia in healthy rats.

Welser-Alves and Milner indicated that microglia, not astrocytes, are the major source of TNF-α and TGFβ1 in postnatal glial cultures, and that microglial production of these antagonistic cytokines is tightly regulated by cytokines such as LPS. Considering the viewpoint of this article, we suggested that oxycodone may ameliorate the whole activation...
degree of primary microglia induced by LPS and continue to keep a relative balance of pro- and anti-inflammatory molecules. And the expression of CD11b, which reflects the activated state of microglia, can also support this explanation. Therefore, relatively high concentrations of oxycodone can both suppress the pro- and anti-inflammatory molecules and associated pathway. Certainly, more inflammatory cytokines need to be tested in future studies. Or rather, our results could be taken into consideration when choosing what analgesic is indicated during perioperative period and postoperative pain treatment. And these findings provide novel insight into the mechanisms by which oxycodone modulates microglia under inflammatory conditions.

Nevertheless, there are some limitations to our study. First and foremost, we did not explore the exact mechanism of regulation of oxycodone in LPS-induced primary microglia. Inflammatory response induced by LPS may be involved in TLK4/NF-κB signaling pathway.\(^3\) We need to further investigate which molecule or mediator was blocked or inhibited by oxycodone in this pathway in LPS-induced primary microglia. In addition, for TGF-β1/smad2/3 signaling, it clearly had an anti-inflammatory effect on activated microglia. So, we need to further evaluate the dual inhibiting effect of oxycodone in activated primary microglia.

Second, several studies documented that plenty of stimuli could activate microglia, such as high glucose and hypoxia.\(^3\) These factors can also be linked to neuroinflammation or diabetic neuropathic pain models in microglia. Whether oxycodone has the dual inhibiting effect on activated microglia induced by different stimuli should be investigated in future studies.

**Conclusion**

In the present study, the findings indicated that oxycodone at 50 and 100 ng/mL suppresses the inflammatory response and inhibits TGF-β1/smad2/3 signaling in LPS-induced primary microglia. Oxycodone might be an analgesic of choice when patients with neuroinflammatory diseases require pain relief.

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**Author contributions**

All authors contributed toward data analysis, drafting and revising the paper and agree to be accountable for all aspects of the work.

**Disclosure**

The authors report no conflicts of interest in this work.

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