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ORIGINAL PAPER

PKC Mediates LPS-Induced IL-1β Expression and Participates in the Pro-inflammatory Effect of $A_{2A}R$ Under High Glutamate Concentrations in Mouse Microglia

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Abstract

Pathogens such as bacterial lipopolysaccharide (LPS) play an important role in promoting the production of the inflammatory cytokines interleukin-1 beta (IL-1β) and tumour necrosis factor-α (TNF-α) in response to infection or damage in microglia. However, whether different signalling pathways regulate these two inflammatory factors remains unclear. The protein kinase C (PKC) family is involved in the regulation of inflammation, and our previous research showed that the activation of the PKC pathway played a key role in the LPS-induced transformation of the adenosine A_{2A} receptor ($A_{2A}R$) from anti-inflammatory activity to pro-inflammatory activity under high glutamate concentrations. Therefore, in the current study, we investigated the role of PKC in the LPS-induced production of these inflammatory cytokines in mouse primary microglia. GF109203X, a specific PKC inhibitor, inhibited the LPS-induced expression of IL-1β messenger ribonucleic acid and intracellular protein in a dose-dependent manner. Moreover, 5 μM GF109203X prevented LPS-induced IL-1β expression but did not significantly affect LPS-induced TNF-α expression. PKC promoted IL-1β expression by regulating the activity of NF-κB but did not significantly impact the activity of ERK1/2. $A_{2A}R$ activation by CGS21680, an $A_{2A}R$ agonist, facilitated LPS-induced IL-1β expression through the PKC pathway at high glutamate concentrations but did not significantly affect LPS-induced TNF-α expression. Taken together, these results suggest a new direction for specific intervention with LPS-induced inflammatory factors in response to specific signalling pathways and provide a mechanism for $A_{2A}R$ targeting, especially after brain injury, to influence inflammation by interfering with $A_{2A}R$.

Keywords Inflammation · Microglial · LPS · IL-1β expression · TNF-α expression · Protein kinase C

Abbreviations

LPS	Lipopolysaccharide
IL-1β	Interleukin-1 beta
TNF-α	Tumour necrosis factor-α
TBI	Traumatic brain injury
NF-κB	Nuclear factor- kappa-B
$A_{2A}R$	Adenosine A2A receptor
ERK1/2	Extracellular regulated protein kinases 1/2
MAPK	Mitogen-activated protein kinase
TLR4	Toll-like receptor 4
MyD88	Myeloid differentiation primary response 88
Iba-1	Ionized calcium-binding adaptor molecule 1
DABK	des-Arginine^9-bradykinin

Sheng-Yu Fu and Ren-Ping Xiong have contributed equally to the research.

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Introduction

Microglia represent a specialized population of macrophage-like cells in the central nervous system that are capable of orchestrating a potent inflammatory response [1–3]. Research has indicated that neuroinflammation is a major contributing factor to the occurrence and development of neurodegenerative diseases such as Alzheimer's

disease and Parkinson's disease and brain injury [3, 4]. Especially in traumatic brain injury (TBI), bacterial lipopolysaccharide (LPS) and injuries are powerful activators of the innate immune system, stimulating microglia, monocytes and macrophages to synthesize an array of cytokines such as interleukin-1 beta (IL-1β) and tumour necrosis factor-α (TNF-α) and initiating the inflammatory response [2, 5]. In addition, it has been shown that LPS directs an immune response primarily through Toll-like receptor 4 (TLR4)- or Myeloid differentiation primary response 88 (MyD88)-mediated activation of the nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK) signalling pathways, resulting in the production of IL-1β and TNF-α in microglia [6, 7]. However, whether the production of IL-1β and TNF-α are mediated through the same or different signalling pathways remains unknown. In particular, the times of peak IL-1β and TNF-α expression differed after TBI; the time of peak TNF-α expression was earlier than the time of peak IL-1β expression [8, 9], indicating that the LPS-induced production of IL-1β and TNF-α may have different signalling pathways in microglia.

Protein kinase C (PKC) is a family of phospholipid-dependent serine/threonine kinases, which can be further classified into three PKC isozymes subfamilies: conventional or classic (PKCα, PKCβI, PKCβII and PKCγ), novel or nonclassic (PKCε, PKCδ, PKCη, PKCθ and PKCμ), and atypical (PKCζ and PKCι/λ) [10]. Numerous in vitro and in vivo studies have suggested that PKCs are involved in proliferation, cell cycle progression, differentiation, and apoptosis, and PKCs are known to have a role in the regulation of host defence and inflammation [11, 12]. In particular, PKCα, PKCβ, PKCδ, PKCγ and PKCζ have been shown to regulate the immune inflammatory response in different inflammatory cells, including macrophages, microglia and other cells [13–16]. Moreover, PKC has been reported to regulate the activity of NF-κB in microglia [17, 18]. Therefore, it can be speculated that PKC may be involved in LPS-induced production of IL-1β or/and TNF-α through the NF-κB signalling pathway in microglia.

The adenosine A_{2A} receptor ($A_{2A}R$), a member of the adenosine receptor family, is expressed in microglia and known to be involved in proliferation, morphological phenotype and mediator release [19]. Additionally, we found that extrasynaptic glutamate levels dictate the switch of $A_{2A}R$ from anti-inflammatory and neuroprotective activity through the PKA pathway to pro-inflammatory activity through the PKC pathway, which also explains the protective effect of $A_{2A}R$ inhibition on the brain in many pathological conditions such as TBI and some neurodegeneration pathologies [20]. However, whether this activation of PKC also promotes the expression of IL-1β or/and TNF-α through NF-κB is not clearly understood.

In this study, we used GF109203X, a specific PKC inhibitor, to inhibit PKC activity because our study mainly explored whether the expression of different inflammatory factors after LPS stimulation was related to the PKC signalling pathway. GF109203X-mediated inhibition is not limited to a specific isoform of PKC; it can antagonize many isoforms of PKC (such as PKCα, PKCβ, PKCδ, PKC γ and PKCζ) and was used in our previous research [20, 21]. We first identified that exposure of primary mouse microglial cells to LPS increases PKC activity and IL-1β and TNF-α expression and that the inhibition of PKC activity by different doses of GF109203X blocked IL-1β expression rather than TNF-α expression. Additionally, we observed that the activity of NF-κB and the signalling factors extracellular regulated protein kinases 1/2 (ERK1/2) were increased after cells were treated with LPS, the activity of NF-κB was decreased by GF109203X, and ERK1/2 was resistant to the PKC inhibitor, indicating that increasing IL-1β expression via PKC involves the NF-κB pathway. Finally, the combination of high concentrations of glutamate and 3-[4[2-[[6-amino-9-[(2R,3R,4S,5S)-5-(ethylcarbamoyl)-3,4-dihydroxy-oxo lan-2-yl]purin-2-yl] amino]ethyl]phenyl]propanoic acid (CGS21680), a selective $A_{2A}R$-specific agonist [22], increased the LPS-induced expression of IL-1β but did not significantly affect the expression of TNF-α; moreover, inhibition of PKC activity blocked the LPS-induced increase in the expression of IL-1β.

Materials and Methods

Antibodies and Reagents

Antibodies specific for the indicated proteins were used. The anti-ERK1/2 antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). The anti-p65 antibody and the HRP-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibodies specific for IL-1β, p-p65 (S536), p-ERK1/2 (T202/Y204), and Iba-1 were purchased from Abcam (Cambridge, MA, USA). The antibodies specific for PKCα, p-PKCα (T638), TNF-α, and GAPDH and fluorophore-conjugated secondary antibodies were purchased from Bioworld Technology, Co., Ltd. (Nanjing, China). The same antibodies were used for both Western blotting and immunofluorescence.

The PKC inhibitor GF109203X and $A_{2A}R$ agonist CGS21680 were purchased from Tocris (Bio-Techne, MN, USA), and LPS and glutamate were purchased from Sigma (St. Louis, MO, USA).

Primary Cell Culture

Mouse microglial cell cultures were prepared as described previously [20]. Cerebral cortices from neonatal C57BL/6 mice (1–2 days of age), provided by the Animal Center at Daping Hospital, Third Military Medical University (Certificate scxk (Yu) 2002–0002, Chongqing, China), were dissected, the meninges were carefully removed, and the samples were digested with 10 ml TrypLE™ Express (Gibco, MD, USA) for 20 min at 37 °C. The digestion process was stopped by adding 2 ml of foetal bovine serum (Biological Industries, Kibbutz Beit Haemek, Israel). The suspension was passed through a 75 μm pore mesh, pelleted and resuspended in DMEM/F12 culture medium containing 10% foetal bovine serum. Cells were seeded at 1.5×10^5 cells/cm^2 and cultured at 37 °C in humidified 5% CO2/95% air until the mixed glial cultures were confluent (10 days). Floating and slightly adhered cells on the mixed culture cell layer were obtained by gentle shaking for 15–20 min. The obtained cell suspension was seeded at $1–2 \times 10^5$ cells/cm^2 in a 12-well plate and allowed to attach for 1 h at 37 °C. Unattached cells were removed, leaving strongly adherent cells, which were primarily microglia. The purity of the microglial cultures was confirmed by immunofluorescence with an Iba-1 monoclonal antibody, a microglial marker. Purified microglial cultures were used for experiments within 2–3 days of isolation. The experimental procedures were performed in accordance with the Animal Ethical and Welfare Committee of Third Military Medical University.

Reagent Treatment

Purified microglial cells were pretreated with 0, 0.05, 0.5, or 5 μM PKC inhibitor GF109203X for 1 h, followed by incubation with 1 μg/ml LPS for 6 h. To elucidate the possible signalling mechanism, 300 μM glutamate and 100 nM $A_{2A}R$ agonist CGS21680 were used. For these cotreatments, CGS21680 was added 10 min before LPS treatment, and glutamate was added to the cultures 30 min after 5 μM GF109203X was added (Fig. 1a). The sequence of the reagent treatment was based on our previous experiment and the literature [20, 21, 23]. Dimethyl sulfoxide (DMSO) was used as the vehicle control.

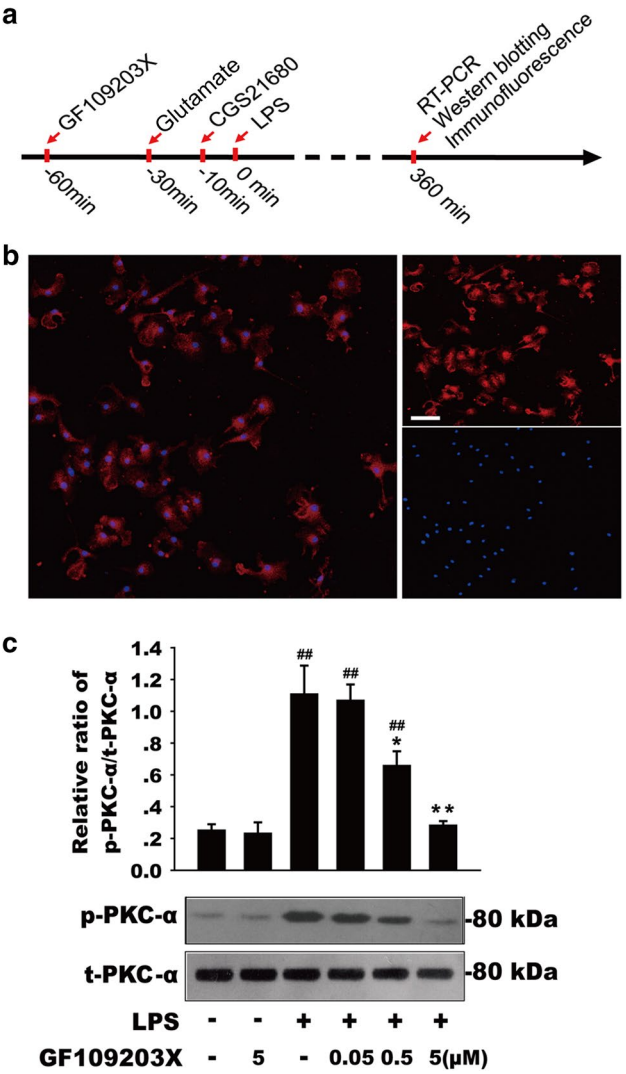

Fig. 1 GF109203X abrogates the level of p-PKCα induced by LPS in primary mouse microglial cells. Schematic of reagent treatment (**a**). Immunofluorescence staining for Iba-1 (**b**) in primary microglia cultures. Nuclei are labelled with DAPI (blue), and Iba-1 is indicated by red fluorescence; scale bar 50 μm. The LPS-induced change in p-PKCα levels in microglia after GF109203X treatment (n = 3). The results were obtained from three independent experiments. *P < 0.05 and **P < 0.01 compared with the LPS stimulation group; #P < 0.05, ##P < 0.01 compared with the untreated control group

RNA Isolation and RT-PCR

Total RNA of primary microglial cells after reagent treatment was isolated using TRIzol reagent (Invitrogen, NY, USA) with a standard method and was then reverse transcribed to cDNA using a kit (Promega, WI, USA). A qPCR master mix kit (Promega, WI, USA) was used for quantitative PCR. The following primers were used to measure the levels of IL-1β and TNF-α: IL-1β, 5′-ACT GTT TCT AAT GCC TT CCC-3′ and 5′-ATG GTT TCT TGT GAC CCT GA-3′; TNF-α, 5′-CTG TGA AGG GAA TGG GTG TT-3′ and 5′-TCA CTG TCC CAG CAT CTT GT-3′; and GAPDH, 5′-AGG TTG TCT CCT GCG ACT TCA-3′ and 5′-TGG TCC AGG GTT TCT TAC TCC-3′. PCR was performed at 95 °C for 10 min followed by 40 cycles at 95 °C for 20 s and 60 °C for 60 s. The relative abundance of the target gene was calculated by normalization to GAPDH, and the data are expressed as ratios relative to the control.

Western Blotting

Cultured cells were lysed in RIPA buffer (Solarbio, Beijing, China) containing protease and phosphatase inhibitors (Thermo Fisher Scientific, CA, USA). Total protein concentration was measured using a BCA kit (Solarbio, Beijing, China). Approximately 20 μg of protein from each sample was resolved on 12% SDS-PAGE gels and then transferred to PVDF membranes (Millipore, MA, USA). The membranes were blocked with 5% bovine serum albumin (Solarbio, Beijing, China) and probed with the following primary antibodies overnight at 4 °C: anti-PKCα (1:600), anti-p-PKCα (1:600), anti-IL-1β (1:1000), anti-TNF-α (1:600), anti-p65 (1:300), anti-p-P65 (1:1000), anti-ERK1/2(1:1000), anti-p-ERK1/2 (1:1000), and anti-GAPDH(1:600). After incubation for 1 h at room temperature with HRP-conjugated goat anti-mouse IgG or goat anti-rabbit IgG secondary antibodies (1:1000), membranes were visualized using Clarity Western ECL Substrate (Bio-Rad, CA, USA). The band intensity was analysed by ImageJ, and the relative quantity of the target protein was normalized to GAPDH or the non-phosphorylated protein for three independent experiments.

Immunofluorescence

After reagent treatment in a 24-well plate, cultured cells were washed with PBS and fixed with 4% paraformaldehyde. Then, the cells were washed with PBS, permeabilized with 0.3% Triton X-100 and incubated with 5% goat serum. Cells were incubated with anti-IL-1β (1:200), anti-TNF-α (1:200) and anti-Iba-1 (1:200) antibodies overnight (4 °C), washed with PBS, and incubated with the appropriate fluorescent dye-conjugated secondary antibody [goat anti-rat IgG (H+L) rhodamine (TRITC) or goat anti-mouse IgG (H + L) Texas Red, [1:200] for 1 h (37 °C) in a dark environment. Nuclei were then stained with DAPI (Solarbio, Beijing, China). After washing with PBS, the samples were observed by a fluorescence microscope (Leica, Wetzlar, Germany). To quantify immunofluorescence staining, the ratio of the integrated optical density (IOD) to the positively stained areas was determined using the Image-Pro Plus image analysis software, version 6.0 (Media Cybernetics, Rockville, MD). The quantitative analysis was independently performed in 5 fields (high-magnification images, magnification of ×400) randomly selected from each well (3 wells per group and 2 independent experiments). Quantitative analysis was independently performed by two observers who were blinded to the treatment conditions. Determination of purity was performed by counting the number of ionized calcium-binding adaptor molecule 1 (Iba-1)-positive cells in at least 5 fields (high-magnification images, ×400 magnification) in at least three separate wells.

To evaluate cell sphericity, considered to be an index of microglial cell activation, single cells (approximately 200) were outlined, and the "shape descriptors" were calculated using ImageJ image analysis software as previously described [24]. For each cell, the software gives a value of sphericity ranging from 0 to 1, the latter representing a perfect sphere. Quantitative analysis was independently performed in 3 fields (high-magnification images, ×400 magnification) randomly selected from each well (3 wells per group and 2 independent experiments).

Statistical Analysis

Data were analysed by one-way analysis of variance (ANOVA) followed by Dunnett's test using Sigma Plot 12.5 statistics software. Values are expressed as the mean ± SE from three independent experiments.

Results

The Inhibitory Effect of GF109203X and Its Effect on the Level of p-PKCα Induced by LPS in Primary Mouse Microglial Cells

To verify the cell culture purity, we performed immunostaining with a microglial marker (Iba-1) (Fig. 1b). The microglial cultures contained 98.13 ± 0.34% Iba-1-positive cells.

Because the level of phosphorylated PKCα not only is inhibited by GF109203X but also is involved in LPS-induced microglial inflammation [25], an antibody specific for phosphorylated PKCα was used as an indicator of the inhibition of PKC by GF109203X. The p-PKCα level in primary microglial cells was significantly increased after 6 h of stimulation with 1 μg/ml LPS compared with that in the control condition; however, GF109203X inhibited the LPS-induced increase in p-PKCα in a dose-dependent manner in the range of 0.05-5 μM. The dose of 5 μM GF109203X markedly impacted the p-PKCα level; this dose reduced the p-PKCα level almost to the control level (Fig. 1c).

Effects of PKC Inhibition on LPS-Induced Expression of IL-1β and TNF-α in Microglia

The messenger ribonucleic acid (mRNA) and intracellular protein levels of IL-1β and TNF-α were significantly increased in microglia after LPS stimulation for 6 h (Fig. 2a–d). Additionally, the LPS-induced increases in the mRNA and intracellular protein levels of IL-1β were inhibited by 0.05–5 μM GF109203X in a dose-dependent manner; 0.05–5 μM GF109203X had no significant effect on the mRNA and intracellular protein levels of TNF-α (Fig. 2a–d).

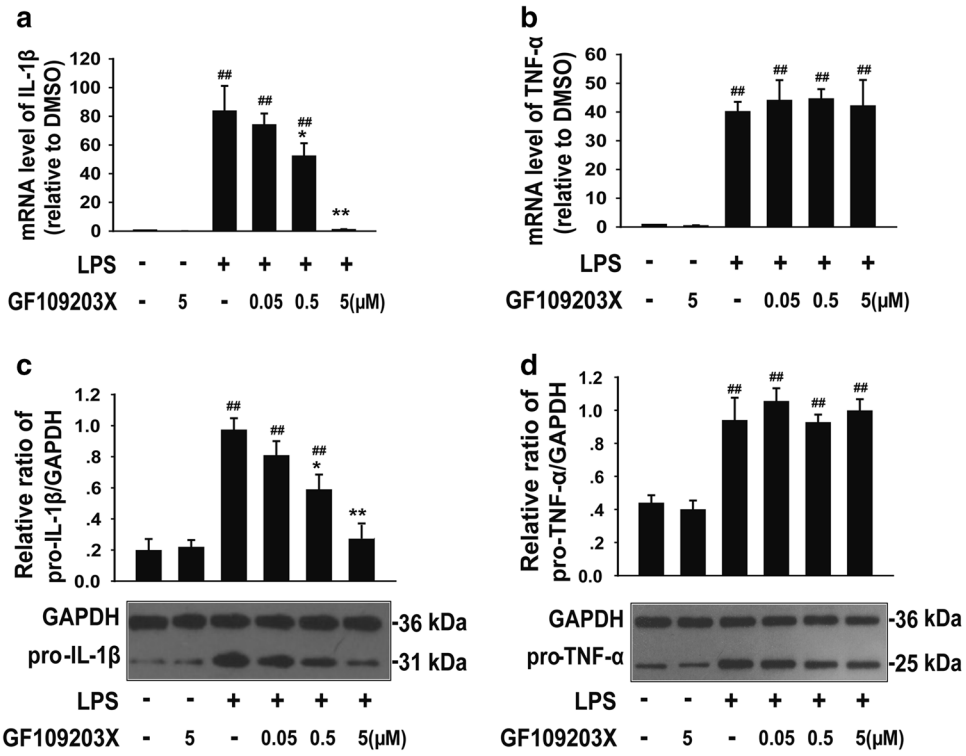

Fig. 2 Inhibition of PKC abrogates LPS-induced IL-1β expression in microglia without affecting TNF-α expression. LPS-induced mRNA expression of IL-1β (**a**) and TNF-α (**b**) in microglia after PKC inhibition (n = 6). *P < 0.05 and **P < 0.01 compared with the LPS stimulation group; #P < 0.05 and ##P < 0.01 compared with the untreated control group. Changes in the LPS-induced intracellular protein levels of IL-1β (**c**) and TNF-α (**d**) in microglia after PKC inhibition (n = 3). Data were obtained from three independent experiments. *P < 0.05 and **P < 0.01 compared with the LPS stimulation group; #P < 0.05 and ##P < 0.01 compared with the untreated control group

Immunofluorescence staining (Fig. 3a–f) showed that Iba-1 was expressed in the cytoplasm and that the fluorescence intensity was significantly increased 6 h after LPS stimulation. Moreover, the microglia changed from a long spindle shape to a round shape after LPS stimulation, and this change was significantly inhibited after the application of 5 μM GF109203X (Supplementary Fig. 1). IL-1β and TNF-α were expressed in the cytoplasm and nucleus, and the changes in the fluorescence intensity associated with these proteins were similar to the fluorescence intensity associated with Iba-1. However, the LPS-induced increase in the fluorescence intensity of IL-1β and Iba-1 was significantly inhibited after the application of 5 μM GF109203X; however, 5 μM GF109203X had no effect on the fluorescence intensity of TNF-α.

Effects of PKC Inhibition on the LPS-Induced Levels of p-P65 and p-ERK1/2 in Microglia

Western blotting (Fig. 4a, b) showed that the levels of p-P65 and p-ERK1/2 after stimulation by LPS were significantly increased in microglia. In the absence of LPS, inhibition of PKC by GF109203X alone did not significantly affect the levels of p-P65 and p-ERK1/2. In contrast, in the presence of LPS, the LPS-induced increase in the level of p-P65 was inhibited by GF109203X in a dose-dependent manner (Fig. 4a), but LPS stimulation did not significantly impact the level of p-ERK1/2 (Fig. 4b).

The Effects of PKC Inhibition on LPS-Induced IL-1β Expression are Mediated by $A_{2A}R$ at High Glutamate Concentrations

Under LPS stimulation, high concentrations of glutamate alone significantly increased the mRNA and intracellular protein levels of IL-1β and TNF-α, while CGS21680 alone decreased the level of only IL-1β (Fig. 5a, b). High concentrations of glutamate combined with CGS21680 significantly facilitated the LPS-induced expression of mRNA and increased the intracellular protein level of IL-1β; however, this treatment did not significantly affect the level of TNF-α (Fig. 5a, b). Inhibition of PKC significantly inhibited the LPS-induced increase in the levels of IL-1β with high concentrations of glutamate alone or high concentrations of glutamate combined with CGS21680, but inhibition of PKC had no significant effect on TNF-α (Fig. 5c, d).

Discussion

LPS Induces IL-1β Expression in Microglial Cells Through PKC and TNF-α Expression Without Affecting the PKC Pathway

Similar to previous studies [26], LPS-stimulated microglia showed morphological changes and increased expression of Iba-1, IL-1β and TNF-α in our research (Fig. 3), indicating that microglia were activated. However, we also found

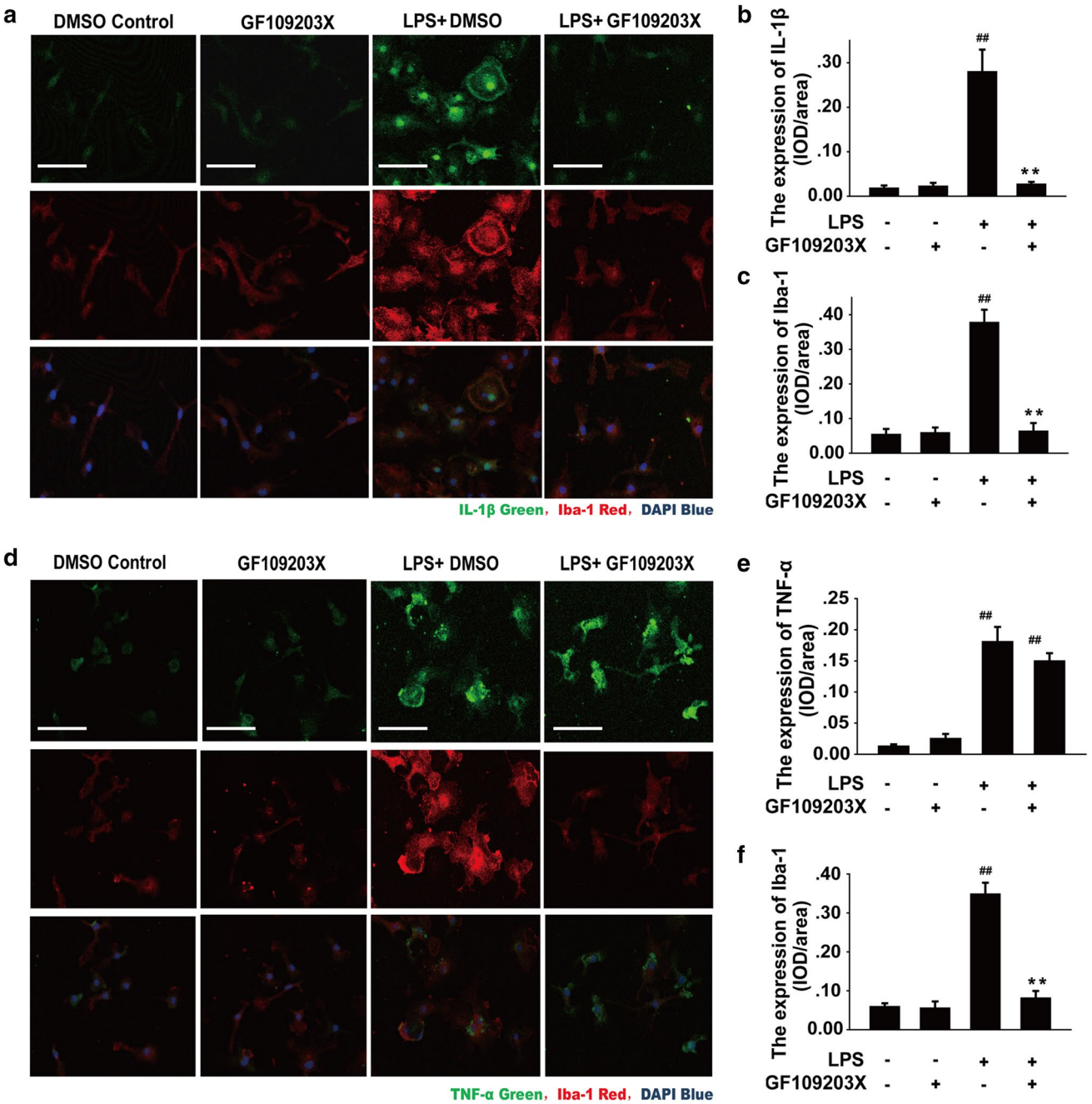

Fig. 3 Immunofluorescence shows that the inhibition of PKC abrogates the LPS-induced increase in IL-1β in microglia without affecting the increase in TNF-α. Immunofluorescence staining for IL-1β and Iba-1 (**a**) and the relative levels of IL-1β (**b**) and Iba-1 (**c**). *P < 0.05 and **P < 0.01 compared with the LPS stimulation group; #P < 0.05 and ##P < 0.01 compared with the untreated control group. Immunofluorescence staining for TNF-α and Iba-1 (**d**) and the relative levels of TNF-α (**e**) and Iba-1 (**f**). *P < 0.05 and **P < 0.01 compared with the LPS stimulation group; #P < 0.05 and ##P < 0.01 compared with the untreated control group. Nuclei are labelled with DAPI (blue), IL-1β and TNF-α are indicated by green fluorescence, and Iba-1 is indicated by red fluorescence; scale bar 50 μm

that PKC antagonists dose-dependently inhibited only LPS-induced IL-1β expression and did not affect TNF-α expression, suggesting that LPS mediated IL-1β expression in microglia through the PKC pathway. Moreover, the finding of LPS-induced IL-1β expression through the PKC pathway by Huang et al., in macrophages and by Jayaprakash et al., in monocytic lymphoma cells [7, 27] is consistent with our results. However, some studies have reported that LPS can promote the secretion of both IL-1β and TNF-α through the PKC pathway in microglia [15, 28]. Because the processes

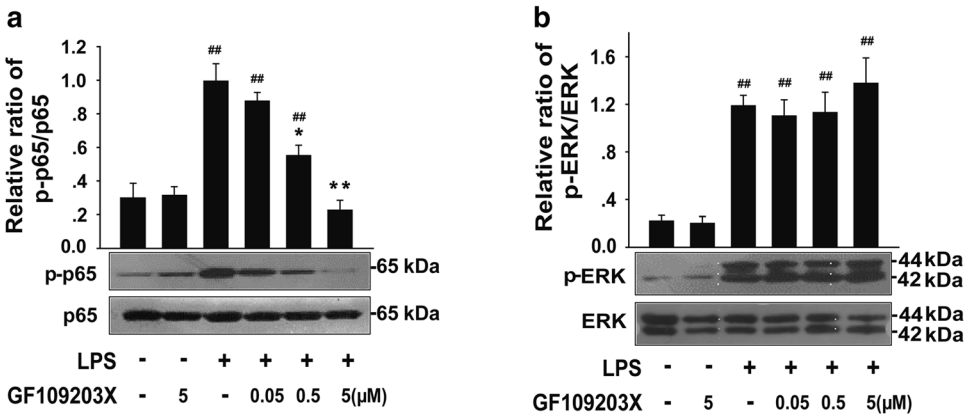

Fig. 4 Inhibition of PKC abrogates LPS-induced p-P65 levels in microglia without affecting p-ERK1/2 levels. Changes in the LPS-induced expression and phosphorylation of P65 (**a**) and ERK1/2 (**b**) in microglia after PKC inhibition (n = 3). The results were obtained from three independent experiments. *P < 0.05 and **P < 0.01 compared with the LPS stimulation group; #P < 0.05 and ##P < 0.01 compared with the untreated control group

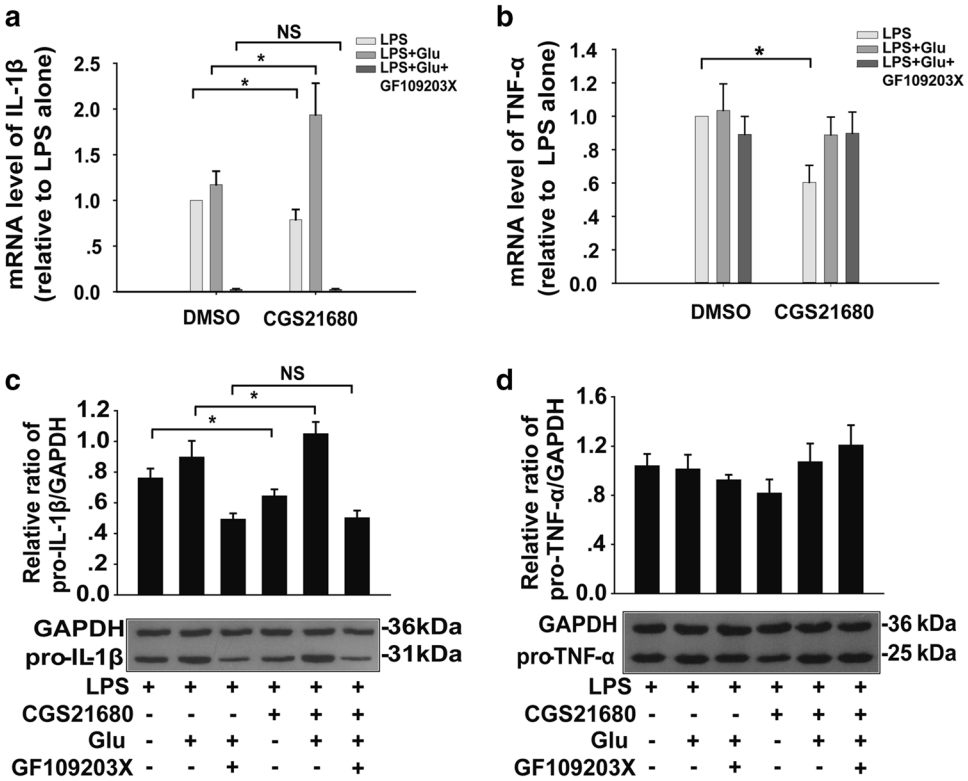

Fig. 5 Inhibition of PKC abrogates the expression of IL-1β induced by $A_{2A}R$ activation after LPS stimulation at high glutamate concentrations. LPS-mediated mRNA expression of IL-1β (**a**) and TNF-α (**b**) in microglia induced by $A_{2A}R$ activation after LPS stimulation at high glutamate concentrations (n = 6). *P < 0.05 and **P < 0.01 compared with the corresponding DMSO control group. Changes in the intracellular protein levels of IL-1β (**c**) and TNF-α (**d**) in microglia induced by $A_{2A}R$ activation after LPS stimulation at high glutamate concentrations (n = 3). The results were obtained from three independent experiments. *P < 0.05 and **P < 0.01 compared with the corresponding control group without CGS21680

of mRNA transcription and secretion should be complementary, resulting in an overall increase in cytokine protein levels, the increased expression of IL-1β and TNF-α in our study may be a possible explanation for their finding. In addition, some studies have examined the effect of the PKC pathway on TNF-α expression [29, 30], but most did not use LPS stimulation. More importantly, the time of the peak TNF-α level in injured tissues after TBI occurred earlier than that of IL-1β in vivo [8, 9]. Moreover, TNF-α expression decreased and IL-1β expression remained increased 48 h after LPS stimulation in vitro [28], suggesting that the LPS-induced expression of IL-1β and TNF-α occurs through different pathways. Furthermore, according to our findings, the PKC pathway regulates IL-1β expression, and previous studies reported [30] that TNF-α can promote PKC activity, suggesting that the peak level of TNF-α can further promote the expression of IL-1β; these results provide a new explanation for the early appearance of the TNF-α peak and the late appearance of the IL-1β peak after LPS stimulation. This finding will also be an important focus in our future research.

In addition, microglial activation, associated with an increase in TNF-α and IL-1β mRNA expression or protein levels [31], was dependent on the expression of Iba-1 [32]. We found that the fluorescence intensity of Iba-1 increased after LPS stimulation, consistent with some reports indicating that LPS stimulation can increase the level of Iba-1 [33, 34]. Additionally, we found that a

PKC inhibitor could reduce the LPS-induced increase in the fluorescence intensity of Iba-1, suggesting that LPS could increase the level of Iba-1 through the PKC pathway. However, due to the lack of research on the signalling pathway that regulates Iba-1 expression, there is no similar research support in the literature. Meotti FC found [35] that GF109203X, a PKC inhibitor, could reduce the expression of the B1 agonist des-Arginine^{9}-bradykinin (DABK) and induce mechanical hyperalgesia. DABK promotes the expression of Iba-1, which is one of the cellular mechanisms that promotes hyperalgesia. Therefore, these results also suggest that the PKC pathway may be involved in the expression of Iba-1.

LPS-Activated PKC Induces IL-1β Expression Through NF-κB

TLR4-MyD88-TAK1-mediated NF-κB and MAPK signalling are two important pathways that induce inflammatory cells to produce inflammatory factors after LPS activation [6, 7, 36]. In our research, LPS stimulation significantly increased the levels of p-P65 and p-ERK1/2 in microglia (Fig. 4a, b), indicating that the NF-κB and MAPK signalling pathways were activated by LPS stimulation. However, inhibition of PKC affected the LPS-induced level of p-P65, with no significant effect on the LPS-induced level of p-ERK1/2 (Fig. 4a, b), suggesting that PKC can regulate the production of inflammatory factors through the NF-κB pathway, consistent with some reports [37, 38]. Although some studies found that the PKC pathway also affects the release of inflammatory factors through the ERK1/2 pathway [39, 40], this finding may be related to the different signalling pathways in different cells and different stimulating factors used. Notably, some studies have shown that PKC induces not only IL-1β but also TNF-α expression through NF-κB [41–43]. However, unlike the method used in our research, the above mentioned use of white blood cells and non-LPS stimulation in previous studies may be the possible reason for the different mechanisms of action of these pathways.

In addition, we found that the LPS stimulation-mediated increase in TNF-α expression in microglia was not related to the PKC/NF-κB pathway and that LPS could promote the phosphorylation of ERK1/2, indicating the activation of the MAPK signalling pathway. Combined with the results of previous studies, our findings indicate that the MAPK signalling pathway can regulate the expression of TNF-α [36, 44], suggesting that LPS stimulation can increase the expression of TNF-α by regulating the MAPK signalling pathway. However, whether this pathway functions in this manner requires further confirmatory research.

$A_{2A}R$ Activation Facilitates LPS-Induced IL-1β Expression Through PKC at High Glutamate Concentrations

$A_{2A}R$ controls neuroinflammation by controlling microglial activation [45], and $A_{2A}R$ activation promotes the inflammatory and cytotoxic phenotype of microglia and the release of TNFα [46]. Moreover, pathological events induce a gain of function of $A_{2A}R$, which then forms heteroreceptor complexes with other receptors, the dopamine- type-2 (D2) receptor and metabotropic glutamate receptor 5 (mGluR5) [47]. Following our previous finding that glutamate concentration was the key factor for $A_{2A}R$ to promote or inhibit inflammation transition [20], we found that the $A_{2A}R$ agonist (CGS21680) inhibited the expression of IL-1β and TNF-α in the absence of glutamate, a result similar to our previous results in vivo using $A_{2A}R$ knockout animals or in vitro using CGS21680 [21]. However, in the presence of a high glutamate concentration, CGS21680 facilitated IL-1β expression through the PKC pathway and did not affect TNF-α expression, indicating that $A_{2A}R$ exerted a pro-inflammatory effect through PKC/IL-1β at high glutamate concentrations. This finding may also explain why adenosine-activated $A_{2A}R$ can increase IL-1β levels [48] and is consistent with our previous finding that CGS21680 can promote IL-1β expression in neutrophils [21]. Although some studies have shown that the inhibition of $A_{2A}R$ can affect both IL-1β and TNF-α [46], the impact of $A_{2A}R$ inhibition may be related to the different signalling pathways involved in the regulation of $A_{2A}R$ in different pathological environments. Furthermore, our study revealed that $A_{2A}R$ promotes inflammation through the PKC/IL-1β pathway at high glutamate concentrations, thus providing a new molecular pathway for the study of the inflammatory mechanism of $A_{2A}R$ at high glutamate concentrations.

In conclusion, we found that LPS induces IL-1β expression in microglia via the PKC pathway and TNF-α expression without affecting the PKC pathway, while LPS-activated PKC induces IL-1β expression via the NF-κB pathway. High levels of glutamate induce the $A_{2A}R$–mGluR5 interaction to trigger a pro-inflammatory effect of $A_{2A}R$ activation via PKC signalling [21], and we found that $A_{2A}R$ activation facilitates IL-1β expression via the PKC pathway at high glutamate concentrations (Fig. 6). This study provides not only experimental support for the existence of different signalling pathways in LPS-induced microglia for IL-1β and TNF-α synthesis but also a new direction for specific intervention with LPS-induced inflammatory factors in response to specific signalling pathways. In addition, this study reveals the pro-inflammatory signalling pathway of $A_{2A}R$ under high glutamate concentrations, which provides support for influencing inflammation through $A_{2A}R$ intervention, especially after brain injury.

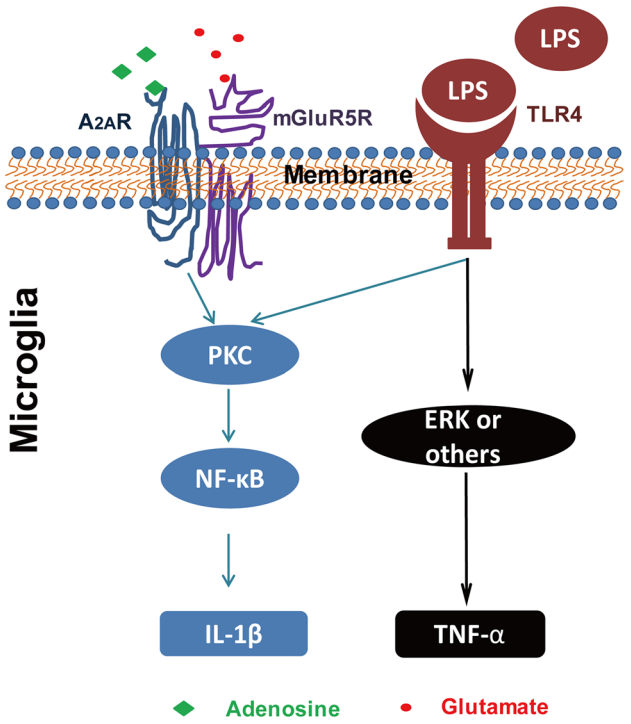

Fig. 6 Schematic representation of the different pathways mediating IL-1β and TNF-α expression in microglia stimulated by LPS and the signalling pathways of LPS-induced IL-1β expression promoted by $A_{2A}R$ activation under high glutamate concentrations. In primary cultured microglia, LPS can promote the expression of IL-1β via the PKC-NF-κB pathway, while TNF-α may be induced by ERK1/2 or other pathways. $A_{2A}R$ activation facilitates the expression of IL-1β in response to LPS via the PKC pathway under high-glutamate conditions, which induces the interaction between $A_{2A}R$ and mGluR5

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflicts of interest.

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