Brilliant blue G attenuates neuro-inflammation via regulating MAPKs and NF-κB signaling pathways in lipopolysaccharide-induced BV2 microglia cells

WEI WANG[1,2*], FEIRAN HUANG[3*], WEIFENG JIANG[4], WEIWEI WANG[1,2] and JIE XIANG[1,2]

[1]Department of Rehabilitation, The Affiliated Hospital of Xuzhou Medical University, Xuzhou, Jiangsu 221002; [2]School of Medical Technology, Xuzhou Medical University, Xuzhou, Jiangsu 221004; [3]Department of Neurology, The Affiliated Hospital of Jiangsu University, Zhenjiang, Jiangsu 212001; [4]Department of Neurology, The Second People's Hospital of Quzhou, Quzhou, Zhejiang, 324000, P.R. China

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Abstract. Previous studies have demonstrated that the P2X purinoceptor 7 (P2X7) receptor (P2X7R) serves a critical role in regulating the inflammatory response of various diseases in the central nervous system. The anti-inflammatory effect of brilliant blue G (BBG), a specific antagonist of the P2X7R, remains unclear in lipopolysaccharide (LPS)-induced BV-2 cells. The present study suggested that BBG attenuated the neuroinflammatory response; the protein levels of inducible oxide synthase and cyclooxygenase-2, and the mRNA and secretion levels of pro-inflammatory cytokines including interleukin (IL)-16, IL-1β and tumor necrosis factor-α (TNF-α), were all decreased in LPS-induced BV2 cells. BBG inhibited the activation of MAPKs by inhibiting the phosphorylation of p38 mitogen-activated protein kinase, c-Jun N-terminal kinase and extracellular signal-regulated kinase. Notably, transcription factor p65 nuclear translocation was also inhibited, thereby leading to the inactivation of NF-κB. The inhibitory effects of BBG on MAPKs and NF-κB were additionally enhanced through the application of MAPK and NF-κB inhibitors. Taken together, the results demonstrated that BBG contributed to the suppression of the inflammatory effects in LPS-induced BV2 cells via the inhibition of NF-κB and MAPKs signaling pathways.

Correspondence to: Dr Jie Xiang, Department of Rehabilitation, The Affiliated Hospital of Xuzhou Medical University, 99 West Huaihai Road, Xuzhou, Jiangsu 221002, P.R. China
E-mail: 18052268386@163.com

*Contributed equally

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Introduction

Neuroinflammation is a common disease-associated event, which may affect the progression of multiple neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson's disease (PD), traumatic brain injury and stroke (1,2). Under normal conditions, microglial cells, the major immune cells of the brain, are the first line of defense in the innate immune responses and tissue repair of the central nervous system (CNS), and maintain CNS homeostasis through their precise activation (3,4). Sustained over-activation of microglial cells may cause neuronal death or tissue damage mediated by the excessive production or release of pro-inflammatory mediators including tumor necrosis factor-α (TNF-α), nitric oxide (NO), interleukin 6 (IL-6), IL-1β, inducible NO synthase (iNOS), reactive oxygen species and cyclooxygenase-2 (COX-2) (5,6). Therefore, inhibiting the excessive activation of microglia may be an effective anti-inflammatory approach for attenuating the progression of multiple neurodegenerative diseases.

Lipopolysaccharide (LPS), as an activator of inflammation, is a major component of the outer membrane in gram-negative bacteria, and is commonly used as a pro-inflammatory agent to generate inflammation models (7). Several signaling pathways are involved in microglia-associated neuroinflammation. In general, NF-κB is located in the cytoplasm and binds to the NF-κB inhibitor α protein. Upon stimulation with LPS, IκBα is phosphorylated rapidly and degraded, which leads to the release of p50/ transcription factor p65 (p65) NF-κB heterodimers. NF-κB dimers move into the nucleus and bind to inflammation-associated genes, resulting in the transcriptional activation of pro-inflammatory mediators (8). In addition, MAPKs have been demonstrated to serve key roles in the inflammatory response and are involved in various cellular processes (9). Research indicates that LPS may induce the phosphorylation of three major MAPK pathways, p38, JNK and ERK, which activates the production of pro-inflammatory cytokines (10). Taken together, the NF-κB and MAPK signaling pathways are crucial for the modulation of inflammation in various neurological diseases (11,12).

The P2X7 receptor (P2X7R), a purinergic receptor, is expressed in the microglia, neurons and astrocytes of the CNS. In various neurodegenerative processes, P2X7R is overactivated due to ATP release, resulting in anion imbalance and triggering of cell death (13,14). The activation of P2X7R is involved in several signaling pathways, including the NF-κB, MAPK and NFAT pathways (15). P2X7 activation leads to microglial activation and facilitates the production of IL-1β, IL-6 and TNF-α, additionally aggravating cell damage in neurodegenerative diseases (16). Recently, Wang *et al* (17) demonstrated that brilliant blue G (BBG), a selective and non-competitive P2X7R antagonist, serves a neuroprotective role by attenuating microglial activation in an LPS-induced PD model. Whether the MAPK/NF-κB pathway is involved in the anti-inflammatory effect of BBG in LPS-induced PD models remains unclear.

Therefore, the aim of the present study was to investigate whether P2X7 may be regarded as a key upstream factor that additionally activates the MAPK/NF-κB signaling pathways that are involved in LPS-induced neuroinflammation.

Materials and methods

Reagents and antibodies. BBG, LPS and MTT were purchased from Sigma-Aldrich; Merck KGaA. Fetal bovine serum (FBS) was obtained from Gibco; Thermo Fisher Scientific, Inc., Dulbecco's modified Eagle's medium (DMEM) was obtained from HyClone; GE Healthcare Life Sciences. The BCA protein assay kit (cat. no. P0012), penicillin/streptomycin (cat. no. C0222), protease inhibitor (cat. no. ST505), phosphorylated protease inhibitor (cat. no. P1096), DAPI (cat. no. C1002), RIPA lysis buffer (cat. no. P0013B), SB203580 (cat. no. S1863), SP600125 (cat. no. S1876), PD98059 (cat. no. S1805) and Nuclear and Cytoplasmic Extraction kit (cat. no. P0027) were obtained from Beyotime Institute of Biotechnology. BAY 11-7082 was purchased from Selleck Chemicals (cat. no. S2913). ELISA kits for TNF-α (cat. no. 70-EK2208-24), IL-6 (cat. no. 70-EK106/2-24) and IL-1β (cat. no. 70-EK101B-24) were purchased from Hangzhou Multi Sciences (Lianke) Biotech Co., Ltd. The primers for the reverse transcription-quantitative polymerase chain reaction (RT-qPCR) were purchased from Shanghai GeneChem Co., Ltd. The following primary antibodies were purchased from Cell Signaling Technology, Inc.: anti-Lamin B (cat. no. 12586), p38 (cat. no. 8690), phosphorylated (p)-p38 (cat. no. 4511), JNK (cat. no. 9252), p-JNK (cat. no. 4668), ERK (cat. no. 4795), p-ERK (cat. no. 4370), NF-κB p65 (cat. no. 8242), COX-2 (cat. no. 4852) and iNOS (cat. no. 13120). Anti-TLR4 was purchased from Abcam (cat. no. 47093). Anti-P2X7R were purchased from Alomone Labs (cat. no. APR-004). Anti-β-actin was purchased from ProteinTech Group, Inc. (cat. no. 66009-1-lg). The anti-mouse IRDye® 680RD-conjugated (cat. no. C70124-05) and anti-rabbit IRDye® 800CW-conjugated (cat. no. C11117-05) secondary antibodies were purchased from LI-COR Biosciences.

Cell culture. BV2 cells were purchased from the China Center for Type Culture Collection. BV2 cells were cultured in DMEM medium supplemented with 10% FBS and 1% penicillin/streptomycin, and incubated at 37˚C in a humidified atmosphere of 5% CO_2. LPS was dissolved in phosphate-buffered saline (PBS) as a stock solution (1 mg/ml), and stored at -20˚C. BV-2 cells ($5x10^4$) were seeded into 96- or 6-well plates, respectively, and exposed to LPS (1 µg/ml) in the presence or absence of 1 µmol/ml BBG for 24 h. The cells were pretreated with different inhibitors (BAY 11-7082, 10 µM; PD98059, 20 µM; SP203580, 20 µM; and SP600125, 20 µM) for 1 h prior to stimulation with 1 µg/ml LPS for 24 h.

MTT assay. BV-2 cells ($5x10^4$) were seeded in 96-well plates and treatment with LPS (1 µg/ml) in the presence or absence of 5 µmol/ml BBG for 24 h. Then, cells were cultured in fresh medium and 5 mg/ml MTT for an additional 4 h at 37˚C. The supernatant then was removed and 150 µl DMSO was added. The optical density of the medium was detected at 570 nm using a microplate reader (BioTek Instruments, Inc.). For relative quantification, the value of absorbance in each group was normalized to that in the control group.

Immunocytochemistry. BV-2 cells ($5x10^6$) were seeded into 12-well plates overnight, and treated with LPS in the presence or absence of BBG for 24 h. Following the treatment, the supernatant was removed via pipette and the cells were fixed with 4% paraformaldehyde (PFA) for 30 min at 37˚C. Then, cells were permeabilized with 0.2% Triton X-100 for 20 min at 37˚C, and blocked with 5% donkey serum (Vicmed) for 30 min at 37˚C. The cells were incubated with monoclonal rabbit NF-κB p65 (1:800) at 4˚C overnight and then incubated with a secondary antibody Alexa Fluor® 594-conjugated donkey anti-rabbit secondary antibodies (1:500; Invitrogen; Thermo Fisher Scientific, Inc.) for 2 h at 37˚C. Nuclei were then stained with 10% DAPI (Beyotime Institute of Biotechnology) for 10 min at 37˚C. Fluorescence images were captured with a fluorescencemicroscope (magnification, x40; Olympus Corporation).

ELISA assays. BV2 cells were seeded in 24-well plate at a density of $1x10^5$ cells/well, treated with 1 µmol/ml BBG and stimulated with LPS for 24 h. Following this treatment step, the culture supernatants were collected by centrifugation (1,000 x g for 5 min; 4˚C). The levels of inflammatory cytokines TNF-α, IL-1β and IL-6 were detected by ELISA according to the manufacturer's protocol.

Western blot analysis. For whole cell lysates, the cells were lysed in 200 µl RIPA lysis buffer and then 1% protease inhibitor and 1% phosphorylated protease inhibitor were added. After incubation on ice for 10 min, the lysates were centrifuged at 12,000 x g for 30 min at 4˚C, and the supernatant was collected. According to the Nuclear and Cytoplasmic Extraction kit instructions, the cytoplasmic and nuclear proteins were extracted. The proteins concentration was quantified using the BCA protein assay kit. Protein samples (50 µg/lane) were loaded on 10-12% SDS-PAGE and transferred to nitrocellulose filter membranes. The membranes were then blocked with 5% skimmed milk for 2 h at room temperature. The following primary antibodies were incubated with: anti-Lamin B1 (1:1,000), NF-κB p65 (1:1,000), p-p38 (1:1,000), p38 (1:1,000), p-JNK (1:1,000), JNK

(1:1,000), p-ERK (1:1,000), ERK (1:1,000), COX-2 (1:1,000), iNOS (1:1,000), P2X7R (1:500), TLR4 (1:500) and β-actin (1:10,000). Following washing with TBST three times, the membranes were incubated with an anti-mouse IRDye® 680RD-conjugated antibody (1:10,000) or an anti-rabbit IRDye® 800CW-conjugated antibody (1:10,000). The bands were determined with Odyssey® Imaging Systems (LI-COR Biosciences) and protein bands were quantified with ImageJ software (version 1.5.2; National Institutes of Health).

RT-qPCR. Total RNA from BV2 cells was extracted using TRIzol® reagent (Thermo Fisher Scientific, Inc.) and then RNA (1 µg) was reverse transcribed to cDNA using HiScriptQ RT SuperMix for qPCR (Vazyme Biotech Co., Ltd.). The cDNA (2 µl) was amplified using a sequence detection system SYBR-Green Master Mix (Vazyme Biotech Co., Ltd.). RT-qPCR was performed using a LightCycler® 480 Real-Time PCR system (Roche Diagnostics) according to the manufacturer's protocol. The cycling parameters were: 95˚C for 5 min, followed by 40 cycles at 95˚C for 10 sec and 60˚C for 30 sec, 95˚C for 15 sec, 60˚C for 60 sec and 95˚C for 15 sec. RT-qPCR primers were as followed: TNF-α forward, CAACGGCATGGATCT CAAAG; TNF-α reverse, GTCGTTGCTTGGTTCTCCTTG; IL-6 forward, TTGCCTTCTTGGGACTGATG; IL-6 reverse, GAATTGCCATTGCACAACTCT; IL-1β forward, GCCCAT CCTCTGTGACTCATG; IL-1β reverse, GTCGTTGCTTGG TTCTCCTTG; GAPDH forward, TGGTGAAGGTCGGTG AAC; and GAPDH reverse, GCTCCTGGAAGATGGTGA TGG. GAPDH was used as the control. The relative RNA expression of TNF-α, IL-6 and IL-1β was analyzed according to the $2^{-\Delta\Delta Cq}$ method (18).

Statistical analysis. Each experiment was repeated three times. Data are expressed as mean ± standard error of the mean. Statistical significance of data was analyzed using analysis of variance followed by Fisher's Least Significant Difference tests. GraphPad Prism 6.0 (GraphPad Software, Inc.) and SPSS 19.0 software (IBM Corp.) were used to generate graphs and perform statistical analysis. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effects of BBG on the viability of BV2 cells. To evaluate the cytotoxic effects of BBG on BV2 cells, cell viability was examined using the MTT assay. The cells were treated with 1 µM BBG in the presence or absence of LPS (1 µg/ml) for 24 h. As demonstrated in Fig. 1, BBG did not affect cell viability with or without LPS, suggesting that BBG is not harmful to BV-2 cells.

BBG inhibits LPS-induced inflammation mediators in BV2 cells. To investigate whether BBG suppresses the production of pro-inflammatory cytokines in LPS-induced BV2 cells, RT-qPCR was used. The results revealed that LPS markedly increased the mRNA and secretion levels of IL-1β, IL-6 and TNF-α. Co-treatment with BBG significantly decreased the level of these molecules (Fig. 2A-C). Similarly, western blot analysis revealed that LPS significantly upregulated the expression of iNOS and COX-2 proteins in BV2 cells, and that

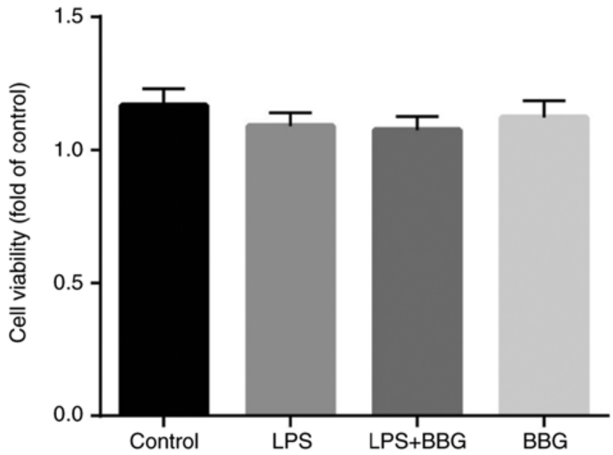

Figure 1. Effect of BBG on the cell viability of BV-2 cells. BV-2 cells were treated with BBG (1 µM/ml) in the absence or presence of LPS (1 µg/ml) for 24 h, and the cell viability was determined by MTT assay. Data are expressed as the mean ± standard error of the mean of three independent experiments. BBG, brilliant blue G; LPS, lipopolysaccharide.

this inflammatory effect was suppressed by BBG (Fig. 2D). Additionally, compared with the control group, the TLR4 protein level was significantly increased in LPS-induced BV2 cells, while BBG treatment significantly downregulated the protein level of TLR4. Furthermore, the TLR4 protein level was not significantly altered following the addition of ATP, a P2X7R agonist (Fig. 2E). BBG markedly inhibited the protein expression of P2X7R in LPS-induced BV2 cells. By contrast, no significant change was detected after the addition of ATP (Fig. 2E).

BBG inhibits NF-κB activation in LPS-induced BV2 cells. As NF-κB is a vital transcription factor contributing to the expression of iNOS and inflammatory responses, the present study investigated whether NF-κB was activated or not under treatment with BBG.

The cytosolic and nuclear proteins were extracted, and western blot analysis indicated that the protein level of p65 was increased in the nuclear fraction of the LPS-treated group; when the cells were additionally treated with BBG, the nuclear translocation was inhibited. However, pretreatment with the NF-κB inhibitor BAY 11-7082 for 1 h further significantly suppressed NF-κB p65 nuclear translocation (Fig. 3A). Furthermore, immunofluorescence analysis suggested that BBG inhibited NF-κB p65 nuclear translocation (Fig. 3B).

BBG inhibits the phosphorylation of MAPKs in LPS-induced BV-2 cells. The present study also investigated whether BBG affects the phosphorylation of MAPKs. As demonstrated in Fig. 4, compared with the control group, the phosphorylation levels of p38, JNK and ERK1/2 were significantly upregulated in LPS-treated group. However, the phosphorylation levels of these proteins were significantly decreased after the addition of BBG. Besides, co-treatment with MAPK inhibitors (p38 inhibitor SP203580, JNK inhibitor SP600125 and ERK inhibitor PD98059) additionally decreased the phosphorylation levels of p38, JNK and ERK.

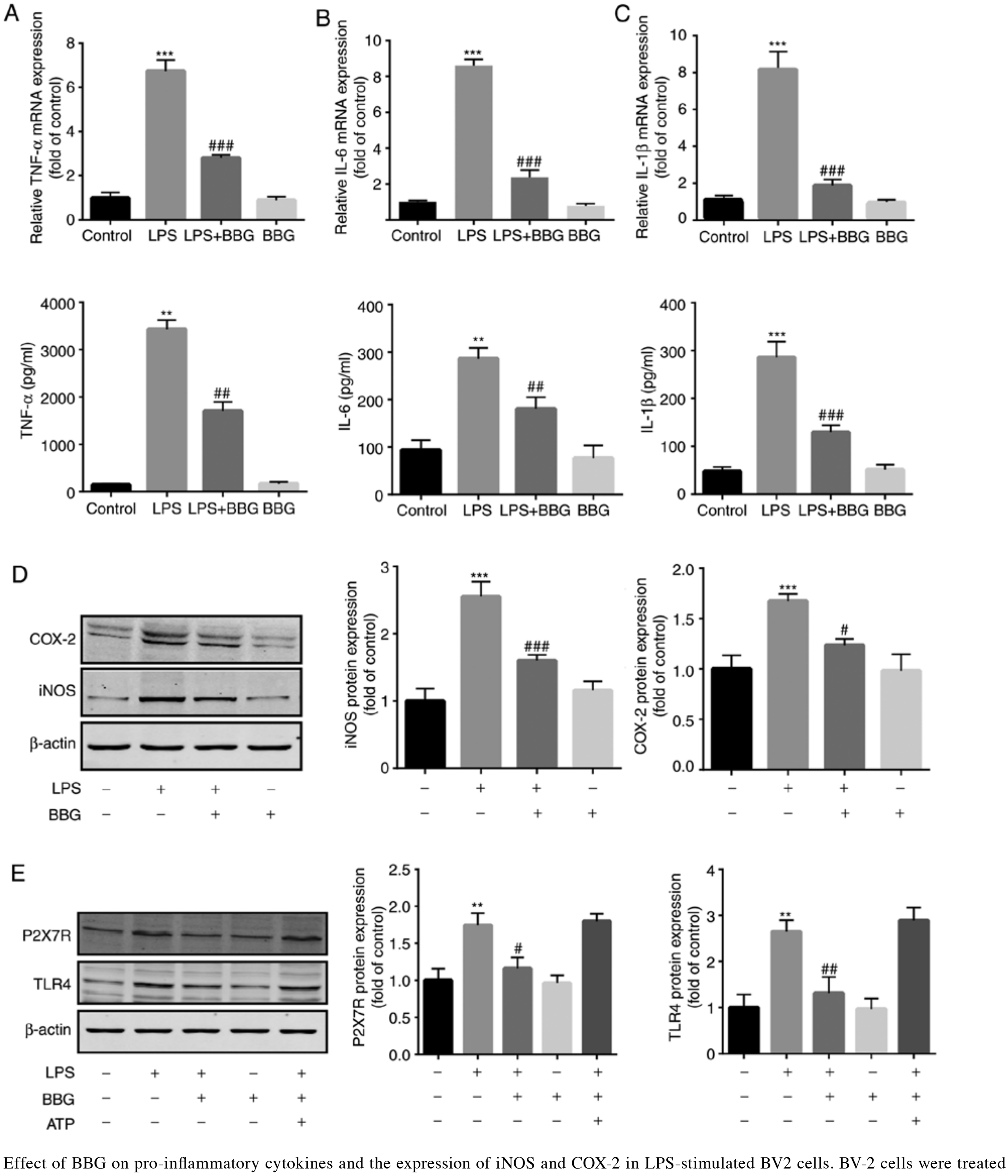

Figure 2. Effect of BBG on pro-inflammatory cytokines and the expression of iNOS and COX-2 in LPS-stimulated BV2 cells. BV-2 cells were treated with 1 μM BBG in the presence or absence of LPS (1 μg/ml) for 24 h. (A-C) The level of (A) TNF-α, (B) IL-1β and (C) IL-6 was measured by reverse transcription-quantitative polymerase chain reaction and ELISA. (D) The protein levels of iNOS and COX-2 were analyzed by western blot analysis. (E) The protein levels of TLR4 and P2X7R were analyzed by western blot analysis. Cells were incubated with LPS for 22 h in the presence or absence of ATP (0.1 mM) for an additional 2 h. Data are expressed as the mean ± standard error of the mean of three independent experiments. **P<0.01 and ***P<0.001 vs. control group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. LPS-induced group. iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2; LPS, lipopolysaccharide; TLR-4, toll-like receptor-4; BBG, brilliant blue G; TNF-α, tumour necrosis factor-α; IL, interleukin; P2X7R, P2x purinoceptor 7 receptor.

Discussion

Neuroinflammation is considered to be an important contributor to the development and progression of several neurodegenerative diseases, including PD and AD (19,20). Over-activated microglial cells trigger neurotoxic effects due to the increase in inflammatory mediators [NO and prostaglandin E2 (PGE2)] and various toxic cytokines (21). Therefore, an effective way to delay the progression of various neurodegenerative diseases is through suppressing the secretion of pro-inflammatory mediators by over-activated microglial cells (22). The present study identified that BBG, a selective and non-competitive P2X7R

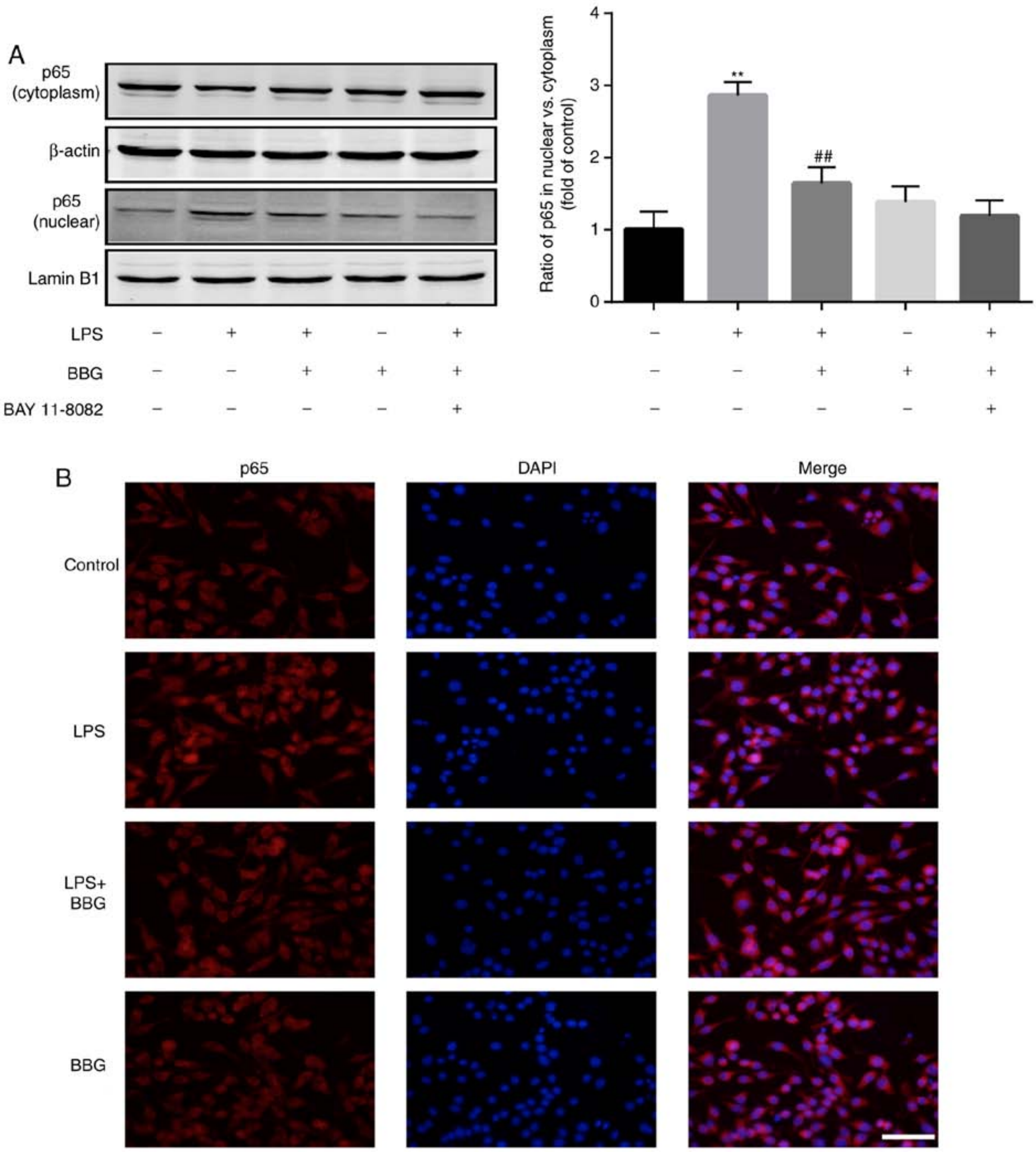

Figure 3. Effect of BBG on the LPS-induced activation of NF-κB in BV2 cells. (A) The expression of NF-κB p65 in the cytoplasm and nucleus were analyzed by western blot analysis. Cells were pretreated with 10 μM NF-κB inhibitor BAY 11-7082 for 1 h prior to treatment with 1 μg/ml LPS for 24 h. (B) Immunofluorescence images demonstrating the translocation of NF-κB/p65. Scale bar, 20 μm. Data are expressed as the mean ± standard error of the mean of three independent experiments. **P<0.01 vs. control group; $^{\#\#}$P<0.01 vs. the LPS-induced group. LPS, lipopolysaccharide; BBG, brilliant blue G; p65, transcription factor 65.

antagonist, notably lessened the production of inflammatory mediators and the release of pro-inflammatory cytokines in an LPS-induced inflammation model. A previous study demonstrated that P2X7R primarily exists in microglia, astrocytes and neuronal cells in the CNS (23). Concomitantly, P2X7R has been demonstrated to be involved in inflammatory responses caused by LPS stimulation, and the activation of P2X7R may directly facilitate the maturation and release of pro-inflammatory cytokines (24). The present study suggested that co-treatment with BBG markedly inhibited the mRNA expression of IL-1β, IL-6 and TNF-α in LPS-stimulated microglial cells. In addition, it has been demonstrated that an excessive production of iNOS and COX-2 in microglia may aggravate inflammatory disorders (25). iNOS and COX-2, as pro-inflammatory enzymes, may promote the generation of PGE2 and NO, impair respiratory chain complexes I and II, and produce multiple deleterious reactive molecules (26). The results suggested that co-treatment with BBG markedly decreased the production of iNOS and COX-2. These data demonstrated that BBG has anti-neuroinflammatory properties by attenuating production of the pro-inflammatory cytokines by LPS-induced microglial cells.

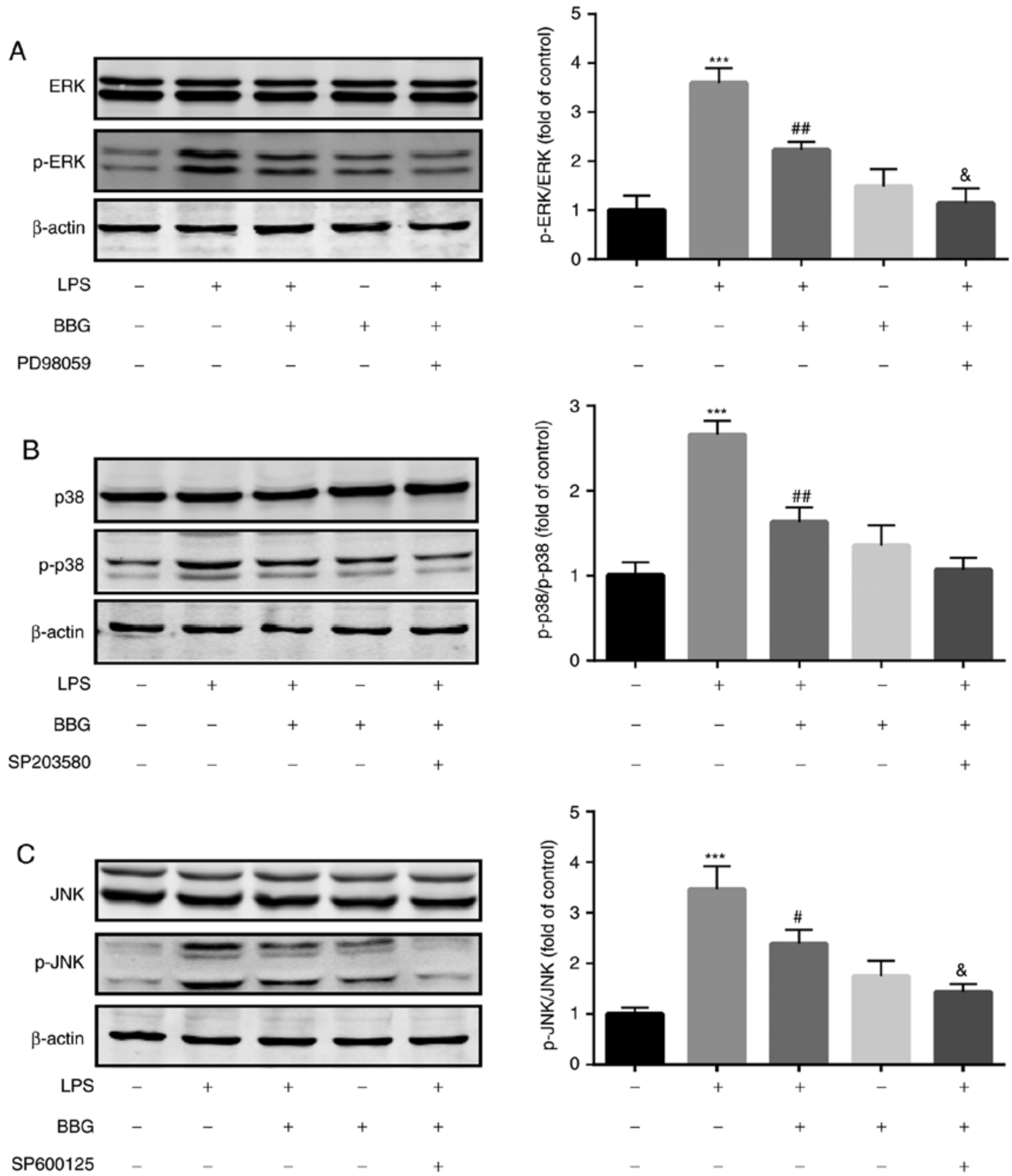

Figure 4. Effect of BBG on LPS-induced activation of MAPK pathways in BV2 cells. The phosphorylation levels of p38, ERK and JNK were determined by western blot analysis. (A-C) Cells were pretreated with (A) ERK inhibitor PD98059 (20 μM), (B) MAPK inhibitor p38 inhibitor SP203580 (20 μM) and (C) JNK inhibitor SP600125 (20 μM) for 1 h prior to treatment with 1 μg/ml LPS for 24 h. Data are expressed as the mean ± standard error of the mean of three independent experiments. $^{***}P<0.001$ vs. control group; $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs. LPS-induced group; $^{\&}P<0.05$ vs. LPS+BBG group. LPS, lipopolysaccharide; BBG, brilliant blue G; p38, p38 mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase.

TLR4 has been demonstrated to be involved in neuroinflammation, and is upregulated in response to nerve injury. TLR4 recognizes exogenous ligands such as LPS, leading to the activation of TLR4 and promoting the production of a variety of inflammatory cytokines. Previous data suggest that inhibiting or knocking out TLR4 effectively reverses neuroinflammation or neuropathic pain (27). Consistent with a previous study (27), the present results suggested that BBG treatment decreased the LPS-induced elevation of the TLR4 protein level. By contrast, no significance change was observed following ATP treatment.

NF-κB is a dimeric transcription factor that regulates the expression of multiple genes and serves a critical role in cellular signaling pathways against immunity, inflammation and cell death (28). It has been suggested that P2X7R may activate the NF-κB signaling pathway, and it has a close association with inflammatory diseases (29). A previous study suggested that P2X7R regulates the matrix metalloproteinase 13 (MMP-13) and NF-κB pathways in cartilage tissue and mediates OA-induced pain and inflammation. Furthermore, NF-κB signaling inhibitors may suppress the expression of P2X7R and MMP-13, and relieve OA-induced pain and inflammation (30). Similar to these data, A438079, a P2X7R antagonist, decreases NF-κB activation, intensifies the caspase-1 expression in lamina propria immune cells and suppresses pro-inflammatory cytokine production in colon tissues, in addition to relieving murine colitis (29). In the present study, the results suggested that BBG significantly inhibited the nuclear translocation of NF-κB p65, and this inhibitory effect was additionally enhanced following pretreatment with the NF-κB inhibitor BAY 11-7082. These results suggested that the NF-κB signaling pathway participates in the regulation of the production of pro-inflammatory mediators by BBG. MAPKs are serine/threonine kinases, and regulate the expression of genes including p38 MAPK, ERK and JNK, which are associated with immune and inflammatory responses and are upregulated in LPS-stimulated macrophages and microglia (31). It has been demonstrated that P2X7R contributes to the phosphorylation of p38 MAPK during intracerebral hemorrhage (32). The activation of p38 MAPK further leads to the production of active caspase-3, and ultimately to cell death (33). Furthermore, the inhibition of P2X7R and MAPKs provided significant neuroprotection in a subarachnoid hemorrhage or intracerebral hemorrhage model (34). Previous studies have also demonstrated the marked activation of p38 MAPK and P2X7R in PD models (35,36). In addition, p38 MAPK signaling pathway inhibitors or P2X7R antagonists provide a significant neuroprotection effect against damage to the substantia nigra and striatum in PD models (35). In the present study, BBG significantly decreased the levels of phosphorylated p38, ERK and JNK in LPS-induced BV2 cells, and co-treatment with MAPK inhibitors (SB203580, SP600125 and PD98059) further suppressed the levels of these kinases, suggesting that the anti-inflammatory effect may be attributed to the suppression of the MAPK signaling pathway.

In conclusion, the results of the present study demonstrated that BBG significantly inhabited the inflammatory response in LPS-induced BV-2 cells. The anti-inflammatory mechanism of BBG may be mediated via the suppression of the activation of the MAPK/NF-κB signaling pathways. These data indicated that BBG may be an effective agent for the treatment of neuroinflammation in neurodegenerative diseases.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JX conceived and designed the experiments. WJ, FH and WWW performed the experiments. WW and FH wrote the manuscript. WW and WJ conducted the data analysis. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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