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Brilliant blue G attenuates lipopolysaccharide-mediated microglial activation and inflammation☆

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Abstract

Previous studies have confirmed that oxidized adenosine triphosphate, a P2X7 receptor antagonist, attenuates lipopolysaccharide-mediated microglial activation and inflammatory expression following neuronal damage in rat brain. NaCl and temperature may affect the potency of oxidized adenosine triphosphate. Brilliant blue G is a derivative of a widely used food additive and has little toxicity. This study explored the effects of brilliant blue G, a selective P2X7 receptor antagonist, on microglial activation and inflammation. Results demonstrated that brilliant blue G inhibited the release of cyclooxygenase-2 and interleukin-6 in BV2 cells. Immunofluorescence displayed that brilliant blue G could suppress lipopolysaccharide-induced microglial activation. This study used RNA interference to block P2X7 receptor expression and found that small interfering RNA also suppressed the release of cyclooxygenase-2 and interleukin-6 in BV2 cells. These results suggested that downregulation of the P2X7 receptor by brilliant blue G was involved in the inhibition of microglial activation and inflammation.

Key Words

neural regeneration; neurodegenerative disease; brilliant blue G; P2X7 receptor; lipopolysaccharide; microglia; inflammatory cytokines; RNA interference; cyclooxygenase-2; interleukin-6; grants-supported paper; photographs-containing paper; neuroregeneration

Research Highlights

(1) This study established the relative specificity of P2X7 receptor expression on microglia from murine brain tissue, providing evidence for the selective regulation of microglia *via* this receptor.
(2) RNA interference and brilliant blue G intervention could inhibit microglial activation and inflammatory reactions by regulating the P2X7 receptor, and provided experimental evidence of a novel way for the prevention and treatment of inflammatory-mediated neurodegenerative disease.
(3) Brilliant blue G is characterized by low toxicity and high selectivity, and can be used as an appropriate drug for blocking microglia-mediated inflammation and degeneration.

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INTRODUCTION

Microglia are resident immune cells of the brain parenchyma that play important roles in the developing and adult central nervous system of mammals. In normal conditions, resting microglia act as supportive glial cells with their ramified processes dynamically monitoring the physiological homeostasis of the extracellular space[1]. When presented with specific stimuli stemming from pathologies, such as infection or trauma, microglia become activated, change their morphology to an amoeboid shape and release proinflammatory cytokines, such as cyclooxygenase-2 and interleukin-6[2]. A growing body of research supports involvement of activated microglia in brain pathologies caused by infectious diseases, trauma, tumors, ischemia, Alzheimer's disease, Parkinson's disease, Down's syndrome, multiple sclerosis and neuro-AIDS[3].

Brilliant blue G is a polysulfonate dye and a very potent noncompetitive selective antagonist of the P2X7 receptor. This compound has a nanomolar affinity for this receptor, and produces an action by inhibiting the initial cationic influx currents through P2X7 receptors. The downstream events associated with the activation of the P2X7 receptor can also be blocked by brilliant blue G[4]. Activation of the P2X7 receptor involves upregulated levels of extracellular adenosine triphosphate and participates in a diversity of microglia-mediated inflammatory responses[5-6]. To observe the role of the P2X7 receptor in microglial activation in the present study, lipopolysaccharide (a traditional microglial activator) was used to induce activation of microglia and to trigger a series of inflammatory cascade reactions. Lipopolysaccharide is known to induce nitric oxide and tumor necrosis factor-α production in microglia through various extracellular signal-regulated kinases, p38 mitogen-activated protein kinase, and c-Jun N-terminal kinase pathways[7-8]. Previous studies have proven that oxidized adenosine triphosphate, a P2X7 receptor antagonist, attenuates lipopolysaccharide-mediated microglial activation and inflammation following neuronal damage in rat brain[9]. Given that NaCl and temperature may affect the potency of oxidized adenosine triphosphate[10], we confirmed that brilliant blue G will exert an inhibitory effect on lipopolysaccharide-induced encephalitis caused by microglial activation.

Currently, no report has been published confirming our hypothesis that the application of brilliant blue G can regulate microglia-mediated neuroinflammation. The current study investigated the microglial inflammatory response in the presence of brilliant blue G both *in vitro* and *in vivo*. Furthermore, this study examined the effect of brilliant blue G in lipopolysaccharide-induced microglial activation and used the findings to characterize the anti-inflammatory effects of brilliant blue G and other P2X7 receptor inhibitors to provide experimental evidence for the prevention and treatment of inflammatory-mediated neurodegenerative disease.

RESULTS

Lipopolysaccharide-induced microglial activation and upregulation of P2X7 receptor expression

Normal microglia were generally in a resting state, with the presence of branch-like forms and with the absence of CD11b expression in immunofluorescence staining (Figure 1A). After stimulation with lipopolysaccharide for 24 hours, microglia were transformed into an early activated condition characterized by large soma and amoeba-like shapes with intense CD11b staining (Figure 1B). P2X7 receptor protein expression in BV2 cells increased in a dose-dependent manner 24 hours after cells were stimulated with lipopolysaccharide, peaking at a lipopolysaccharide concentration of 100 ng/mL (Figure 2).

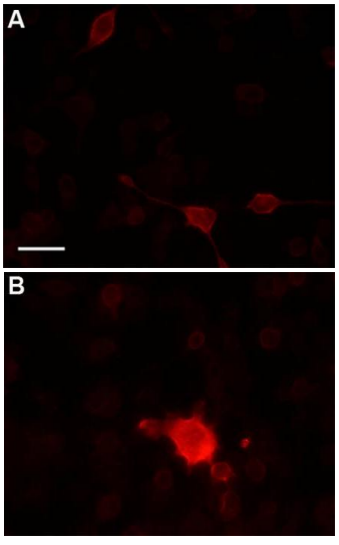

Figure 1 Effect of lipopolysaccharide on the morphology of microglia (immunofluorescence staining, Cy3 labeled, scale bar: 20 μm).

The BV2 cells were treated with 100 ng/mL lipopolysaccharide for 24 hours.

(A) Control group: BV2 cells in a resting state.

(B) Lipopolysaccharide group: the morphological transformation of activated microglia with an amoeba-like shape.

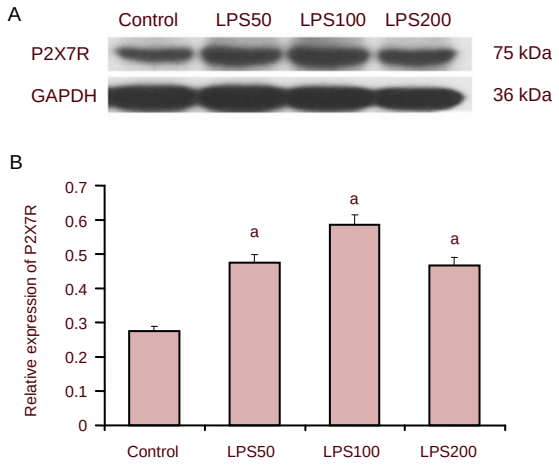

Figure 2 Effects of different concentrations of lipopolysaccharide on the expression of the P2X7 receptor (P2X7R) in BV2 cells.

(A) Western blot demonstrated the trend in P2X7R expression in BV2 cells after 24 hours following increasing concentrations of lipopolysaccharide stimulation (0, 50, 100, 200 ng/mL equate to Control, LPS50, LPS100, LPS200, respectively).

(B) Relative absorbance ratios of P2X7R compared with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) calculated by densitometric quantification of the bands. Data are expressed as mean ± SD, $n = 3$. $^{a}P < 0.05$, *vs*. control group one-way analysis of variance followed Student's *t*-test.

Brilliant blue G inhibited lipopolysaccharide-induced microglial activation *in vitro*

This study examined morphological changes of lipopolysaccharide-stimulated BV2 cells in the presence of brilliant blue G, a P2X7 receptor inhibitor. Brilliant blue G was added to culture medium of BV2 cells 30 minutes before lipopolysaccharide treatment. Immunofluorescence staining was performed at 24 hours after lipopolysaccharide stimulation. More activated microglia, characterized by round cells with abundant cytoplasm and CD11b-positive staining, were present in the lipopolysaccharide group (Figure 3B) compared with the control group (Figure 3A). The trend was reversed in the lipopolysaccharide plus brilliant blue G group (Figure 3C). After brilliant blue G treatment, the expression of CD11b decreased and the morphology of the BV2 cells approached the "resting" ramified state. Fluorescence intensity of the control group and lipopolysaccharide plus brilliant blue G group was similarly weaker than the lipopolysaccharide group (Figure 3).

Brilliant blue G inhibited lipopolysaccharide-induced microglial activation and downregulated P2X7 receptor expression in mouse brain

Fifteen BALB/c mice were randomly divided into control, lipopolysaccharide, and lipopolysaccharide plus brilliant blue G groups. After 3 consecutive days of intervention, immunofluorescence staining was used to observe CD11b and P2X7 receptor expression. Results showed that the number of microglia (CD11b-positive cells) in the cerebral cortex of the lipopolysaccharide-stimulated brain at day 3 was more than that seen in the control and lipopolysaccharide plus brilliant blue G groups (Figure 4A). Double immunostaining for CD11b and P2X7 receptor revealed a predominant colocalization of CD11b-positive cells with the P2X7 receptor in each group. Moreover, the number of P2X7 receptor/CD11b-positive cells in the cortex of mice was significantly greater in the lipopolysaccharide group than in the control and lipopolysaccharide plus brilliant blue G groups (Figure 4B; $P < 0.05$).

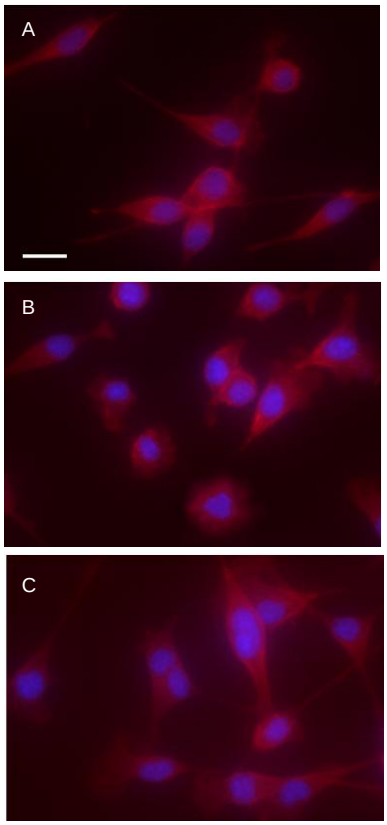

Figure 3 Effect of brilliant blue G on lipopolysaccharide-induced morphologic changes of BV2 cells (fluorescence microscope, scale bar: 20 μm).

The immunofluorescence staining of CD11b (red, Cy3 labeled) showed that activated microglia after lipopolysaccharide stimulation (B) became amoeba-like compared with the control group (A), while lipopolysaccharide plus brilliant blue G group (C) took on a greater number of branch-like cells compared with the lipopolysaccharide group. Nuclei of BV2 cells were counterstained with 4',6-diamidino-2-phenylindole (blue).

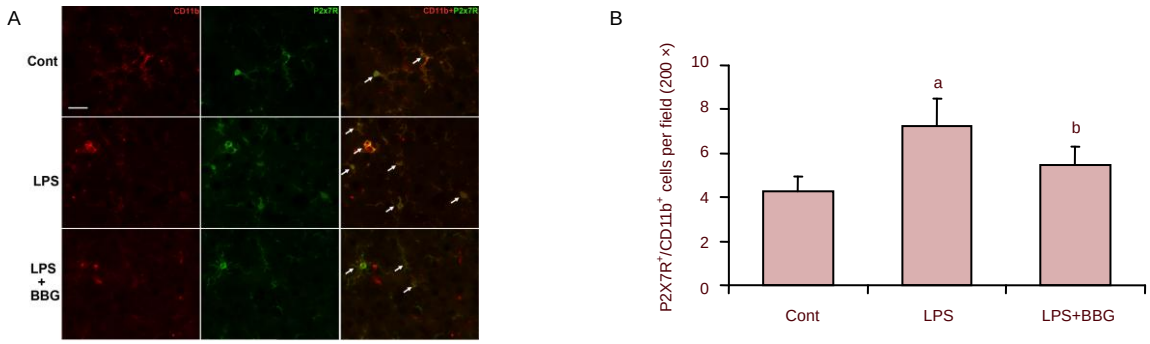

Figure 4 Effect of brilliant blue G (BBG) on CD11b and P2X7 receptor (P2X7R) expression in the cortex of lipopolysaccharide (LPS)-treated mice.

(A) Double-immunofluorescence staining of CD11b and P2X7R in the cortex of mice for the characterization of the location of the P2X7R (green) on microglial cells (red) in the cerebral cortex of BALB/c mice. CD11b/P2X7R colocalized cells were marked with arrows. Scale bar: 100 μm.

(B) Quantification of P2X7R/CD11b-positive cells in the cortex of BALB/c mice. Data are presented as mean ± SD, $n = 3$. $^{a}P < 0.05$, *vs.* control group (Cont); $^{b}P < 0.05$, *vs.* LPS group (one-way analysis of variance followed by Student-Newman-Keuls test).

Inhibition of the P2X7 receptor by brilliant blue G or small interfering RNA (siRNA) alleviated the inflammatory response induced by lipopolysaccharide

The expression of P2X7 receptor and relevant inflammatory mediators was analyzed at 24 hours post lipopolysaccharide stimulation. Brilliant blue G had a distinct inhibitory effect on P2X7 receptor expression in BV2 cells, both at the protein and mRNA levels (Figure 5; $P < 0.05$). Western blot assay demonstrated that the expression of cyclooxygenase-2 protein was significantly elevated after 24 hours of lipopolysaccharide stimulation and this upregulation was distinctly reversed by brilliant blue G intervention (Figure 5C; $P < 0.05$). Real-time PCR displayed that brilliant blue G decreased the expression of proinflammatory cytokines, including interleukin-6 and cyclooxygenase-2 (Figure 5D; $P < 0.05$).

To further confirm the role of P2X7 receptors in the production of proinflammatory factors, siRNA was delivered to BV2 cells to confirm the specific block of P2X7 receptor expression. Negative control siRNA had no effects on the level of P2X7 receptor, cyclooxygenase-2 and interleukin-6 mRNA (Figure 6; $P > 0.05$). BV2 cells pretreated with specific siRNA for P2X7 receptors were stimulated with lipopolysaccharide, whereupon the levels of proinflammatory cytokines were estimated. As seen with brilliant blue G, genetic silencing of P2X7 receptor with a Kv1.3-specific siRNA inhibited lipopolysaccharide-induced increase in cyclooxygenase-2 and interleukin-6 (Figure 6; $P < 0.05$).

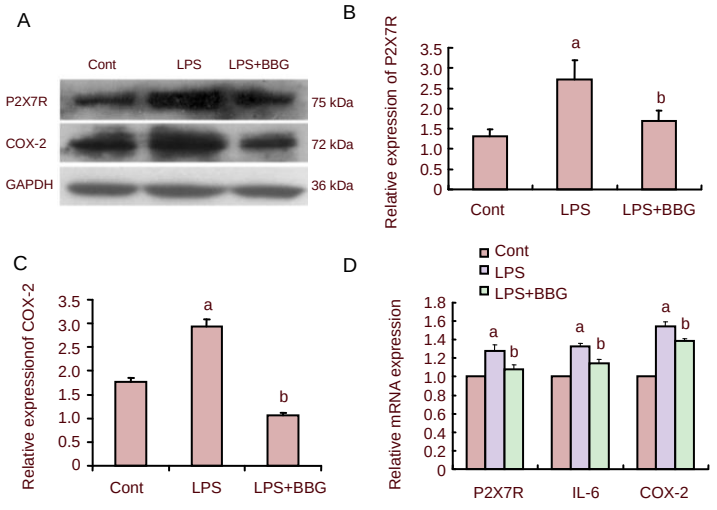

Figure 5 Effect of brilliant blue G (BBG) on cyclooxygenase-2 (COX-2) and interleukin-6 production in lipopolysaccharide (LPS)-activated microglia.

BV2 cells were treated with BBG (100 μM) or isovolumetric PBS buffer for 30 minutes, followed by the addition of LPS (100 ng/mL) for 24 hours.

(A) P2X7 receptor (P2X7R), COX-2 or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression detected by western blot assay.

(B, C) Relative ratios of P2X7R and COX-2 compared with GAPDH calculated by absorbance level of the bands.

(D) mRNA levels of P2X7R, interleukin-6 (IL-6) and COX-2 in each group were assayed using real-time PCR. GAPDH served as an internal control.

Data are presented as mean ± SD, $n = 3$. $^{a}P < 0.05$, *vs.* control (Cont) group; $^{b}P < 0.05$, *vs.* LPS group (one-way analysis of variance followed by Student-Newman-Keuls test).

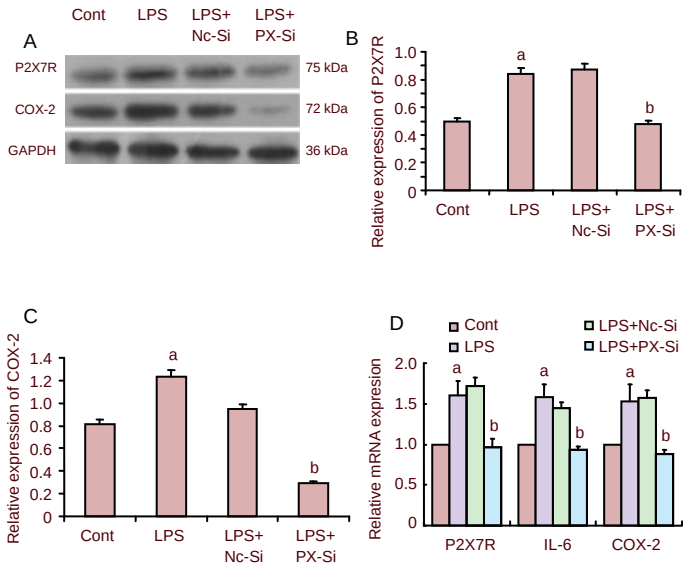

Figure 6 Effect of P2X7 receptor (P2X7R) siRNA on cyclooxygenase-2 (COX-2) and interleukin-6 (IL-6) production in lipopolysaccharide (LPS)-activated microglia.

BV2 cells were treated with P2X7R siRNA (LPS + PX-Si), negative control siRNA (LPS + NC-Si) or PBS for 6 hours, followed by an addition, or omission, of 100 ng/mL LPS for 24 hours.

(A) P2X7R, COX-2 or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression was detected using western blot.

(B, C) The relative ratios of P2X7R and COX-2 compared with GAPDH, calculated by absorbance level of the bands.

(D) mRNA levels of P2X7R, IL-6 and COX-2 in each group were assayed in real-time PCR. GAPDH served as an internal control.

Data are presented as mean ± SD, $n = 3$. $^{a}P < 0.05$, *vs*. control (Cont) group; $^{b}P < 0.05$, *vs*. LPS group (one-way analysis of variance followed by Student-Newman-Keuls test).

DISCUSSION

Proinflammatory cytokines released by activated microglia are contributing factors to pathological processes seen in many neurological disorders[11]. It is commonly accepted that chronic and progressive neurodegeneration is associated with neurogenic inflammation, mainly mediated by microglia, the resident macrophage population in the brain. Control of inflammation *via* regulation of microglial activation has become an area of interest in the study of neurodegenerative disease. Specifically, a recent focus has turned to the involvement of ion channels in microglial activation[12]. Various microglial ion channels have been shown to be involved in modulating membrane potential, cell volume, and intracellular ion concentrations to change proliferation, cell morphology, migration, and cytokine production[13]. More than five probable paths provoke microglial activation with relevant signaling mediators and/or receptors, including fractalkine, interferon-g, monocyte chemoattractant protein-1, P2X4 and Toll-like receptor 4[14]. Delayed-rectifier potassium voltage-gated channel kv1.3 and a21-activated K1 channel KCNN4/KCa3.1, for instance, play a vital role in mediating microglial activation[15-16]. Additionally, microglial inflammatory activities are induced by P2X7 receptor activation via its downstream p38 mitogen-activated protein kinase-early growth response pathway[17].

The P2X7 receptor is a member of the family of nonselective cationic channels gated by extracellular adenosine triphosphate. This receptor has unique properties with respect to other P2X members, such as its low affinity for adenosine triphosphate and its ability to be activated in murine cells by ADP-ribosylation through ADP-ribosyltransferase and nicotinamide adenine dinucleotide[18-19]. Unlike other P2X receptors, the P2X7 receptor forms macropores when exposed to continuous or high-level applications of adenosine triphosphate. These macropores allow the passage of 900-Da molecules (as Lucifer Yellow) to induce cell death[20-21]. This process seems to involve both dilation of the channel pore and the opening of additional channels, such as pannexin-1[22]. Although P2X7 receptor expression has been detected in various tissues, including neurons, glial cells, fibroblasts, smooth muscle, endothelial and epithelial cells[21], it is also in immune cells such as monocytes, macrophages, dendritic cells and T cells, where P2X7 receptor function has been extensively studied[23]. Past studies have shown that the P2X7 receptor drives microglial activation and proliferation, therefore identification of extracellular adenosine triphosphate and the P2X7 receptor is a crucial factor in amyloid-β-dependent microglia activation[24]. Abundant evidence reinforces this receptor as a crucial player for induction of inflammatory responses caused by microglial activation. In accordance with previous studies, our findings demonstrated that lipopolysaccharide-induced microglial activation had an apparent effect on upregulation of the P2X7 receptor.

This study demonstrated an anti-inflammatory effect for

brilliant blue G on lipopolysaccharide-stimulated microglia *in vitro* and *in vivo*. Brilliant blue G exhibited potent inhibitory activity on P2X7 receptor, cyclooxygenase-2 and interleukin-6 production in lipopolysaccharide-stimulated microglia. This study confirmed that direct inhibition of P2X7 receptor expression by P2X7 receptor siRNA in lipopolysaccharide-stimulated microglia led to the same downregulation of cyclooxygenase-2 and interleukin-6 production, which indicated that the P2X7 receptor is a key regulatory point in the microglial inflammatory response. Double-immunofluorescence staining for CD11b and P2X7 receptor in this study, and a previous study of immunofluorescence colocalization[9], confirmed that the P2X7 receptor was mainly present in lipopolysaccharide-stimulated microglia but less so in astrocytes and neurons. This observation increases the feasibility of P2X7 receptor-mediated microglial regulation.

It is known that microglia play a key role in mediating inflammatory processes in the central nervous system, which are associated with various neurodegenerative diseases[25-26]. Lipopolysaccharide, a glycolipid derived from the membrane surface of gram-negative bacteria (an endotoxin), has been used extensively in inflammatory studies[27-28]. Lipopolysaccharide activates microglia and exerts neurotoxic effects in both *in vitro* and *in vivo* systems[29-30]. This study demonstrated that lipopolysaccharide pretreatment activated microglia, which was indicated by morphological changes and a dramatic increase in levels of CD11b, a microglial cell marker, in lipopolysaccharide-treated BV2 cells. Lipopolysaccharide-treated microglia exhibited an increase in levels of interleukin-6 and cyclooxygenase-2, indicating that brain microglia release these proinflammatory cytokines when activated. Therefore, lipopolysaccharide-treated microglia in culture provide a feasible model for studying the roles of microglia in brain inflammation.

Brilliant blue G, an inhibitor of the P2X7 receptor, is a derivative of a commonly used blue food coloring, which can traverse the blood-brain barrier. Systemic administration of brilliant blue G may comprise a readily feasible approach for inflammatory disease in human brains. This study inferred that administration of brilliant blue G reduced the severity of brain inflammation without evident toxicity. Currently, more than 1 million pounds of blue food color are consumed yearly in the United States, corresponding to a daily intake of 16 mg per person[31-32] and has no known toxicity[33], except for the potential of metabolic acidosis in septic patients. This favorable toxicity profile, accompanied with its potent P2X7 receptor inhibition[34], prompted us to test the neuroprotective actions of brilliant blue G in brain inflammation. This analysis showed that brilliant blue G significantly reduced brain inflammation, with its only notable side effect being the transient acquisition of a blue tint to the skin. Brilliant blue G targeted resident microglial cells because they express high levels of P2X7 receptors. Brilliant blue G strongly inhibited microglial activation at 24 hours after lipopolysaccharide treatment. As such, the suppression of reactive gliosis in mice treated with brilliant blue G might be secondary to the inhibition of the P2X7 receptor on microglia.

A critical difference in the anti-inflammatory mechanism of brilliant blue G compared with both minocycline and methylprednisolone is that P2X7 receptors are activated within seconds when cytosolic adenosine triphosphate flows out of traumatized cells, whereas both steroids and minocycline target inflammatory processes downstream to P2X7 receptor activation[35]. Furthermore, the expression of the P2X7 receptor was chiefly increased in lipopolysaccharide-stimulated microglia and brilliant blue G only has a single cellular target with few effects on neurons and astrocytes. Brilliant blue G may have a unique therapeutic profile, capable of suppressing the earliest steps in microglia-mediated inflammatory cascades. Since the deleterious effect of activated microglia on neurogenesis is probably mediated through the action of proinflammatory cytokines, such as interleukin-1, interleukin-6, and tumor necrosis factor-α[36], brilliant blue G may be inferred to restore neurogenesis in inflammation, though this has not yet been confirmed by scientific evidence.

In summary, brilliant blue G has indirect potency exerting substantial anti-inflammatory effects on activated microglial cells, although the exact mechanisms responsible for these actions are not well understood. Results suggest that anti-inflammatory activity of brilliant blue G may be mediated, at least in part, by inhibition of P2X7 receptor expression in microglia. Thus, brilliant blue G may serve as a potential alternative drug for microglial inflammatory- mediated neurodegenerative disorders. However, the detailed anti-inflammatory mechanisms of brilliant blue G in microglial activation will require further investigations.

MATERIALS AND METHODS

Design

A randomized, controlled experiment.

Time and setting

Experiments were performed at the Research Center of Medicine, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, China from June 2010 to December 2011.

Materials

Adult male BALB/c mice (*n* = 15, 6–8 weeks of age) weighing 18–20 g were bought from the Laboratory Animal Center of Sun Yat-sen University in China (license No. 0094467). All protocols were conducted in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China[37].

Methods

Cell stimulation and treatment

BV2 cells, an immortalized murine microglial cell line that exhibits phenotypic and functional properties of reactive microglial cells, were obtained from the Cell Center of Peking Union Medical College in China. The BV2 cell line has been used as a suitable model for the study of resting and activated microglia *in vitro* [38]. BV2 microglial cells were cultured in high-glucose Dulbecco's Modified Eagle's Medium-F12 (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (Gibco), 100 μg/mL streptomycin and 100 U/mL penicillin at 37°C in a humidified incubator with air/CO_2 (95%/5%, v/v). Cells were passaged every 2–3 days. BV2 cells were pretreated with 50, 100 and 200 ng/mL lipopolysaccharide (Sigma, St. Louis, MO, USA) for 24 hours. Cells pretreated with 100 ng/mL[39] lipopolysaccharide were used for morphological experiments. Cell culture medium without lipopolysaccharide was used as a control.

Animal preparation and drug intervention

Mice from the lipopolysaccharide plus brilliant blue G group were injected intraperitoneally with brilliant blue G (45 mg/kg per day; Sigma)[40] dissolved in normal saline 30 minutes prior to lipopolysaccharide injection. A single dose of lipopolysaccharide (5 mg/kg; Sigma)[41] was intraperitoneally injected in mice from the lipopolysaccharide and lipopolysaccharide plus brilliant blue G groups. Brilliant blue G was administered to mice of the lipopolysaccharide plus brilliant blue G group for 3 consecutive days. Simultaneously, the lipopolysaccharide group received an equal volume of normal saline.

Immunofluorescence staining

Immunohistochemistry: After administration for 3 consecutive days, animals were anesthetized with 10% chloral hydrate (4 mL/kg） and then received intracardiac perfusion with PBS and 4% paraformaldehyde. After perfusion, brains were quickly removed, post-fixed overnight with 4% paraformaldehyde and then dehydrated in gradient sucrose solution (10%–20%–30%) at 4°C. Serial sections of mouse brain (10 μm) were cut using a cryostat microtome and mounted onto microscope slides. These slides were stored temporarily in cryoprotectant solution at –30°C. Frozen sections were blocked in solution containing 5% normal goat serum for 1 hour after rinsing in 0.3% Triton X-100 for 30 minutes at 37°C. After several washes, sections were simultaneously incubated overnight at 4°C with the following primary antibodies: monoclonal rat anti-mouse CD11b (1:100; AbD Serotec, Kidlington, Oxford, UK) and polyclonal rabbit anti-P2X7 receptor (1:200; Alomone Labs, Jerusalem, Israel). Primary antibodies were detected with Alexa-488-conjugated goat anti-rabbit and Alexa-594-conjugated goat anti-rat antibodies (1:500; Jackson ImmunoResearch, Jacksonville, PA, USA) for 1 hour at room temperature. Sections were coverslipped with glycerol and fluorescent signals were then detected using an Olympus BX5 microscope (Tokyo, Japan). P2X7 receptor$^+$/CD11b$^+$ cells were counted in five random 200 × fields in the cortex for each repeated experiment. Five coronal slices per animal, including the hippocampus (five mice in each group), were counted. The average of all fields for one mouse was included in statistical analysis.

Immunocytochemistry: Prior to staining, BV2 cultures were washed twice with PBS. Cells were fixed with 4% paraformaldehyde for 20 minutes, and then blocked with 5% goat serum albumin for 1 hour. Afterwards, cells were incubated with monoclonal rat anti-mouse CD11b (1:100; AbD Serotec) at 4°C overnight. After repeated washing with PBS three times, cells were incubated with rabbit anti-rat Cy3-conjugated secondary antibodies (1:200; Millipore, Billerica, MA, USA) for 1 hour at 37°C in a thermostat incubator and coverslipped in anti-fluorescence-quenching reagent (Boster, Wuhan, Hubei Province, China). After 4',6-diamidino-2-phenylindole (Sigma) staining, fluorescent images were detected and analyzed, as mentioned above.

Inhibiting expression of P2X7 receptor by siRNA

BV2 microglia cells were maintained in proliferating medium in 6-well plates for 2 days, at which time they reached 30–50% confluence and were used for transfection of siRNA. The siRNAs targeting mouse P2X7 receptor sequence were chemically synthesized by GenePharma: forward and reverse RNA strands (5'-CCG UAC UCA UCA AGA AUA ATT-3'; 5'-UUA UUC UUG

AUG AGU ACG GTT-3'). Control non-specific sequences were as follows: 5'-UUC UCC GAA CGU GUC ACG UTT-3'; 5'-ACG UGA CAC GUU CGG AGA ATT-3'. Briefly, 5 μL of transfectamine (Invitrogen, Carlsbad, CA, USA) was added to Opti-MEM I (Gibco) reduced serum medium to a final volume of 250 μL. A volume of 10 μL of siRNA was added to Opti-MEM I to a final volume of 250 μL and incubated for 5 minutes. The serene and transfectamine solutions were combined and mixed by gentle pipetting and incubated at room temperature for 20 minutes. Cells were washed with Opti-MEM I, and 1 500 μL of fresh Opti-MEM I was added to each well. The transfection agent/siRNA complex was added dropwise into the cells and incubated at 37°C. BV2 cells were treated with P2X7 receptor siRNA or a negative control siRNA for 6 hours, followed by an addition of lipopolysaccharide (100 ng/mL) for 24 hours. Gene and protein expression were detected by real-time PCR and western blot assay, respectively.

Expression of P2X7 receptor, inteleukin-6 and cyclooxygenase-2 mRNA in BV2 cells

P2X7 receptor, interleukin-6 and cyclooxygenase-2 mRNA expression was determined by real-time PCR. BV2 cells were treated and total RNA was extracted using an RNeasy mini kit [TaKaRa Biotechnology (Dalian) Co., Ltd., Dalian, Liaoning Province, China]. RNA samples were used for first-strand cDNA synthesis using a SuperScript cDNA kit (TaKaRa). Real-time PCR was performed using a Roche Lightcycle 480 (Roche, Welwyn Garden, Hertfordshire, UK) and a SYBR green real-time kit (TaKaRa). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. The ratios of P2X7 receptor/GAPDH, interleukin-6/GAPDH and cyclooxygenase-2/GAPDH for every sample were first calculated, and results were expressed as fold changes compared with control. Primer sequences are shown in Table 1.

Table 1 Primer sequences used for quantitative real-time PCR

Gene	Primer (5'–3')
Glyceraldehyde-3-phosphate dehydrogenase	Forward: ATC TTC TTG TGC AGT GCC AG Reverse: CGT TGA TGG CAA CAA TCT CC
Cyclooxygenase-2	Forward: GCC CGA CAC CTT CAA CAT T Reverse: GCC TTT GCC ACT GCT TGT
Interleukin-6	Forward: GTC ACA GAA GGA GTG GCT AAG GA Reverse: TAA CGC ACT AGG TTT GCC GAG TAG
P2X7 receptor	Forward: TTA TGG CAC CGT CAA GTG G Reverse: TCT CCG TCA CCT CTG CTA TG

Western blot analysis for P2X7 receptor and cyclooxygenase-2 expression in BV2 cells

Protein expression of P2X7 receptor and cyclooxygenase-2 was monitored in microglia by western blot assay. Total protein extracts were prepared and quantified from BV2 cells in radioimmunoprecipitation assay lysates. In brief, 60 μg of total proteins for each lane were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane using an electrophoretic transfer apparatus. The membrane was then incubated with primary antibodies, including polyclonal rabbit anti-P2X7 receptor (1:400; Alomone Labs), polyclonal rabbit anti-cyclooxygenase-2 (1:300; Cell Signaling Technology, Beverly, MA, USA), and polyclonal rabbit anti-GAPDH (1:500; Cell Signaling Technology) at 4°C overnight. Subsequently the membrane was incubated with the secondary antibody: goat anti-rabbit IgG-horseradish peroxidase (1:5 000; Cell Signaling Technology) for 1 hour at room temperature. After washing in Tris-buffered saline Tween-20, blots were developed with enhanced chemiluminescence detection reagents (Millipore). The relative absorbance values of bands were analyzed using a gel imaging analysis system (Genetics, Campbell, CA, USA).

Statistical analysis

Statistical analysis was performed using SPSS 16.0 software (SPSS, Chicago, IL, USA). At least three independent experiments were performed for each experimental procedure. Measurement data are expressed as mean ± SD. Differences were assessed using one-way analysis of variance for multiple comparisons followed by the Student-Newman-Keuls test for intergroup comparisons or Student's *t*-test for intergroup comparisons. Two-tailed values of $P < 0.05$ were considered statistically significant.

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REFERENCES

[1] Nimmerjahn A, Kirchhoff F, Helmchen F. Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. Science. 2005;308(5726):1314-1318.

[2] Brown GC, Neher JJ. Inflammatory neurodegeneration and mechanisms of microglial killing of neurons. Mol Neurobiol. 2010;41(2-3):242-247.

[3] Mayer AM. Therapeutic implications of microglia activation by lipopolysaccharide and reactive oxygen species generation in septic shock and central nervous system pathologies: a review. Medicina (B Aires). 1998;58(4): 377-385.

[4] Jiang LH, Mackenzie AB, North RA, et al. Brilliant blue G selectively blocks ATP-gated rat P2X(7) receptors. Mol Pharmacol. 2000;58(1):82-88.

[5] Gudipaty L, Munetz J, Verhoef PA, et al. Essential role for Ca^{2+} in regulation of IL-1beta secretion by P2X7 nucleotide receptor in monocytes, macrophages, and HEK-293 cells. Am J Physiol Cell Physiol. 2003;285(2): C286-299.

[6] Witting A, Walter L, Wacker J, et al. P2X7 receptors control 2-arachidonoylglycerol production by microglial cells. Proc Natl Acad Sci U S A. 2004;101(9): 3214-3219.

[7] Waetzig V, Czeloth K, Hidding U, et al. c-Jun N-terminal kinases (JNKs) mediate pro-inflammatory actions of microglia. Glia. 2005;50(3):235-246.

[8] Bhat NR, Fan F. Adenovirus infection induces microglial activation: involvement of mitogen-activated protein kinase pathways. Brain Res. 2002;948(1-2):93-101.

[9] Choi HB, Ryu JK, Kim SU, et al. Modulation of the purinergic P2X7 receptor attenuates lipopolysaccharide-mediated microglial activation and neuronal damage in inflamed brain. J Neurosci. 2007;27(18):4957-4968.

[10] Hibell AD, Thompson KM, Xing M, et al. Complexities of measuring antagonist potency at P2X(7) receptor orthologs. J Pharmacol Exp Ther. 2001;296(3):947-957.

[11] Zindler E, Zipp F. Neuronal injury in chronic CNS inflammation. Best Pract Res Clin Anaesthesiol. 2010; 24(4):551-562.

[12] Farber K, Kettenmann H. Physiology of microglial cells. Brain Res Brain Res Rev. 2005;48(2):133-143.

[13] Eder C. Regulation of microglial behavior by ion channel activity. J Neurosci Res. 2005;81(3):314-321.

[14] Graeber MB. Changing face of microglia. Science. 2010;330(6005):783-788.

[15] Schlichter LC, Kaushal V, Moxon-Emre I, et al. The Ca^{2+} activated SK3 channel is expressed in microglia in the rat striatum and contributes to microglia-mediated neurotoxicity in vitro. J Neuroinflammation. 2010;7:4.

[16] Kaushal V, Koeberle PD, Wang Y, et al. The Ca^{2+}-activated K^+ channel KCNN4/KCa3.1 contributes to microglia activation and nitric oxide-dependent neurodegeneration. J Neurosci. 2007;27(1):234-244.

[17] Friedle SA, Brautigam VM, Nikodemova M, et al. The P2X7-Egr pathway regulates nucleotide-dependent inflammatory gene expression in microglia. Glia. 2011; 59(1):1-13.

[18] Koch-Nolte F, Haag F, Guse AH, et al. Emerging roles of NAD^+ and its metabolites in cell signaling. Sci Signal. 2009;2(57):mr1.

[19] Haag F, Adriouch S, Brass A, et al. Extracellular NAD and ATP: Partners in immune cell modulation. Purinergic Signal. 2007;3(1-2):71-81.

[20] North RA. Molecular physiology of P2X receptors. Physiol Rev. 2002;82(4):1013-1067.

[21] Surprenant A, North RA. Signaling at purinergic P2X receptors. Annu Rev Physiol. 2009;71:333-359.

[22] Pelegrin P, Surprenant A. Pannexin-1 mediates large pore formation and interleukin-1beta release by the ATP-gated P2X7 receptor. EMBO J. 2006;25(21):5071-5082.

[23] Chen L, Brosnan CF. Regulation of immune response by P2X7 receptor. Crit Rev Immunol. 2006;26(6):499-513.

[24] Sanz JM, Chiozzi P, Ferrari D, et al. Activation of microglia by amyloid {beta} requires P2X7 receptor expression. J Immunol. 2009;182(7):4378-4385.

[25] Jamin N, Junier MP, Grannec G, et al. Two temporal stages of oligodendroglial response to excitotoxic lesion in the gray matter of the adult rat brain. Exp Neurol. 2001; 172(1):17-28.

[26] Dehmer T, Lindenau J, Haid S, et al. Deficiency of inducible nitric oxide synthase protects against MPTP toxicity in vivo. J Neurochem. 2000;74(5):2213-2216.

[27] Carter DA, Dick AD. Lipopolysaccharide/interferon-gamma and not transforming growth factor beta inhibits retinal microglial migration from retinal explant. Br J Ophthalmol. 2003;87(4):481-487.

[28] Maekawa S, Aibiki M, Si QS, et al. Differential effects of lowering culture temperature on mediator release from lipopolysaccharide-stimulated neonatal rat microglia. Crit Care Med. 2002;30(12):2700-2704.

[29] Hughes EH, Schlichtenbrede FC, Murphy CC, et al. Minocycline delays photoreceptor death in the rds mouse through a microglia-independent mechanism. Exp Eye Res. 2004;78(6):1077-1084.

[30] Nakamura Y, Si QS, Kataoka K. Lipopolysaccharide-induced microglial activation in culture: temporal profiles of morphological change and release of cytokines and nitric oxide. Neurosci Res. 1999;35(2):95-100.

[31] Borzelleca JF, Depukat K, Hallagan JB. Lifetime toxicity/carcinogenicity studies of FD & C Blue No. 1 (brilliant blue FCF) in rats and mice. Food Chem Toxicol. 1990;28(4):221-234.

[32] Hess SM, Fitzhugh OG. Absorption and excretion of certain triphenylmethane colors in rats and dogs. J Pharmacol Exp Ther. 1955;114(1):38-42.
[33] Remy M, Thaler S, Schumann RG, et al. An in vivo evaluation of Brilliant Blue G in animals and humans. Br J Ophthalmol. 2008;92(8):1142-1147.
[34] Jiang LH, Mackenzie AB, North RA, et al. Brilliant blue G selectively blocks ATP-gated rat P2X(7) receptors. Mol Pharmacol. 2000;58(1):82-88.
[35] Seabrook TJ, Jiang L, Maier M, et al. Minocycline affects microglia activation, Abeta deposition, and behavior in APP-tg mice. Glia. 2006;53(7):776-782.
[36] Ekdahl CT, Claasen JH, Bonde S, et al. Inflammation is detrimental for neurogenesis in adult brain. Proc Natl Acad Sci U S A. 2003;100(23):13632-13637.
[37] The Ministry of Science and Technology of the People's Republic of China. Guidance Suggestions for the Care and Use of Laboratory Animals. 2006-09-30
[38] Kim WK, Hwang SY, Oh ES, et al. TGF-beta1 represses activation and resultant death of microglia via inhibition of phosphatidylinositol 3-kinase activity. J Immunol. 2004; 172(11):7015-7023.
[39] Kim HY, Park EJ, Joe EH, et al. Curcumin suppresses Janus kinase-STAT inflammatory signaling through activation of Src homology 2 domain-containing tyrosine phosphatase 2 in brain microglia. J Immunol. 2003; 171(11):6072-6079.
[40] Diaz-Hernandez M, Diez-Zaera M, Sanchez-Nogueiro J, et al. Altered P2X7-receptor level and function in mouse models of Huntington's disease and therapeutic efficacy of antagonist administration. FASEB J. 2009;23(6): 1893-1906.
[41] Kaushik DK, Gupta M, Das S, et al. Kruppel-like factor 4, a novel transcription factor regulates microglial activation and subsequent neuroinflammation. J Neuroinflammation. 2010;7:68.

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