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Full Paper

Anti-neuroinflammatory Activity of a Novel Cannabinoid Derivative by Inhibiting the NF-κB Signaling Pathway in Lipopolysaccharide-Induced BV-2 Microglial Cells

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Abstract. Microglial-mediated neuroinflammation has recently been implicated as one of the important mechanisms responsible for the progression of neurodegenerative diseases. Activated microglia cells produce various neurotoxic factors that are harmful to neurons. Therefore, suppression of the inflammatory response elicited by activated microglia is considered a potential therapeutic target for neurodegenerative diseases. The cannabinoid (CB) system is widespread in the central nervous system and is very crucial for modulating a spectrum of neurophysiological functions such as pain, appetite, and cognition. In the present study, we synthesized and investigated a novel CB derivative (CD-101) for its ability to suppress lipopolysaccharide (LPS)-mediated activation of BV-2 microglial cells and subsequent release of various inflammatory mediators. CD-101 significantly inhibited the production of inflammatory markers such as nitric oxide, cyclooxygenase-2, and pro-inflammatory cytokines such as tumor necrosis factor-α, interleukin-1β, and interleukin-6. The anti-neuroinflammatory effect of this novel cannabinoid derivative occurred by inhibiting p38MAPK phosphorylation and by decreasing nuclear translocation of p65 subunit of nuclear factor kappa-B in LPS-stimulated BV-2 microglial cells. These results suggest that the use of the cannabinoid derivative CD-101 might be a potential therapeutic target against neuroinflammatory disorders.

Keywords: lipopolysaccharide, microglia, neuroinflammation, cannabinoid signaling

Introduction

Neuroinflammation plays an important role in the pathogenesis of several neurodegenerative diseases such as Parkinson's disease (PD), Alzheimer's disease (AD), and multiple sclerosis (1 – 3). Microglia are resident immune cells in the central nervous system (CNS) (4, 5) and serve the role of immune surveillance and host defense under physiological conditions (6). Activated microglia and damaged neuronal cells release various proinflammatory mediators such as nitric oxide (NO), inducible NO synthase (iNOS), cyclooxygenase-2 (COX-2), prostaglandin E2, interleukin-6 (IL-6), interleukin-1β (IL-1β), and tumor necrosis factor-α (TNF-α) (7 – 9) in various neuroinflammatory diseases. Therefore, suppression of microglia mediated inflammation would be an ideal therapeutic approach to alleviate neuroinflammatory diseases. The endocannabinoid system refers to a group of neuromodulatory lipids and their receptors that are involved in a variety of physiological processes, including appetite, pain-sensation, mood, and memory. Until now, two G-protein–coupled receptors in the CNS, namely, cannabinoid (CB) CB1 and CB2, have been cloned (10). Among the CB receptors, CB1-receptor expression occurs in the olfactory bulb, cortex, cerebellum, hypothalamus, basal ganglia, nucleus accumbens, spinal cord, and microglial cells. CB1 receptors are also present on peripheral tissues such as the gastrointestinal tract,

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adipose tissue, skeletal muscle, testes, uterus, placenta, and bone (11). In contrast, CB2 receptors are primarily localized in cells involved in immune and inflammatory responses (10, 12). Targeting the CB1 receptor has multiple effects such as changes in cognition, anxiety, control of appetite, emesis, and motor behavior, as well as sensory, autonomic, and neuroendocrine responses (13, 14). Antagonizing CB1 receptors has been reported to augment the antiparkinsonian action of levodopa in 1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine–treated rhesus monkeys (15). The CB1-receptor antagonist rimonabant is also reported to be adjunctively therapeutic as well as monotherapeutic in an animal model of PD (16). A recent report has revealed that modulation of neuroinflammation is one of the physiological functions of the CB signaling system (12). Growing evidence indicates that the CB system is altered in common neurodegenerative conditions (17, 18). Therefore, the CB system is a promising target for therapeutic approaches, particularly for those neurodegenerative diseases wherein neuroinflammation is one of the major components. Based on the recent knowledge of CB signaling, we have synthesized and identified a novel and efficacious cyclic amine derivative using microglial cell-based screening.

In the present study, we report the anti-neuroinflammatory effect of a novel synthetic CB derivative in lipopolysaccharide (LPS)-stimulated BV-2 microglial cells. Microglial activation through mitogen activated protein kinase (MAPK), nuclear factor kappa-B (NF-κB), protein kinase C, and tyrosine kinases, resulting in release of proinflammatory cytokines and neurotoxic mediators, can be induced by treatment with LPS both in vitro and in vivo (19, 20). LPS-stimulated BV-2 cells, which simulate inflammation, have been demonstrated to be a good cellular model for screening potential therapeutic compounds for neuroinflammatory disorders (21). Our novel synthesized cannabinoid derivative (CD-101) inhibited the production of inflammatory markers, including NO, COX-2, and pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6. Subsequent mechanistic studies revealed that the anti-neuroinflammatory effect of this novel CB derivative occurred by inhibiting p38MAPK phosphorylation and by decreasing nuclear translocation of p65 subunit of NF-κB in LPS-stimulated BV-2 microglial cells.

Materials and Methods

Reagents and antibodies

The chemical structure of CD-101 is shown in Fig. 1. LPS (*Escherichia coli* 0111:B4; Sigma, St. Louis, MO, USA), Tween-20, bovine serum albumin, dimethyl sulfoxide (DMSO), sodium nitrite, sulfanilamide, *N*-(1-naphthyl) ethylenediamine dihydrochloride, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma. The 6-well and 96-well tissue culture plates and 100-mm culture dishes were purchased from Nunc, Inc. (Aurora, IL, USA). Fetal bovine serum (FBS) was purchased from PAA Laboratories, Inc. (Etobicoke, Ontario, Canada). Dulbecco's modified Eagle medium (DMEM), containing 4,500 mg/L of D-glucose, 84 mg/L of L-arginine, 110 mg/L of sodium pyruvate, and phosphate-buffered saline (PBS) as well as other cell culture reagents, was obtained from

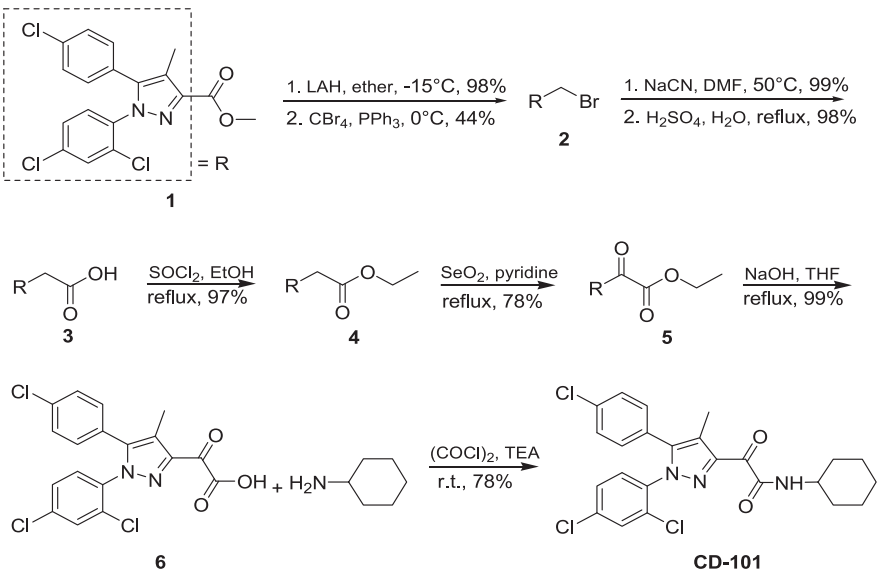

Fig. 1. Synthesis of the novel cannabinoid derivative CD-101.

Gibco/Invitrogen (Carlsbad, CA, USA). The protease inhibitor cocktail tablets and phosphatase inhibitor cocktail tablets were supplied by Roche (Indianapolis, IN, USA). Antibodies to COX-2, p65NF-κB, and nucleolin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and antibodies for iNOS, p38, phospho-p38, IκB-α, p-IκB-α, and β-actin were supplied by Cell Signaling Technology (Danvers, MA, USA).

Cell culture

BV-2 cells (a mouse microglial cell line), originally developed by Dr. V. Bocchini (University of Perugia, Perugia, Italy), were used as reported previously (20, 22). The immortalized murine BV-2 cell line, which exhibits both phenotypic and functional properties of reactive microglia cells (23), were grown and maintained in DMEM supplemented with 5% heat-inactivated FBS and 50 μg/mL penicillin-streptomycin and maintained in a humidified incubator at 37°C with 5% CO_2.

NO assay

NO production was assayed by measuring the levels of nitrite (stable NO metabolite) in culture medium. Accumulated levels of nitrites were measured in the cell supernatant using the Griess reaction (24). BV-2 cells (2.5×10^4 cells/mL) were plated in 96-well plates in 200 μL culture medium, pre-treated with different concentrations of CD-101 (0.1, 1, and 2.5 μM) for 60 min, and then stimulated with LPS (100 ng/mL) for 24 h. Briefly, 50 μL of culture supernatant was reacted with an equal volume of Griess reagent (1 part 0.1% naphthylethylenediamine and 1 part 1% sulfanilamide in 5% H_3PO_4) in 96-well plates at room temperature. Nitrite concentrations were determined using standard solutions of sodium nitrite prepared in cell culture medium. The absorbance was determined at 540 nm using a microplate reader (Tecan Trading AG, Mannedorf, Switzerland). Results are representative of three independent experiments.

Cytotoxicity assay

Viability of cultured cells was determined by measuring the reduction of MTT to formazan. Briefly, BV-2 cells (2.5×10^4 cells/mL) were pre-treated with different concentrations of CD-101 (0.1, 1, and 2.5 μM) for 60 min in 96-well plates and 200 μL culture medium and then stimulated with LPS (100 ng/mL) for 24 h. Then, 0.5 mg/mL of MTT solution was added to each well. Following 4 h incubation at 37°C, the supernatants were removed, and the formed formazan crystals were dissolved in DMSO. The absorbance at 550 nm was determined using a microplate reader. Results are representative of three independent experiments.

Measurement of PGE_2 levels

BV-2 cells (50×10^4 cells/mL) were sub-cultured in 6-well plates and incubated for 24 h without or with CD-101 (0.1, 1, and 2.5 μM) in the presence of LPS (100 ng/mL). BV-2 cells untreated with LPS were included for comparison. The culture medium supernatant was collected by centrifugation and the PGE_2 concentration was determined by an EIA kit (Cayman Chemical Co., Ann Arbor, MI, USA). The absorbance was read at 420 nm using a microplate reader (Tecan Trading AG). Each experiment was performed in triplicate.

Isolation of total RNA and reverse transcription–polymerase chain reaction (RT-PCR)

BV-2 cells (50×10^4 cells/mL) were cultured in 6-well plates, and total RNA was isolated by extraction with TRIzol (Invitrogen). For RT-PCR, 2.5 μg of total RNA was reverse transcribed using a First-Strand cDNA Synthesis kit (Invitrogen). Then, cDNA was amplified by PCR using specific primers as mentioned previously (20, 22). The PCR was performed using the above-prepared cDNA as a template for respective targets, as described previously (25, 26). PCR products were analyzed on 1% agarose gels. Results are representative of three independent experiments.

Western blot analysis

Cells were washed twice with ice cold PBS, and total cell lysates were obtained by adding 50 or 100 μL of RIPA buffer (1 × PBS, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS, containing fresh protease inhibitor cock-tail) to BV-2 cells (50×10^4 cells/mL) cultured in 6-well plates. Electrophoresis and immunoblotting procedures followed a previous report (20). PVDF membranes were tagged by incubating them overnight with anti-iNOS (1:1000), anti-IκB-α (1:1000), anti-p-IκB-α (1:1000), anti-p38 (1:1000), anti-phospho-p38 (1:1000), anti-β-actin (1:2000), anti-COX-2 (1:1000), anti-phospho p65NF-κB (1:500), and anti-nucleolin (1:500) antibodies followed by a 1 h incubation with horseradish peroxidase–conjugated secondary antibodies (1:1000 – 2000) (Cell Signaling Technology). The optical densities of the antibody specific bands were analyzed with a Luminescent Image Analyzer, LAS-3000 (Fuji, Tokyo). In a parallel experiment, nuclear proteins were extracted using the method provided by the nuclear and cyto plasmic extraction kit provided by Thermo Scientific (Rockford, IL, USA). Results are representative of three independent experiments.

Immunofluorescence assay

BV-2 microglia cells (5×10^4 cells/well in 24-well plate) were cultured on sterile cover slips in 24-well

plates and treated with different concentrations of CD-101 (0.1, 1, and 2.5 μM) for 60 min followed by LPS (100 ng/mL) for 30 min to detect the intracellular location of the NF-κB p65 sub-unit. At 30 min after the LPS treatment, the cells were fixed with methanol for 30 min at −20°C and washed with PBS for 5 min. The fixed cells were then permeabilized with 1% Triton X-100 in PBS for 10 min at room temperature, washed with PBS for 5 min, and then treated with 10% goat serum in PBS for 60 min. The permeabilized cells were then treated with monoclonal mouse anti-human NF-κB (p65) (1:200) (Santa Cruz Biotechnology) for 60 min at room temperature and washed with in PBS for 5 min. The cells were then incubated in a 1:200 dilution of Alexa Fluor 568-labeled goat anti-mouse antibody (Invitrogen) for 60 min at room temperature and washed in PBS for 5 min. Cells were stained with 1 μM Hoechst staining solution for 30 min at 37°C and then washed. Finally, the cover slips with cells were dried at 55°C in an oven for 10 – 12 min and mounted in a 1:1 mixture of xylene and malinol.

Statistical analyses

Statistical analyses were conducted using GraphPad software version 5 (GraphPad, La Jolla, CA, USA). Values are presented as the mean ± standard error. Significant differences between the groups were determined using a one-way analysis of variance followed by Tukey's post hoc analysis. *P*-values < 0.05 were considered significant.

Results

CD-101 synthesis

The novel cannabinoid derivative CD-101 (Fig. 1), containing an oxoacetamide group, was produced (compound 1) according to a previously reported procedure (27) by reducing with LAH in diethyl ether. The resulting methyl alcohol was then converted into bromomethyl compound 2 with triphenylphosphine and tetrabromide via an Appel reaction. Substitution of the bromo group in 2 with the nitrile group using sodium cyanide in DMF at 50°C to give acetonitrile was followed by hydrolysis of the nitrile group under the sulfuric acid afforded by acetic acid compound 3. After the acid group was protected with ethanol using thionyl chloride, the resulting ethyl ester 4 was oxidized using selenium dioxide in pyridine to produce oxoacetate 5, which was hydrolyzed under basic conditions to give oxoacetic acid 6. Finally, after compound 6 (600 mg, 1.46 mmol) was converted into its corresponding acid chloride using oxalyl chloride (377 μL, 4.39 mmol) in dichloromethane (5 mL), the acid chloride was coupled with cyclohexylamine (507 μL, 4.39 mmol) to give CD-101 as a white solid (390 mg, 54%). The characteristics of the compound are as follows: Mp 190°C; IR (KBr, cm^{-1}) 3295, 1678; ^{1}H nuclear magnetic resonance (NMR) ($CDCl_3$) δ 1.30 – 1.40 (m, 2H), 1.35 – 1.45 (m, 2H), 1.58 – 1.75 (m, 4H), 1.90 – 2.00 (m, 2H), 2.32 (s, 3H), 3.90 – 4.00 (m, 1H), 7.00 – 7.08 (m, 2H), 7.24 – 7.34 (m, 4H), 7.40 – 7.50 (t, 1H, J = 2.0 Hz), 7.70 – 7.80 (d, 1H, J = 6.8 Hz); ^{13}C NMR ($CDCl_3$) δ 10.03, 24.63, 25.54, 32.64, 48.51, 120.61, 126.33, 127.80, 128.88, 130.24, 130.23, 130.72, 132.50, 135.12, 135.34, 136.06, 142.62, 146.71, 159.98, 182.79; MS *m/z* 489.95, 491.95 [$(M + H)^+$ for $^{35}Cl_3$ and $^{35}Cl_2{}^{37}Cl$].

Effect of CD-101 on NO production in LPS stimulated BV-2 microglial cells

We performed the MTT assay to determine whether the CD-101 treatment was cytotoxic to BV-2 microglia cells. Viability of control cells was used as the control value of 100%. BV-2 cells were treated with LPS (100 ng/mL) with or without different concentrations of CD-101 (0.1, 1, and 2.5 μM) for 24 h. As a result, treatment with LPS (100 ng/mL) and CD-101 did not result in any significant cytotoxicity. CD-101 in combination with LPS (100 ng/mL) also did not cause any significant change in BV-2 cell viability (Fig. 2A). Among various inflammatory mediators produced by activated microglia, NO production has been widely used as a representative measure of inflammatory activation of microglial cells (28, 29). Therefore, the inhibitive effect of CD-101 on NO production in LPS-stimulated microglia was examined. BV-2 microglial cells were pre-treated for 30 min with different concentrations of CD-101 (0.1, 1, and 2.5 μM), followed by LPS (100 ng/mL) treatment for 24 h. The accumulated nitrite in the culture medium estimated by the Griess reaction was used as an index of NO synthesis. Our data indicated that the control and CD-101 treatment did not induce an increase in NO levels. As shown in Fig. 2B, LPS treatment markedly induced NO production (9.18 ± 0.37 μM) as compared to that in the control. Pre-treatment with CD-101 at 0.1, 1, and 2.5 μM significantly reduced NO levels in LPS-induced BV-2 microglial cells in a dose-dependent manner to 8.85 ± 0.36 μM, 5.80 ± 0.33 μM, and 4.84 ± 0.23 μM, respectively. Hence, our results indicate that CD-101 potently inhibits increased NO production in LPS-stimulated BV-2 microglial cells.

Effect of CD-101 on LPS-stimulated iNOS and COX-2 mRNA, protein expression, and PGE_2 levels in LPS-stimulated BV-2 microglial cells

To support the effect of CD-101 on LPS-stimulated microglia activation and LPS-induced nitrite production, iNOS and COX-2 mRNA expression and protein levels were detected by RT-PCR and western blot analyses. BV-2 microglia cells were pretreated with CD-101 (0.1,

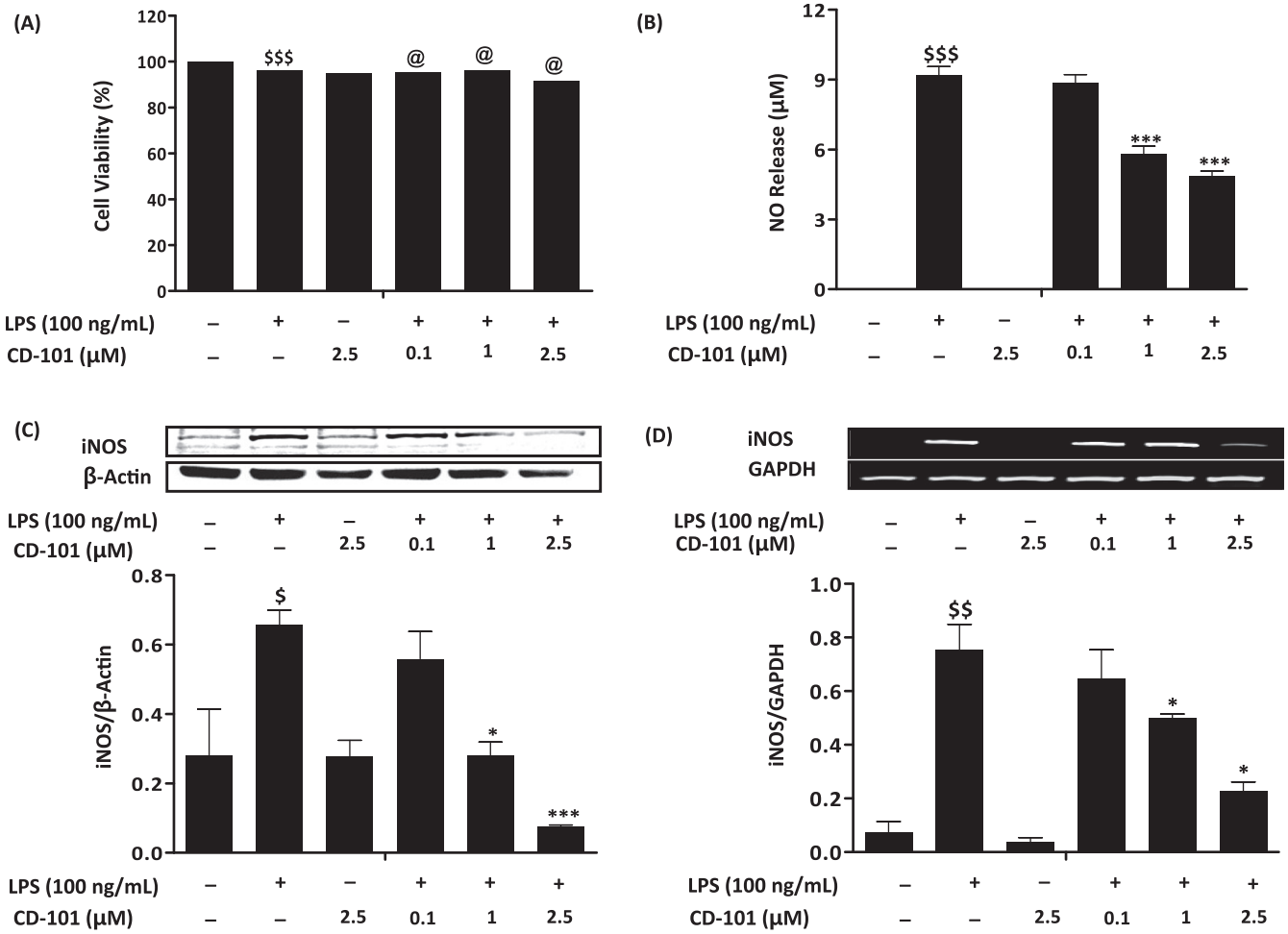

Fig. 2. Effect of CD-101 and/or lipopolysaccharide (LPS) on viability of BV-2 microglial cells using the MTT assay and the effect of CD-101 on nitric oxide (NO) release and inducible nitric oxide synthase (iNOS) protein and mRNA expression in BV-2 microglial cells. A: BV-2 cells were incubated with 0.1, 1, and 2.5 μM CD-101 for 1 h followed by LPS treatment (100 ng/mL) for 24 h. Results are shown as a percentage of control samples. Data are reported as the mean ± standard error (S.E.M.) for three independent experiments. $^{\$\$\$}$Not significantly different as compared to the control group. $^{@}$Not significantly different from the LPS-treated group. B: BV-2 cells were pre-treated with different concentrations of CD-101 (0.1, 1, and 2.5 μM) for 60 min before incubating with LPS (100 ng/mL) for 24 h. Nitrite levels were measured in the culture media using the Griess reaction. C: In a parallel experiment, BV-2 microglial cells were pre-treated with different concentrations (0.1, 1, and 2.5 μM) of CD-101 for 60 min before incubating them with LPS (100 ng/mL) for 18 h. The lysates were analyzed by immunoblotting with an anti-iNOS antibody. Quantification data are shown in the lower panel. Results are expressed as a ratio of iNOS and β-actin. D: BV-2 microglial cells were pre-treated with the indicated concentrations of CD-101 for 60 min before incubating with LPS (100 ng/mL) for 6 h. Total RNA was extracted and further analyzed by RT-PCR. GAPDH was used as the control gene. Quantification data are shown in the lower panel. Results are expressed as a ratio of iNOS and GAPDH. Data are the mean ± S.E.M. of three independent experiments. $^{\$\$\$}P < 0.001$, $^{\$\$}P < 0.01$, and $^{\$}P < 0.05$, compared with the control group; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, compared with the LPS-treated group. Significance was determined by ANOVA followed by Tukey's multiple comparison test.

1, and 2.5 μM) for 1 h and then stimulated with LPS (100 ng/mL) for another 6 and 24 h. As shown in Fig. 2, C and D, pre-treatment with CD-101 at various concentrations (0.1, 1, and 2.5 μM) significantly inhibited LPS-induced iNOS protein and mRNA expression in a dose-dependent manner. COX-2 is another important inflammatory enzyme that is upregulated by cytokines, endotoxins, and various neurodegenerative diseases (30). Therefore, we investigated the effects of CD-101 on COX-2 in LPS-activated BV-2 microglial cells. We found that treatment of BV-2 cells with LPS (100 ng/mL) significantly increased COX-2 mRNA and protein expression. Pre-treatment with CD-101 at various concentrations (0.1, 1, and 2.5 μM) significantly inhibited LPS-induced COX-2 protein level and mRNA expression in BV-2 microglial cells in a dose-dependent manner

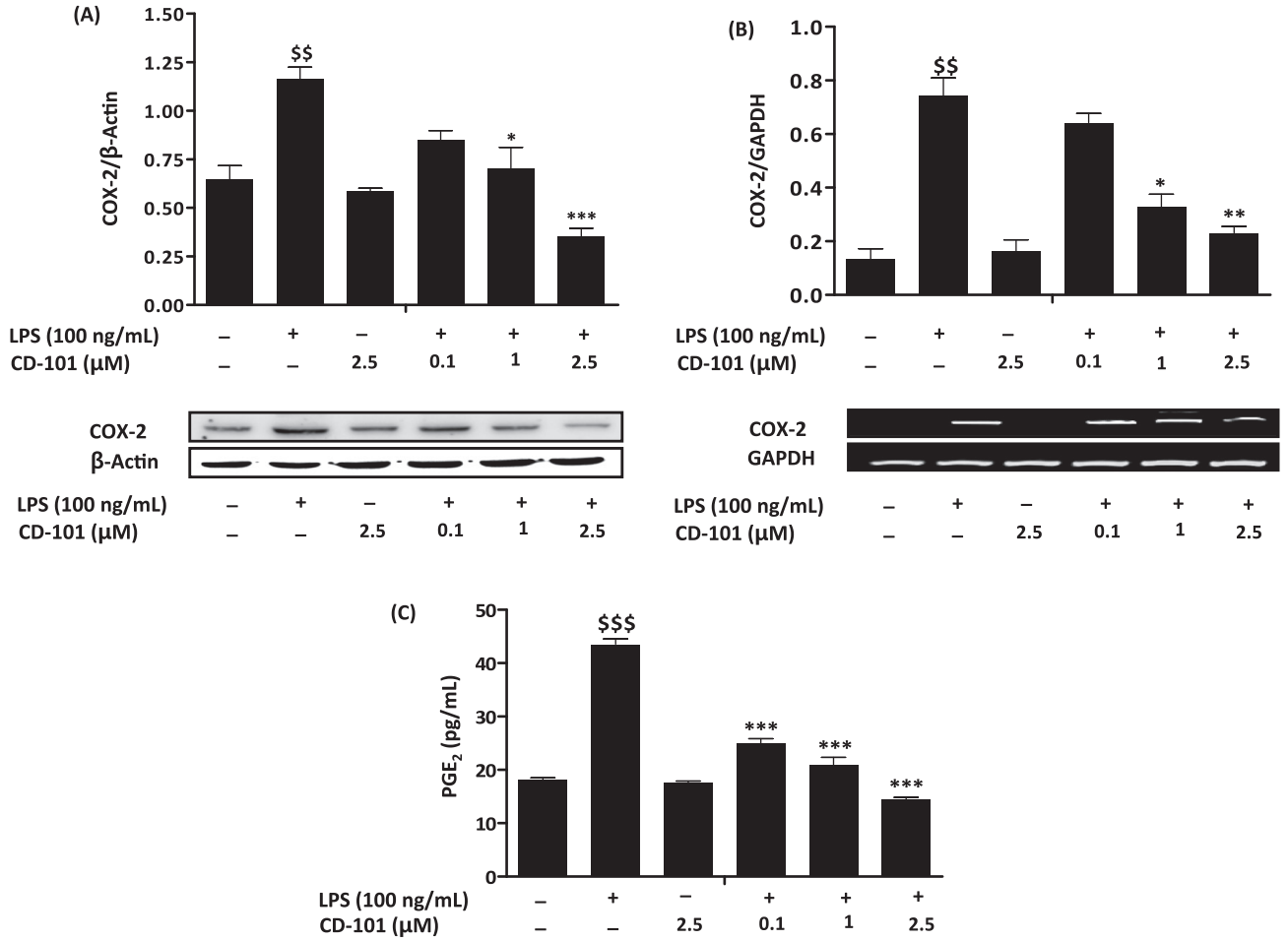

Fig. 3. Effect of CD-101 on lipopolysaccharide (LPS)-induced cyclooxygenase-2 (COX-2) mRNA, protein expression in BV-2 microglial cells, and PGE_2 levels. A: BV-2 microglial cells were pre-treated with different concentrations (0.1, 1, and 2.5 μM) of CD-101 for 60 min before incubating with LPS (100 ng/mL) for 18 h. Cell lysates were electrophoresed, and COX-2 protein expression was detected with a specific antibody. β-Actin was used as an internal control. Quantification data are shown in the lower panel. Results are expressed as a ratio of COX-2 and β-actin. B: Cells were pre-treated with the indicated concentrations of CD-101 for 60 min and then stimulated with LPS (100 ng/mL) for 6 h. Total RNA was extracted and COX-2 gene expression from LPS-stimulated BV-2 microglia was assessed by RT-PCR analysis. GAPDH was used as an internal control. Quantification data are shown in the lower panel. Results are expressed as a ratio of COX-2 and GAPDH. C: BV-2 microglia cells 5×10^4 cells/well in a 6-well plate were incubated LPS (100 ng/mL) in the presence or absence of the indicated concentrations of CD-101 (0.1, 1, and 2.5 μM) for 18 h. The amounts of PGE_2 in the supernatants of BV-2 microglia cells were measured using a competitive enzyme immunoassay kit. The absorbance was measured at 420 nm. Data are the mean ± standard error for three independent experiments. $^{\$\$}P < 0.01$ and $^{\$\$\$}P < 0.001$, compared with the control group; $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$, compared with the LPS-treated group. Significance was determined by ANOVA followed by Tukey's multiple comparison test.

(Fig. 3: A and B). These results indicate that CD-101 had an inhibitory effect on inflammatory markers such as iNOS and COX-2 at the transcriptional and translational levels. PGE_2 is another downstream inflammatory enzyme produced by COX-2 (31). In our experiments we observed that CD-101 (0.1, 1, and 2.5 μM) significantly reduced the LPS-induced PGE_2 production in BV-2 microglia cells (Fig. 3C).

Effect of CD-101 on the production of the LPS-induced pro-inflammatory cytokines in BV-2 microglia cells

Based on the rationale that proinflammatory cytokines are responsible for neuronal death in microglia (32 – 34), we investigated the potential effects of CD-101 on the production of pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1β. BV-2 cells were treated with CD-101 (0.1, 1, and 2.5 μM) for 1 h in the presence or absence of LPS (100 ng/mL) for 6 h. An expression analysis was used to determine whether suppression of TNF-α, IL-6,

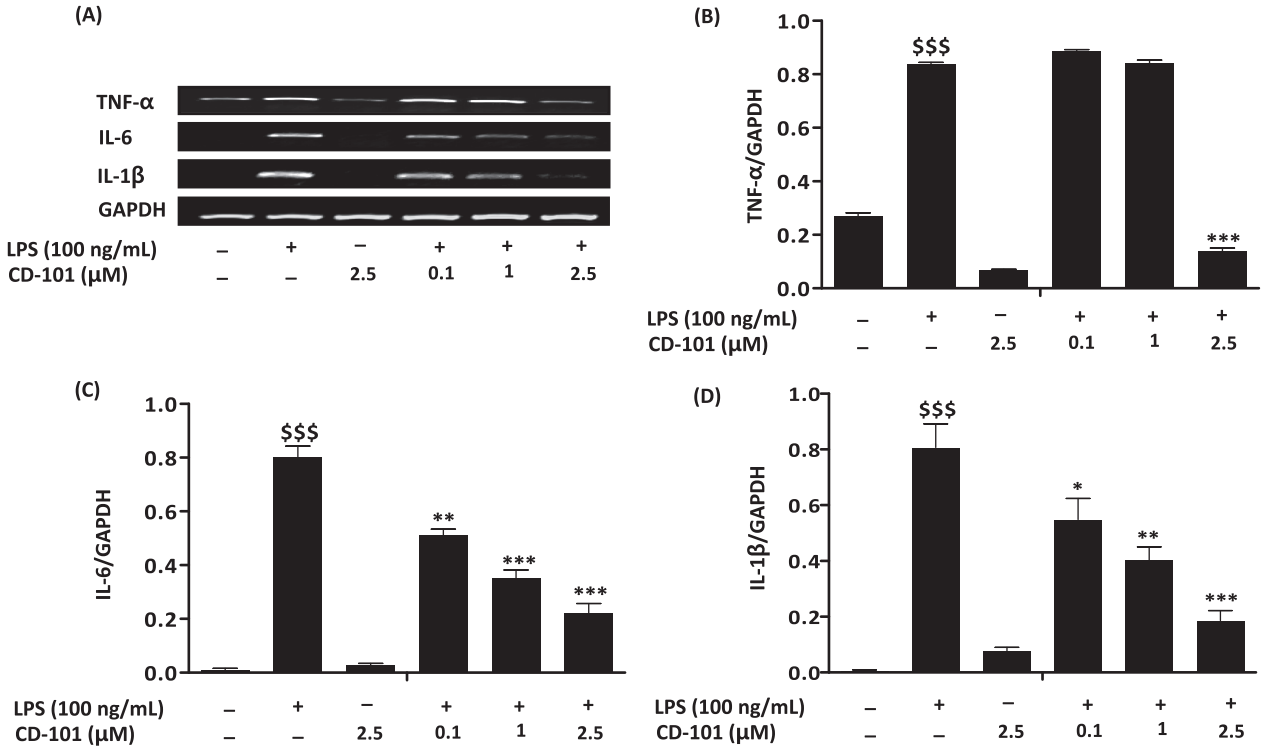

Fig. 4. Effect of CD-101 on pro-inflammatory cytokines in lipopolysaccharide (LPS)-stimulated BV-2 microglial cells. A: Cells were pre-treated with the indicated doses of CD-101 for 60 min before LPS (100 ng/mL). The levels of tumor necrosis factor (TNF)-α (B), interleukin (IL)-6 (C), and IL-1β (D) mRNA expression were determined by RT-PCR analysis after 6 h of LPS treatment. GAPDH were used as an internal control. Results are expressed as a ratio of TNF-α/GAPDH, IL-6/GAPDH, and IL-1β/GAPDH. $^{\$\$\$}P < 0.001$, compared with the control group; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, compared with the LPS-treated group. Significance was determined by ANOVA followed by Tukey's multiple comparison test.

and IL-1β by CD-101 was due to a decrease in their respective mRNA levels in BV-2 microglial cells. Exposing BV-2 microglial cells to LPS (100 ng/mL) for 6 h resulted in a significant increase in the production of TNF-α, IL-6, and IL-1β as compared to that in the control (Fig. 4A). Pre-treatment with CD-101 at various concentrations (0.1, 1, and 2.5 μM) significantly decreased LPS-induced mRNA expression of IL-6 and IL-1β in a dose-dependent manner (Fig. 4: C and D). In contrast, we found a non-significant decrease in LPS-induced TNF-α gene expression levels at 0.1 and 1 μM, whereas the dose of 2.5 μM showed a significant decrease in TNF-α gene expression level (Fig. 4B). These results suggest that CD-101 suppresses the production of TNF-α, IL-6, and IL-1β at the transcriptional level.

Effects of CD-101 on LPS-induced IκB-α phosphorylation and NF-κB activation in BV-2 microglial cells

NF-κB plays an essential role in LPS-induced expression of iNOS and COX-2 genes and the production of pro-inflammatory cytokines (35 – 37). Activation of NF-κB includes IκB-α phosphorylation and subsequent nuclear translocation of the p65 subunit of NF-κB into the nucleus. Therefore, BV-2 microglial cells were treated with various concentrations of CD-101 (0.1, 1, and 2.5 μM) for 1 h with or without the LPS (100 ng/mL) for 30 min to examine whether CD-101 regulates NF-κB pathways, and the levels of the NF-κB p65 subunit and p-IκB-α were examined. As shown in Fig. 5A, LPS treatment significantly induced IκB-α phosphorylation in BV-2 cells. CD-101 did not induce a significant increase in IκB-α phosphorylation in BV-2 cells. However CD-101 at 1 and 2.5 μM showed a significant and dose-dependent decrease in LPS-induced IκB-α phosphorylation in BV-2 microglial cells (Fig. 5A). As shown in Fig. 5B, stimulation with LPS (100 ng/mL) for 30 min significantly increased nuclear translocation of the p65 subunit of NF-κB in BV-2 microglial cells. This nuclear translocation of p65NF-κB was significantly inhibited by CD-101 at 1 and 2.5 μM; nucleolin was used as the internal control (Fig. 5B). Additionally, CD-101 treatment did not induce any significant increase in nuclear translocation of p65NF-κB. In a parallel immunofluorescence assay, we confirmed translocation of p65NF-κB into the

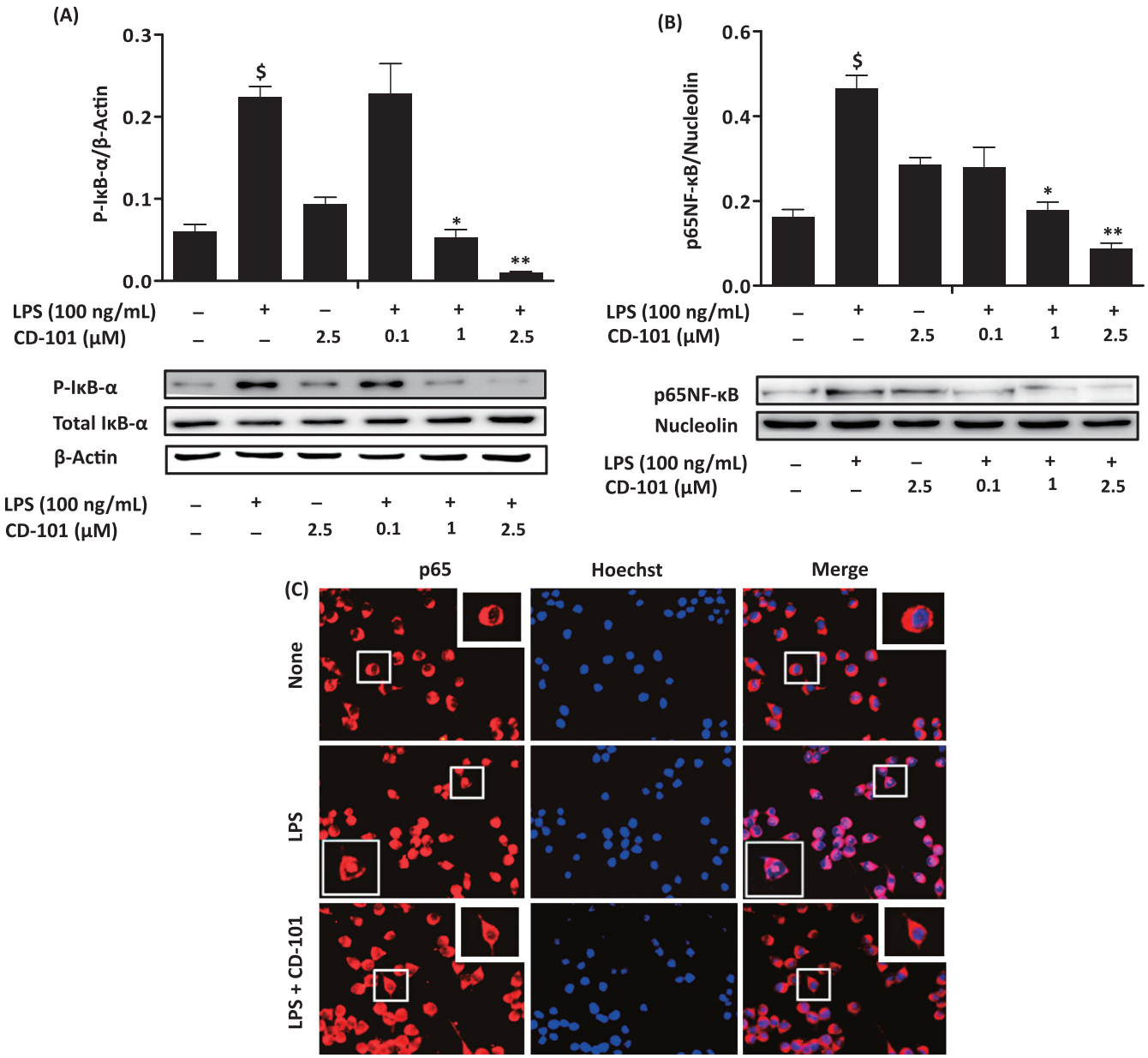

Fig. 5. Effect of CD-101 on inhibition of p65 nuclear factor (NF)-κB activation and inhibition of p-IκB-α phosphorylation in lipopolysaccharide (LPS)-stimulated BV-2 microglial cells. Cells were treated with the indicated dose (0.1, 1, and 2.5 μM) of CD-101 30 min before LPS (100 ng/mL) treatment. Total nuclear protein was subjected to 10% SDS-PAGE followed by western blotting using anti-p-IκB-α (A) and p65NF-κB (B). Densitometric analysis of p-IκB-α and p65NF-κB is shown in the lower panel. Results are expressed as a ratio of p-IκB-α/β-actin for A and p65NF-κB/nucleolin for B. C: Cells were stimulated with LPS (100 ng/mL) in the absence or presence of CD-101 (0.1, 1, and 2.5 μM) added 1 h before the stimulation. At 30 min after the LPS addition, sub-cellular location of the NF-κB p65 subunit was determined by immunofluorescence assay. Data are presented as the mean ± S.E.M. (n = 3) for three independent experiments. $^{\$}P < 0.05$, compared with the control group; $^{*}P < 0.05$ and $^{**}P < 0.01$, compared with the LPS-treated group. Significance was determined by ANOVA followed by Tukey's multiple comparison test.

nucleus within 30 min of LPS stimulation, which was reversed by treatment with 2.5 μM CD-101 (Fig. 5C).

Effects of CD-101 on LPS-induced activation of p38MAP kinase in LPS-stimulated BV-2 microglial cells

Release of pro-inflammatory mediators by activated microglia is controlled by MAPK signaling pathways (38, 39). Therefore, we determined whether CD-101 influences the p38MAPK pathways in BV-2 microglia cells. BV-2 cells were pre-treated with CD-101 at various concentrations (0.1, 1, and 2.5 μM) for 60 min and then stimulated with LPS (100 ng/mL) for 30 min. As a result,

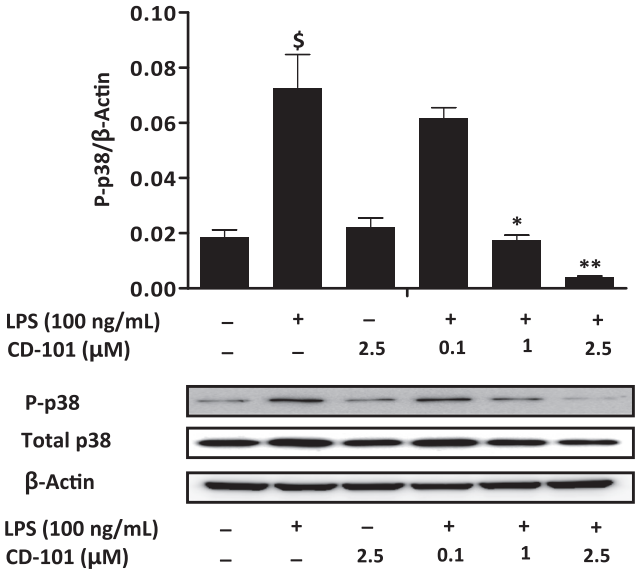

Fig. 6. Effect of CD-101 on lipopolysaccharide (LPS)-induced p38MAPK phosphorylation in BV-2 microglial cells. BV-2 microglial cells were treated with the three different doses (0.1, 1, and 2.5 μM) of CD-101 60 min before LPS treatment (100 ng/mL) for 30 min. Total protein was separated using 10% SDS-PAGE. Proteins were analyzed by immunoblotting with an anti-phospho-p38MAPK. Densitometric analysis of the p38MAPK band is shown in the lower panel. Results are expressed as a ratio of phospho-p38 (p-p38)/β-actin. Data are the mean ± standard error for three independent experiments. $^{\$}P < 0.05$, compared with the control group; $^{*}P < 0.05$ and $^{**}P < 0.01$, compared with the LPS-treated group. Significance was determined by ANOVA followed by Tukey's multiple comparison test.

the LPS treatment significantly increased p38MAPK phosphorylation at 30 min compared to that in the control. Pretreatment with CD-101 significantly inhibited LPS-stimulated upregulation of p38MAPK phosphorylation in a dose-dependent fashion (Fig. 6), indicating that CD-101 is capable of downregulating a vital signal transduction pathway involved in LPS-stimulated BV-2 microglial cells and subsequently abates the production of pro-inflammatory mediators.

Discussion

Microglia are critical immune surveillance and defense cells (40) in a healthy brain and have a quiescent phenotype that can undergo activation due to brain damage or immunological stimuli (41) and shows harmful effects on neuronal cell survival (42) through the production of various neurotoxic factors (43, 44), resulting in adverse chronic inflammation in the CNS (41, 45). Therefore, regulating microglial activation is a major concern to suppress neuroinflammation related to neuroinflam matory diseases. Microglial cells express different receptors that recognize pathogens, toxins, and molecules released by damaged cells including endocannabinoids. Thus, depending on the extent and stimulation of such receptors, expression of specific genes is primed, and their respective products will determine the fate of microglia towards becoming anti-inflammatory or pro-inflammatory (46). Similarly, various pro-inflammatory cytokines, chemokines, and chemically similar metabolites such as endocannabinoids are released by activated immune cells in response to neuroinflammatory stimuli. Increased levels of endocannabinoids basically act as chemotactic agents by recruiting inflammatory cells and facilitating the release of cytokines such as TNF-α and IL-6 in response to an antigen. In addition to the induction of endocannabinoid, activation of immune cells by LPS also increases CB1 receptor expression (47). Over-activation of CB1 receptors has been linked to PD pathology (48). Additionally, stimulation of the CB1 receptor by endocannabinoids decreases intracellular cAMP concentrations and leads to phosphorylation of various MAPKs, including p38MAPK, further resulting in expression of immediate early genes (49 – 51).

Targeting of the CB1 receptor is useful for reducing bradykinesia and delaying L-dopa–induced dyskinesia (52). Various studies have reported the anti-inflammatory activity of CB1-receptor antagonists (53 – 55) in animal models but the anti-neuroinflammatory activity of these molecules has not been evaluated with respect to neuroinflammatory disorders. In our study, the novel synthetic cannabinoid derivative CD-101 was investigated to explore its potential as a therapeutic candidate against inflammation in a LPS-stimulated microglial cell model. Our data revealed that CD-101 inhibited inflammatory activation of BV-2 microglia primarily by blocking the release of hallmark inflammatory markers such as iNOS, COX-2, TNF-α, IL-6, and IL-1β at the transcriptional and translational levels. We also found that CD-101 mediated its anti-inflammatory responses by blocking the p38MAPK and NF-κB pathways in BV-2 microglial cells.

Activated microglia produces several potentially neurotoxic mediators such as iNOS and COX-2 (56 – 61). In our study, CD-101 attenuated increased iNOS and COX-2 mRNA expression and protein level, which was evident by the dose-dependent reduction of nitrite and PGE_2 production in LPS stimulated BV-2 cells, suggesting that CD-101 exerts its potent anti-neuroinflammatory effect by inhibiting iNOS and COX-2 expression. Pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α are crucial mediators in the process of neuroinflammation (4, 62). In our experiments, we observed that CD-101 significantly attenuated the mRNA expression of pro-inflammatory cytokines including TNF-α, IL-1β, and IL-6 in a dose-dependent fashion. Our results are

in accordance with previous studies reporting ablation of the inflammatory response by KR-31360 in LPS-stimulated BV-2 microglial cells (63).

The transcription factors p50 and p65NF-κB play a critical role in the onset of inflammatory responses by regulating the expression of pro-inflammatory genes such as iNOS, COX-2, and TNF-α (64 – 67). Under normal conditions, NF-κB is present in the cytoplasm of microglial cells in its inactive form bound to IκB-α/β. Upon exposure to immunological stimuli, the upstream signaling molecule IκB kinase induces IκB-α/β phosphorylation, which, in turn, results in NF-κB release (68). The released NF-κB translocates to the nucleus and promotes the expression of pro-inflammatory markers such as iNOS, COX-2, and cytokines (69). NF-κB activation and IκB-α phosphorylation are involved in LPS-induced iNOS, COX-2, and TNF-α expression (70). In this study, we observed that CD-101 inhibited LPS-stimulated translocation of p65NF-κB and IκB-α phosphorylation in a dose-dependent manner, suggesting that CD-101 suppresses LPS-induced NF-κB activity to regulate iNOS, COX-2, and TNF-α expression.

MAPK signaling pathways regulate a variety of cellular activities such as proliferation, differentiation, survival, and neuronal death. Deviation from the fine tuning of MAPK signaling pathways has been implicated in the development of many neurodegenerative diseases such as AD, PD, and amyotrophic lateral sclerosis (ALS). Hyper phosphorylation of MAPK molecules ultimately activates the transcription factor NF-κB and subsequent production of inflammatory molecules (26, 71). Therefore, in addition to our data showing that CD-101 regulates LPS-induced NF-κB activation, we further evaluated the effect of CD-101 on the upstream p38MAPK signaling pathway. We found that CD-101 significantly attenuated LPS-stimulated phosphorylation of p38MAPK in a dose-dependent fashion. Our results agree with previous studies wherein persistent activation of the p38MAPK signaling pathway has been suggested to mediate neuronal apoptosis in AD, PD, and ALS (72 – 74). Furthermore, several other studies (38, 75 – 78) have also reported that the NF-κB and p38MAPK pathways are probably crucial targets of therapeutics designed to control CNS inflammation. Although other MAPK pathways are also involved in controlling microglia-mediated inflammation, we have found that inhibition of p38MAPK is the major one.

In conclusion, our results demonstrate that CD-101, a novel synthetic CB derivative, exerted potent anti-inflammatory effects on an LPS-stimulated model of microglial cells. Both NF-κB and p38MAPK signaling pathways were largely involved in CD-101–mediated modulation of inflammatory mediators. Due to the promising potential of CD-101 to regulate microglia-mediated neuroinflammatory events, CD-101 could be further explored for its beneficial role in suppressing neuroinflammation. Hence, our findings may provide new insight for the development of therapeutics from CD-101 to act against inflammation in neuroinflammatory diseases.

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