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Original Research Article

Inhibition of Nitric Oxide and Prostaglandin E_2 Expression by Methanol Extract of *Polyopes affinis* in Lipopolysaccharide-stimulated BV2 Microglial Cells through Suppression of Akt-dependent NF-κB Activity and MAPK Pathway

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Abstract

Purpose: *To determine whether the methanol extract of Polyopes affinis (MEPA) down-regulates the expression of pro-inflammatory mediators in lipopolysaccharide (LPS)-stimulated BV2 microglial cells.*
Methods: *The production of nitric oxide (NO) and prostaglandin E_2 (PGE_2) was measured by the Griess reagents and enzyme-linked immunosorbent assay (ELISA), respectively. Expression levels of mRNA and protein in LPS-stimulated BV2 microglial cells were assessed by reverse transcription-polymerase (RT-PCR) and Western blot analysis. Activation of nuclear factor-κB (NF-κB) was detected by electrophoretic mobility shift assay (EMSA).*
Results: *MEPA inhibited the expression of LPS-induced pro-inflammatory mediators, NO and PGE_2, as well as their respective genes, iNOS and COX-2, at both protein and mRNA levels, without any accompanying cytotoxicity. Moreover, treatment with MEPA significantly suppressed the LPS-induced DNA-binding activity of NF-κB, which is known as a main transcription factor for the regulation of pro-inflammatory genes, as well as the nuclear translocation of its subunit p65 and p50, by degrading IκBα. MEPA increased Akt dephosphorylation which leads to suppression of the DNA-binding activity of NF-κB in LPS-stimulated BV2 microglial cells and suppressed phosphorylation of ERK and JNK, which are involved in the mitogen-activated protein kinase (MAPK) signaling pathway for regulating pro-inflammatory genes.*
Conclusion: *Our results indicate that MEPA down-regulates pro-inflammatory mediators such as NO and PGE_2 by suppressing Akt-dependent NF-κB activity as well as phosphorylation of ERK and JNK in LPS-stimulated BV2 microglial cells.*

Keywords: *Polyopes affinis, Nitric oxide, Prostaglandin E_2, Nuclear factor-κB*

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INTRODUCTION

Several seaweed extracts are well-known to regulate immune and inflammatory responses. Marine algae, have been extensively studied since they are a rich source of novel structural compounds that exhibit biological activities against cardiac, vascular and hepatic disease; high blood pressure; senility; and diabetes. Compared to other algae such as green and brown algae, red algae contain a higher amount of proteins, carbohydrates and other nutrients [1,2]. Many researchers are initiating attempts to seek potent compounds derived from marine algae, which have anti-inflammatory characteristics [3–5]. Although *Polyopes affinis*, which is a marine red algae, has been used to treat diseases in adults in North-Eastern Asia, studies investigating its anti-inflammatory effects in lipopolysaccharide (LPS)-stimulated BV2 microglial cells have not been carried out.

Inflammation is the biological response to harmful stimuli such as autoimmune diseases, pathogenic infections, damaged cells and irritants. Thus inflammation initiates the healing process [6]. Inflammation increases the expression of inflammatory mediators such as nitric oxide (NO) and prostaglandin E_2 (PGE_2), which are regulated by inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), respectively [7,8]. These inflammatory mediators are required for the regulation of cellular pathways involved in protecting against organ disorder, such as ischemic damage [9,10]. However, excessive inflammatory response can lead to overexpression of pro-inflammatory mediators, which can result in severe inflammatory disorders such as septic shock, rheumatoid arthritis, Alzheimer's disease and Parkinson's disease [10,11]. Therefore, agents that attenuate pro-inflammatory mediators in microglial cells could represent promising strategies to tackle brain injury and neurodegenerative diseases.

Cell signaling pathways involve the production of various cytokines and inflammatory mediators. The nuclear factor-κB (NF-κB) pathway, in particular, may play a key role in such pathways due to its rapid activation and potency as a transcriptional activator [12]. Thus, aberrant NF-κB activation is known to be a potential target associated with various inflammatory diseases [13]. According to recent studies, Akt and mitogen-activated protein kinases (MAPKs) lead to NF-κB activation by proteasome-dependent IκB degradation [14,15]. Therefore, inhibition of NF-κB activity through the Akt and MAPK pathways results in anti-inflammatory effects.

In the present study, we investigated the methanol extract of *P. affinis* (MEPA)-mediated inhibition of NO and PGE_2 expression by assessing the suppression of NF-κB activity in LPS-stimulated BV2 microglial cells. Regulation of NF-κB activity by MEPA is also associated with the inhibition of phosphorylation of Akt and MAPK in an LPS-induced anti-inflammatory reaction.

EXPERIMENTAL

Reagents and chemicals

LPS and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Rabbit anti-human antibodies against iNOS, COX-2, p65, p50, phospho (p)-IκBα, and IκBα were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The antibody against β-actin and c23 were obtained from Sigma. Rabbit anti-human antibodies against p-ERK, ERK, p-p38, p38, p-JNK, JNK, p-Akt and Akt were obtained from cell signaling (Beverly, MA). Peroxidase-labeled goat anti-rabbit immunoglobulin was purchased from KOMA Biotechnology (Seoul, Republic of Korea). LY294002 was purchased from Calbiochem (San Diego, CA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from WelGENE Inc. (Daegu, Republic of Korea). MEPA was purchased from Jeju HI-Tech Industry Development Institute (Jeju, Republic of Korea). Other chemicals were purchased from Sigma.

Cell culture and viability

BV2 microglia cells were cultured at 37 ^{o}C in 5% CO_2 in DMEM supplemented with 5% FBS and antibiotics (WelGENE Inc., Daegu, Republic of Korea). In all experiments, cells were pre-treated with the indicated concentrations of MEPA for 1 h before the addition of LPS (1 μg/ml) in serum-free DMEM. MTT assay was used to determine cell viability.

NO assay

BV2 microglial cells (2×10^5 cells/ml) were plated onto 24-well plates and pre-treated with the indicated concentrations of MEPA for 1 h prior to stimulation with 1 μg/ml of LPS for 24 h. The cell supernatants were collected and assayed for NO production using Griess reagent. The samples were mixed with equal volume of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride) and then incubated at room temperature for 5

min. The absorbance was measured at 540 nm on a microplate reader. Nitrite concentration was determined from a sodium nitrite standard curve.

Measurement of PGE_2

The expression levels of PGE_2 were measured by enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. BV2 microglial cells (2×10^5 cells/ml) were plated in 24-well plates and pretreated with the indicated concentrations of MEPA for 1 h prior to stimulation with 1 µg/ml of LPS for 24 h. One hundred microliters of culture media were collected for the determination of PGE_2 concentrations by ELISA.

Reverse transcriptase polymerase chain reactions (RT-PCR)

Total RNA was extracted from BV2 microglial cells using Easy-blue reagent (iNtRON Biotechnology, Sungnam, Republic of Korea) according to the manufacturer' instructions. One microgram of RNA was reverse-transcribed using MMLV reverse transcriptase (Bioneer, Daejeon, Republic of Korea). Then, cDNA was amplified by PCR using specific primer iNOS (forward 5'-cctcctccaccctaccaagt-3' and reverse 5'-cacccaaagtgcttcagtca-3'), COX- 2 (forward 5'-aagacttgccaggctgaa ct-3' and reverse 5'-cttctgcagtccaggttcaa-3') and β-Actin (forward 5'-tgtgatggtgggaatgggtcag-3' and reverse 5'-tttgatgtcacgcacgatttcc-3'). The following PCR conditions were applied: COX-2 and iNOS, 25 cycles of denaturation at 94 ºC for 30 s, annealing at 59 ºC for 30 s and extended at 72 ºC for 30 s; β-actin, 23 cycles of denaturation at 94 ºC for 30 s, annealing at 57 ºC for 30 s and extended at 72 ºC for 30 s. β-actin was used as an internal control to evaluate relative expression of COX-2 and iNOS.

Western blot analysis

Cells were harvested by scraping from the wells and washed twice with cold PBS. Total cell extracts were prepared using PRO-PREP protein extraction solution (iNtRON Biotechnology, Sungnam, Republic of Korea). Briefly, after treatment with the indicated concentrations of MEPA, cells were harvested, washed once with ice-cold PBS and gently lysed for 30 min in 100 µl of ice-cold PRO-PREP lysis buffer. Lysates were centrifuged at 14,000 *g* at 4 ºC for 10 min to obtain the supernatants. Supernatants were collected and protein concentrations determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). Samples were stored at -80°C or immediately used for Western blot analysis. The proteins were separated on SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH). Proteins were detected using an enhanced chemiluminescence detection system (Amersham, Arlington Heights, IL).

Electrophoretic mobility shift assay (EMSA)

EMSA was carried out with the nuclear extract. Briefly, the preparation of nuclear extracts was conducted using NE-PER nuclear extraction reagents (Pierce, Rockford, IL). Synthetic complementary NF-κB-binding oligonucleotides (5'-AGT TGA GGG GAC TTT CCC AGG C-3') (Santa Cruz Biotechnology) were 3'-biotinylated using a biotin 3'-end DNA labeling kit (Pierce; Rockford, IL) according to the manufacturer's instructions. Binding reactions were carried out for 20 min at room temperature in the presence of 50 ng/µl poly(dI-dC), 0.05% Nonidet P-40, 5 mM $MgCl_2$, 10 mM EDTA, and 2.5% glycerol in 1× binding buffer (LightShift™ chemiluminescent EMSA kit, Pierce) using 20 fmol of biotin-end-labeled target DNA and 10 µg of nuclear extract. Assays were loaded onto native 4% polyacrylamide gels pre-electrophoresed for 60 min in 0.5× Tris borate/EDTA and electrophoresed at 100 V before being transferred onto a positively charged nylon membrane (Hybond™N+) in 0.5Tris borate/EDTA at 100V for 30 min. Transferred DNAs were cross-linked to the membrane at 120 mJ/cm^2 and detected using horseradish peroxidase-conjugated streptavidin (LightShift™ chemiluminescent EMSA kit) according to the manufacturer's instructions.

Statistical analysis

All data were derived from triplicate determinations. Statistical analyses were conducted using SigmaPlot software (version 11.0) Values were presented as mean ± SE. Significant differences between the groups were determined using two-way ANOVA. Statistical significance was set at $p < 0.05$.

RESULTS

Effect of MEPA on the viability of BV2 microglial cells

MTT assay was used to evaluate the cytotoxic effects of MEPA in BV2 microglial cells. Cells were treated with MEPA (0-100 µg/ml) in the presence or absence of LPS for 24 h, following which cell viability was measured. Concentrations of MEPA of up to 100 µg/ml

showed no difference in cell viability (Fig. 1). However, a dose of 150 µg/ml of MEPA resulted in 20% cytotoxicity (data not shown). These results indicated that MEPA does not produce cytotoxic effects in LPS-induced BV2 microglia cells at concentrations of 100 µg/ml, which was the dosage used in all subsequent experiments.

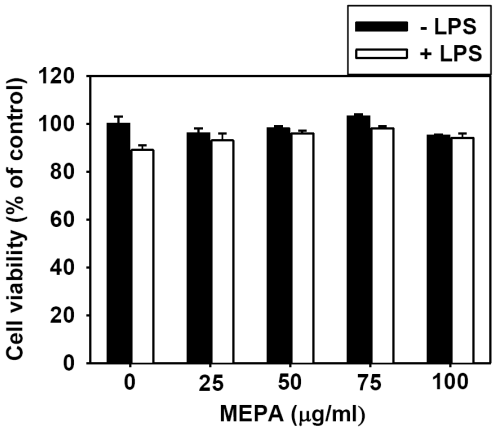

Fig 1: Effect of MEPA on the viability of BV2 microglial cells. Each value indicates mean ± SE (n = 3).

Inhibition of NO production and iNOS expression by MEPA in LPS-stimulated BV2 microglial cells

NO production in LPS-stimulated BV2 microglial cells showed a ~7-fold increase (13.5 ± 1.2 µM), compared to the control (1.9 ± 0.2 µM). Pretreatment with MEPA reduced LPS-induced NO production in a dose-dependent manner (Fig. 2A), 100 µg/ml of MEPA producing the strongest effect (3.2 ± 0.9 µM). Since NO production is regulated by iNOS, we next performed western blot and RT-PCR to assess the iNOS protein and mRNA levels at 24 h and 6 h, respectively, after treatment with LPS. Consistent with the results of NO production, we found that MEPA reduces levels of iNOS protein (Fig. 2B) and mRNA (Fig. 2C), when compared to treatment with LPS alone. These results indicate that MEPA inhibits NO production by suppressing iNOS.

Inhibition of PGE_2 production and COX-2 expression by MEPA in LPS-stimulated BV2 microglial cells

Treatment with LPS alone significantly increased the levels of PGE_2 production (987 ± 26 pg/ml) when compared to the control (103 ± 13 pg/ml) (Fig. 3A). Pretreatment of cells with MEPA substantially attenuated the LPS-induced increase in PGE_2 expression in a dose-dependent manner, with maximal suppression observed at a concentration of 100 µg/ml (301 ± 21 pg/ml). Next, to investigate the levels of COX-2 protein and mRNA, western blot analysis and RT-PCR were performed. We observed a significant increase in the expression of COX-2 protein and mRNA at 24 h and at 6 h, respectively (Fig. 3B and 3C), after treatment with LPS. Upon pretreatment of cells with MEPA for 1 h, LPS-induced COX-2 expression was significantly downregulated. These results indicate that MEPA inhibits PGE_2 expression by suppressing COX-2.

Inhibition of LPS-induced NF-*κ*B activation by MEPA in BV2 microglial cells

To evaluate MEPA-mediated regulation of NF-κB activity, we determined the DNA-binding activity of NF-κB and nuclear translocation of its subunits p65 and p50 in BV2 microglial cells. EMSA results showed that treatment with LPS caused a significant increase in the DNA-binding activity of NF-κB, while treatment with MEPA results in downregulation of LPS-stimulated NF-κB activity.

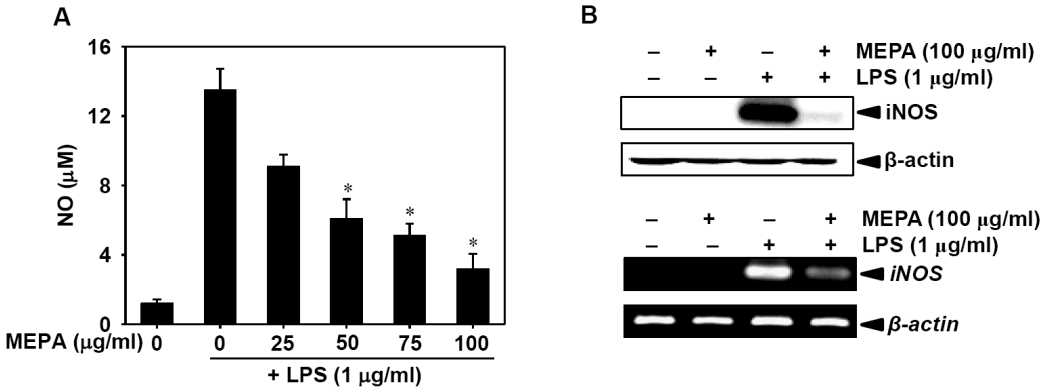

Fig 2: Effect of MEPA on LPS-induced NO and iNOS expression. Cells were seeded at 2 × 10^5 cells/ml and incubated with the indicated concentrations of MEPA 1 h before treatment with LPS (1.0 µg/ml) for 24 h (A and B) or 6 h (C). (A) NO expression was determined by the Griess assay; *p < 0.01. (B) Western blot analysis was performed with antibodies against iNOS. (C) RT-PCR analysis of iNOS was performed on total RNA isolated at 6 h. β-Actin was used as an internal control for western blot analysis and RT-PCR; n = 3

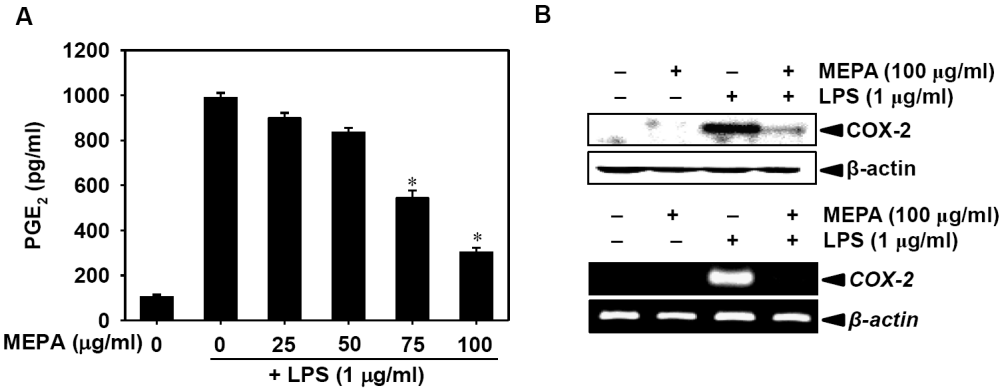

Fig 3: Effect of MEPA on LPS-induced PGE_2 and COX-2 expression. Cells were seeded at 2×10^5 cells/ml and incubated with the indicated concentrations of MEPA 6 h before treatment with LPS (1.0 μg/ml) for 24 h (A and B) or 6 h (C). (A) PGE_2 expression was determined by ELISA performed according to the manufacturer's instructions; *$p < 0.01$. (B) Western blot analysis was performed with antibodies against COX-2. (C) RT-PCR analysis of COX-2 was performed on total RNA isolated at 6 h. β-Actin was used as an internal control for western blot analysis and RT-PCR; n = 3

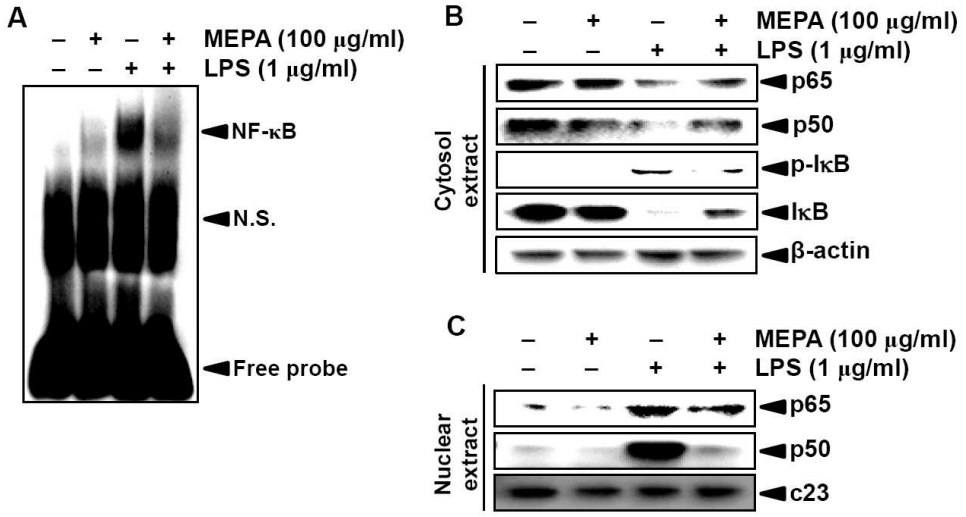

Fig 4: Effect of MEPA on NF-κB activity in LPS-stimulated BV2 microglial cells. Cells were pretreated with vehicle or the indicated concentrations of MEPA 1 h before stimulation with LPS (1.0 μg/ml) for 30 min. (A) Nuclear extracts were prepared and analyzed for DNA-binding activity of NF-κB using EMSA. Cytosolic (B) and nuclear (C) extracts were prepared to determine the levels of p65, p50, p-IκBα and IκBα by western blot analysis; β-Actin and c23 were internal controls. A result shown is representative of 3 independent experiments.

To further investigate whether MEPA regulates nuclear translocation of p65 and p50, as well as IκB phosphorylation and degradation, we performed western blot analysis to evaluate protein expression levels. Treatment with LPS caused a significant decrease in levels of p65 and p50 in the cytosolic extract (Fig. 4B), while increasing levels in the nuclear extract (Fig. 4C), suggesting that LPS induces nuclear translocation of p65 and p50. However, MEPA treatment significantly sustained expression levels of p65 and p50 in the cytosolic extract by suppressing phosphorylation and degradation of IκB. These results indicate that MEPA inhibits LPS-induced activation of NF-κB by inhibiting the phosphorylation and degradation of IκB.

Suppression of Akt-mediated NF-κB activation and MAPKs by MEPA in LPS-stimulated BV2 microglial cells

MEPA significantly decreased LPS-induced phosphorylation of Akt, ERK and JNK, but not p38 MAPK (Fig. 5A and 5B). To further ascertain whether NF-κB activation is involved in the MEPA-induced dephosphorylation of Akt, EMSA was performed after cells were treated with the *Akt inhibitor LY294002. Treatment with LY294002 significantly reduces the DNA-binding activity of* NF-κB (Fig. 5C). These results indicate that MEPA-induced NF-κB inactivation is associated with Akt, as well as with the ERK and JNK pathway.

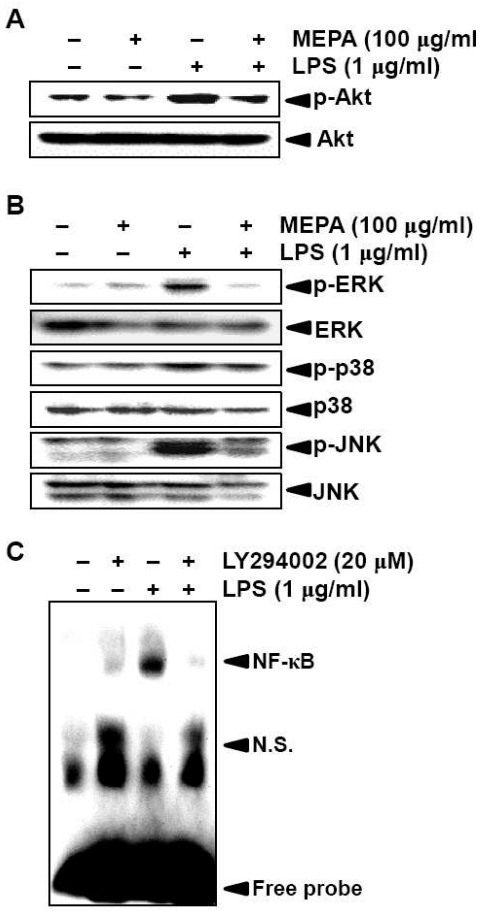

Fig 5: Effect of MEPA on LPS-induced phosphorylation of Akt and MAPKs. (A and B) Cells were treated with vehicle or the indicated concentrations of MEPA 1 h before stimulation with LPS (1.0 μg/ml) for 15 min. Total protein was subjected to 10% SDS-PAGE followed by western blot analysis with antibodies specific to the phosphorylated forms of Akt (A) and MAPKs (B). (C) In a parallel experiment, cells were incubated with LY294002 (20 μM) for 30 min 1 h before LPS treatment, nuclear extracts were prepared and analyzed for DNA-binding activity of NF-κB using EMSA; n = 3

DISCUSSION

Pathological agents such as bacteria and fungi stimulate microglia and macrophages resulting in the activation of mediators involved in inflammation [16]. Therefore, regulation of abnormal activation of microglia cells is a potential therapeutic target. In recent years, several researchers have attempted to identify compounds from marine seaweed, which exhibit anti-inflammatory effects in LPS-stimulated macrophages and microglial cells [17,18]. In this regard, in an attempt to identify novel anti-inflammatory compounds from seaweeds, we have investigated the use of MEPA as a possible pharmaceutical candidate against inflammatory response. First, we found that MEPA reduces expression of the pro-inflammatory mediators NO and PGE_2, as well as their regulatory genes *iNOS* and *COX-2*, in LPS-stimulated cells. In addition, suppression of LPS-induced NF-κB activation via the Akt signal pathway and MAPK pathways induced the anti-inflammatory effects of MEPA.

NF-κB is known to be the most important transcription factor in the regulation of pro-inflammatory genes such as *iNOS* and *COX-2* [19]. It is a ubiquitous protein that displays a rapid cellular response upon activation by LPS under various pathological conditions. In its inactive state, NF-κB exists as a heterodimer of p65 and p50 subunits in the cytoplasm. Once activated, NF-κB translocates into the nucleus through the phosphorylation and degradation of IκB, and proceeds to transcribe pro-inflammatory genes such as *iNOS* and *COX-2* [15]. Our findings show that MEPA downregulates the LPS-stimulated expression of *iNOS* and *COX-2* by inhibiting the nuclear translocation of p65 and p50, resulting in the suppression of NF-κB activity. In a recent study, it was shown that the Akt pathway is directly involved in regulating NF-κB activity to attenuate inflammatory responses through the degradation of IκB [20]. In this study, we also found that MEPA suppresses the Akt-dependent activation of NF-κB, suggesting that MEPA affects the regulation of the inflammatory signal cascade. Nevertheless, further work is required to substantiate these observations in light of the study that revealed the role of the transcriptional factor AP-1 expression of pro-inflammatory genes [21].

The MAPK signaling pathway is involved in inflammatory responses occurring activated microglial cells [22]. However, there are several inconsistencies in the expression of proteins of the MAPK family such as ERK, p38 and JNK. Reports suggest that ERK and p38 are essential for regulating LPS-induced NF-κB activation [23,24]. Other investigations revealed the crucial effects of JNK on inducing pro-inflammatory mediators via the NF-κB pathway [25,26]. Interestingly, there are yet other reports suggesting that none of the MAPK family has any influence on NF-κB activation [27]. Therefore, we speculate that such discrepancies arise from the individual properties of each compound. In this study, we show that MEPA significantly inhibits the LPS-induced phosphorylation of ERK, p38 and JNK. However, further functional studies of MEPA-mediated suppression of LPS-induced MAPKs in the regulation of NF-κB activity are needed.

In summary, we have established that MEPA inhibits the expression of NO and PGE_2 in LPS-

stimulated BV2 microglial cells through the suppression of their regulatory genes. In addition, we also show that the inhibitory effects of MEPA are associated with the suppression of Akt-dependent NF-κB activation as well as the MAPK pathway.

CONCLUSION

Our data demonstrate that MEPA down-regulates pro-inflammatory mediators NO and PGE_2, and their respective regulatory genes iNOS and COX-2 in LPS-stimulated BV2 microglial cells. This anti-inflammatory effect results from suppression of Akt-dependent NF-κB activity as well as phosphorylation of ERK and JNK in LPS-stimulated BV2 microglial cells. Collectively, we conclude that MEPA could be a potential novel anti-inflammatory candidate.

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COMPETING INTERESTS

The authors report no conflicts of interest and they alone are responsible for the content.

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