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Anti-neuroinflammatory effect of aurantiamide acetate from the marine fungus *Aspergillus* sp. SF-5921: Inhibition of NF-κB and MAPK pathways in lipopolysaccharide-induced mouse BV2 microglial cells

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ABSTRACT

In the course of a search for anti-neuroinflammatory metabolites from marine fungi, aurantiamide acetate (**1**) was isolated from marine-derived *Aspergillus* sp. as an anti-neuroinflammatory component. Compound **1** dose-dependently inhibited the production of nitric oxide (NO) and prostaglandin E_2 (PGE_2) in BV2 microglial cells. It also attenuated inducible NO synthase (iNOS), cyclooxygenase-2 (COX-2), and other pro-inflammatory cytokines, such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α). In a further study designed to elucidate the mechanism of its anti-neuroinflammatory effect, compound **1** was shown to block the activation of nuclear factor-kappa B (NF-κB) in lipopolysaccharide (LPS)-induced BV2 microglial cells by inhibiting the phosphorylation of the inhibitor kappa B-α (IκB)-α. In addition, compound **1** decreased the phosphorylation levels of c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinases (MAPKs). These results suggest that compound **1** has an anti-neuroinflammatory effect on LPS stimulation through its inhibition of the NF-κB, JNK and p38 pathways.

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1. Introduction

Neuroinflammation, a chronic inflammation in the brain, leads to neurodegenerative disorders such as dystrophic neuronal growth. Microglial cells, a type of primary immune cells in the normal brain, play an important role in the natural immune response in the central nervous system (CNS) [1–4]. These cells have been regarded as brain macrophages, which are activated in response to brain damage and release various bioactive molecules, including nitric oxide (NO), prostaglandin E_2 (PGE_2), monocyte chemo-attractant protein-1 (MCP-1), and pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6) [5,6]. These cytokines and pro-inflammatory mediators can cause cell death and brain injury [7,8]. In addition, inducible nitric oxide synthase (iNOS) catalyzes the development of excessive NO, which can lead to neuroinflammation [9]. Cyclooxygenase-2 (COX-2), an inducible enzyme, can produce many pro-inflammatory cytokines like prostaglandin E_2 (PGE_2) [10]. Recent studies have shown that high levels of iNOS and COX-2, induced by inflammatory stimulants, such as LPS and pro-inflammatory cytokines, increase neuronal disease by disturbing the balance in immune response [11]. Accordingly, the control of microglial activation could be a therapeutic approach for the treatment of such neurodegenerative disorders as Alzheimer's disease (AD), Parkinson's disease (PD), HIV-associated dementia (HAD), stroke, and multiple sclerosis (MS) [12–14].

The transcription factor NF-κB is implicated in the regulation of many genes that encode mediators of immune, acute-phase, and inflammatory responses. NF-κB, the original name of which is the p50–p65 heterodimer, has been considered a core mediator of the immune response for more than a decade. NF-κB remains inactive in the cytosol because of its interaction with IκB. However, in response to inflammatory signals such as LPS, IκB-α is phosphorylated and subsequently degraded, resulting in the activation and nuclear translocation of NF-κB [15,16]. In addition, mitogen-activated protein kinases (MAPKs) are serine/threonine protein kinases that adjust basic biological responses, by reacting to extracellular stress signals. MAPKs are composed of p38, JNK and ERK1/2 (p44/p42). These molecules have a crucial role in cell proliferation, differentiation, and death [17,18]. NF-κB and MAPK signaling pathways are involved in regulating the inflammatory

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process, and several studies have shown that natural products can exert anti-neuroinflammatory effects by inhibiting the NF-κB and MAPK signaling pathways [19]. For example, glaucocalyxin-A from *Rabdosia japonica* was reported to have a powerful anti-neuroinflammatory effect through its inhibition of the aforementioned signaling pathways. Phlorofucofuroeckol A from *Ecklonia stolonifera* is another example of a secondary metabolite that has similar biological effects [20,21].

Marine microorganisms as sources of pharmacologically active metabolites have recently attracted significant interest, and a number of marine fungal secondary metabolites have been suggested as potential lead compounds for new drugs [22–25]. In the course of searching for bioactive natural products from marine microorganisms, aurantiamide acetate (**1**) was isolated from marine-derived *Aspergillus* sp. SF5921 as an anti-inflammatory component. In the present study, we report isolation, structure elucidation and anti-neuroinflammatory effect of compound **1** in LPS-induced BV2 microglial cells. In addition, we report on our investigation of the molecular mechanism that produces the anti-neuroinflammatory effects of this metabolite.

2. Materials and methods

2.1. Fungal materials and isolation of aurantiamide acetate (*1*)

Aspergillus sp. SF-5921 (deposited at the College of Medical and Life Sciences fungal strain repository, Silla University) was isolated from the unidentified sponge that was collected in the Sea of Ross (S 76°06.258′, E 169°12.756′) at a depth of 375 m in February, 2011. This fungus was identified based on the analysis of its ribosomal RNA (rRNA) sequence. A GenBank search with the 28S rRNA gene of SF-5921 (GenBank accession number KF647561) indicated *Aspergillus penicillioides* (U81265), *Eurotitum chevalieri* (JN938915), *A. proliferans* (FR848827) and A. *glaucus* (JF922029) as the closest matches, with sequence identities of 99.87%, 97.94%, 97.81% and 97.81% respectively. Therefore, the marine-derived fungal strain SF-5921 was characterized as *Aspergillus* sp. The fungal strain was cultured on ten Petri dishes (90 mm), each containing 20 mL of potato dextrose agar medium [0.4% (*w*/*v*) potato starch, 2% (*w*/*v*) dextrose, 3% (*w*/*v*) NaCl, and 1.5% (*w*/*v*) agar]. The plates were individually inoculated with 2 mL of seed cultures of the fungal strain, and they were incubated at 25 °C for a period of 14 days. The extraction of the agar media with EtOAc (500 mL) provided an organic phase, which was then concentrated in vacuo to yield 21 mg of extract. The EtOAc extract of the fungus was purified by HPLC using a reversed phase with a gradient from 50% to 100% MeOH in H_2O over 50 min to obtain compound **1** [4.2 mg, 20% of extract, $t_R = 33.3$ min].

Aurantiamide acetate (**1**): white solid, $[\alpha]_D -44°$ (*c* 0.075, CH_2Cl_2); ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.71 (2H, d, *J* = 7.7 Hz, H-16/H-20), 7.52 (1H, t, *J* = 7.7 Hz, H-18), 7.43 (2H, t, *J* = 7.7 Hz, H-17/H-19), 7.28 (2H, d, *J* = 7.0 Hz, H-23/H-27), 7.26 (2H, d, *J* = 7.0 Hz, H-24/H-26), 7.23 (1H, d, *J* = 5.6 Hz, H-25), 7.16 (2H, brs, H-5/H-9), 7.14 (1H, brs, H-7), 7.06 (2H, d, *J* = 6.9 Hz, H-6/H-8), 6.74 (1H, d, *J* = 7.7 Hz, N-Hb), 5.94 (1H, d, *J* = 8.4 Hz, N-Ha), 4.75 (1H, q, *J* = 5.5 Hz, H-13), 4.34 (1H, m, H-2), 3.92 (1H, dd, *J* = 11.6, 4.8 Hz, H-10b), 3.80 (1H, dd, *J* = 11.3, 4.0 Hz, H-10a), 3.22 (1H, dd, *J* = 13.7, 5.5 Hz, H-21b), 3.05 (1H, dd, *J* = 13.6, 8.4 Hz, H-21a), 2.75 (1H, dd, *J* = 11.8, 1.8 Hz, H-3b), 2.72 (1H, s, H-3a), 2.02 (3H, s, H-12); ^{13}C NMR (100 MHz, $CDCl_3$): δ 170.9 (C-11), 170.3 (C-1), 167.2 (C-14), 137.8 (C-22), 133.7 (C-15), 136.7 (C-4), 132 (C-18), 129.4 (C-24/C-26), 129.2 (C-23/C-27), 128.9 (C-6/C-8), 128.7 (C-5/C-9), 128.6 (C-17/C-19), 127.2 (C-25), 127.1 (C-16/C-20), 126.8 (C-7), 64.6 (C-10), 55.1 (C-13), 49.5 (C-2), 38.5 (C-21), 37.5 (C-3), 20.9 (C-12); and HRESIMS: *m*/*z* 445.2122 $[M + H]^+$ (calcd for $C_{27}H_{29}N_2O_4$, 445.2127).

2.2. Instruments

ESIMS data were obtained using a Q-TOF micro LC–MS/MS instrument (Waters, Manchester, U.K.). Optical rotations were recorded using a Jasco p-2000 digital polarimeter. NMR spectra (1D and 2D) were recorded in $CDCl_3$ with a JEOL JNM ECP-400 spectrometer, and the chemical shifts were referenced relative to the residual solvent peaks ($\delta_H/\delta_C = 7.26/77.0$). HSQC and HMBC experiments were optimized for $^1J_{CH} = 140$ Hz and $^nJ_{CH} = 8$ Hz, respectively. HPLC (YOUNGLIN-YL9100, Younglin, Anyang, Korea) separation was performed using a Phenomenex Synergi 4u Polar-RP 80A, AXIA packed column (21.2 × 150 mm, 5-μm particle size) with a flow rate of 5 mL/min and the solvents used for HPLC were all analytical grade.

2.3. Chemicals and reagents

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and other tissue culture reagents were purchased from Gibco BRL Co. (Grand Island, NY, USA). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Primary antibodies, including mouse/goat/rabbit anti-COX-2, iNOS, IκB-α, p-IκB-α, p50, p65, p-IKKα/β/γ, IKKα/β/γ as well as secondary antibodies were purchased from Santa Cruz Biotechnology (Heidelberg, Germany) and p-ERK, ERK, p-JNK, JNK, p-p38, and p38 antibodies were obtained from Cell Signaling Technology (Cell Signaling, Danvers, MA, USA). Enzyme-linked immunosorbent assay (ELISA) kits for PGE_2, TNF-α, and IL-1β were purchased from R&D Systems, Inc. (Minneapolis, MN, USA).

2.4. Cell culture and viability assay

BV2 microglia cells were received from Prof. Hyun Park at Wonkwang University (Iksan, Korea). BV2 microglia cells were maintained at 5×10^5 cells/mL in DMEM medium supplemented with 10% heat-inactivated FBS, penicillin G (100 U/mL), streptomycin (100 mg/L), and L-glutamine (2 mM), and they were incubated at 37 °C in a humidified atmosphere containing 5% CO_2 and 95% air. The cell viability was determined by adding 100 mg/mL of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) to 1 mL of a cell suspension (1×10^5 cells per 1 mL in 96-well plates) and incubated for 4 h. The formazan formed was dissolved in acidic 2-propanol, and the optical density was measured at 540 nm.

2.5. Preparation of cytosolic and nuclear fractions

BV2 microglial cells were homogenized in PER-Mammalian Protein Extraction Buffer (1:20, w:v) (Pierce Biotechnology, Rockford, IL, USA) containing freshly added protease inhibitor cocktail I (EMD Biosciences, San Diego, CA, USA) and 1 mM phenylmethylsulfonlyfluoride (PMSF). The cytosolic fraction of the cells was prepared by centrifugation at 16,000 × *g* for 5 min at 4 °C. The nuclear and cytoplasmic cell extracts were prepared with NE-PER nuclear and cytoplasmic extraction reagents (Pierce Biotechnology, Rockford, IL, USA), respectively.

2.6. Nitrite (NO production) determination

The nitrite concentration in the medium, an indicator of NO production, was measured with the Griess reaction. Each supernatant (100 μL) was mixed with an equal volume of the Griess reagent (Solution A: 222488; Solution B: S438081, Sigma), and the absorbance of the mixture at 525 nm was determined using an ELISA plate reader.

2.7. PGE_2 assay

BV2 microglial cells were cultured in 24-well plates, pre-incubated for 3 h with different concentrations of aurantiamide acetate (**1**), and then stimulated for 24 h with LPS. Supernatant from the culture (100 μL) was collected to determine the PGE_2 concentration using an ELISA kit (R & D Systems, Minneapolis, MN, USA).

2.8. TNF-α and IL-1β assays

In 24-well plates, BV2 microglial cells were pre-incubated for 3 h in various concentrations of aurantiamide acetate (**1**), and then stimulated for 24 h with LPS. From the culture supernatants, the concentration of TNF-α and IL-1β were determined using ELISA kits (R & D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

2.9. Western blot analysis

BV2 microglial cells were harvested and pelleted by centrifugation at 16000 rpm for 15 min. The cells were then washed with PBS and lysed with 20 mM Tris-HCl buffer (pH 7.4) containing a protease inhibitor mixture (0.1 mM PMSF, 5 mg/mL aprotinin, 5 mg/mL pepstatin A, and 1 mg/mL chymostatin). The protein concentration was determined with the Lowry protein assay kit (P5626; Sigma). An equal amount of protein from each sample was resolved using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then electrophoretically transferred onto a Hybond enhanced chemiluminescence (ECL) nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). The membrane was blocked with 5% skimmed milk and sequentially incubated with the primary antibody (Santa Cruz Biotechnology, CA, USA) and horseradish peroxidase-conjugated secondary antibody followed by ECL detection (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

2.10. DNA binding activity of NF-κB

Microglia were pretreated for 3 h with the indicated concentrations of compound **1** and then stimulated for 30 min with LPS (1 μg/mL). The DNA-binding activity of NF-κB in nuclear extracts was measured using the TransAM kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer's instructions.

2.11. Statistical analysis

The data are expressed as the mean ± S.D. of at least three independent experiments. To compare three or more groups, one-way analysis of the variance was used, followed by Newman–Keuls post hoc test. The statistical analysis was performed with GraphPad Prism software, version 3.03 (GraphPad Software Inc., San Diego, CA, USA).

3. Results

3.1. Isolation and structure determination of aurantiamide acetate (1) and cell viability in BV2 microglia

To identify the anti-neuroinflammatory component(s) in the organic extract of culture media of the marine fungus *Aspergillus* sp. SF-5921, purification step with HPLC was employed, and this led to the isolation of a dipeptide-type fungal metabolite, aurantiamide acetate (**1**) (Fig. 1A). The structure of the isolated compound was identified by analysis of NMR and MS data, along with a comparison of its spectral data to those reported in the literature [26,27]. The purity of aurantiamide acetate was estimated to be more than 97% in HPLC analysis (Fig. S3). The cytotoxicity of compound **1** to BV2 microglia was assessed by the MTT assay. Cell viability was not significantly affected in 10 μM to 200 μM range of compound **1**, suggesting that compound **1** was not toxic to BV2 microglial cells in the concentrations tested (Fig. 1B). Thus, for the further experiments, the concentration of compound **1** used to treat the cells was kept with the range of 10–100 μM.

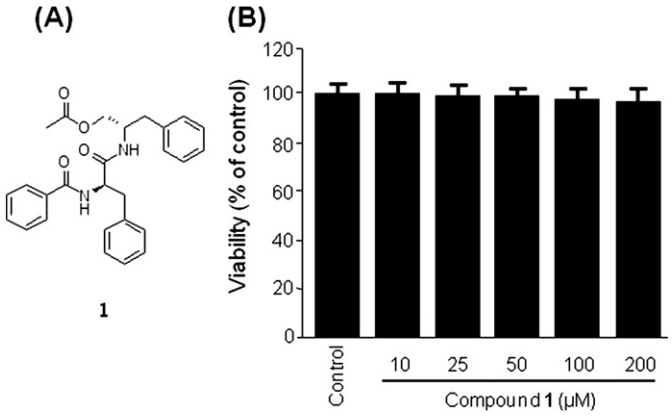

Fig. 1. Chemical structure of aurantiamide acetate (A) and effects of compound **1** on cell viability (B). BV2 microglia were incubated for 24 h with various concentrations of compound **1** (10–200 μM). Cell viability was determined as described under Materials and methods. Data represent the mean values of three experiments ± SD. $^{*}p < 0.05$ compared to the group treated with LPS alone.

3.2. Effects of aurantiamide acetate (1) on the productions of pro-inflammatory mediators and cytokines in BV2 microglia stimulated with LPS

To evaluate the anti-neuroinflammatory effects of aurantiamide acetate (**1**) on LPS-stimulated BV2 cells, the concentrations of pro-inflammatory mediators such as NO and PGE_2 were measured in both the presence and absence of compound **1** at non-cytotoxic concentrations ranging from 10 to 100 μM. BV2 cells were pretreated with compound **1** for 3 h, followed by stimulation with LPS (1 μg/mL) for 24 h. As shown in Fig. 2, the LPS treatment triggered an approximate 10-fold increase in nitrite concentration in the culture media, compared to that of the untreated group. However, pre-treatment of the microglial cells with compound **1** for 3 h decreased the production of NO as indicated by the nitrite concentration in a concentration-dependent manner, with an IC_{50} value of 49.70 μM (Fig. 2A). Under the same conditions, compound **1** also suppressed PGE_2 production in a concentration-dependent manner, with an IC_{50} value of 51.53 μM (Fig. 2B). These results indicated that compound **1** suppressed the LPS-induced pro-inflammatory mediators. This observation led to further investigation, by enzyme immunoassay, of the effects of compound **1** on the production of LPS-induced pro-inflammatory cytokines such as TNF-α and IL-1β. As shown in Fig. 2, compound **1** diminished the IL-1β production in a concentration-dependent manner, with an IC_{50} value of 40.36 μM, when the cells were pre-treated with compound **1** for 3 h treatment. On the other hand, compound **1** showed no effect on the production of TNF-α.

3.3. Effects of aurantiamide acetate (1) on the expression of pro-inflammatory enzymes in BV2 microglia stimulated with LPS

The effects of compound **1** on LPS-induced iNOS and COX-2 expressions were evaluated by western blot analysis (Fig. 3). BV2 microglial cells were challenged with LPS (1 μg/mL) in the presence or absence of compound **1** at non-cytotoxic concentrations ranging from 10 to 100 μM. Western blotting analysis revealed that compound **1** suppressed both LPS-induced iNOS (Fig. 3A) and COX-2 (Fig. 3B) expressions, in a dose-dependent manner. These results suggested that compound **1** inhibited pro-inflammatory cytokines and mediators by the reduction of iNOS and COX-2 protein levels.

3.4. Effects of aurantiamide acetate (1) on NF-κB activation in BV2 microglia stimulated with LPS

Accumulating data indicate that NF-κB is a major regulatory component of the inflammatory responses mediated by LPS or pro-inflammatory cytokines. The iNOS and COX-2 promoter regions contain

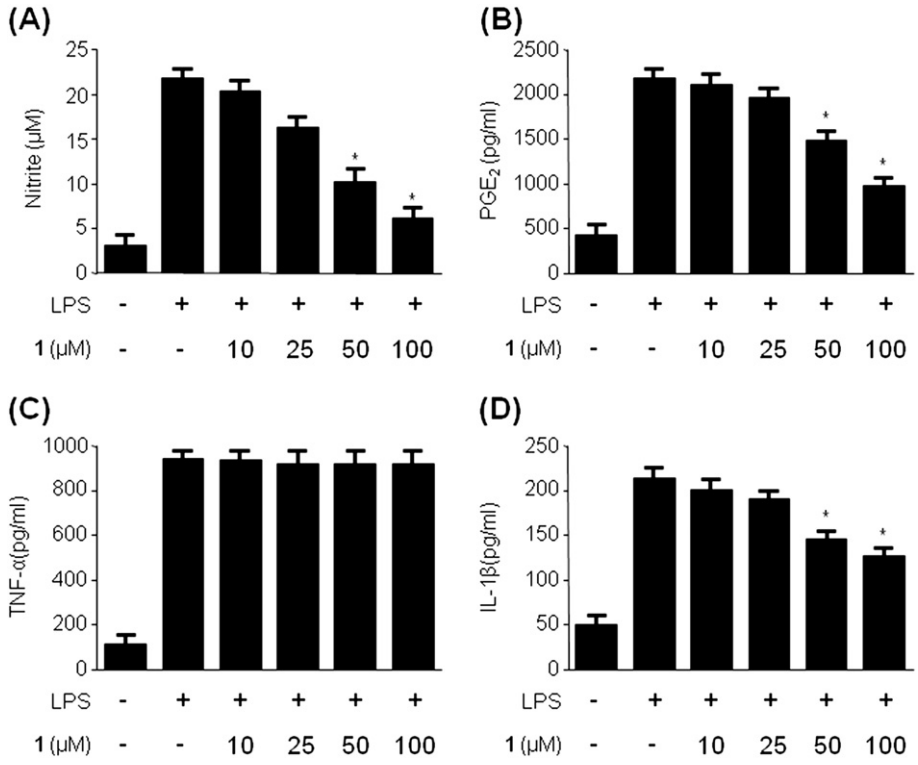

Fig. 2. Effects of aurantiamide acetate (**1**) on nitrite (A), PGE_2 (B), TNF-α (C), and IL-1β (D) production in BV2 microglia stimulated with LPS. Cells were pre-treated for 3 h with indicated concentrations of **1**, and then stimulated for 24 h with LPS (1 μg/mL). The concentrations of nitrite (A), PGE_2 (B), TNF-α (C), and IL-1β (D) were determined as described under Materials and methods. Data represent the mean values of three experiments ± SD. $^*p < 0.05$ compared to the group treated with LPS.

NF-κB sites, which are necessary for inducing the expression of these genes [28,29]. Thus, we further explored whether compound **1** regulates the NF-κB pathway. Treating the cells with LPS markedly increased the translocation of p65 and p50 to the nucleus, but pretreatment with compound **1** significantly suppressed this nuclear translocation (Fig. 4A). In addition, IκB-α phosphorylation was increased after LPS treatment, but pretreatment with compound **1** significantly reduced this phosphorylation (Fig. 4B). Furthermore, compound **1** significantly inhibited LPS-induced IκB-α degradation (Fig. 4B). In line with these results, compound **1** also suppressed the DNA binding activity of NF-κB in nuclear extracts from BV2 microglia stimulated with LPS for 30 min. As shown in Fig. 4D, the treatment of cells with LPS markedly increased NF-κB binding activity by approximately 3-fold; however, compound **1** inhibited this activity in a dose-dependent manner. Therefore, it was suggested that compound **1** prevents NF-κB-DNA binding by inhibiting the phosphorylation and degradation of IκB-α, and the consequent

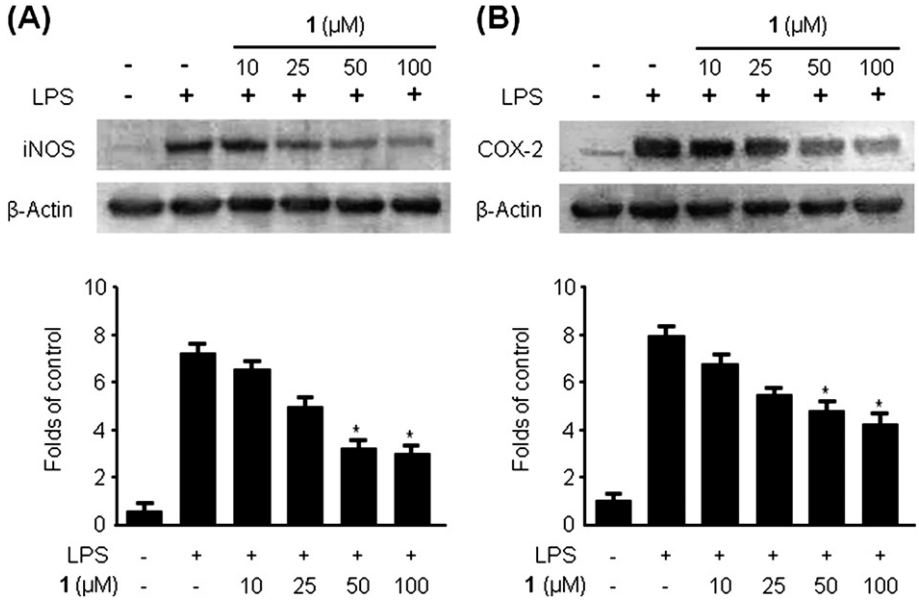

Fig. 3. Effects of aurantiamide acetate (**1**) on protein iNOS (A) and COX-2 (B) expression in BV2 microglia stimulated with LPS. Cells were pre-treated for 3 h with indicated concentrations of **1**, and then for 24 h with LPS (1 μg/mL). Western blot analyses (A, B) were performed as described in Materials and methods; representative blots of three independent experiments are shown. Data presented here represent the mean values of three experiments ± S.D. $^*p < 0.05$ compared to the group treated with LPS alone.

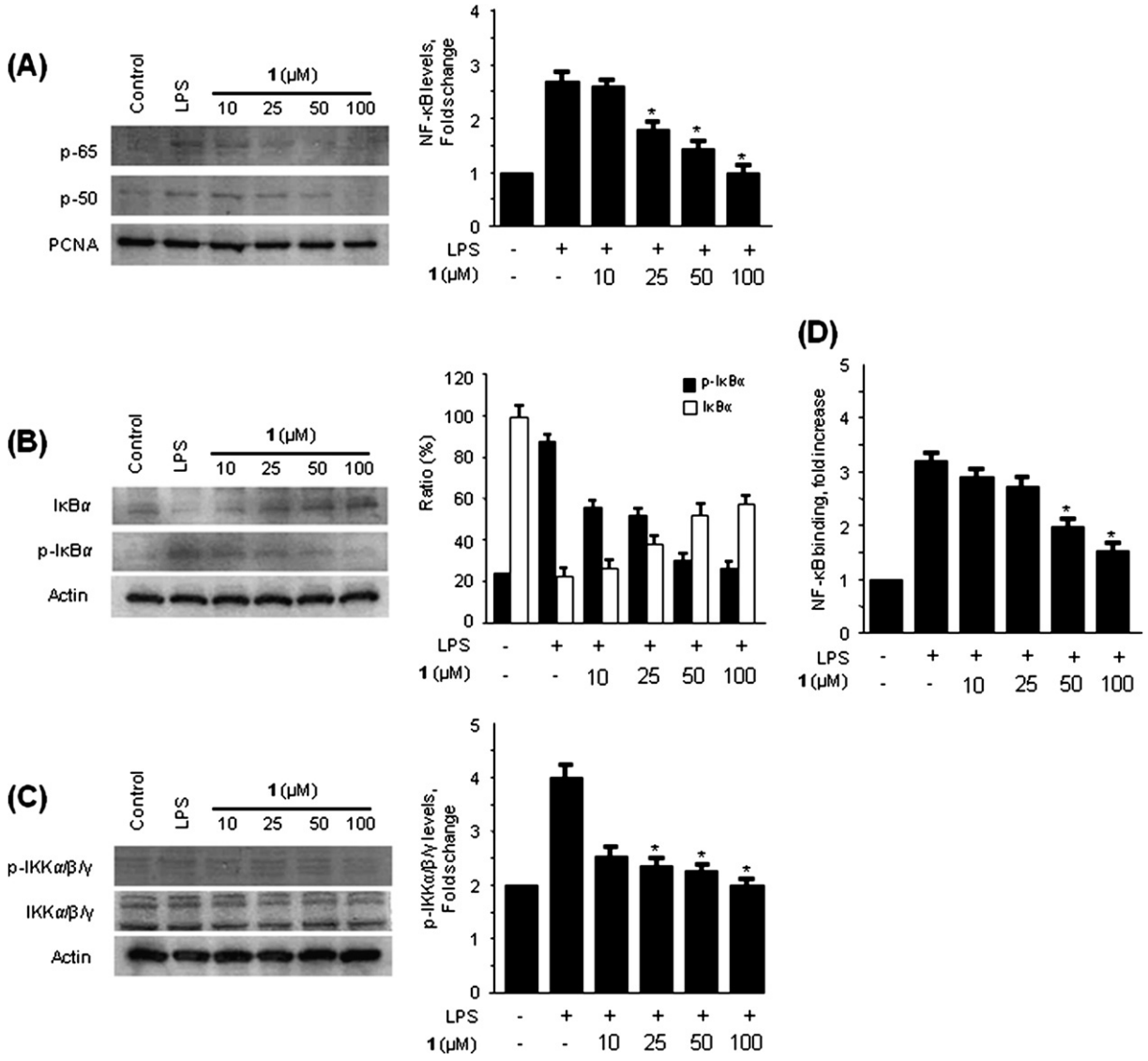

Fig. 4. Effects of aurantiamide acetate (**1**) on LPS-induced NF-κB activation. (A) Nuclear extracts were prepared for a Western blot of p65 and p50 of NF-κB, using specific anti-p65 and anti-p50 monoclonal antibodies. (B) A commercially available NF-κB ELISA (Active Motif) was used to test the nuclear extracts and determine the degree of NF-κB binding. (C) Following pretreatment with **1** (10, 25, 50, and 100 μM) for 3 h, cells were treated with LPS for 30 min. Total proteins were prepared and the western blot analysis was performed using specific IκB-α and p-IκB-α antibodies. (D) Following pretreatment with **1** (10, 25, 50, and 100 μM) for 3 h, cells were treated with LPS (1 μg/mL) for 30 min. Total cellular proteins (30 μg) were resolved by SDS-PAGE, transferred to NC membranes, and detected with specific p-IKKα/β/γ, IKK α/β/γ antibodies. The data shown, representative of three independent experiments, are the mean values of three experiments ± S.D. $^{*}p < 0.05$ compared to the group treated with LPS alone.

nuclear translocation of p65/p50. The inhibition of the IκB-α phosphorylation could be related to the inhibition of the corresponding IκB kinase (IKK) [30]. Therefore, we used western blot analysis to examine the effect of compound **1** on the LPS-induced activation of IKKα/β/γ. As shown in Fig. 4C, LPS strongly induced IKKα/β/γ phosphorylation, whereas pretreatment with compound **1** (50 or 100 μM) significantly reduced this phosphorylation without affecting total cellular IKKα/β/γ level. Collectively, our data demonstrated that compound **1** was able to inhibit LPS-induced NF-κB activation by preventing the processes leading to the activation of the IKK complex.

*3.5. Effect of aurantiamide acetate (**1**) on the phosphorylation of MAPKs in BV2 microglia stimulated with LPS*

The MAPK pathway is known to be involved in the inflammatory process, and inhibition of the MAPK pathway is the prominent route to block the induction of pro-inflammatory mediators by LPS. Therefore, various MAPK family proteins, particularly ERK, JNK and p38 are regarded as an important target for the development of anti-inflammatory agents implicated in the regulation of inflammatory processes [31,32]. To investigate how aurantiamide acetate (**1**) suppresses inflammatory reactions that the MAPK pathway mediates, we assessed the effect of compound **1** on the LPS-induced phosphorylation of ERK, JNK, and p38 in BV2 microglial cells. As shown in Fig. 5, the phosphorylation levels of ERK, JNK, and p38 increased after 30 min of treatment with LPS. However, pre-treatment with aurantiamide acetate (**1**) for 3 h, at 10 to 100 μM, significantly inhibited the LPS-induced phosphorylation of p38 and JNK in a dose-dependent manner (Fig. 5B and C), while ERK phosphorylation was not affected. On the other hand, neither LPS nor compound **1** affected the expressions of ERK, JNK, and p38. These data suggested that aurantiamide acetate (**1**) regulated inflammatory reactions by inhibiting JNK and p38 MAPK signaling pathways.

4. Discussion

Microglial cells are brain macrophage cells that exist in the central nervous system (CNS) [33,34]. They are activated by extracellular stimuli, including LPS, interferon-γ (IFN-γ), and β-amyloid, and that

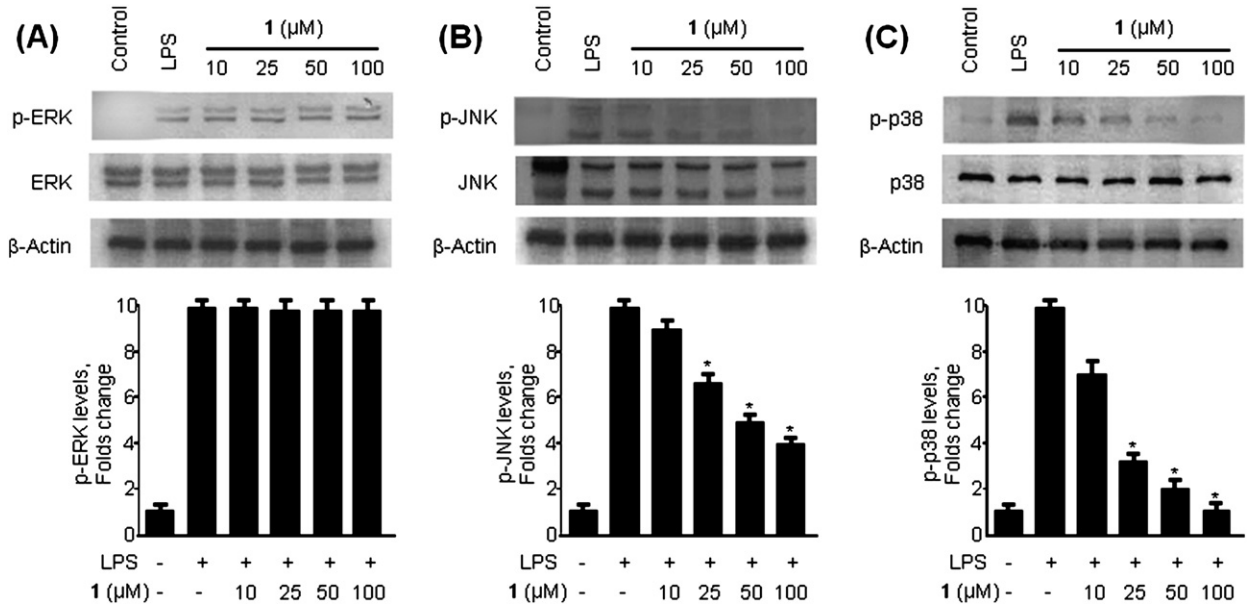

Fig. 5. Effects of aurantiamide acetate (**1**) on ERK, JNK, and p38 MAPK phosphorylation and protein expression. Cells were pre-treated for 3 h with the indicated concentrations of **1** and stimulated for 30 min with LPS (500 ng/mL) (A, B, and C). The levels of (A) phosphorylated-ERK (p-ERK), (B) phosphorylated-JNK (p-JNK), and (C) phosphorylated-p38 MAPK (p-p38 MAPK) were determined by western blotting. Representative blots from three independent experiments with similar results and densitometric evaluations are shown. Data shown represent the mean values of three experiments ± S.D. $^{*}p < 0.05$ compared to the group treated with LPS alone.

activation initiates several crucial cellular responses that play important roles in the pathogenesis of neuroinflammation [5,6]. The various pro-inflammatory cytokines and neurotoxic mediators produced by activated microglia are thought to contribute to neuronal injury and the pathogenesis of the neuroinflammatory diseases. Thus, controlling the production of pro-inflammatory mediators would be a potential target for neuroinflammation-related diseases [7,8]. In the present study, aurantiamide acetate (**1**) was shown to inhibit the production of NO and PGE_2 in LPS-stimulated BV2 microglial cells. To understand the mechanism of this inhibitory effect of compound **1**, we examined its influence on the protein levels of iNOS and COX-2 enzymes, which are responsible for the production of NO and PGE_2. The results showed that compound **1** suppressed the protein expression of iNOS and COX-2 in a dose-dependent manner, indicating that the inhibitory effects of compound **1** on NO and PGE_2 production correlate with the inhibition of iNOS and COX-2 protein expression. In addition, the aurantiamide acetate (**1**) inhibited NO production and iNOS and COX-2 protein expression in both pre- and post- treatment conditions (Fig. S1). This result demonstrated that the anti-inflammatory effects of aurantiamide acetate (**1**) are not subject to the cell treatment condition of **1**. To address whether the anti-inflammatory effects of aurantiamide acetate (**1**) are confined to microglial cells, the effects of compound **1** on the NO production and iNOS/COX-2 expression in LPS-stimulated RAW 264.7 cells were evaluated. As shown in Supplementary information, aurantiamide acetate (**1**) also reduced the NO production (Fig. S2B), and iNOS and COX-2 expression in LPS-stimulated RAW 264.7 cells (Fig. S2C, S2D).

Nuclear factor-kappa B (NF-κB), the principal transcription factor that controls inflammatory response, regulates the inflammatory gene expressions such as iNOS, COX-2, TNF-α, IL-1β, and IL-6 [15,35]. In normal cell conditions, NF-κB exists as a complex with its inhibitory protein (IκB) in the cytoplasm. Once the microglial cells have been activated by various stimulations, the IκB protein is released and degraded resulting in free p50 and p65, which then translocate to the nucleus [36]. Since the protein expressions of iNOS and COX-2 are regulated by certain transcription factors such as NF-κB, we examined whether the inhibitory effects of compound **1** on pro-inflammatory mediators are exerted via the NF-κB pathway. Compound **1** was found to inhibit the phosphorylation of IκB-α and IKKα/β/γ in LPS-stimulated BV2 microglial cells. Moreover, compound **1** was shown to reduce significantly the DNA binding activity of NF-κB in nuclear extracts taken from LPS-stimulated BV2 microglial cells. In addition to the production of NO and PGE_2 by INOS and COX-2, a variety of pro-inflammatory cytokines are also regulated by NF-κB, and this was consistent with the finding that compound **1** reduced the production of IL-1β in LPS-stimulated microglial cells.

The MAPK pathway plays a role in the LPS-stimulated induction of various pro-inflammatory mediators as well as in the activation of NF-κB [19–21]. Therefore, the effects of aurantiamide acetate (**1**) on the LPS-induced phosphorylation of three MAPK family proteins, p38, JNK, and ERK were assessed. The results indicated that phosphorylation of JNK and p38 was inhibited by pre-treatment with compound **1**, suggesting that the inhibition of MAPK pathway is also involved in the anti-inflammatory effects of compound **1**. In addition, the inhibition of NF-κB activation by compound **1** might be medicated through changes in the phosphorylation of MAPKs.

Members of the fungal genus *Aspergillus* are known to produce various types of secondary metabolites with interesting bioactivities such as cytotoxic, antimicrobial and miscellaneous effects. Accordingly, this species is recognized as a rich source of pharmacologically valuable new compounds [37]. In the course of our continuing search for bioactive secondary metabolites from marine-derived fungi, aurantiamide acetate (**1**) was encountered as an anti-neuroinflammatory principle from the cultures of marine-derived fungus belongs to the genus *Aspergillus*. Compound **1** was originally isolated from *Piper aurantiacum*, and it has been isolated from various biological resources, such as algae, fungi, and plants [26,38–40]. Various biological effects of this compound, such as antioxidant, antimicrobial [39], cathepsin inhibitory [40], anti-inflammatory, and analgesic [41], have also been reported. In particular, compound **1** has been shown to inhibit the production of TNF-α and IL-2 in whole blood cytokine assays of rat [41]. However, the anti-inflammatory effects of compound **1** in LPS-stimulated microglial cells and the molecular mechanism involved in these effects were not reported.

In summary, the present study demonstrated that aurantiamide acetate (**1**) regulated anti-neuroinflammatory activity, by suppressing the NF-κB, JNK and p38 activation in BV2 microglia cells and, in turn, inhibiting significantly such LPS-induced pro-inflammatory mediators and cytokines such as NO, PGE_2 and IL-1β.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.intimp.2014.10.006.

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