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Bee venom and melittin reduce proinflammatory mediators in lipopolysaccharide-stimulated BV2 microglia

Dong-Oh Moon [a,1], Sung-Yong Park [b,1], Kyeong-Jun Lee [a], Moon-Soo Heo [a], Ki-Cheon Kim [a], Mun-Ock Kim [c], Jae-Dong Lee [c], Yung Hyun Choi [d], Gi-Young Kim [a,*]

[a] *Faculty of Applied Marine Science, Cheju National University, Jeju-si, Jeju Special Self-Governing Province 690-756, South Korea*
[b] *Department of Food Science and Technology, Chung-Ang University, Ansung 456-756, South Korea*
[c] *Department of Microbiology, College of Natural Sciences, Pusan National University, Busan 609-735, South Korea*
[d] *Department of Biochemistry, Dongeui University College of Oriental Medicine, Busan 614-054, South Korea*

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Abstract

Bee venom (BV), well known as a traditional Oriental medicine, has been shown to exhibit anti-arthritic and anti-carcinogenic effects. However, the molecular mechanisms responsible for the anti-inflammatory activity of BV have not been elucidated in microglia. In the present study, we investigated the anti-inflammatory effect of BV and its major component, melittin (MEL), on lipopolysaccharide (LPS)-stimulated BV2 microglia. Our results indicate that BV and MEL suppress LPS-induced nitric oxide (NO) and inducible NO synthase (iNOS) expression in a dose-dependent manner, without causing cytotoxicity in BV2 microglia. Moreover, BV and MEL suppressed LPS-induced activation of nuclear factor kappa B (NF-κB) by blocking degradation of IκBα and phosphorylation of c-Jun N-terminal kinase (JNK) and Akt, which resulted in inhibition of iNOS expression. Our data also indicate that BV and MEL exert anti-inflammatory effects by suppressing the transcription of cyclooxygenase (COX)-2 genes and proinflammatory cytokines, such as interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF)-α. BV and MEL also attenuated the production of prostaglandin E_2 (PGE_2). These results demonstrate that BV and MEL possess a potent suppressive effect on proinflammatory responses of BV2 microglia and suggest that these compounds may offer substantial therapeutic potential for treatment of neurodegenerative diseases that are accompanied by microglial activation.

Keywords: Bee venom; Melittin; Nitric oxide; Cyclooxygenase-2; Nuclear factor kappa B; c-Jun N-terminal kinase; Akt

1. Introduction

Microglia, resident macrophage-like population of brain cells, are believed to play a major role in host defense and tissue repair in the central nervous system (CNS) [1]. However, overactivation of microglia can occur immediately after brain injury, such as inflammatory, ischemic, and neurodegenerative pathologies of the CNS, resulting in deleterious effects [2]. Chronic microglial activation and consequent overproduction of proinflammatory mediators such as nitric oxide (NO), inducible NO synthase (iNOS), prostaglandin E2 (PGE2), cyclooxygenase (COX)-2 and proinflammatory cytokines [interleukin (IL)-1β, IL-6 and

* Corresponding author. Tel.: +82 64 754 3427; fax: +82 64 756 3493.
E-mail address: immunkim@cheju.ac.kr (G.-Y. Kim).
[1] Equally contributed to this work.

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tumor necrosis factor (TNF)-α] are a histopathological hallmark of various neurological diseases, including Alzheimer's disease, Parkinson's disease, multiple sclerosis, and cerebral ischemia [3,4]. Previous studies have also demonstrated that reduction of proinflammatory mediators in microglia may attenuate the severity of these disorders [5,6]. Therefore, mechanisms to regulate microglial activation may have important therapeutic potential for the treatment of neurodegenerative diseases that are accompanied by microglial activation.

Bee venom (BV) consists of several biologically active peptides, including melittin (MEL), apamin, adolapin and mast cell degranulating peptide (MCDP) [7]. Recent reports have indicated that acupuncture and administration of BV can significantly impart an anti-arthritic response mediated by inhibition of inflammation mediators, similar to non-steroidal anti-inflammatory drugs [8,9]. Jang and his colleagues reported that BV has anti-inflammatory effects in Raw264.7 macrophage cell line which is ascribed to only transcriptional downregulation of iNOS, COX-2, nuclear factor kappa B (NF-κB) and mitogen-activated protein kinases (MAPKs) [10]. Our previous study also showed that BV induces apoptosis in human leukemic U937 cells through the modulation of caspase-3 and Bcl-2 [11]. Nevertheless, no reports have investigated the anti-inflammatory effects and molecular mechanisms of BV and MEL in LPS-stimulated microglia.

In this study, we first report that pretreatment with BV or MEL significantly inhibit iNOS production and NO expression in LPS-stimulated BV2 microglia through the inhibition of NF-κB, c-Jun N-terminal kinase (JNK) and Akt signaling pathway. In addition, BV and MEL pretreatment significantly inhibited the expression of COX-2, PGE_2 and proinflammatory cytokines (IL-1β, IL-6 and TNF-α) in LPS-stimulated BV2 microglia. The present results may have clinical implications and suggest that BV and MEL may offer potential for the treatment and prevention of inflammatory disease.

2. Materials and methods

2.1. Materials

BV, MEL, LPS, Tween-20, pyrrolidine dithiocarbamate (PDTC) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO). Rabbit anti-human iNOS, COX-2, p65, phospho (p)-IκBα and IκBα-polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against ERK, p-ERK, p38, p-p38, JNK, pJNK, Akt and p-Akt were purchased from PharMingen (San Diego, CA). The antibody against β-actin was from Sigma. Peroxidase-labeled goat anti-rabbit immunoglobulin was purchased from Amersham (Arlington Heights, IL). PD98059, SB203580,

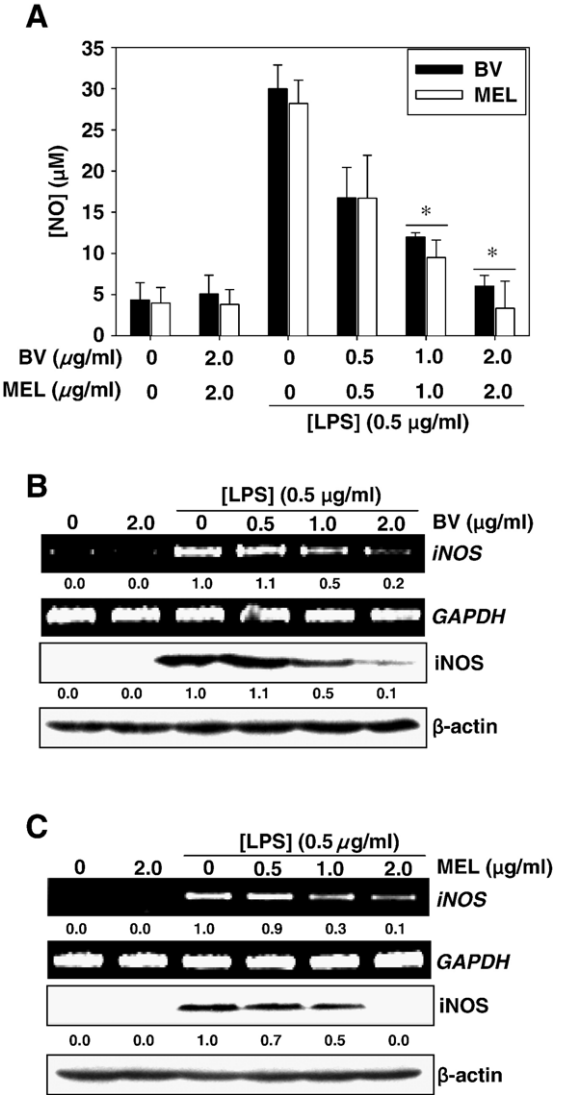

Fig. 1. Effect of BV and MEL on the levels of NO and iNOS production by LPS-stimulated BV2 microglia. (A) BV2 microglia were pretreated for 1 h with BV and MEL prior to stimulation with LPS. After 24 h, the amounts of NO were determined using Griess reagent and a standard curve created using $NaNO_2$ in culture medium. In parallel experiments, the expression of levels of iNOS mRNA and protein were measured by RT-PCR and Western blot analysis after 6 h of LPS treatment in the presence of BV (B) and MEL (C). Results are representative of those obtained from three independent experiments and the densitometric data (arbitrary) shown under the immunoblots and RT-PCR are presented as fold changes as compared with their respective control. Values are expressed as means±S.D. from three independent experiments. *$P<0.05$ indicates a significant difference from cells treated with LPS in the absence of BV or MEL.

SP600125 and LY294002 were obtained from Calbiochem (San Diego, CA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and other tissue culture reagents were purchased from Gibco (Grand Island, NY).

2.2. Cells culture

The BV2 immortalized murine microglial cell line was provided by Dr. E. J. Choi (Korea University, South Korea). BV2 cells were constructed by infecting primary microglia with a v-raf/v-myc oncogene-carrying retrovirus (J2). The murine BV2 microglia were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin, and were maintained in a humidified incubator with 5% CO_2. In all experiments, cells were incubated in the presence of the indicated concentrations of BV or MEL before the addition of LPS (0.5 μg/ml) in serum-free DMEM.

2.3. Cytotoxicity assay

Cell viability was evaluated by the MTT reduction assay. In brief, cells (1×10^5 cells/ml) were seeded in 24 well plates and treated with various reagents for the indicated time periods. After various treatments, the medium was removed and the cells were incubated with 0.5 mg/ml of MTT solution. After incubation for 3 h at 37 °C and 5% CO_2, the supernatant was removed and formation of formazan was measured at 540 nm with a microplate reader.

2.4. Isolation of total RNA and RT-PCR

The total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacture's instructions. Total RNA was isolated according to the manufacture's instructions. RNA (3 μg) was reverse-transcribed using M-MLV reverse

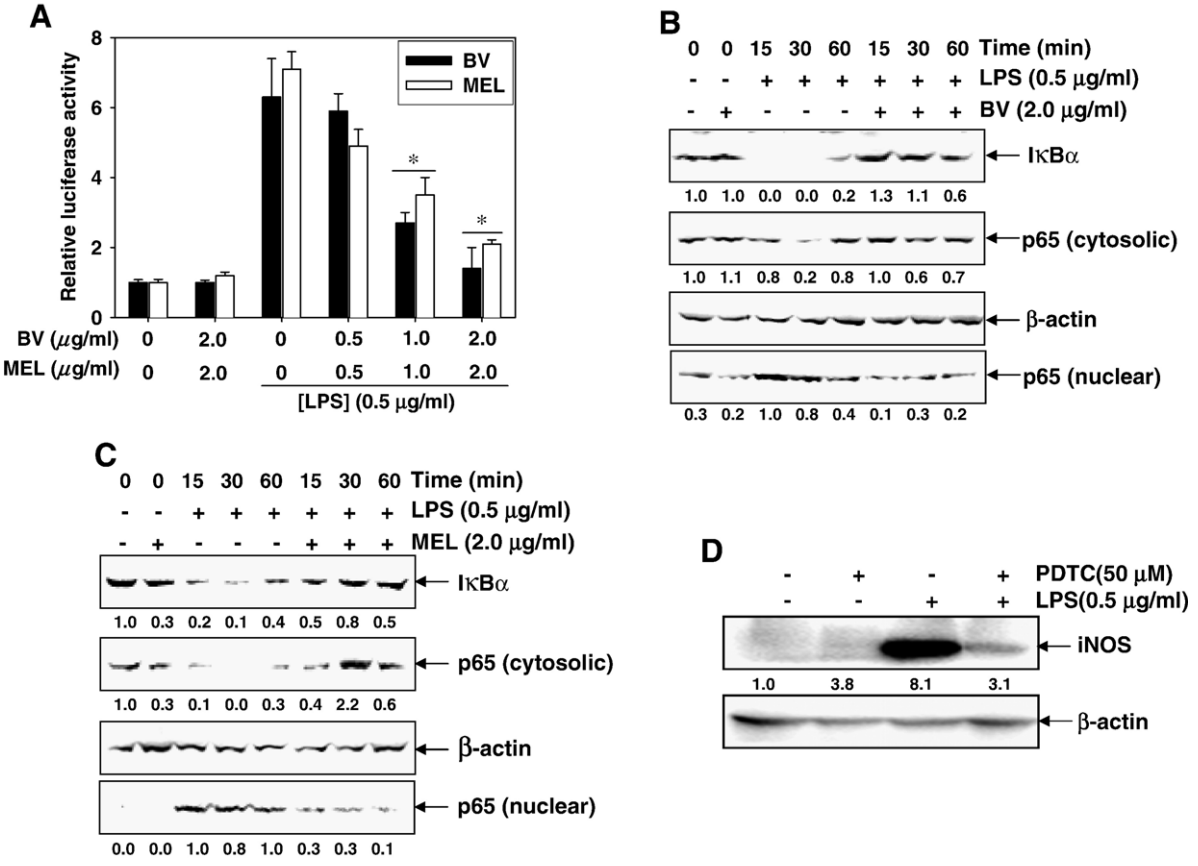

Fig. 2. Effect of BV and MEL on NF-κB activation and IκBα degradation induced by LPS in microglia. (A) Transfected BV2 microglia were pretreated with BV or MEL for 1 h and then stimulated with LPS for 6 h. The NF-κB activity was expressed as relative luciferase activity. BV2 cells were also treated with the indicated doses of BV (B) and MEL (C) for 1 h before LPS treatment for the indicated times. (D) In a parallel experiment, BV2 cells were treated with 50 μM PDTC for 1 h prior to LPS treatment for 6 h. Total protein was subjected to 10% SDS-PAGE followed by immunoblots using anti-IκBα, anti-NF-κB p65 and anti-iNOS. Results are representative of those obtained from three independent experiments and the densitometric data (arbitrary) shown under the immunoblots are presented as fold changes as compared with their respective control. Values are expressed as means ± S.D. from three independent experiments. *$P < 0.05$ indicates a significant difference from the value in cells treated with LPS in the absence of BV or MEL.

transcriptase (Promega, Madison, WI). Single stranded cDNA was amplified by PCR with primers for iNOS, COX-2, glyceraldhyde-3-phosphate dehydrogenase (GAPDH), IL-1β, IL-6 and TNF-α, whose primer sequences have been described in previous studies [12,13]. The following PCR conditions were applied: GAPDH, 18 cycles of denaturation at 94 °C for 30 s, annealing at 57 °C for 30 s and extended at 72 °C for 30 s; iNOS, COX-2, IL-1β, IL-6 and TNF-α, 25 cycles of denaturation at 94 °C for 30 s, annealing at 52 °C for 30 s, and extended at 72 °C for 30 s. The GAPDH was used as an internal control to evaluate relative expression of COX-2 and iNOS, IL-1β, IL-6 and TNF-α. Densitometry analysis of bands was performed by Scion image (Scion, Frederic, MD).

2.5. Immunoblot analysis

The cells were washed with PBS three times, placed at 4 °C, and lysed for 30 min in lysis buffer (1% Triton X-100, 1% deoxycholate, and 0.1% NaN_3). Lysates were then centrifuged at 14,000×*g* at 4 °C. The supernatants were collected for further analysis. Equal amounts of protein were separated electrophoretically using 10% SDS-PAGE and then the gel was transferred to 0.45 μm polyvinylidene fluoride (PVDF: Millipore, Bedford, MA). The membranes were soaked in blocking buffer (5% skimmed milk), incubated overnight with primary antibodies, incubated with followed by horseradish peroxidase conjugated antibodies, and immune complexes were then visualized by the enhanced chemiluminescence (ECL) detection system according to the recommended procedure (Amersham). In a parallel experiment, nuclear protein was prepared using lysis buffer (10 mM Tris–Cl, pH 7.4, 3 mM $CaCl_2$, 2 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 mM PMSF and protease inhibitors) for 15 min at 4 °C. After centrifugation and washing of the nuclei pellet, ice-cold hypertonic extraction buffer (20 mM HEPES–KOH, pH 7.9, 25% glycerol, 420 mM NaCl, 0.2 mM EDTA, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol and protease inhibitors) was added and samples were incubated at 4 °C for 30 min with constant shaking. The nuclear protein extracts were isolated by centrifugation at 14,000×*g* for 30 min. Densitometry analysis of bands was performed by Scion image.

2.6. Nitrite assays (Griess assay)

NO levels in the culture supernatants were measured by a Griess reaction. After cells (5×10^5 cells/ml) were stimulated in 24 well plates for 24 h, 100 μl of each cultured medium was mixed with the same volume of the Griess reagent (1% sulfanilamide/0.1% *N*-(1-naphthyl)-ethylenediamine dihydrochloride/2.5% H_3PO_4). NO concentration was determined by measuring the absorbance at 540 nm with a Vmax 96-well microplate spectrophotometer. Nitrite concentration was calculated with reference to a standard curve of sodium nitrite generated by known concentrations.

2.7. Measurement of PGE_2

BV2 cells were subcultured in 6-well plates (5×10^5 cells/ml) and incubated with PIC (0, 10, and 20 μM) in the presence or absence of LPS (0.5 μ/ml) for 24 h. One hundred microliters of culture-medium supernatant was collected for determination of PGE_2 concentration by ELISA (Cayman, MI).

2.8. Enzyme-linked immunosorbent assay (ELISA)

IL-1β, IL-6 and TNF-α, were measured by ELISA kits (R & D, Minneapolis, MN). The absorbance at 450 nm was determined using a microplate reader.

2.9. NF-κB luciferase assay

A total of 1×10^6 BV2 cells were transfected with 2 μg NF-κB-Luciferase reporter plasmids and pCMV-Gal control vector (BD Sciences, San Jose, CA) using Lipofectamine according

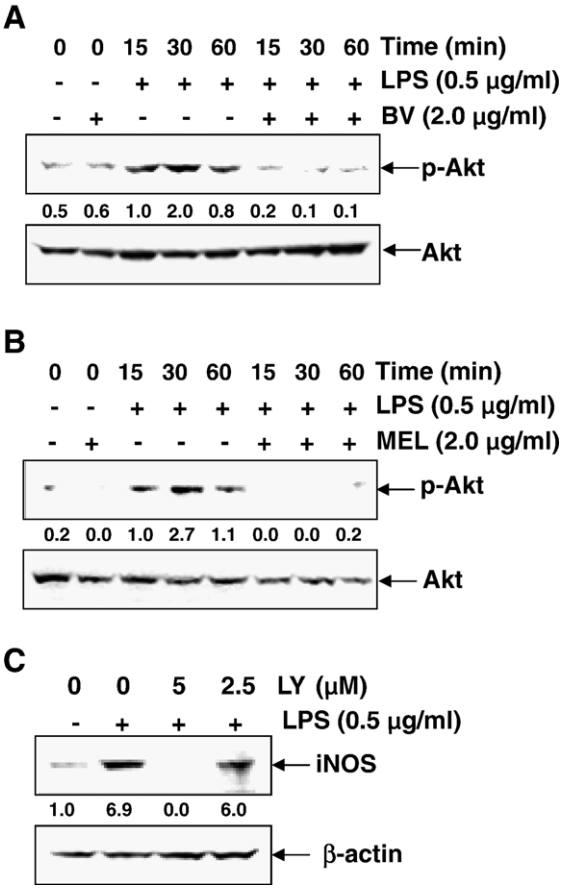

Fig. 3. Effect of BV and MEL on Akt activation induced by LPS in BV2 microglia. BV2 microglia were treated with the indicated doses of BV (A) and MEL (B) 1 h before LPS treatment for the indicated times. (C) In a parallel experiment, BV2 microglia were treated with 2.5 μM or 5 μM LY294002 (a specific Akt inhibitor) 1 h before LPS treatment for the indicated times. Total protein was subjected to 10% SDS-PAGE followed by Western blotting using anti-Akt. Results are representative of those obtained from three independent experiments and the densitometric data (arbitrary) shown under the immunoblots are presented as fold changes, as compared with their respective control.

to the manufacturer's protocol (Gibco BRL). After incubating with DNA-Lipofectamine mixtures, the cells were preincubated in the presence or in the absence of BV or MEL before being stimulated with LPS for 6 h. After stimulation, the cells were then washed twice with PBS and lysed with reporter lysis buffer (Luciferase Assay System kit; Promega, Madison, WI). After vortexing and centrifugation at 12,000×*g* for 1 min 4 °C, the supernatant was stored −70 °C for the luciferase assay. After 20 μl of the cell extract was mixed with 100 μl of the luciferase assay reagent at room temperature, the mixture was measured a microplate luminometer LB96V (Perkin-Elmer, Foster City, CA). Luciferase activity was normalized by dividing the mean luciferase RLU by the mean β-galactosidase RLU.

2.10. Statistical analysis

Data is presented as the mean±S.D. of at least three separate experiments. Comparisons between two groups were analyzed using Student's *t*-test. *P* values less than 0.05 were considered to be statistically significant.

3. Results

3.1. BV and MEL attenuate NO secretion through regulation of transcriptional levels

LPS has been reported to induce the release of NO release in microglia through the de novo synthesis of iNOS [14].

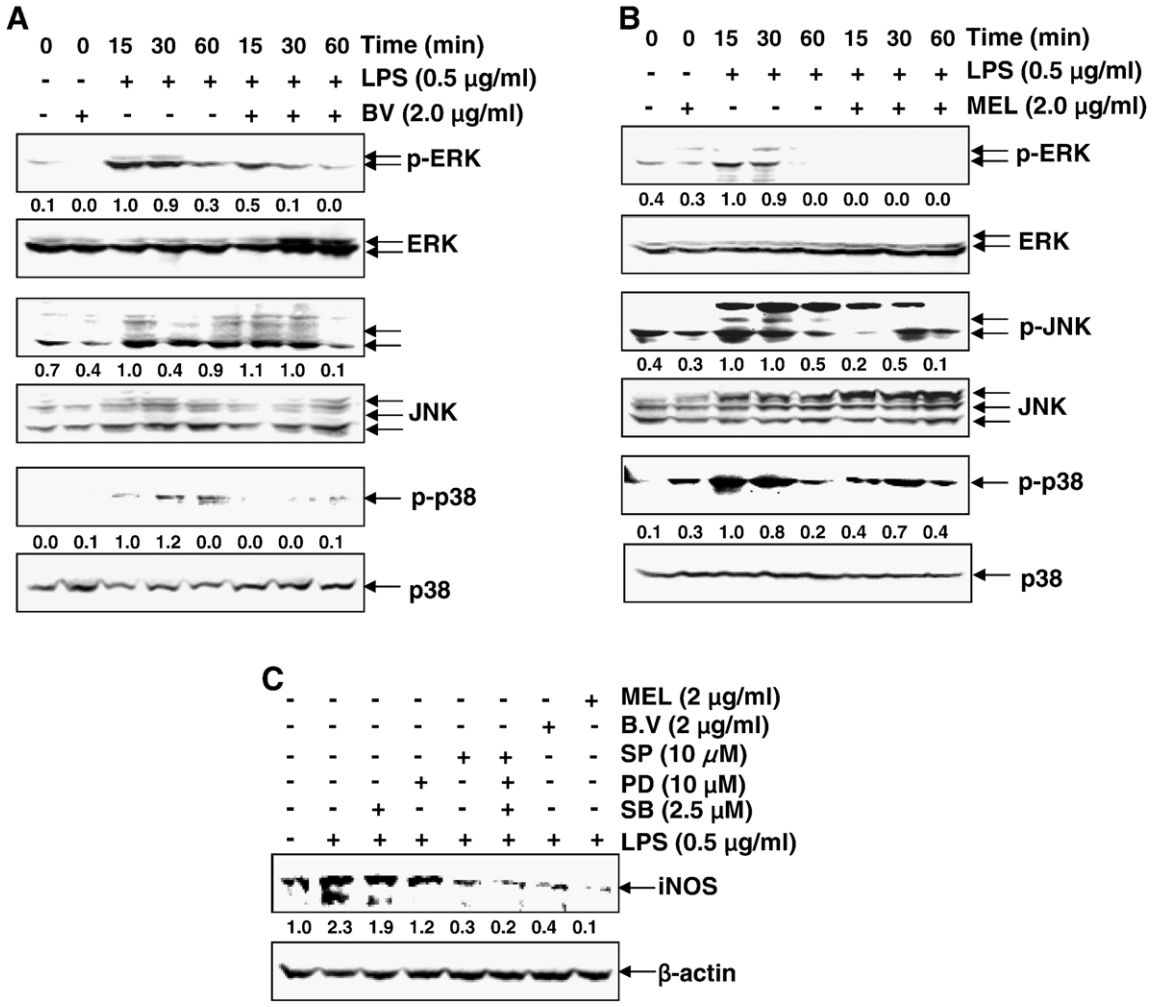

Fig. 4. Effect of BV and MEL on ERK, p38 MAPK and JNK activation induced by LPS in BV2 microglia. BV2 cells were treated with the indicated doses of BV (A) or MEL (B) 1 h prior to LPS treatment for the indicated times. (C) In a parallel experiment, BV2 cells were treated with 2.5 μM SB203580 (SB), 10 μM PD98059 (PD) and 10 μM SP600125 (SP) for 1 h prior to LPS treatment for the indicated times. Total protein was subjected to 10% SDS-PAGE followed by Western blotting using anti-ERK and anti-p38 MAPK (A), and anti-Akt. (B) Results are representative of those obtained from three independent experiments and the densitometric data (arbitrary) shown under the immunoblots are presented as fold changes, as compared with their respective control.

Therefore, we initially investigated the effect of BV and MEL on NO and iNOS expression in LPS-stimulated BV2 microglia. To analyze NO production, BV2 microglia were pretreated either BV or MEL (0.5, 1.0 and 2.0 μg/ml) for 1 h prior to stimulation with LPS (0.5 μg/ml). Following 24 h of LPS stimulation, the levels of NO in the culture media were determined using a series of the Griess assays. As shown in Fig. 1A, in the absence of BV or MEL treatment, LPS stimulation resulted in a marked induction of NO production (29±3 μM) as compared to untreated control cells (5±2 μM). However, pretreatment of BV or MEL significantly reduced the levels of LPS-induced NO production in a dose-dependent manner. Pretreatment of BV2 microglia with BV or MEL (each 2.0 μM) for 1 h prior to LPS stimulation markedly reduced NO production to 6±2 μM and 4±3 μM, respectively. We next investigated the effect of BV and MEL on iNOS expression. Exposure to LPS for 6 h resulted in a significant induction of both iNOS mRNA and protein in LPS-stimulated BV2 microglia. BV (Fig. 1B) and MEL (Fig. 1C) both significantly inhibited LPS-induced iNOS mRNA and protein expression. This inhibition generally occurred in a dose-dependent manner. Control GAPDH mRNA and β-actin protein levels were not affected by LPS, BV or MEL treatment. The results showed that BV and MEL inhibited NO transcription through a pathway upstream of the iNOS gene.

It is possible that the inhibition of NO production in LPS-stimulated cells could be ascribed to cytotoxic effects of BV and MEL. In order to exclude this possibility, we investigated the effect of BV and MEL in BV2 microglia viability. Under the experimental conditions described above, cell viability was determined to be >93%, as determined by MTT assays. Therefore, the inhibitory effect of BV and MEL on LPS-stimulated NO and iNOS production was not due to cytotoxic action on BV2 microglia.

3.2. BV and MEL inhibit LPS-induced NF-κB activation through regulation of IκB degradation

NF-κB, one of the Rel family transcription factors, is implicated in the regulation of iNOS [15]. In a previous study, LPS stimulation was shown to increase NF-κB activation through IκBα degradation [16]. Therefore, we performed luciferase assays and Western blot analysis to determine whether pretreatment with BV or MEL inhibited LPS-induced NF-κB activation and IκBα degradation. As shown in Fig. 2A, LPS stimulation significantly enhanced NF-κB activity by 6- to 7-fold over basal levels. Furthermore, BV and MEL pretreatment significantly inhibited LPS-induced NF-κB activity in a dose-dependent manner, corresponding to 7% and 12% inhibition at 0.5 μM, 56% and 51% at 1.0 μM, and 78% and 71% at 2.0 μM, respectively. We next investigated the effect of BV and MEL on LPS-induced NF-κB activation and IκBα degradation. BV2 microglia were pretreated with BV and MEL for 1 h and then stimulated with LPS for the indicated times. As shown in Fig. 2B and C, IκBα underwent significant degradation 15 min after LPS treatment. LPS treatment also induced translocation of the NF-κB p65 subunit into the nucleus, however the effect of LPS was significantly inhibited by pretreatment of BV (Fig. 2B) and MEL (Fig. 2C). We next investigated whether NF-κB inhibition can induce expression of iNOS in LPS-stimulated BV2 microglia. As

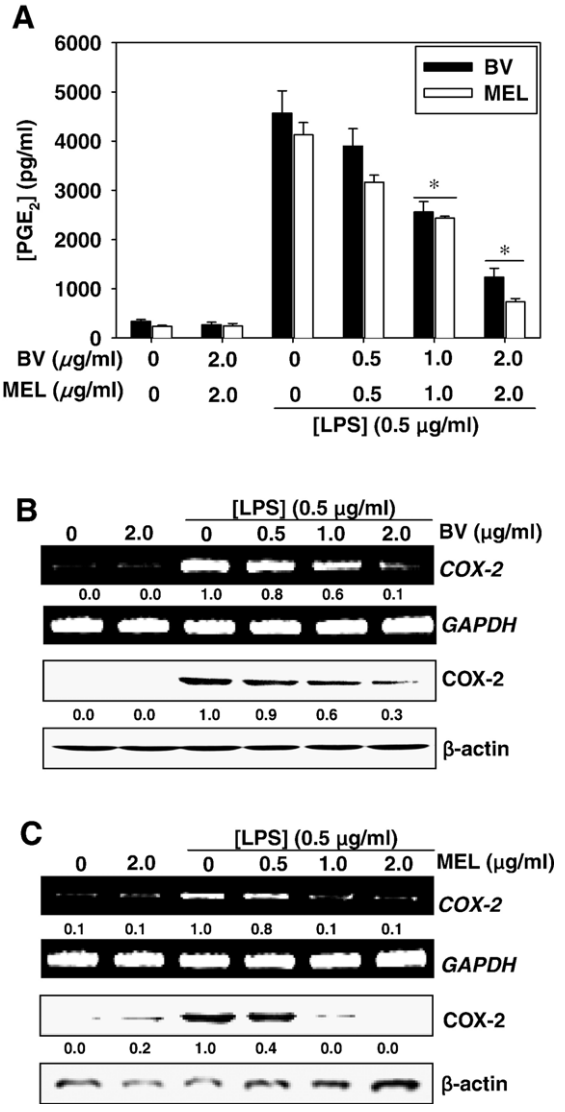

Fig. 5. Effect of BV and MEL on the levels of PGE_2 and COX-2 production by LPS-stimulated BV2 microglia. (A) BV2 microglia were stimulated with LPS for 24 h following a 1 h pretreatment with BV or MEL pretreatment and incubated for 24 h. The amounts of PGE_2 were determined using ELISA in culture medium. In a parallel experiment, after LPS treatment for 6 h after BV (B) and MEL (C) preincubation, the expression of levels of COX-2 mRNA and protein were measured by RT-PCR and immunoblots. Results are representative of those obtained from three independent experiments and the densitometric data (arbitrary) shown under the immunoblots and RT-PCR are presented as fold changes as compared with their respective control. Values are expressed as means±S.D. from three independent experiments. *$P<0.05$ indicates a significant difference from cells treated with LPS in the absence of BV or MEL.

shown in Fig. 2D, pretreatment of NF-κB inhibitor (PDTC) prevented the increase in iNOS expression by LPS. These results indicate the potential role of NF-κB as a possible mechanism for BV and MEL mediated suppression NO expression.

3.3. BV and MEL reduce LPS-induced NO production through downregulation of Akt and JNK

The Akt signaling molecule is known to regulate NF-κB activation through IκB degradation [17]. Therefore, the effect of BV and MEL on LPS-induced Akt activation was examined. As shown in Fig. 3A and B, phosphorylation of Akt was markedly increased 15 min after LPS stimulation, however BV and MEL pretreatment significantly inhibited LPS-induced Akt phosphorylation. To next evaluate the potential contribution of the Akt signal pathway in LPS-stimulated iNOS expression, we used the selective inhibitor LY294002 to block PI3K, an upstream molecule of Akt. Our results showed that the LPS-stimulated iNOS expression was inhibited by LY294002 treatment in a dose-dependent manner (Fig. 3C). The results show that BV and MEL significantly inhibit iNOS expression in LPS-stimulated BV2 microglia through downregulation of Akt phosphorylation.

MAPKs are the most important signaling molecules involved in regulating the synthesis and release of NO by activated microglia [18]. Therefore, it is possible that BV and MEL inhibit LPS-induced NO expression by downregulating MAPK signaling. To further evaluate the role of MAPK signaling pathways in iNOS expression, we investigated the ability of LPS to activate MAPKs in BV2 microglia. The results showed that BV and MEL pretreatment significantly inhibited the phosphorylation of ERK, JNK, and p38 MAPK in LPS-stimulated BV2 microglia (Fig. 4A and B). This suggests that BV and MEL are capable of disrupting the key signal transduction pathways activated by LPS in BV2 microglia, which subsequently prevents the production of iNOS. We also used a series of specific MAPK inhibitors, including PD98059, which has been shown to block activation of MAPK kinase 1 (the direct activator of ERK), SB203580, which is a specific inhibitor of p38 MAPK, and SP600125, which is a specific inhibitor of JNK. As shown in Fig. 4C, SP600125 treatment decreased LPS-induced iNOS expression, while SB203580 and PD98059 did not. The results indicate that JNK, but not ERK or p38 MAPK, is involved in LPS-stimulated NO production in BV2 microglia.

3.4. BV and MEL suppress the expression of COX-2 mRNA and protein, and PGE2 in LPS-stimulated BV2 microglia

We next attempted to assess whether BV and MEL could inhibit production of LPS-induced COX-2 and PGE_2 expression in BV2 microglia. Cells were pretreated with BV or MEL

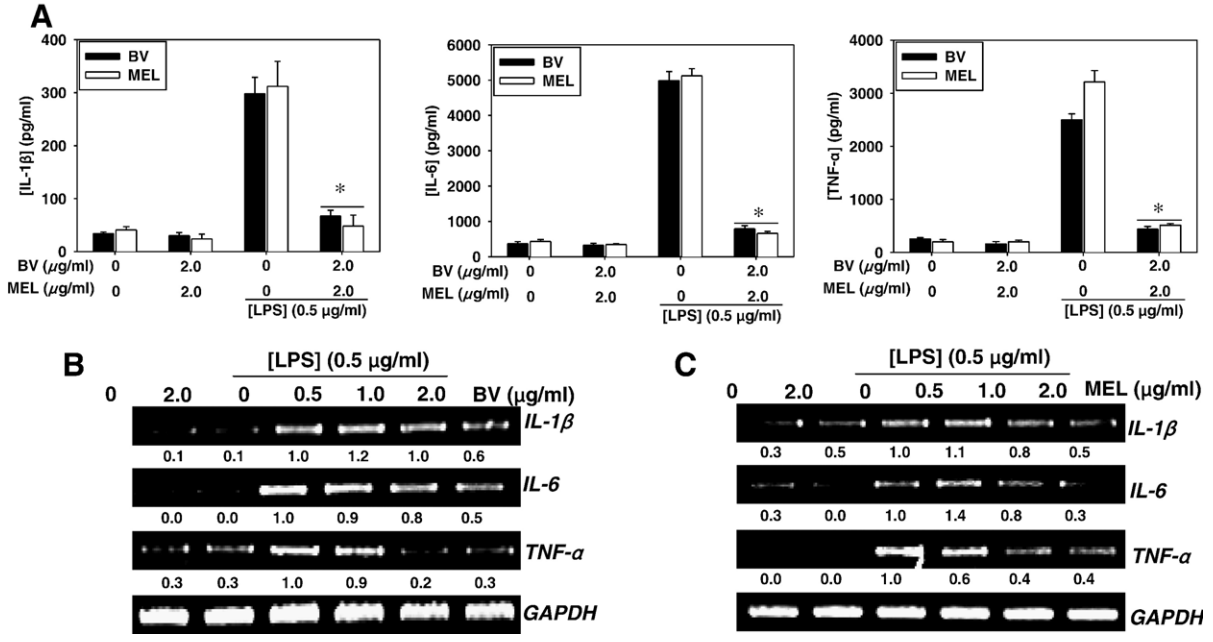

Fig. 6. Effect of BV and MEL on proinflammatory cytokines in LPS-stimulated microglia. BV2 cells were treated with the indicated concentrations of BV or MEL for 1 h prior to LPS treatment. Total RNA and supernatants were isolated at 6 h or 24 h after LPS treatment, respectively. (A) After incubation for 24 h, the supernatants were taken and the amounts of IL-1β, IL-6 and TNF-α were measured. (B) After 6 h of incubation following BV (B) or MEL (C) preincubation, the levels of IL-1β, IL-6 and TNF-α mRNA were determined by RT-PCR. Results are representative of those obtained from three independent experiments and the densitometric data (arbitrary) shown under the RT-PCR are presented as fold changes as compared with their respective control. Values are expressed as means ± S.D. from three independent experiments. *$P<0.05$ indicates a significant difference from cells treated with LPS in the absence of BV or MEL.

for 1 h and then stimulated with 0.5 μg/ml LPS. After incubation for 24 h, the cell culture medium was harvested, and the production of PGE_2 was measured using ELISA. As shown in Fig. 5A, the amount of PGE_2 in the culture medium increased from 267±53 pg/ml to 4567±456 pg/ml after 24 h of exposure to LPS. PGE_2 synthesis was decreased to 2561± 212 pg/ml and 1236±182 pg/ml after treatment with 1.0 μM and 2.0 μM of BV, respectively and to 2434±39 pg/ml and 734±67 pg/ml after treatment with at 1.0 μM and 2.0 μM of MEL, respectively. To further elucidate the effects of BV or MEL on COX-2, RT-PCR and immunoblot analyses were performed to determine the COX-2 mRNA and protein levels in BV2-stimulated microglia. In the absence of LPS stimulation, BV and MEL treatment did not result in detectable levels of COX-2 mRNA or protein, however, these levels were significantly induced after LPS treatment. As shown in Fig. 5B and C, BV or MEL elicited a concentration-dependent inhibition of LPS-stimulated COX-2 mRNA and protein. The results indicated that BV and MEL suppress LPS-induced PGE_2 synthesis through downregulation of COX-2 mRNA and protein.

3.5. BV and MEL repress expression of proinflammatory cytokines in LPS-stimulated BV2 microglia

We next incubated BV2 microglia with BV and MEL on the production of proinflammatory cytokines, including IL-1β, IL-6 and TNF-α. Following 24 h of LPS treatment, the levels of the cytokines in the culture media were measured by ELISA. BV and MEL alone had no effect on the production of IL-1β, IL-6 and TNF-α in normal BV2 microglia. However, the levels of IL-1β, IL-6 and TNF-α levels were increased in the culture media of LPS-stimulated BV2 microglia. Pretreatment with BV or MEL resulted in a dose-dependent decrease of cytokine production (Fig. 6A). We next determined whether BV and MEL could regulate the transcription of IL-1β, IL-6 and TNF-α mRNA. Consistent with the results obtained from the cytokine production immunoassay, the LPS-mediated increase in IL-1β, IL-6, and TNF-α mRNA levels were reduced by BV or MEL pretreatment (Fig. 6B and C), suggesting that BV or MEL negatively regulate the production of IL-1β, IL-6 and TNF-α at the transcriptional level.

4. Discussion

In this study, we first evaluated whether BV and MEL regulate NO and iNOS expression in LPS-stimulated BV2 microglia. The results of this study show that BV and MEL prevent LPS-induced NO and iNOS expression and NF-κB activation via IκBα regulation and Akt downregulation. BV and MEL also induced the downregulation of iNOS expression associated with inhibition of the JNK and Akt pathways. In addition, our results indicate that BV and MEL effectively inhibit the response to LPS stimulation. This inhibition is accompanied by a dose-dependent decrease in COX-2 mRNA and protein levels, which was evident by the reduction of PGE_2. BV and MEL also effectively inhibited transcription of LPS-induced proinflammatory cytokines, such as IL-1β, IL-6 and TNF-α. Furthermore, treatment with BV and MEL did not result in significant cytotoxicity in LPS-stimulated BV2 microglia, even at the highest concentration. This result suggests that the anti-inflammatory effects of BV and MEL were not due to cell death.

NF-κB is an important transcription factor for proinflammatory mediators [19,20]. The intracellular signaling mechanisms related to LPS stimulation have previously been studied in microglia [21]. Systemic injection of a sublethal LPS dose induces acute inflammation in susceptible strains of mice [22]. Furthermore, blockage of NF-κB transcriptional activity in the microglial nucleus can also suppress expression of iNOS, COX-2 and proinflammatory cytokines, such as IL-1β, IL-6 and TNF-α [23,24]. Moreover, BV and MEL significantly inhibited sodium nitroprusside, TNF-α or LPS-induced IκB release and NF-κB activity as well as the expressions of iNOS and COX-2, and the generation of NO and PGE_2 in macrophages and synoviocytes through binding of IκB kinases [25]. The promoter region of the murine gene encoding iNOS and COX-2 contains NF-κB binding sites [26,27], which suggests that the inhibitory effect of inflammatory gene expression is related to the inhibition of the DNA binding activity of NF-κB [25]. The activation of NF-κB has been also reported to be the most essential process iNOS by LPS stimulation in macrophages and synoviocytes [27]. Also, several antioxidant polyphenol compounds have been shown to directly inhibit the expression of NF-κB-dependent iNOS expression [28–30]. Therefore, we investigated the effect of BV and MEL on NF-κB activation and IκB degradation in BV2 microglia. Our data was supported by results showing that BV and MEL significantly inhibited the degradation of IκB and the subsequent production of iNOS in LPS-stimulated BV2 microglia. Previous data indicated that Akt mediates degradation of IκB through phosphorylation of IκB kinase and subsequently increases NF-κB activity [31]. In our previous studies, Akt-IκB-NF-κB signal cascades are essential for LPS-stimulated BV2 microglia in inflammatory responses [32,33]. Our data also showed that BV and MEL inhibit LPS-induced NO production and NF-κB activation via the Akt signal pathway.

MAPKs have previously been reported to regulate the production of iNOS and NO in LPS-stimulated

microglia [18,34]. This response is presumed to involve p38 MAPK, but not ERK, in various cell types [35], including microglia [36]. This response likely involves the activation of transcription factors that positively regulate the induction of inflammatory genes [37]. Inhibition of p38 MAPK is expected to be beneficial for injuries that involve microglia activation and inflammation. Specific inhibitors of p38 MAPK have proven to reduce inflammation, slow down microglia activation and provide neuroprotective effects [37]. This study explored possible anti-inflammatory mechanisms related to MAPKs and inhibition of iNOS expression by BV and MEL in LPS-stimulated BV2 microglia. We showed that BV and MEL significantly inhibit all MAPKs studied in this report, including ERK, p38 MAPK and JNK. Unlike previous studies, treatment with the p38 MAPK inhibitor (SB203580) could not inhibit LPS-induced iNOS expression. We found that a specific JNK inhibitor (SP600125) significantly inhibited iNOS expression in LPS-stimulated BV2 microglia. Nevertheless, a recent study reported that JNK is the major mediator of iNOS and inflammatory cytokine induction and inflammatory signaling in glial cells [38]. Although the reason for this discrepancy in the p38 and JNK pathway is not fully understood, our results implicate JNK as a major mediator of iNOS induction and inflammatory signaling in BV2 microglia.

In summary, to the best of our knowledge this study is the first to show that BV and MEL inhibit the production of NO, PGE2, and cytokines in LPS-stimulated BV2 microglia. This anti-inflammatory effect occurs through a downregulation of NF-κB activation via regulation of the IκBα pathway. We also show that BV and MEL attenuate NO production via inhibition of the JNK and Akt pathway. These findings portend the clinical importance of BV and MEL as compounds for treating LPS-mediated sepsis syndrome and inflammation disease, however further in vivo investigation of this activity is necessary to elaborate the mechanisms and permit full exploitation of its promise.

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References

[1] Kreutzberg GW. Microglia: a sensor for pathological events in the CNS. Trends Neurosci 1996;19:312–8.
[2] Chao CC, Hu S, Peterson PK. Glia, cytokines, and neurotoxicity. Crit Rev Neurobiol 1995;9:189–205.
[3] Gonzalez-Scarano F, Baltuch G. Microglia as mediators of inflammatory and degenerative diseases. Annu Rev Neurosci 1999;22:219–40.
[4] Nakajima K, Kohsaka S. Functional roles of microglia in the brain. Neurosci Res 1993;17:187–203.
[5] Eikelenboom P, van Gool WA. Neuroinflammatory perspectives on the two faces of Alzheimer's disease. J Neural Transm 2004;111:281–94.
[6] Liu B, Hong JS. Role of microglia in inflammation-mediated neurodegenerative diseases: mechanisms and strategies for therapeutic intervention. J Pharmacol Exp Ther 2003;304:1–7.
[7] Lariviere WR, Melzack R. The bee venom test: a new tonic-pain test. Pain 1996;66:271–7.
[8] Park HJ, Lee SH, Son DJ, Oh KW, Kim KH, Song HS, et al. Antiarthritic effect of bee venom: inhibition of inflammation mediator generation by suppression of NF-κB through interaction with the p50 subunit. Arthritis Rheum 2004;50:3504–15.
[9] Lee JD, Kim SY, Kim TW, Lee SH, Yang HI, Lee DI, et al. Anti-inflammatory effect of bee venom on type II collagen-induced arthritis. Am J Chin Med 2004;32:361–7.
[10] Jang HS, Kim SK, Han JB, Ahn HJ, Bae H, Min BI. Effects of bee venom on the pro-inflammatory responses in RAW264.7 macrophage cell line. J Ethnopharmacol 2005;99:157–60.
[11] Moon DO, Park SY, Heo MS, Kim KC, Park C, Ko WS, et al. Key regulators in bee venom-induced apoptosis are Bcl-2 and caspase-3 in human leukemic U937 cells through downregulation of ERK and Akt. Int Immunopharmacol 2006;6:1796–807.
[12] Tha KK, Okuma Y, Miyazaki H, Murayama T, Uehara T, Hatakeyama R, et al. Changes in expressions of proinflammatory cytokines IL-1β, TNF-α and IL-6 in the brain of senescence accelerated mouse (SAM) P8. Brain Res 2000;885:25–31.
[13] Kang JH, Sung MK, Kawada T, Yoo H, Kim YK, Kim JS, et al. Soybean saponins suppress the release of proinflammatory mediators by LPS-stimulated peritoneal macrophages. Cancer Lett 2005;230:219–27.
[14] Corradin SB, Mauel J, Donini SD, Quattrocchi E, Ricciardi-Castagnoli P. Inducible nitric oxide synthase activity of cloned murine microglial cells. Glia 1993;7:255–62.
[15] Baeuerle PA, Henkel T. Function and activation of NF-κB in the immune system. Annu Rev Immunol 1994;12:141–79.
[16] Ghosh S, Karin M. Missing pieces in the NF-κB puzzle. Cell 2002;109:S81–96.
[17] Madrid LV, Wang CY, Guttridge DC, Schottelius AJ, Baldwin Jr AS, Mayo MW. Akt suppresses apoptosis by stimulating the transactivation potential of the Rel A/p65 subunit of NF-κB. Mol Cell Biol 2000;20:1626–38.
[18] Koistinaho M, Koistinaho J. Role of p38 and p44/42 mitogen-activated protein kinases in microglia. Glia 2002;40:175–83.
[19] Siebenlist U, Franzoso G, Brown K. Structure, regulation and function of NF-κB. Annu Rev Cell Biol 1994;10:405–55.
[20] Kuprash DV, Udalova IA, Turetskaya RL, Rice NR, Nedospasov SA. Conserved kappa B element located downstream of the tumor necrosis factor alpha gene: distinct NF-κB binding pattern and enhancer activity in LPS activated murine macrophages. Oncogene 1995;11:97–106.
[21] Ozato K, Tsujimura H, Tamura T. Toll-like receptor signaling and regulation of cytokine gene expression in the immune system. Biotechniques 2002:66–72 [Suppl.].

[22] Rahman I, Macnee W. Role of transcription factors in inflammatory lung diseases. Thorax 1998;53:601–12.
[23] Petrova TV, Akama KT, van Eldik LJ. Cyclopentenone prostaglandins suppress activation of microglia: down-regulation of inducible nitric oxide synthase by 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2. Proc Natl Acad Sci U S A 1999;96:4668–73.
[24] Ye SM, Johnson RW. Regulation of interleukin-6 gene expression in brain of aged mice by nuclear factor κB. J Neuroimmunol 2001;117:87–96.
[25] Park HJ, Son DJ, Lee CW, Choi MS, Lee US, Song HS, et al. Melittin inhibits inflammatory target gene expression and mediator generation via interaction with IκB kinase. Biochem Pharmacol 2007;73:237–47.
[26] Tak PP, Gerlag DM, Aupperle KR, Geest DA, Overbeek M, Bennett BL, et al. Inhibitor of nuclear factor kappaB kinase beta is a key regulator of synovial inflammation. Arthritis Rheum 2001;44:1897–907.
[27] Xie QW, Kashiwabara Y, Nathan C. Role of transcription factor NF-κB/Rel in induction of nitric oxide synthase. J Biol Chem 1994;269:4705–8.
[28] Park JE, Kang YJ, Park MK, Lee YS, Kim HJ, Seo HG, et al. Enantiomers of higenamine inhibit LPS-induced iNOS in a macrophage cell line and improve the survival of mice with experimental endotoxemia. Int Immunopharmacol 2006;6: 226–33.
[29] Hseu YC, Wu FY, Wu JJ, Chen JY, Chang WH, Lu FJ, et al. Anti-inflammatory potential of Antroida camphorate through inhibition of iNOS, COX-2 and cytokines via the NF-κB pathway. Int Immunopharmacol 2005;5:1914–25.
[30] Bi XL, Yang JY, Dong YX, Wang JM, Cui YH, Ikeshima T, et al. Resveratrol inhibits nitric oxide and TNF-α production by lipopolysaccharide-activated microglia. Int Immunopharmacol 2005;5:185–93.
[31] Zimmermann S, Moelling K. Phosphorylation and regulation of Raf by Akt (protein kinase B). Science 1999;286:1741–4.
[32] Moon DO, Chi YH, Kim ND, Park YM, Kim GY. Anti-inflammatory effects of β-lapachone in lipopolysaccharide-stimulated BV2 microglia. Int Immunopharmacol 2007;7:506–14.
[33] Moon DO, Kim KC, Jin CY, Han MH, Park C, Lee KJ, et al. Inhibitory effects of eicosapentaenoic acid on lipopolysaccahride-induced activation in BV2 microglia. Int Immunopharmacol 2007;7:222–9.
[34] Pyo H, Jou I, Jung S, Hong S, Joe EH. Mitogen-activated protein kinases activated by lipopolysaccharide and beta amyloid in cultured rat microglia. Neuroreport 1998;9:871–4.
[35] Chen CC, Wang JK. p38 but not p44/p42 mitogen-activated protein kinase is required for nitric oxide synthase induction mediated by lipopolysaccharide in RAW 264.7 macrophages. Mol Pharmacol 1999;55:481–8.
[36] Bhat NR, Zhang P, Lee JC, Hogan EL. Extracellular signal-regulated kinase and p38 subgroups of mitogen-activated protein kinase regulate inducible nitric oxide synthase and tumor necrosis factor-α gene expression in endotoxin-stimulated primary glial cultures. J Neurosci 1998;18:1633–41.
[37] Wesselborg S, Bauer MK, Vogt M, Schmitz ML, Schulze-Osthoff K. Activation of transcription factor NF-κB and p38 mitogen-activated protein kinase is mediated by distinct and separate stress effector pathways. J Biol Chem 1997;272:12422–9.
[38] Pawate S, Bhat NR. c-Jun N-terminal kinase (JNK) regulation of iNOS expression in glial cells: predominant role of JNK1 isoform. Antioxid Redox Signal 2006;8:903–9.