

7,8-Dihydroxyflavone attenuates the release of pro-inflammatory mediators and cytokines in lipopolysaccharide-stimulated BV2 microglial cells through the suppression of the NF- κ B and MAPK signaling pathways

HYE YOUNG PARK¹, CHEOL PARK², HYE JIN HWANG^{3,5}, BYUNG WOO KIM^{4,5}, GI-YOUNG KIM⁶, CHEOL MIN KIM⁷, NAM DEUK KIM¹ and YUNG HYUN CHOI^{5,8}

¹Department of Pharmacy, Busan National University, Busan 609-735; Departments of ²Molecular Biology, ³Food and Nutrition and ⁴Life Science and Biotechnology, and ⁵Anti-Aging Research Center and Blue-Bio Industry RIC, Donggeui University, Busan 614-714; ⁶Laboratory of Immunobiology, Department of Marine Life Sciences, Jeju National University, Jeju 690-756; ⁷Research Center for Anti-Aging Technology Development and Department of Biochemistry, Busan National University College of Medicine, Yangsan 626-870; ⁸Department of Biochemistry, Donggeui University College of Oriental Medicine, Busan 614-052, Republic of Korea

Received October 9, 2013; Accepted February 3, 2014

DOI: 10.3892/ijmm.2014.1652

Abstract. 7,8-Dihydroxyflavone (7,8-DHF), a member of the flavonoid family, has received considerable attention as a selective tyrosine kinase receptor B agonist. However, the pharmacological mechanisms responsible for its anti-inflammatory activities in microglial cells have yet to be elucidated. In this study, we evaluated the anti-inflammatory effects of this compound on the production of inflammatory mediators and cytokines in lipopolysaccharide (LPS)-stimulated murine BV2 microglial cells. At non-toxic concentrations, 7,8-DHF attenuated the production of nitric oxide (NO) and prostaglandin E₂ (PGE₂), by inhibiting inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2) expression, respectively. Furthermore, the release and expression of inflammatory cytokines, including tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β), were inhibited by 7,8-DHF. In addition, 7,8-DHF suppressed nuclear factor- κ B (NF- κ B) translocation and its transcriptional activity by blocking I κ B (I κ B)- α degradation; in addition, it exerted suppressive effects on the phosphorylation of mitogen-activated protein kinases (MAPKs). These results indicate that 7,8-DHF possesses therapeutic potential against neurodegenerative diseases that involve microglial activation.

Introduction

Microglia are resident macrophage-like cells in the brain, and have been suggested to play a major role in host defense and tissue repair in the central nervous system (CNS) (1,2). Under pathological conditions, activated microglia release pro-inflammatory mediators, including nitric oxide (NO), prostaglandin E₂ (PGE₂), reactive oxygen species (ROS) and pro-inflammatory cytokines (2,3). The overproduction of these inflammatory mediators and cytokines causes severe forms of various neurodegenerative diseases, such as Alzheimer's disease (AD), cerebral ischemia, multiple sclerosis and trauma. Not surprisingly, activated microglia have been shown to be a major cellular source of pro-inflammatory and/or cytotoxic factors that cause neuronal damage in the CNS (4-6). Thus, a decrease in the number of pro-inflammatory mediators and cytokines in microglial cells may attenuate the severity of these disorders (7-9).

Flavonoids are polyphenolic compounds that are present in high concentrations in fruits and vegetables (10-12). They have multiple pharmacological activities, such as antioxidant, anti-inflammatory, immunomodulatory and antitumor effects (13-15). A number of studies have demonstrated that flavonoids protect against neuronal cell death, enhance existing neuronal function and stimulate neuronal regeneration (16-18). Among these flavonoids, 7,8-dihydroxyflavone (7,8-DHF) is a selective tyrosine kinase receptor B (TrkB) agonist that can cross the blood-brain barrier (19-21). In a recent study, this flavonoid was demonstrated to have neurotrophic activities in various neurological diseases, such as stroke and Parkinson's disease (19). Although several studies have indicated that 7,8-DHF has antioxidant and anti-inflammatory activities (21-24), to our knowledge, no studies to date have investigated the molecular mechanisms underlying its anti-inflammatory effects in microglial cells.

Correspondence to: Dr Yung Hyun Choi, Department of Biochemistry, Donggeui University College of Oriental Medicine, San 45, Yangjung-dong, Busanjin-gu, Busan 614-052, Republic of Korea
E-mail: choiyh@deu.ac.kr

Key words: 7,8-dihydroxyflavone, BV2 microglia, anti-inflammation, nuclear factor- κ B, mitogen-activated protein kinases

In the present study, we investigated the inhibitory effects of 7,8-DHF on the production of inflammatory mediators and the mechanisms through which it induces these anti-inflammatory effects on lipopolysaccharide (LPS)-stimulated murine BV2 microglial cells.

Materials and methods

Materials. 7,8-DHF was obtained from Professor Jin Won Hyun of Jeju National University (Jeju, Korea). LPS, Tween-20, bovine serum albumin (BSA) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Rabbit anti-inducible nitric oxide synthase (iNOS), cyclooxygenase (COX)-2, nuclear factor- κ B (NF- κ B) p65 and I κ B- α polyclonal antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Antibodies against lamin B, extracellular signal-regulated kinase (ERK), phosphorylated (p)-ERK, p38 mitogen-activated protein kinase (MAPK), p-p38 MAPK, c-Jun N-terminal kinase (JNK), and p-JNK were purchased from Cell Signaling Technology (Danvers, MA, USA). The peroxidase-labeled donkey anti-rabbit immunoglobulin and peroxidase-labeled sheep anti-mouse immunoglobulin were purchased from Amersham Corp. (Arlington Heights, IL, USA). Dulbecco's modified Eagle's medium (DMEM) containing l-glutamine (200 mg/l), fetal bovine serum (FBS), penicillin and streptomycin, Triton X-100, and all other chemicals were purchased from Gibco (Grand Island, NY, USA).

Cell culture. BV2 microglial cells were maintained in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 μ g/ml streptomycin at 37°C in a humidified incubator with 5% CO₂. Confluent cultures were passaged by trypsinization. Cells used in the experiments were washed twice with warm DMEM (without phenol red) and treated in serum-free medium for at least 4 h prior to the treatments. In all experiments, cells were treated with various concentrations of 7,8-DHF for the indicated times prior to the addition of LPS (0.5 μ g/ml).

Cell viability assay. Cell viability was measured based on the formation of blue formazan that was metabolized from colorless MTT by mitochondrial dehydrogenases, which are active only in live cells. In brief, BV2 cells (3 \times 10⁵ cells/well) were seeded and treated with various reagents for the indicated periods of time. After the various treatments, the medium was removed and the cells were incubated with 0.5 mg/ml of MTT solution. Following incubation for 2 h at 37°C and 5% CO₂, the supernatant was removed and the formation of formazan was measured at 540 nm using a microplate reader (Dynatech MR-7000; Dynatech Laboratories, Chantilly, VA, USA).

NO production. Concentrations of NO in the culture supernatants were determined by measuring nitrite, which is a major stable product of NO, using Griess reagent (Sigma-Aldrich Chemical Co.). Cells (5 \times 10⁵ cells/ml) were stimulated in 24-well plates for 24 h, and then 100 μ l of each culture medium was mixed with an equal volume of Griess reagent (1% sulfanilamide/0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride/2.5% H₃PO₄). Nitrite levels were determined using an ELISA plate reader at 540 nm, and nitrite concentrations

were calculated by reference to a standard curve generated by known concentrations of sodium nitrite (25).

RNA isolation and RT-PCR. Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA (1.0 μ g) obtained from the cells was reverse-transcribed using M-MLV reverse transcriptase (Promega, Madison, WI, USA) to produce cDNAs. The *iNOS*, *COX-1*, *COX-2*, *IL-1 β* and *TNF- α* genes were amplified from the cDNA by PCR. The PCR primers were as follows: mouse *iNOS* (5'-ATG TCC GAA GCA AAC ATC AC-3' and 5'-TAA TGT CCA GGA AGT AGG TG-3'); *COX-2* (5'-CAG CAA ATC CTT GCT GTT CC-3' and 5'-TGG GCA AAG AAT GCA AAC ATC-3'); *IL-1 β* (5'-ATG GCA ACT GTT CCT GAA CTC AAC T-3' and 5'-TTT CCT TTC TTA GAT ATG GAC AGG AC-3'); and *TNF- α* (5'-ATG AGC ACA GAA AGC ATG ATC-3' and 5'-TAC AGG CTT GTC ACT CGA ATT-3'). Following amplification, the PCR reactions were electrophoresed on 1% agarose gels.

Protein extraction and western blot analysis. The cells were washed 3 times with phosphate-buffered saline (PBS) and lysed in lysis buffer (1% Triton X-100, 1% deoxycholate and 0.1% NaN₃) containing protease inhibitor cocktail tablets (Roche Diagnostics GmbH, Mannheim, Germany). In a parallel experiment, nuclear proteins were prepared using nuclear extraction reagents (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. Equal amounts of protein were separated on SDS-polyacrylamide gels, transferred onto nitrocellulose membranes (Schleicher and Schuell, Keene, NH, USA) by electroblotting, and subsequently blocked in 5% bovine serum albumin (BSA)-Tris-buffered saline Tween-20 (TBST, 100 mM Tris, pH 8.0, 150 mM NaCl and 0.1% Tween-20) for 1 h at room temperature. Following incubation with the appropriate primary antibodies for 1 h, the membranes were incubated for 1 h at room temperature with secondary antibodies conjugated to horseradish peroxidase. Following 3 washes in TBST, immunoreactive bands were visualized using an ECL detection system (Pierce).

Cytokine assays. The levels of IL-1 β and TNF- α were measured by ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Briefly, the BV2 microglial cells (5 \times 10⁵ cells/ml) were plated in 24-well plates and pre-treated with the indicated concentrations of 7,8-DHF for 1 h prior to treatment with 0.5 μ g/ml LPS for 24 h. One hundred microliters of culture-medium supernatants were collected for determination of the IL-1 β and TNF- α concentrations by ELISA, as previously described (26).

NF- κ B luciferase assay. A total of 1 \times 10⁶ BV2 cells were transfected with 2 μ g NF- κ B-luciferase reporter plasmids (BD Biosciences, San Jose, CA, USA) using Lipofectamine according to the manufacturer's instructions (Gibco). Following incubation with DNA-Lipofectamine mixtures, the cells were pre-incubated in the presence or absence of 7,8-DHF for 1 h prior to being stimulated with LPS for 0.5 or 1 h. The cells were then washed twice with PBS and lysed with reporter lysis buffer (Promega). After vortexing and centrifugation at 12,000 \times g for 1 min at 4°C, the supernatant was stored at -70°C for use in the luciferase assay. After 20 μ l of the cell extract was mixed with

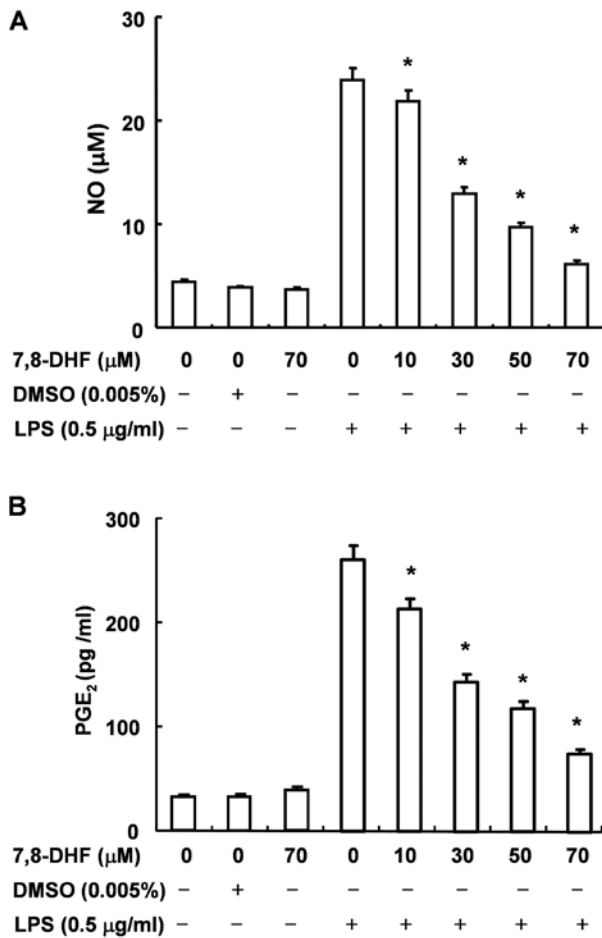


Figure 1. Inhibition of nitric oxide (NO) and prostaglandin E₂ (PGE₂) production by 7,8-dihydroxyflavone (7,8-DHF) in lipopolysaccharide (LPS)-stimulated BV2 microglial cells. BV2 microglial cells were pre-treated with various concentrations of 7,8-DHF (10-70 µM) for 1 h prior to incubation with LPS (0.5 µg/ml) for 24 h. (A) Nitrite content was measured using Griess reaction and (B) PGE₂ concentration was measured in culture medium using a commercial ELISA kit. Each value indicates the mean ± standard deviation (SD) and is representative of results obtained from 3 independent experiments. *P<0.05, significant difference from the value obtained for cells treated with LPS in the absence of 7,8-DHF.

100 µl of the luciferase assay reagent at room temperature, the mixture was measured on a microplate luminometer LB96V (Perkin-Elmer, Foster City, CA, USA).

Statistical analyses. Data values represent the means ± standard deviation (SD). Statistical significance was determined using an analysis of variance that was followed by a Student's t-test. A value of P<0.05 was considered to indicate a statistically significant difference.

Results

7,8-DHF attenuates NO and PGE₂ production in LPS-stimulated BV2 microglial cells. To evaluate the inhibitory effects of 7,8-DHF on LPS-stimulated NO production in BV2 microglial cells, NO levels in the cell culture medium were measured by Griess assay. For this experiment the, BV2 microglial cells were pre-treated with various concentrations of 7,8-DHF (10-70 µM) for 1 h prior to being stimulated with LPS (0.5 µg/ml). According to the NO detection assay, LPS

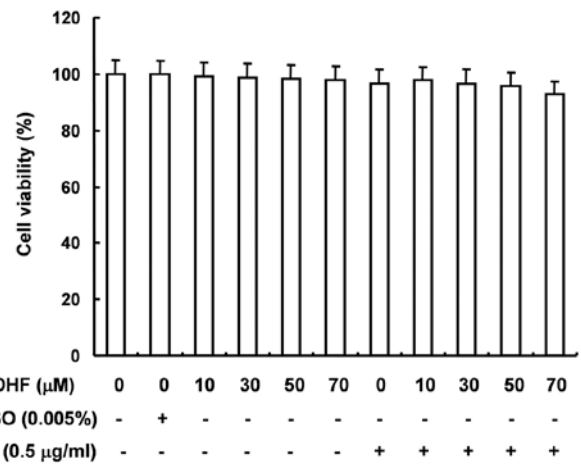


Figure 2. Effects of 7,8-dihydroxyflavone (7,8-DHF) and lipopolysaccharide (LPS) on the viability of BV2 microglial cells. Cells were treated with the indicated concentrations of 7,8-DHF or LPS (0.5 µg/ml) alone, or pre-treated with the indicated concentrations of 7,8-DHF for 1 h prior to exposure to LPS. After 24 h, cell viability was assessed using MTT reduction assays. Each value indicates the mean ± standard deviation (SD) and is representative of results obtained from 3 independent experiments.

alone was able to markedly induce NO production by the cells. Pre-treatment with 7,8-DHF significantly repressed the levels of NO production in LPS-stimulated BV2 microglial cells in a concentration-dependent manner (Fig. 1A).

As PGE₂ represents another important inflammatory mediator that is produced from the conversion of arachidonic acid by COXs, we then evaluated the inhibitory effects of 7,8-DHF on PGE₂ levels present in the supernatant by ELISA under the same conditions. The amount of PGE₂ present in the culture medium increased from the initial levels after 24 h of exposure to LPS alone. A marked repression was observed following the administration of 7,8-DHF (Fig. 1B).

In order to exclude the cytotoxic effects of 7,8-DHF on the growth of BV2 microglial cells, the cells were exposed to various concentrations of 7,8-DHF for 24 h in the presence or absence of LPS, and cell viability was then measured by MTT assay. The concentrations of 7,8-DHF used to inhibit NO and PGE₂ production did not affect cell viability (Fig. 2). These results clearly indicated that the inhibition of NO and PGE₂ production in LPS-stimulated BV2 cells was not due to a cytotoxic action of 7,8-DHF.

7,8-DHF inhibits LPS-stimulated iNOS and COX-2 expression in LPS-stimulated BV2 microglial cells. We performed RT-PCR and western blot analyses to determine whether the inhibition of NO and PGE₂ production by 7,8-DHF in LPS-stimulated BV2 cells was associated with the decreased levels of iNOS and COX-2 expression. The levels of iNOS and COX-2 proteins were markedly induced after 24 h of exposure to LPS, and 7,8-DHF significantly inhibited iNOS and COX-2 protein expression in the LPS-stimulated BV2 microglial cells in a concentration-dependent manner (Fig. 3A). Next, to determine whether or not 7,8-DHF suppresses the LPS-mediated induction of iNOS and COX-2 at the transcriptional level, the effects of 7,8-DHF on iNOS and COX-2 mRNA expression were evaluated. The RT-PCR data showed that the reduction in iNOS and COX-2 mRNAs correlated with the reduction in the

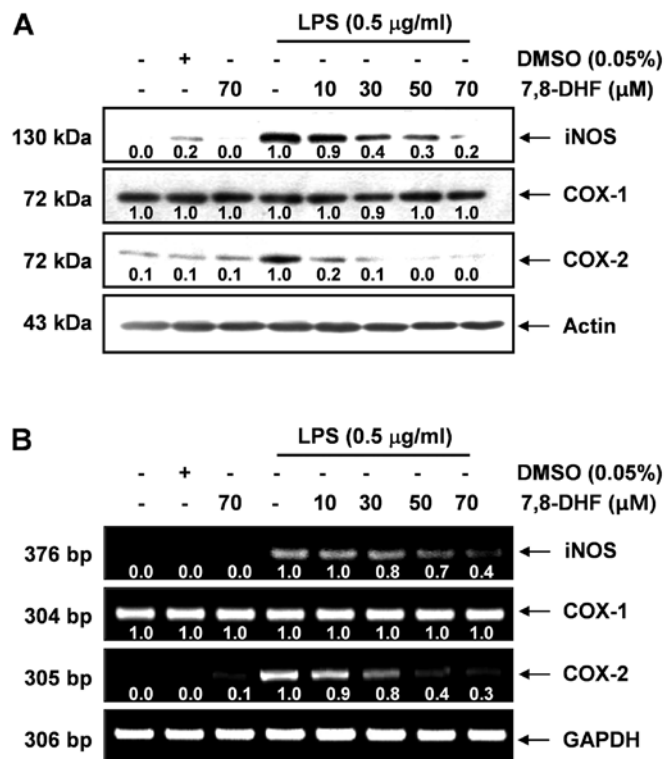


Figure 3. Inhibition of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) expression by 7,8-dihydroxyflavone (7,8-DHF) in lipopolysaccharide (LPS)-stimulated BV2 microglial cells. (A) BV2 microglial cells were pre-treated with the indicated concentrations of 7,8-DHF 1 h prior to incubation with LPS (0.5 µg/ml) for 24 h. Cell lysates were then prepared and western blot analysis was performed using antibodies specific for murine iNOS and COX-2. (B) Following exposure to LPS for 6 h, total RNA was prepared for RT-PCR of iNOS and COX-2 gene expression in LPS-stimulated BV2 microglial cells. Actin and GAPDH were used as internal controls for western blot analysis and RT-PCR, respectively. The numbers represent the average densitometric analyses as compared with (A) actin and (B) GAPDH in, at a minimum, 2 or 3 different experiments.

corresponding protein levels (Fig. 3B). These results suggested that the 7,8-DHF-induced reductions in the expression of *iNOS* and *COX-2* were the cause of the inhibition of NO and PGE₂ production.

7,8-DHF suppresses LPS-induced IL-1β and TNF-α production in LPS-stimulated BV2 microglial cells. Next, we analyzed the effects of 7,8-DHF on the production of pro-inflammatory cytokines, such as IL-1β and TNF-α. The levels of IL-1β and TNF-α production were markedly increased in the culture medium of LPS-stimulated BV2 microglial cells (Fig. 4). Pre-treatment with 7,8-DHF resulted in a significant decrease in the release of these pro-inflammatory cytokines in a concentration-dependent manner. In a parallel experiment, using RT-PCR, we investigated the effects of 7,8-DHF on LPS-induced *IL-1β* and *TNF-α* mRNA expression. *IL-1β* and *TNF-α* mRNA transcription also decreased following treatment with 7,8-DHF (Fig. 5). These results suggest that 7,8-DHF is effective in the suppression of pro-inflammatory cytokine production through the alteration of the transcription levels of *IL-1β* and *TNF-α* in activated microglial cells.

7,8-DHF blocks LPS-induced NF-κB activity in LPS-stimulated BV2 microglial cells. NF-κB is one of the important transcription

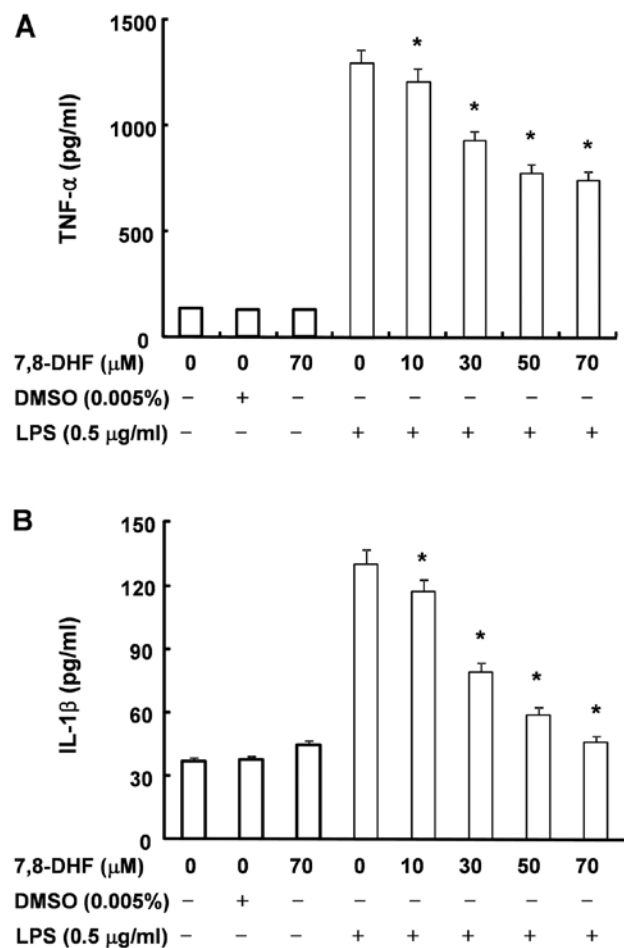


Figure 4. Effect of 7,8-dihydroxyflavone (7,8-DHF) on lipopolysaccharide (LPS)-stimulated interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) production in BV2 microglial cells. BV2 microglial cells were pre-treated with various concentrations of 7,8-DHF for 1 h prior to exposure to LPS (0.5 µg/ml). Following incubation for 24 h, the levels of (A) IL-1β and (B) TNF-α present in the supernatants were measured. Each value indicates the mean ± standard deviation (SD) and is representative of results obtained from 3 independent experiments. *P<0.05, significant difference from the value obtained for cells treated with LPS in the absence of 7,8-DHF.

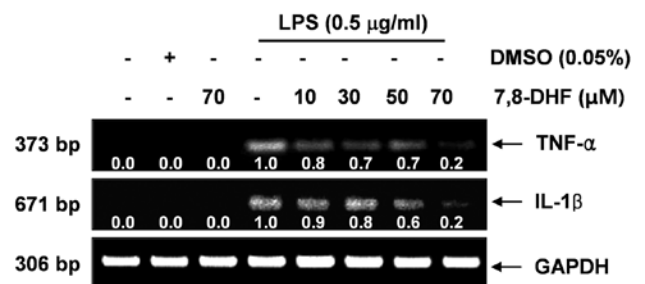


Figure 5. Effect of 7,8-dihydroxyflavone (7,8-DHF) on lipopolysaccharide (LPS)-stimulated interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) expression in BV2 microglial cells. BV2 microglial cells were pre-treated with various concentrations of 7,8-DHF for 1 h prior to exposure to LPS (0.5 µg/ml), and total RNA was isolated at 6 h following exposure to LPS. The levels of IL-1β and TNF-α mRNA were determined by RT-PCR. GAPDH was used as an internal control. The numbers represent the average densitometric analyses as compared with GAPDH in, at a minimum, 2 or 3 different experiments.

factors that regulate the gene expression of pro-inflammatory mediators; therefore, we wished to determine whether 7,8-DH

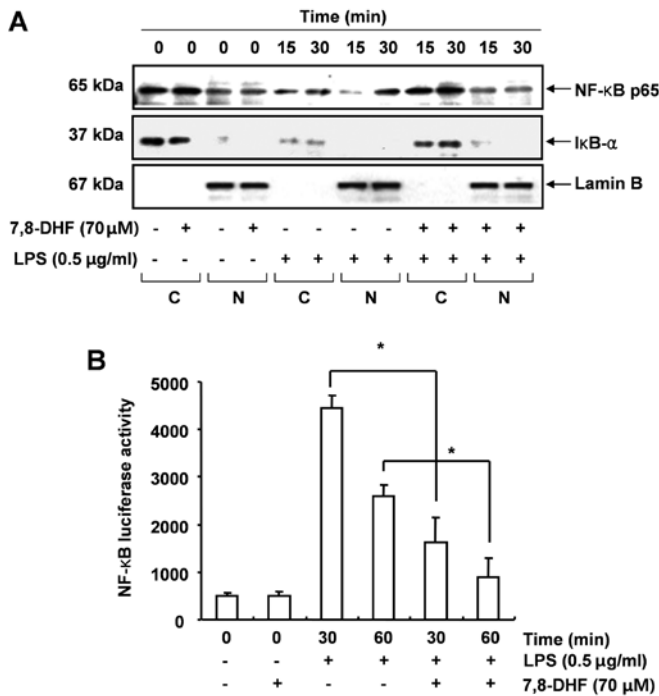


Figure 6. Effects of 7,8-dihydroxyflavone (7,8-DHF) on lipopolysaccharide (LPS)-induced NF-κB activation in BV2 microglial cells. (A) Cells were treated with 70 μM 7,8-DHF for 1 h prior to exposure to LPS (0.5 μg/ml) for the indicated times. Nuclear (N) and cytosolic (C) proteins were subjected to 10% SDS-polyacrylamide gels followed by western blot analysis using anti-NF-κB p65 and anti-IκB-α antibodies. Results are representative of those obtained from 2 independent experiments. Lamin B was used as an internal control for the nuclear fraction. (B) Transfected BV2 microglial cells with NF-κB-luciferase reporter plasmids were pre-treated with 70 μM 7,8-DHF for 1 h and then stimulated with LPS (0.5 μg/ml) for 0.5 or 1 h. NF-κB activity was expressed as luciferase activity. Each value indicates the mean ± standard deviation (SD) and is representative of results obtained from 3 independent experiments. *P<0.05, significant difference from cells treated with LPS in the absence of 7,8-DHF.

affects NF-κB activity. The results from immunoblot analysis shown in Fig. 6A revealed that the amount of NF-κB p65 in the nucleus was markedly increased following exposure to LPS alone. LPS-induced p65 levels in the nuclear fractions were reduced by 7,8-DHF pre-treatment (Fig. 7A). In addition, IκB-α was markedly degraded at 15 min following exposure to LPS; however this LPS-induced IκB-α degradation was significantly reversed by 7,8-DHF. These results suggest that 7,8-DHF inhibits NF-κB activation in BV2 microglial cells through the suppression of IκB degradation and the nuclear translocation of NF-κB.

We then tried to confirm the inhibition of LPS-induced NF-κB activation by 7,8-DHF using a luciferase assay. For this experiment, the BV2 cells transfected with NF-κB-luciferase reporter plasmids were pre-treated with 7,8-DHF for 1 h and stimulated with LPS for 0.5 or 1 h, and then luciferase activity was measured. LPS markedly enhanced NF-κB activity up to approximately 8-fold over the basal level, while 7,8-DHF significantly inhibited the LPS-induced increase in NF-κB activity (Fig. 6B). Taken together, the above findings show that the anti-inflammatory effects of 7,8-DHF in LPS-stimulated BV2 cells involve the NF-κB pathway.

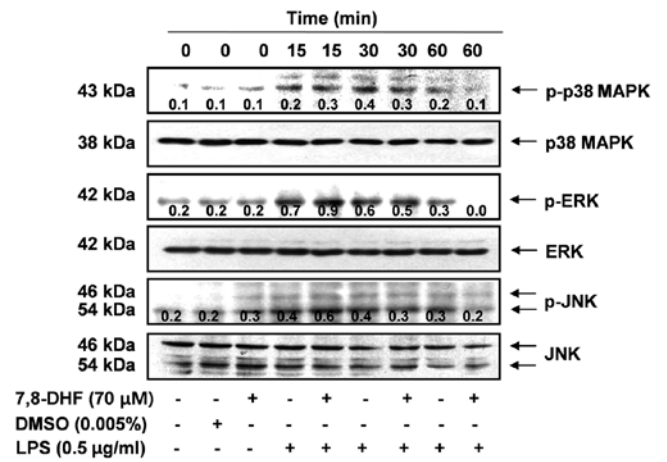


Figure 7. Effect of 7,8-dihydroxyflavone (7,8-DHF) on lipopolysaccharide (LPS)-induced mitogen-activated protein kinase (MAPK) activation in BV2 microglial cells. BV2 microglial cells were pre-treated with 70 μM 7,8-DHF for 1 h prior to exposure to LPS (0.5 μg/ml), and total proteins were isolated at the indicated times following exposure to LPS. The proteins were subjected to SDS-polyacrylamide gels followed by western blot analysis using the indicated antibodies, and an ECL detection system. Results are representative of those obtained from 2 independent experiments. The numbers represent the average densitometric analyses as compared with total p38 MAPK, extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) in, at a minimum, 2 or 3 different experiments.

7,8-DHF reduces the LPS-induced phosphorylation of MAPKs in LPS-stimulated BV2 microglial cells. MAPK pathways are known to be important for the expression of pro-inflammatory mediators and cytokines. Therefore, MAPKs act as specific targets for inflammatory responses. To examine whether the inhibition of inflammation by 7,8-DHF is mediated through MAPK pathways, we examined the effects of 7,8-DHF on the LPS-induced phosphorylation of p38 MAPK, ERK and JNK in BV2 microglial cells by western blot analysis. 7,8-DHF attenuated the LPS-induced phosphorylation of these kinases (Fig. 7). By contrast, the levels of total MAPK proteins were unaffected by either LPS or 7,8-DHF treatment. These results suggest that the activation of MAPKs may be involved in the inhibitory effects of 7,8-DHF on LPS-induced pro-inflammatory mediators in BV2 microglial cells.

Discussion

The present results reveal that 7,8-DHF inhibits the levels of the pro-inflammatory mediators, iNOS and COX-2, induced by LPS, as well as the production of cytokines, such as IL-1β and TNF-α in activated murine BV2 microglial cells through the blockade of NF-κB and of MAPK signaling pathways.

Inflammation in the brain caused by activated microglia plays an important role in the pathology of neurodegenerative disorders, such as AD, cerebral ischemia, multiple sclerosis and amyotrophic lateral sclerosis (7,27). Neuroinflammation with prolonged microglial activation leads to an increase in pro-inflammatory mediators and neurotoxic compounds, including NO, PGE₂, ROS, complement factors and pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α, and subsequently contributes to neuronal dysfunction and neuronal loss (28,29). Thus, the suppression of neuroinflammation and microglial activation process would theoretically attenuate the progres-

sion of neurodegenerative diseases. Accordingly, the inhibition of pro-inflammatory mediators and cytokines by 7,8-DHF shown in the present study may play a beneficial role in the treatment of neurodegenerative diseases.

Among pro-inflammatory mediators released by microglia, NO and PGE₂ are the main cytotoxic mediators participating in the innate immune response in mammals. NO is synthesized from three different isoforms of NOS: endothelial NOS (eNOS), neuronal NOS (nNOS) and iNOS. Among these, iNOS is not usually expressed in the brain. However, activated microglial cells are a major cellular source of iNOS in the brain. The excessive release of NO by activated microglial cells correlates with the progression of neurodegenerative disorders (30,31). COXs are the enzymes that catalyze the conversion of arachidonic acid to prostaglandin H₂ (PGH₂), which is the precursor of a variety of biologically active mediators, such as PGE₂, prostacyclin and thromboxane A₂ (32). COXs exist as two major isozymes: COX-1, a constitutive COX, and COX-2, an isoform that is induced during the response to many stimulants and is activated at the site of the inflammation (33). Several studies have reported that COX-2 is associated with cytotoxicity in brain diseases as the inhibition of COX-2 induction and/or activity reduces brain injury following ischemia and attenuates the progression of neurodegenerative disorders (34-36). Thus, agents that inhibit the production of these inflammatory mediators have been previously considered as potential candidates for anti-inflammatory agents. The results of this study demonstrated that 7,8-DHF inhibited NO and PGE₂ production through the suppression of iNOS and COX-2 expression, respectively, which appears to be due to the suppression of these genes at the transcriptional level (Figs. 1 and 3). The inhibitory effects of 7,8-DHF on the LPS-induced production of NO and PGE₂ were not due to the cytotoxicity of 7,8-DHF, as assessed by MTT assays (Fig. 2).

Our results also revealed that 7,8-DHF significantly attenuated the production of pro-inflammatory cytokines, including TNF- α and IL-1 β (Fig. 4). Since the neuroinflammatory response in activated microglia produces elevated levels of these cytokines, and they have been shown to induce neuronal cell damage, suppressing their production is important for the prevention of neurodegenerative diseases (37-39). TNF- α is primarily produced by activating monocytes, macrophages and T cells. The major producers of TNF- α in the brain are microglial cells, and they may play a role in many pathological conditions in the brain (40,41). Moreover, TNF- α overexpression has been implicated in the pathogenesis of several human CNS disorders (3,42,43). IL-1 β is also a potent pro-inflammatory cytokine that acts through the IL-1 receptors found on numerous cell types, including neurons and microglia. This cytokine is an important mediator of neuroimmune interactions that participate directly in neurodegeneration (44,45). IL-1 β may interact with other molecules that are either released or induced in response to damage, or it may affect only compromised neurons. Thus, the inhibition of cytokine production or function serves as a key mechanism in the control of neurodegeneration. Taken together, our results indicate that 7,8-DHF could be a promising therapeutic candidate for neurodegenerative diseases caused by microglial activation in the brain.

Various intracellular signaling pathways are involved in the modulation of inflammatory responses. NF- κ B and MAPK pathways are amongst the most important signaling molecules

involved in the production of pro-inflammatory mediators and cytokines (46,47). NF- κ B, as a result of its key role in several pathologic conditions, is a major drug target in a variety of diseases. NF- κ B is also a primary regulator of genes that are involved in the production of pro-inflammatory cytokines and enzymes involved in the inflammatory process. It is well known that the blockade of NF- κ B transcriptional activity in the microglial nucleus can also suppress the expression of iNOS, COX-2 and pro-inflammatory cytokines (48,49). In unstimulated cells, NF- κ B is retained in the cytoplasm by binding to I κ B inhibitors. Upon activation by stimuli, I κ B is sequentially phosphorylated by I κ B kinases and then degraded by the proteasome (50,51). This process provides for the availability of free NF- κ B in the cytoplasm and allows for the translocation of NF- κ B proteins from the cytoplasm to the nuclei. In this study, 7,8-DHF significantly attenuated LPS-induced I κ B- α degradation, and inhibited the nuclear translocation of the p65 subunit of NF- κ B and transcriptional activity of NF- κ B in BV2 microglia (Fig. 6). Therefore, the inhibition of NF- κ B signaling pathways in microglial cells by 7,8-DHF may result in the downregulation of pro-inflammatory mediators, resulting in an anti-inflammatory effect.

As major signaling pathways, MAPKs are known to be involved in the LPS-induced production of COX-2 and iNOS through the control of NF- κ B activation in microglial cells (51,52). LPS has been demonstrated to activate three types of MAPKs, including p38 MAPK, ERK and JNK in microglia. According to accumulating data, MAPKs signaling pathways are involved in LPS-induced IL-1 β transcription and production in microglia and other cells (53,54). Furthermore, COX-2 was upregulated by IL-1 β via MAPKs signaling pathways in various cell types (7), and IL-1 β induced iNOS and NO production in C6 astrocytoma cells (54). Hence, in this study, we further evaluated the effect of 7,8-DHF on the activation of three MAPKs induced by LPS in microglial cells. LPS increased the activation of MAPKs, including p38 MAPK, ERK and JNK, whereas 7,8-DHF decreased the LPS-induced activation of these kinases (Fig. 7). These results suggest that the 7,8-DHF-mediated attenuation of pro-inflammatory mediators and cytokines is associated with the inactivation of MAPK signaling pathways, suggesting that the inhibition of MAPKs by 7,8-DHF may partially explain the anti-inflammatory mechanisms of 7,8-DHF.

In conclusion, we found that 7,8-DHF significantly attenuated the levels of neurotoxic pro-inflammatory mediators and cytokines, including NO, PGE₂, TNF- α and IL-1 β in LPS-stimulated microglial cells. The inhibitory action of 7,8-DHF was accompanied by the attenuation of NF- κ B activity through the prevention of NF- κ B translocation from the cytoplasm to the nucleus and by the inhibition of I κ B-degradation. In addition, levels of phosphorylated MAPKs were significantly decreased by pre-treatment with 7,8-DHF in LPS-stimulated microglial cells. These results indicate that 7,8-DHF exerts its anti-inflammatory effects inhibiting the activation of the NF- κ B signaling pathway and the phosphorylation of MAPKs. As a result of the findings presented in this report, 7,8-DHF may ultimately prove useful in the treatment of inflammatory diseases and may be an effective form of therapy for the treatment of several neurodegenerative diseases which accompany microglial activation.

Acknowledgements

This study was supported by the R&D program of MKE/KEIT (10040391, Development of Functional Food Materials and Device for Prevention of Aging-associated Muscle Function Decrease) and Blue-Bio Industry Regional Innovation Center (RIC08-06-07) at Donggeui University as a RIC program under MKE and Busan city, Republic of Korea.

References

- Napoli I and Neumann H: Microglial clearance function in health and disease. *Neuroscience* 158: 1030-1038, 2009.
- Loane DJ and Byrnes KR: Role of microglia in neurotrauma. *Neurotherapeutics* 7: 366-377, 2010.
- Meda L, Cassatella MA, Szendrei GI, Otvos L Jr, Baron P, Villalba M, Ferrari D and Rossi F: Activation of microglial cells by β -amyloid protein and interferon- γ . *Nature* 373: 647-650, 1995.
- Eikelenboom P and van Gool WA: Neuroinflammatory perspectives on the two faces of Alzheimer's disease. *J Neural Transm* 111: 281-294, 2004.
- Gao HM, Liu B, Zhang W and Hong JS: Novel anti-inflammatory therapy for Parkinson's disease. *Trends Pharmacol Sci* 24: 395-401, 2003.
- Liu B and Hong JS: Role of microglia in inflammation-mediated neurodegenerative diseases: mechanisms and strategies for therapeutic intervention. *J Pharmacol Exp Ther* 304: 1-7, 2003.
- Rock RB and Peterson PK: Microglia as a pharmacological target in infectious and inflammatory diseases of the brain. *J Neuroimmune Pharmacol* 1: 117-126, 2006.
- Kaur C and Ling EA: Antioxidants and neuroprotection in the adult and developing central nervous system. *Curr Med Chem* 15: 3068-3080, 2008.
- Deng YY, Lu J, Ling EA and Kaur C: Role of microglia in the process of inflammation in the hypoxic developing brain. *Front Biosci (Schol Ed)* 3: 884-900, 2011.
- Ramos S: Cancer chemoprevention and chemotherapy: dietary polyphenols and signalling pathways. *Mol Nutr Food Res* 52: 507-526, 2008.
- Guo W, Kong E and Meydani M: Dietary polyphenols, inflammation, and cancer. *Nut Cancer* 61: 807-810, 2009.
- Chen S: Natural products triggering biological targets—a review of the anti-inflammatory phytochemicals targeting the arachidonic acid pathway in allergy asthma and rheumatoid arthritis. *Curr Drug Targets* 12: 288-301, 2011.
- García-Lafuente A, Guillamón E, Villares A, Rostagno MA and Martínez JA: Flavonoids as anti-inflammatory agents: implications in cancer and cardiovascular disease. *Inflamm Res* 58: 537-552, 2009.
- González-Gallego J, García-Mediavilla MV, Sánchez-Campos S and Tuñón MJ: Fruit polyphenols, immunity and inflammation. *Br J Nut* 104 (Suppl 3): S15-S27, 2010.
- Magrone T and Jirillo E: Potential application of dietary polyphenols from red wine to attaining healthy ageing. *Curr Top Med Chem* 11: 1780-1796, 2011.
- Juurink BH and Paterson PG: Review of oxidative stress in brain and spinal cord injury: suggestions for pharmacological and nutritional management strategies. *J Spinal Cord Med* 21: 309-334, 1998.
- Ramassamy C: Emerging role of polyphenolic compounds in the treatment of neurodegenerative diseases: a review of their intracellular targets. *Eur J Pharmacol* 545: 51-64, 2006.
- Campos-Esparza MdelR and Torres-Ramos MA: Neuroprotection by natural polyphenols: molecular mechanisms. *Cent Nerv Syst Agents Med Chem* 10: 269-277, 2010.
- Jang SW, Liu X, Yepes M, Shepherd KR, Miller GW, Liu Y, Wilson WD, Xiao G, Blanche B, Sun YE and Ye K: A selective TrkB agonist with potent neurotrophic activities by 7,8-dihydroxyflavone. *Proc Natl Acad Sci USA* 107: 2687-2692, 2010.
- Andero R, Heldt SA, Ye K, Liu X, Armario A and Ressler KJ: Effect of 7,8-dihydroxyflavone, a small-molecule TrkB agonist, on emotional learning. *Am J Psychiatry* 168: 163-172, 2011.
- Chen J, Chua KW, Chua CC, Yu H, Pei A, Chua BH, Hamdy RC, Xu X and Liu CF: Antioxidant activity of 7,8-dihydroxyflavone provides neuroprotection against glutamate-induced toxicity. *Neurosci Lett* 499: 181-185, 2011.
- Zhang R, Kang KA, Piao MJ, Ko DO, Wang ZH, Chang WY, You HJ, Lee IK, Kim BJ, Kang SS and Hyun JW: Preventive effect of 7,8-dihydroxyflavone against oxidative stress induced genotoxicity. *Biol Pharm Bull* 32: 166-171, 2009.
- Kozics K, Valovicova Z and Slamenova D: Structure of flavonoids influences the degree inhibition of Benzo(a)pyrene-induced DNA damage and micronuclei in HepG2 cells. *Neoplasma* 58: 516-524, 2011.
- Park HY, Kim GY, Hyun JW, Hwang HJ, Kim ND, Kim BW and Choi YH: 7,8-Dihydroxyflavone exhibits anti-inflammatory properties by downregulating the NF- κ B and MAPK signaling pathways in lipopolysaccharide-treated RAW264.7 cells. *Int J Mol Med* 29: 1146-1152, 2012.
- Kyung J, Kim D, Park D, Yang YH, Choi EK, Lee SP, Kim TS, Lee YB and Kim YB: Synergistic anti-inflammatory effects of *Laminaria japonica* fucoidan and *Cistanche tubulosa* extract. *Lab Anim Res* 28: 91-97, 2012.
- Lee SH, Kim DW, Eom SA, Jun SY, Park M, Kim DS, Kwon HJ, Kwon HY, Han KH, Park J, Hwang HS, Eum WS and Choi SY: Suppression of 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced skin inflammation in mice by transduced Tat-Annexin protein. *BMB Rep* 45: 354-359, 2012.
- McGeer PL, Yasojima K and McGeer EG: Inflammation in Parkinson's disease. *Adv Neurol* 86: 83-89, 2001.
- Leone S, Ottani A and Bertolini A: Dual acting anti-inflammatory drugs. *Curr Topics Med Chem* 7: 265-275, 2007.
- Mariani MM and Kielian T: Microglia in infectious diseases of the central nervous system. *J Neuroimmune Pharmacol* 4: 448-461, 2009.
- Saha RN and Pahan K: Regulation of inducible nitric oxide synthase gene in glial cells. *Antioxid Redox Signal* 8: 929-947, 2006.
- Brown GC and Neher JJ: Inflammatory neurodegeneration and mechanisms of microglial killing of neurons. *Mol Neurobiol* 41: 242-247, 2010.
- Hawkey CJ: COX-2 inhibitors. *Lancet* 353: 307-314, 1999.
- Vane JR, Mitchell JA, Appleton I, Tomlinson A, Bishop-Bailey D, Croxtall J and Willoughby DA: Inducible isoforms of cyclooxygenase and nitric-oxide synthase in inflammation. *Proc Natl Acad Sci USA* 91: 2046-2050, 1994.
- Giovannini MG, Scali C, Prosperi C, Bellucci A, Pepeu G and Casamenti G: Experimental brain inflammation and neurodegeneration as model of Alzheimer's disease: protective effects of selective COX-2 inhibitors. *Int J Immunopathol Pharmacol* 16 (Suppl 2): S31-S40, 2003.
- Minghetti L and Pocchiari M: Cyclooxygenase-2, prostaglandin E₂, and microglial activation in prion diseases. *Int Rev Neurobiol* 82: 265-275, 2007.
- Choi SH, Aid S and Bosetti F: The distinct roles of cyclooxygenase-1 and -2 in neuroinflammation: implications for translational research. *Trends Pharmacol Sci* 30: 174-181, 2009.
- Boka G, Anglade P, Wallach D, Javoy-Agid F, Agid Y and Hirsch EC: Immunocytochemical analysis of tumor necrosis factor and its receptors in Parkinson's disease. *Neurosci Lett* 172: 151-154, 1994.
- Hunot S, Dugas N, Faucheux B, Hartmann A, Tardieu M, Debre P, Agid Y, Dugas B and Hirsch EC: FcepsilonR2/CD23 is expressed in Parkinson's disease and induces, in vitro, production of nitric oxide and tumor necrosis factor-alpha in glial cells. *J Neurosci* 19: 3440-3447, 1999.
- De Nardin E: The role of inflammatory and immunological mediators in periodontitis and cardiovascular disease. *Ann Periodontol* 6: 30-40, 2001.
- Sawada M, Kondo N, Suzumura A and Marunouchi T: Production of tumor necrosis factor-alpha by microglia and astrocytes in culture. *Brain Res* 491: 394-397, 1989.
- Botchkina GI, Meistrell ME III, Botchkina IL and Tracey KJ: Expression of TNF and TNF receptors (p55 and p75) in the rat brain after focal cerebral ischemia. *Mol Med* 3: 765-781, 1997.
- Akassoglou K, Bauer J, Kassiotis G, Pasparakis M, Lassmann H, Kollias G and Probert L: Oligodendrocyte apoptosis and primary demyelination induced by local TNF/p55TNF receptor signaling in the central nervous system of transgenic mice: models for multiple sclerosis with primary oligodendroglialopathy. *Am J Pathol* 153: 801-813, 1998.
- Sriram K, Matheson JM, Benkovic SA, Miller DB, Luster MI and O'Callaghan JP: Mice deficient in TNF receptors are protected against dopaminergic neurotoxicity: implications for Parkinson's disease. *FASEB J* 16: 1474-1476, 2002.

44. Gougeon PY, Lourens S, Han TY, Nair DG, Ropeleski MJ and Blennerhassett MG: The pro-inflammatory cytokines IL-1 β and TNF α are neurotrophic for enteric neurons. *J Neurosci* 33: 3339-3351, 2013.
45. Rothwell N, Allan S and Toulmond S: The role of interleukin 1 in acute neurodegeneration and stroke: pathophysiological and therapeutic implications. *J Clin Invest* 100: 2648-2652, 1997.
46. Haddad JJ: The role of inflammatory cytokines and NF-kappaB/MAPK signaling pathways in the evolution of familial Mediterranean fever: current clinical perspectives and potential therapeutic approaches. *Cell Immunol* 260: 6-13, 2009.
47. Hamilton T, Novotny M, Pavicic PJ Jr, Herjan T, Hartupee J, Sun D, Zhao C and Datta S: Diversity in post-transcriptional control of neutrophil chemoattractant cytokine gene expression. *Cytokine* 52: 116-122, 2010.
48. Baldwin AS Jr: The NF-kappa B and I kappa B proteins: new discoveries and insights. *Ann Rev Immunol* 14: 649-683, 1996.
49. Moon DO, Choi YH, Kim ND, Park YM and Kim GY: Anti-inflammatory effects of beta-lapachone in lipopolysaccharide-stimulated BV2 microglia. *Int Immunopharmacol* 7: 506-514, 2007.
50. Sarkar FH and Li Y: NF-kappaB: a potential target for cancer chemoprevention and therapy. *Front Biosci* 13: 2950-2959, 2008.
51. Wertz IE and Dixit VM: Signaling to NF-kappaB: regulation by ubiquitination. *Cold Spring Harb Perspect Biol* 2: a003350, 2010.
52. Mattson MP and Camandola S: NF-kappaB in neuronal plasticity and neurodegenerative disorders. *J Clin Invest* 107: 247-254, 2001.
53. Nakajima K and Kohsaka S: Characterization of brain microglia and the biological significance in the central nervous system. *Adv Neurol* 60: 734-743, 1993.
54. Kim YJ, Hwang SY, Oh ES, Oh S and Han IO: IL-1beta, an immediate early protein secreted by activated microglia, induces iNOS/NO in C6 astrocytoma cells through p38 MAPK and NF-kappaB pathways. *J Neurosci Res* 84: 1037-1046, 2006.