**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-1β (IL-1β), were inhibited by 7,8-DHF. In addition, 7,8-DHF suppressed nuclear factor-κB (NF-κB) translocation and its transcriptional activity by blocking IκB (IκB)-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.

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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-xB (NF-xB) p65 and IкB-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 µg/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 µg/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 (3x10⁵ 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 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 (5x10⁵ cells/ml) were stimulated in 24-well plates for 24 h, and then 100 µl 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 µg) 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-1β and TNF-α genes were amplified from the cDNA by PCR. The PCR primers were as follows: mouse iNOS (5'-ATG GCC ACA 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 CCA AAG AAT GAT CCA AAG ATC-3'); IL-1β (5'-ATG GCC 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 ATC-3' and 5'-TAC AGG CTT GTC ACT CCA 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% NaCl) 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-1β 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 (5x10⁵ 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 µg/ml LPS for 24 h. One hundred microliters of culture-medium supernatants were collected for determination of the IL-1β and TNF-α concentrations by ELISA, as previously described (26).

**NF-xB luciferase assay.** A total of 1x10⁵ BV2 cells were transfected with 2 µg NF-xB-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 x g for 1 min at 4°C, the supernatant was stored at -70°C for use in 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 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 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).

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 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.

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.
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 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. (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 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 then 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.

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 stimuli 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-1β (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-1β 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-1β 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-κB and MAPK pathways are amongst the most important signaling molecules involved in the production of pro-inflammatory mediators and cytokines (46,47). NF-κB, as a result of its key role in several pathologic conditions, is a major drug target in a variety of diseases. NF-κB 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-κB 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-κB is retained in the cytoplasm by binding to IκB inhibitors. Upon activation by stimuli, IκB is sequentially phosphorylated by IκB kinases and then degraded by the proteasome (50,51). This process provides for the availability of free NF-κB in the cytoplasm and allows for the translocation of NF-κB proteins from the cytoplasm to the nuclei. In this study, 7,8-DHF significantly attenuated LPS-induced IκB-α degradation, and inhibited the nuclear translocation of the p65 subunit of NF-κB and transcriptional activity of NF-κB in BV2 microglia (Fig. 6). Therefore, the inhibition of NF-κB 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-κB 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-1β transcription and production in microglia and other cells (53,54). Furthermore, COX-2 was upregulated by IL-1β via MAPKs signaling pathways in various cell types (7), and IL-1β 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-1β in LPS-stimulated microglial cells. The inhibitory action of 7,8-DHF was accompanied by the attenuation of NF-κB activity through the prevention of NF-κB translocation from the cytoplasm to the nucleus and by the inhibition of IκB-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-κB 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.
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