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# Synthetic 3′,4′-Dihydroxyflavone Exerts Anti-Neuroinflammatory Effects in BV2 Microglia and a Mouse Model

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## Abstract

Neuroinflammation is an immune response within the central nervous system against various proinflammatory stimuli. Abnormal activation of this response contributes to neurodegenerative diseases such as Parkinson disease, Alzheimer's disease, and Huntington disease. Therefore, pharmacologic modulation of abnormal neuroinflammation is thought to be a promising approach to amelioration of neurodegenerative diseases. In this study, we evaluated the synthetic flavone derivative 3',4'-dihydroxyflavone, investigating its anti-neuroinflammatory activity in BV2 microglial cells and in a mouse model. In BV2 microglial cells, 3',4'-dihydroxyflavone successfully inhibited production of chemokines such as nitric oxide and prostaglandin $E_2$ and proinflammatory cytokines such as tumor necrosis factor alpha, interleukin 1 beta, and interleukin 6 in BV2 microglia. It also inhibited phosphorylation of mitogen-activated protein kinase (MAPK) and nuclear factor (NF)-κB activation. This indicates that the anti-inflammatory activities of 3',4'-dihydroxyflavone might be related to suppression of the proinflammatory MAPK and NF-κB signaling pathways. Similar anti-neuroinflammatory activities of the compound were observed in the mouse model. These findings suggest that 3',4'-dihydroxyflavone is a potential drug candidate for the treatment of microglia-related neuroinflammatory diseases.

**Key Words:** Microglia, Neuroinflammation, Lipopolysaccharide, BV2 microglia, Flavone

## INTRODUCTION

Neuroinflammation is an immune response to proinflammatory stimuli within the central nervous system (CNS). It is a major contributing factor to neurodegenerative diseases, including multiple sclerosis, Parkinson disease, Alzheimer disease, and Huntington disease (Kim and Choi, 2015). Microglia are the resident macrophages of the CNS, with important roles in immune regulation and brain homeostasis (Biber *et al*., 2016). In the normal immune response to various factors such as xenobiotics, microglia can be activated to release neurotrophic factors and support neurogenesis, helping maintain CNS homeostasis. However, abnormal activation of microglia may lead to neuronal injury and production of neurotoxic molecules, such as prostaglandin $E_2$ ($PGE_2$) and nitric oxide (NO), and proinflammatory cytokines, such as interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α (Block and Hong, 2005). Activated microglia also migrate toward damaged areas and further promote neuroinflammation (de Haas *et al*., 2008), and the microglia themselves or microglia-derived inflammatory products eventually accelerate neurodegeneration (Cherry *et al*., 2014; Kim and Choi, 2015). Novel therapeutic agents are needed to inhibit the production of neurotoxic chemokines and cytokines by microglia, blocking their contribution to neuroinflammatory and neurodegenerative conditions.

The anti-inflammatory effects of flavonoids have been reported from *in vitro* and *in vivo* studies, as well as clinical trials (Perez-Cano and Castell, 2016; Zeinali *et al*., 2017). Flavones, a type of flavonoid, are natural products isolated from a number of medicinal plants. Synthetic derivatives of these compounds are based on the 2-phenylchromen-4-one backbone (An *et al*., 2017). The anti-inflammatory activities of fla-

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vones have been investigated in many studies, but most have been focused on natural products such as luteolin. In an effort to identify more potent anti-inflammatory flavones, we synthesized a number of flavone derivatives and evaluated their abilities to inhibit NO and $PGE_2$ production in a cell-based assay using RAW 264.7 cells (An *et al*., 2017). A novel and potent anti-inflammatory flavone derivative identified in that study was 3',4'-dihydroxyflavone. In the present study, we evaluated the antineuroinflammatory effects of 3',4'-dihydroxyflavone and investigated the mechanisms by which it might counteract the effects of lipopolysaccharide (LPS) stimulation in BV2 microglial cells. To confirm the *in vitro* results, we also examined the antineuroinflammatory effects of 3',4'-dihydroxyflavone in the brains of mice injected with LPS.

## MATERIALS AND METHODS

### Chemicals and reagents

We synthesized 3',4'-dihydroxyflavone and flavone (Fig. 1A) using our previously reported procedure (An *et al*., 2017). LPS from *Escherichia coli* serotype O55:B5 (L6529) and luteolin (L9283) were purchased from Sigma-Aldrich (MO, USA). HyClone (GE Healthcare) culture media and other materials were used for cell culture. Rabbit antibodies against p38 (#9212S), phosphor-p38 (#9215S), JNK (#9252S), phosphor-JNK (#9251S), ERK (#9101L), phosphor-ERK (#9102), *i*NOS (#2982S) and COX-2 (#4842S) were purchased from Cell Signaling Technology (MA, USA). Antibodies against β-actin (SC-47778 HRP) and NF-κB (rabbit, SC-372) were purchased from SantaCruz Biotechnology (CA, USA). A rabbit antibody against anti-ionized calcium-binding adapter molecule 1 (Iba-1, #019-19741) was purchased from Wako (Tokyo, Japan). Secondary antibodies were purchased from Bio-Rad Laboratories (CA, USA), SantaCruz Biotechnology and Invitrogen (CA, USA). Mouse TNF-α and IL-6 ELISA kits were purchased from BD. A mouse IL-1β kit was purchased from Invitrogen.

### Cell culture and assessment of cytotoxicity

Murine BV2 microglial cells were cultured in DMEM with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified 5% $CO_2$ atmosphere. Cell viability and toxicity were determined by the WST-1 assay (Roche, Mannheim, Germany). Briefly, BV2 cells in sterile plates were incubated with the indicated concentrations of 3',4'-dihydroxyflavone for 24 h. After replacing the culture media with fresh medium, WST-1 solution was added to each well and the cells were incubated for 2 h. Absorbance at 450 nm was then measured with a plate reader. In some experiments, the cells were incubated with different concentrations of 3',4'-dihydroxyflavone and then incubated with LPS (500 ng/ml) for 24 h.

### Measurement of NO and $PGE_2$

BV2 cells were incubated with various concentrations of 3',4'-dihydroxyflavone for 2 h prior to incubation with or without LPS (500 ng/ml) for a further 22 h. The NO and $PGE_2$ concentrations in culture supernatants were assessed using the Griess reaction kit (#KA1342; Abnova, Taipei City, Taiwan) and the enzyme immune assay kit (#ADI-900-001; Enzo Life Sciences, NY, USA), respectively, according to the manufacturer's instructions.

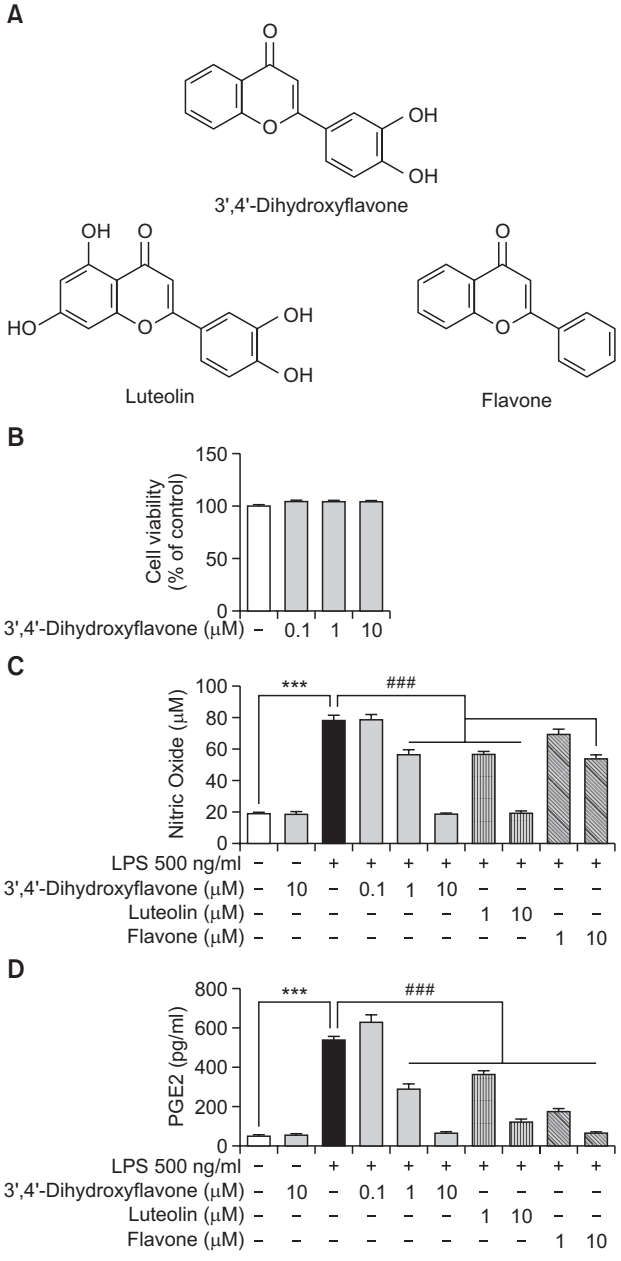


**Fig. 1.** Inhibitory effects of 3',4'-dihydroxyflavone on LPS-induced production of NO and $PGE_2$ in BV2 cells. (A) Chemical structure of 3',4'-dihydroxyflavone, flavone, and luteolin. (B) Effect of 3',4'-dihydroxyflavone on BV2 cell viability. Cells were treated with different concentrations (0-10 μM) of 3',4'-dihydroxyflavone for 24 h. Cell viability was measured with a WST-1 assay (n=6 per group). (C, D) Inhibition of LPS-mediated NO and $PGE_2$ activation by 3',4'-dihydroxyflavone. BV2 cells were pretreated with different concentrations (0-10 μM) of 3',4'-dihydroxyflavone for 2 h, after which the cells were treated with LPS (500 ng/ml) for 22 h. Culture supernatants were collected and production of NO (C) and $PGE_2$ (D) was measured (n=3-6 per group). Statistical analysis included one-way analysis of variance and Tukey's post hoc test. ***$p$<0.001 compared with the control group, $^{###}p$<0.001 compared with the LPS-only group. All error bars indicate standard error of the mean.

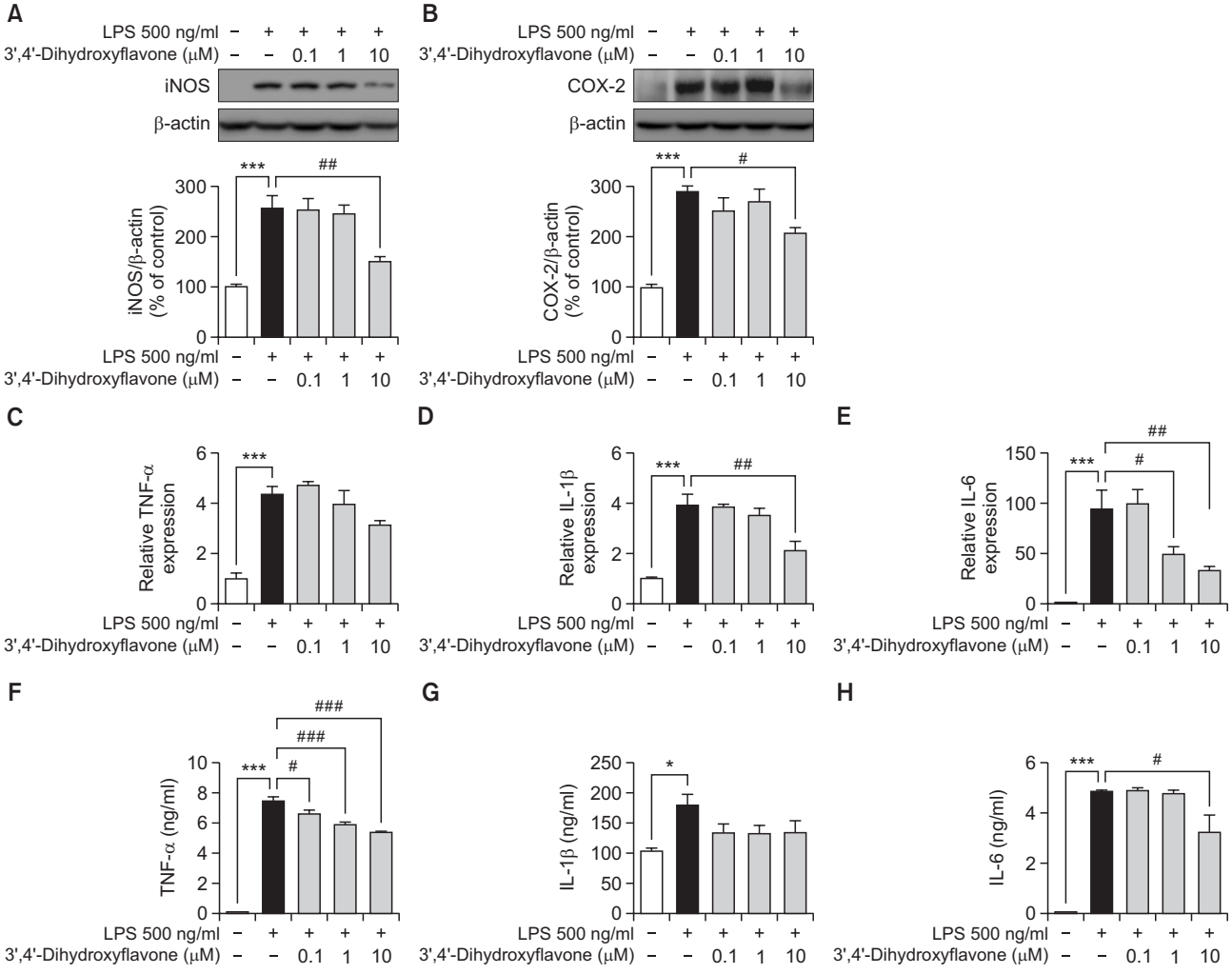


**Fig. 2.** Effects of 3',4'-dihydroxyflavone on LPS-induced proinflammatory mediator expression in BV2 cells. (A, B) Inhibition of LPS-mediated *i*NOS and COX-2 expression by 3',4'-dihydroxyflavone. BV2 cells were pretreated with different concentrations (0-10 μM) of 3',4'-dihydroxyflavone for 2 h, then the cells were treated with LPS (500 ng/ml) for 22 h. The levels of *i*NOS (A) and COX-2 (B) were measured by immunoblotting in whole cell lysates (n=3 per group). Total mRNA was harvested and mRNA expression levels of TNF-α (C), IL-1β (D), and IL-6 (E) were measured by qRT-PCR (n=3-4 per group). GAPDH was used as an internal control. Protein levels of TNF-α (F), IL-1β (G), and IL-6 (H) were determined in the culture medium (n=3 per group). Statistical analysis included one-way analysis of variance and Tukey's post hoc test. **p*<0.05, ****p*<0.001 compared with control group, #*p*<0.05, ##*p*<0.01, and ###*p*<0.001 compared with LPS- only group. All error bars indicate standard error of the mean.

### RNA isolation and quantitative reverse-transcription real-time polymerase chain reaction (qRT-PCR)

mRNA transcription of cytokines was analyzed by qRT-PCR. Total RNA was extracted from BV2 cells and mouse brains using the RNeasy Plus Mini kit and RNeasy Lipid Tissue Mini kit (Qiagen, North Rhine-Westphalia, Germany), respectively, according to the manufacturer's instructions. RNA samples (5 μg) were subjected to cDNA synthesis using an RNA to cDNA EcoDry Premix kit (Takara, Shiga, Japan). cDNA was subjected to qRT-PCR using SYBR Green Mix (Toyobo, Osaka, Japan) and the CFX Connect real-time PCR system (Bio-Rad Laboratories). Primers, synthesized at Cosmo Genetech, were as follows; TNF-α: forward, 5'-GATTATGGCTCAGGGTCCAA-3', reverse, 5'-GCTCCAGTGAATTCGGAAAG-3'; IL-1β: forward, 5'-CCCAAGCAATACCCAAAGAA-3', reverse, 5'-GCTTGTGCTCTGCTTGTGAG-3'; IL-6: forward, 5'-CCGGAGAGGAGACTTCACAG-3', reverse, 5'-TTGCCATTGCACAACTCTTT-3'; GAPDH: forward, 5'-TGAATACGGCTACAGCAACA-3', reverse, 5''-AGGCCCCTCCTGTTATTATG-3'.

### Western blotting

Western blot analysis was performed as previously described (Lee *et al*., 2010). Briefly, following incubation with 3',4'-dihydroxyflavone for 2 h and with LPS for 22 h, cells were collected and washed in ice-cold PBS and then lysed in RIPA buffer (Cell Signaling Technology). Lysates were sonicated for 10 seconds, centrifuged at 13,000 rpm for 10 min at 4°C, and the supernatant was collected. Cortical tissues from mouse brains were weighed and sonicated in a 10X volume of RIPA buffer, homogenized, and centrifuged; the supernatant was collected. Protein concentrations were determined with the Bradford technique (Bio-Rad Protein Assay, Bio-Rad). Equal

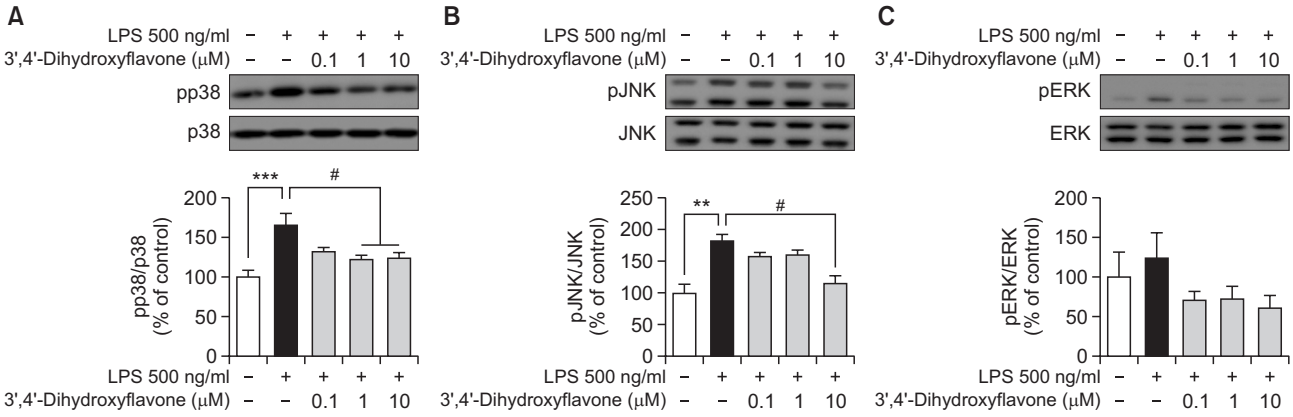


**Fig. 3.** Effects of 3',4'-dihydroxyflavone on LPS-induced phosphorylation of MAPKs in BV2 cells. (A-C) Inhibition of LPS-mediated phosphorylation of p38 (A), JNK (B), and ERK1/2 (C) by 3',4'-dihydroxyflavone. BV2 cells were pretreated with different concentrations (0-10 μM) of 3',4'-dihydroxyflavone for 2 h and were then treated with LPS (500 ng/ml) for 1 h. The levels of p38, JNK, and ERK1/2 and each corresponding phosphorylated form were measured by immunoblotting in whole cell lysates (n=3 per group). Statistical analysis included one-way analysis of variance and Tukey's post hoc test. ***p*<0.01 and ****p*<0.001 compared with control group, #*p*<0.05 compared with LPS-only group. All error bars indicate standard error of the mean.

amounts of proteins were separated by SDS-PAGE and transferred to PVDF membranes. Immunoblotting was carried out with antibodies against phosphor-p38 (1:1000), p38 (1:1000), phosphor-JNK (1:1000), JNK (1:1000), phosphor-ERK (1:1000), ERK (1:1000), *i*NOS (1:1000), COX-2 (1:1000), and β-actin (1:4000). The membranes were incubated with corresponding secondary antibodies. Western blots were developed with an enhanced chemiluminescence detection system (ECL; Amersham Biosciences, Somerset, UK). Densitometric quantification was performed using ImageJ software (National Institutes of Health, MD, USA).

### Luciferase assay

BV2 microglial cells were cultured in 24-well plates for 24 h and then transfected with TK-renilla and NF-κB firefly luciferase constructs (Stratagene, La Jolla, CA, USA) using polyethylenimine according to the manufacturer's instructions. Briefly, transfected cells were pretreated with 3',4'-dihydroxyflavone for 2 h and then stimulated with LPS for 12 h. The cells were then washed twice with ice-cold PBS, and 100 μL of 1x passive lysis buffer was added. After centrifugation at 12,000 ×g for 5 min at 4°C, a 20 μL aliquot of the supernatant was analyzed using a luminometer (Molecular Devices, CA, USA). Luciferase activity was normalized to renilla luciferase activity.

### Animals and treatments

Male C57BL/6 mice (8 weeks old, 20-25 g) were purchased from Daehan Biolink Co., Ltd (Eumseong, Korea). A total of 24 mice were randomly divided into 3 groups (n=8 in each group). A 5 mg/kg dose of 3',4'-dihydroxyflavone r dissolved in saline was administered orally once daily for 3 consecutive days. LPS (5 mg/kg) was injected intraperitoneally (i.p) at on the third day. Analysis was done at 4 h or 24 h after LPS injection. Mice were housed at a 12 h day/night cycle with free access to tap water and food pellets. The mouse study was approved by the Kyung Hee University Institutional Animal Care and Use Committee (IACUC).

### Immunofluorescence

The mice were anesthetized with 2.5% avertin in PBS and immediately afterwards, the hearts were perfused with 4% paraformaldehyde in PBS. After perfusion, the brains were excised, post-fixed overnight at 4°C, and incubated in 30% sucrose at 4°C until equilibrated. Sequential 30-μm coronal sections were made with a cryostat (CM30 50S; Leica, Hessen, Germany) and stored in cryoprotectant (25% ethylene glycol, 25% glycerol, and 0.02 M PB) at 4°C until use. Brain sections were rinsed in PBS and then incubated with anti-Iba-1 antibody (1:1000 dilution) for 24 h at 4°C in the presence of blocking buffer (0.3% triton X-100, 1% NGS and 1% bovine serum albumin). We have quantified Iba-1 immunopositive cells in four sections of cortex per animals. The quantification was done by a researcher who did not know the treatment of each animal. To detect the intracellular location of NF-κB/p65, BV2 cells were fixed with 4% paraformaldehyde in PBS after the treatments described above. The cells were rinsed and then incubated with NF-κB/p65 antibody (1:1000 dilution) for 24 h at 4°C. For visualization, the primary antibody was developed by incubating with Alexa Fluor 488-conjugated secondary antibodies for 1 h at room temperature. Images were captured with an Olympus BX51 microscope and analyzed with ImageJ software (National Institutes of Health).

### Statistical analysis

All data are expressed as the mean ± standard error of the mean using Graph Pad Prism 5.0 software (Graph Pad software Inc., San Diego, CA, USA). The results were analyzed statistically by one-way analysis of variance followed by Tukey's *post hoc* test. A *p*-value less than 0.05 was considered statistically significant.

## RESULTS

### 3′,4′-Dihydroxyflavone inhibits LPS-induced NO and $PGE_2$ production in microglia

To examine the potential cytotoxic effect of 3',4'-dihydroxy-

flavone on microglia, the cells were incubated with various concentrations of 3',4'-dihydroxyflavone for 24 h and cell viability was determined by the WST-1 assay. As shown in Fig. 1B, 3',4'-dihydroxyflavone had no cytotoxic effect on BV2 cells within the range of concentrations used. In our previous study, 3',4'-dihydroxyflavone showed inhibitory effects on NO and $PGE_2$ production in murine macrophage RAW 264.7 cells (An *et al*., 2017). To confirm this in microglia, LPS, a well-known stimulant of inflammation by way of toll-like receptor 4, was added to BV2 cells for 24 h in the presence or absence of 3',4'-dihydroxyflavone, and culture supernatants were analyzed. Similar to our previous results, NO and $PGE_2$ production induced by LPS were significantly lower in BV2 cells preincubated with 10 μM of 3',4'-dihydroxyflavone (Fig. 1C, 1D). In NO inhibitory experiment, 3',4'-dihydroxyflavone was significantly more potent than simple flavone (Fig. 1A), a known natural anti-inflammatory flavonoid, and almost equipotent with luteolin (Fig. 1A), one of the most potent natural anti-inflammatory products. In the case of $PGE_2$ inhibition assay, 3',4'-dihydroxyflavone decreased the production of $PGE_2$, even at a concentration of 1 μM, consistent with the previous finding that 3',4'-dihydroxyflavone inhibited production of $PGE_2$ ($IC_{50}$=0.99 μM) more potently than that of NO ($IC_{50}$=6.29 μM). These results in LPS-stimulated microglia suggest that 3',4'-dihydroxyflavone can reduce neuroinflammation without causing cytotoxic effects.

### 3′,4′-Dihydroxyflavone reduces production of proinflammatory mediators in LPS-stimulated BV2 microglia

Since the production of NO and $PGE_2$ in microglia is primarily regulated by the *i*NOS and COX-2 enzymes, we performed western blot analysis to determine whether the suppression of NO and $PGE_2$ by 3',4'-dihydroxyflavone was associated with modulation of these enzymes. We found that 3',4'-dihydroxyflavone significantly inhibited *i*NOS expression (Fig. 2A) in LPS-stimulated BV2 cells pretreated with 3',4'-dihydroxyflavone. COX-2 expression was also reduced by 3',4'-dihydroxyflavone pretreatment (Fig. 2B). Activated microglia are a major source of inflammatory cytokines such as TNF-α, IL-1β, and IL-6, which can be upregulated and detected in various acute and chronic inflammatory diseases (Block *et al*., 2007). To determine the effect of 3',4'-dihydroxyflavone on the levels of proinflammatory cytokines, BV2 cells were pretreated with 3',4'-dihydroxyflavone for 2 h and then stimulated with LPS for 22 h. The mRNA expression levels of TNF-α, IL-1β, and IL-6 were increased by LPS treatment, but pretreatment with 3',4'-dihydroxyflavone significantly diminished that effect in a concentration-dependent manner (Fig. 2C-2E). In a parallel experiment, we analyzed the production of TNF-α, IL-1β and IL-6 using ELISA. As with mRNA expression, 3',4'-dihydroxyflavone pretreatment significantly reduced TNF-α and IL-6 protein levels in the culture medium of LPS-stimulated BV2 cells as compared with levels in LPS-stimulated cells without pretreatment (Fig. 2F, 2H). IL-1β levels were also diminished by 3',4'-dihydroxyflavone pretreatment, although the difference was not statistically significant (Fig. 2G). Collectively, these results suggest that 3',4'-dihydroxyflavone significantly suppresses LPS-triggered activation of microglia *in vitro*.

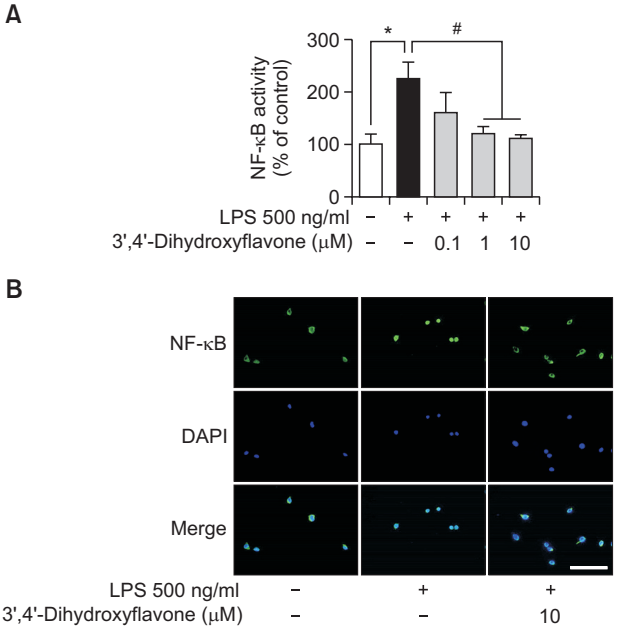


**Fig. 4.** Effects of 3',4'-dihydroxyflavone on NF-κB activation in LPS-stimulated BV2 cells. (A) NF-κB luciferase assay. Cells were transfected with TK-renilla (pRL-TK) and NF-κB firefly luciferase (pNF-κB-Luc) constructs. Transfected cells were pretreated with different concentrations (0-10 μM) of 3',4'-dihydroxyflavone for 2 h and then stimulated with LPS for 12 h. NF-κB-dependent transcriptional activity was determined. (B) Representative images of NF-κB localization. BV2 cells were pretreated with 3',4'-dihydroxyflavone for 2 h and were then treated with LPS (500 ng/ml) for 2 h. Cells were stained by anti-NF-κB/p65 antibody and DAPI. Statistical analysis included one-way analysis of variance and Tukey's post hoc test. *$p$<0.05 compared with control group, $^{\#}p$<0.05 compared with LPS-only group. All error bars indicate standard error of the mean.

### 3′,4′-Dihydroxyflavone blocks LPS-induced mitogen-activated protein kinase signaling activation

A number of studies have demonstrated that activation of mitogen-activated protein kinase (MAPK) signaling cascades, including p38, c-Jun N-terminal kinase (JNK) and extracellular-signal-regulated kinase (ERK1/2), play an important role in modulating the expression of proinflammatory mediators in LPS-stimulated microglia (Kim *et al*., 2004). Therefore, anti-inflammatory effects of 3',4'-dihydroxyflavone in BV2 cells might result from modulation of these signaling pathways. To explore the underlying mechanism by which 3',4'-dihydroxyflavone reduced the inflammatory response in LPS-stimulated microglia, its effects on activation of MAPK pathways were investigated. As shown in Fig. 3, stimulation of BV2 cells by LPS resulted in increased phosphorylation of p38 and JNK, an effect suppressed by 3',4'-dihydroxyflavone (Fig. 3A, 3B). ERK1/2 phosphorylation was slightly increased by LPS stimulation, which again was inhibited by 3',4'-dihydroxyflavone (Fig. 3C). Our results revealed that 3',4'-dihydroxyflavone suppressed LPS-mediated p38, JNK, and ERK1/2 activation in BV2 microglial cells.

### 3′,4′-Dihydroxyflavone blocks LPS-induced NF-κB activation

The NF-κB signaling pathway is another important mediator of the LPS-induced inflammatory response in BV2 cells (Kim

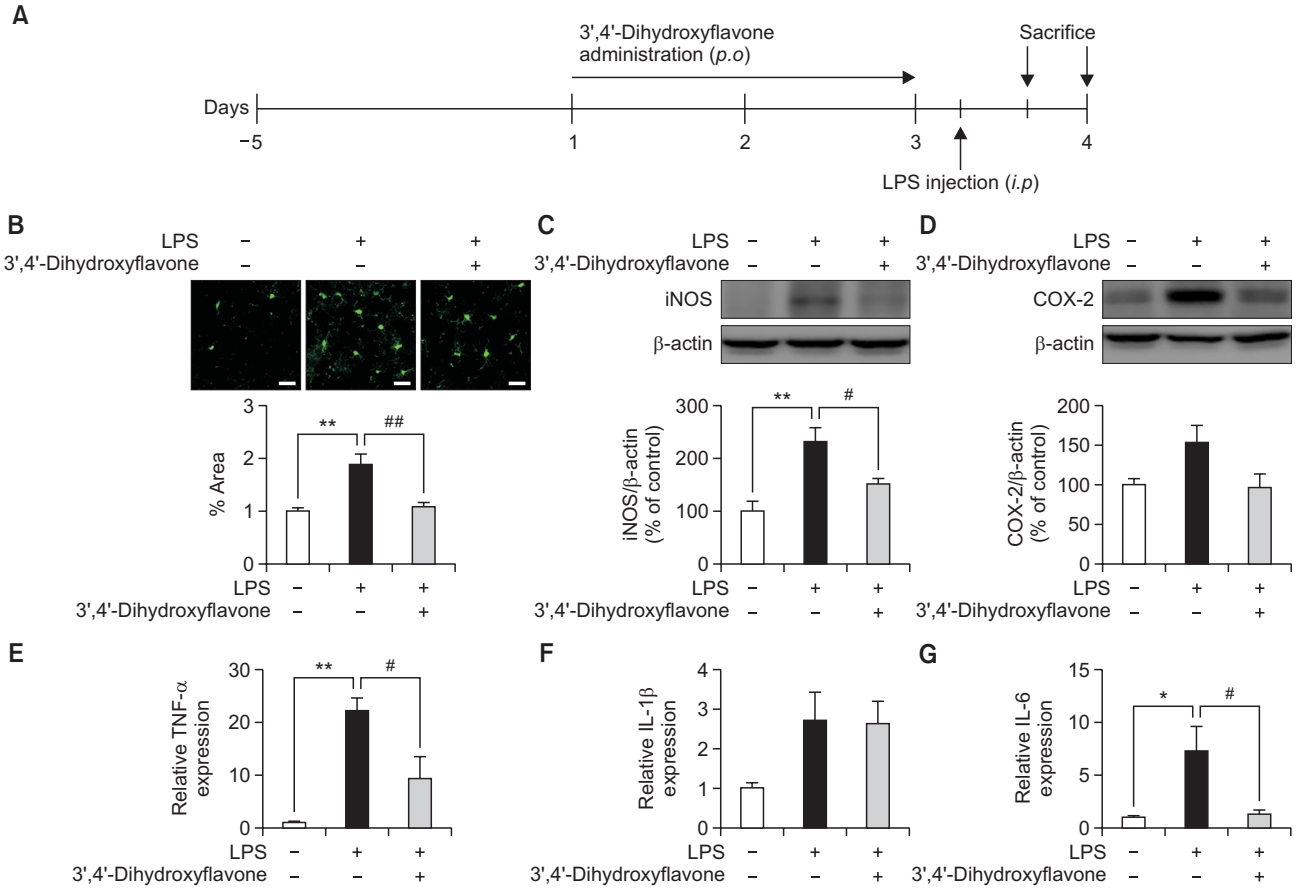


**Fig. 5.** 3',4'-Dihydroxyflavone reduces LPS-induced neuroinflammation in the mouse brain. (A) Experimental protocol. Mice were pretreated with 3',4'-dihydroxyflavone, 5 mg/kg, once daily for 3 consecutive days. LPS, 5 mg/kg, was injected on the third day. Analysis was done at 4 h and 24 h after LPS injection. (B) Representative image and quantification of Iba-1 in LPS-induced neuroinflammation in the mouse brain (n=4 per group, scale bar, 50 μm). (C, D) Inhibition of LPS-mediated iNOS and COX-2 expression by 3',4'-dihydroxyflavone. Immunoblotting was done 24 h after LPS injection (n=4 per group). (E-G) Inhibition of proinflammatory cytokine expression by 3',4'-dihydroxyflavone in the mouse brain. mRNA expression levels of TNF-α (E), IL-1β (F), and IL-6 (G) in mouse brain cortex (n=4 per group). Statistical analysis included one-way analysis of variance and Tukey's post hoc test. *$p$<0.05, **$p$<0.01 compared with control group, $^{\#}p$<0.05, $^{\#\#}p$<0.01 compared with LPS-only group. All error bars indicate standard error of the mean.

*et al*., 2017), and we investigated whether 3',4'-dihydroxyflavone would block this pathway. First, we observed that 3',4'-dihydroxyflavone significantly reduced LPS-stimulated NF-κB transcriptional activity in BV2 cells in a concentration-dependent manner (Fig. 4A). Immunofluorescence analysis further demonstrated that LPS-induced translocation of p65 from the cytosol to the nucleus was prevented by 3',4'-dihydroxyflavone treatment (Fig. 4B). These finding imply that 3',4'-dihydroxyflavone hinders LPS-mediated release of proinflammatory cytokines in BV2 cells by inhibiting the NF-κB pathway.

### 3′,4′-Dihydroxyflavone ameliorates LPS-induced neuroinflammation in the brain of mice

Finally, to confirm whether 3',4'-dihydroxyflavone reduces neuroinflammation *in vivo*, we injected LPS into mice, a well-known animal model of neuroinflammation cause by microglial activation (Tai *et al*., 2017; Zhang and Xu, 2017). We administered 3',4'-dihydroxyflavone to mice orally for 3 days prior to the i.p. injection of LPS (Fig. 5A). Immunofluorescence analysis using Iba-1 antibody on brain cortex sections taken 4 h after LPS injection indicated that systemic LPS treatment markedly increased the number of Iba-1-positive cells in the cortex. Pretreatment with 3',4'-dihydroxyflavone attenuated this Iba-1-reactive cell response to LPS stimulation (Fig. 5B). Western blot analysis indicated that LPS injection elevated *i*NOS and COX-2 expression levels in the mouse brains, but 3',4'-dihydroxyflavone effectively lowered these expression levels, with the exception of COX-2, for which the difference did not reach statistical significance (Fig. 5C, 5D). TNF-α and IL-6 mRNA expression levels were significantly increased by LPS treatment, an effect attenuated by of 3',4'-dihydroxyflavone pretreatment (Fig. 5E, 5G). Although IL-1β expression levels were also increased in LPS-treated mice, they were not reduced by 3',4'-dihydroxyflavone (Fig. 5F). Finally, we evaluated whether 3',4'-dihydroxyflavone amelioration of microglial activation by was related to suppression of the MAPK signaling pathway. Pretreatment with 3',4'-dihydroxyflavone did significantly suppress the phosphorylation of both JNK and ERK1/2 but not of p38 MAPK (Fig. 6). Although the reason for the discrepancy between the results for p38 MAPK *in vitro* and *in vivo* remains unclear, it might relate both to the *in vivo* cortical tissue environment and to the timing of evaluation in the

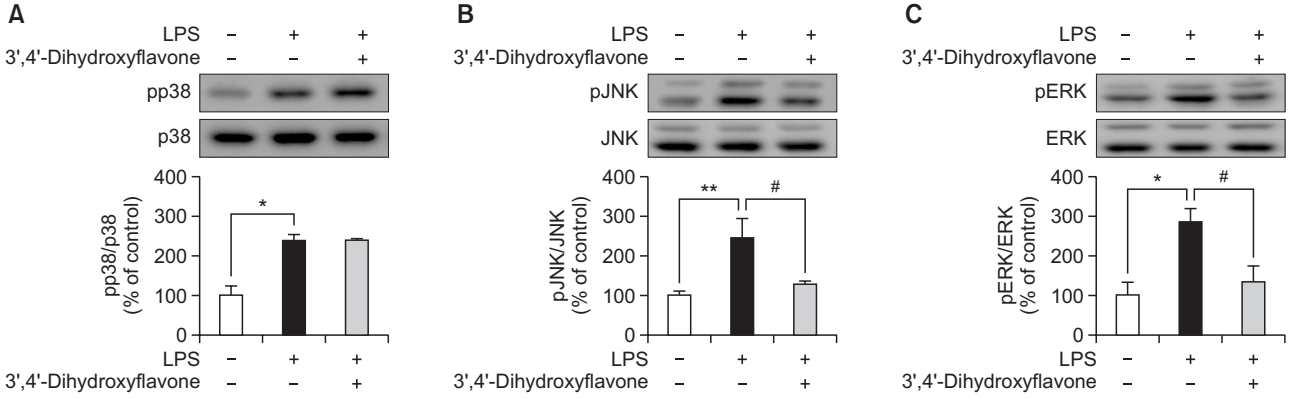


**Fig. 6.** Effects of 3',4'-dihydroxyflavone on LPS-induced phosphorylation of MAPKs in the mouse brain. (A-C) Inhibition of LPS-mediated phosphorylation of p38, JNK, and ERK1/2 by 3',4'-dihydroxyflavone. Mice were pretreated with 3',4'-dihydroxyflavone, 5 mg/kg, once daily for 3 consecutive days. LPS, 5 mg/kg, was injected on the third day. Immunoblotting was done 24 h after LPS injection. Immunoblotting of p38, JNK, and ERK1/2, and each phosphorylated form in samples derived from mouse brains (n=4 per group). Statistical analysis included one-way analysis of variance and Tukey's post hoc test. *$p$<0.05, **$p$<0.01 compared with control group, $^{\#}p$<0.05 compared with LPS-only group. All error bars indicate standard error of the mean.

different experiments. These results suggest that treatment with 3',4'-dihydroxyflavone suppresses microglial activation in the brains of mice by regulating MAPK signal pathways, thereby reducing neuroinflammation.

## DISCUSSION

In an effort to identify novel anti-inflammatory agents, a wide range of studies have been conducted to investigate the biological functions of flavones, compounds that are readily available and relatively nontoxic. However, most investigations have focused on the natural flavones such as luteolin, with relatively few synthetic flavone studies as of yet. In particular, it is not known whether a synthetic flavone such as 3',4'-dihydroxyflavone can inhibit neuroinflammation in microglia (Perez-Cano and Castell, 2016; Zeinali *et al*., 2017). Therefore, the present study was designed to investigate a synthetic flavone derivative, assessing mechanisms by which it might inhibit neuroinflammation. We showed for the first time that 3',4'-dihydroxyflavone, a novel synthetic flavone derivative, significantly suppressed cellular neuroinflammatory processes *in vitro*, reducing the secretion of proinflammatory cytokines and mediators by LPS-stimulated BV2 microglia. This was partly due to inhibition of the MAPK and NF-κB signal pathways. Furthermore, we confirmed that the compound suppressed neuroinflammation in the LPS-stimulated mouse brain, also with inhibition of the MAPK signaling pathway.

Microglia play important roles in neuroinflammation. When activated by CNS injury or other stimuli, microglia contribute to neurodegeneration through release of a variety of inflammatory mediators and cytokines. These inflammatory factors are known to be involved in the pathogenesis of acute and chronic neurodegenerative diseases, such as Alzheimer disease and Parkinson disease (Bodea *et al*., 2014). Therefore, it is thought that suppression of these microglia-derived factors might ameliorate the development of neurodegenerative diseases. The mechanisms by which these cells contribute to neuronal degeneration are the subject of intense researches aimed at finding novel therapies (Cunningham, 2013).

Uncontrolled microglial activation results in quantifiable release of inflammatory mediators such as NO and $PGE_2$ shown to be directly toxic to neurons (Kim *et al*., 2007). Excessive production of NO and $PGE_2$ by increased expression of *i*NOS and COX2 is associated with various types of CNS injury and disease (Choi *et al*., 2009; Ransohoff and Brown, 2012; Chinta *et al*., 2013). In this study, we showed that 3',4'-dihydroxyflavone inhibits production of NO and $PGE_2$ in LPS-stimulated microglia. In particular, 3',4'-dihydroxyflavone was proven to be more potent than flavone and almost equipotent to luteolin. This indicates that 3',4'-dihydroxyflavone is worth further investigation to assess possible additional anti-inflammatory activity in microglia. We also confirmed that 3',4'-dihydroxyflavone-mediated inhibition of NO and $PGE_2$ production resulted from suppression of *i*NOS and COX-2 expression. Thus, these results suggest that 3',4'-dihydroxyflavone is a potential therapeutic agent to treat neuroinflammation in various CNS diseases, analogous to the effects of non-steroidal anti-inflammatory drugs that also inhibit COX-2. The latter have been clinically tested and shown to decrease neuroinflammation (Lichtenstein *et al*., 2010).

Neuroinflammation is further mediated by microglial secretion of proinflammatory cytokines, such as TNF-α, IL-1β, and IL-6. Elevated levels of these cytokines result in worse outcomes from brain injury (Kim *et al*., 2004). The present study demonstrated that LPS stimulation of BV2 cells caused a significant increase in TNF-α, IL-1β, and IL-6 mRNA expression, an effect which was clearly decreased by 3',4'-dihydroxyflavone. This suggests that the compound inhibits production of these cytokines by downregulating their gene expression. We also evaluated signal transduction pathways related to LPS-induced neuroinflammation, including MAPKs (including p38, JNK and ERK1/2) and NF-κB. These are reported to have crucial roles in neuroinflammation because of the production of chemokines such as $PGE_2$ and cytokines including TNF-α (Boje, 2004; Liu *et al*., 2016). Therefore, inhibition of these signaling pathways might elucidate certain underlying mechanisms in microglial activation-mediated neuroinflammation (Bachstetter and Van Eldik, 2010). In our current study, LPS-stimulated activation of MAPKs and NF-κB in microglia was

markedly reduced by 3',4'-dihydroxyflavone, indicating that its effect of suppressing neuroinflammation occurs partly through inhibition of these signaling pathways. The inhibitory effects of 3',4'-dihydroxyflavone on expression of proinflammatory cytokines and the related signaling pathways were also confirmed in our *in vivo* mouse experiments. Our finding of 3',4'-dihydroxyflavone-mediated inhibition of cytokine production further supports the agent's potential as an anti-neuroinflammatory agent.

The anti-neuroinflammatory effects of 3',4'-dihydroxyflavone we found in the mouse experiments might indicate that the compound can penetrate the blood-brain barrier (BBB). Since 3',4'-dihydroxyflavone is a small molecule with a low molecular weight (MW: 254.24) and has appropriate physicochemical properties, including lipophilicity (cLogP: 2.35), it might be expected to cross the BBB and penetrate the CNS even though it has a slightly polar catechol moiety. In addition, dinitrocatechol, an inhibitor of catechol-*O*-methyltransferase, has been shown to cross the BBB, which suggests that other catechol compounds might do so as well (Polak *et al*., 2014). However, there are a number of issues that still need to be addressed to confirm the ability of 3',4'-dihydroxyflavone to penetrate the BBB.

In conclusion, we demonstrated the anti-inflammatory effects of 3',4'-dihydroxyflavone in LPS-induced microglia and an *in vivo* mouse model. The compound reduced the levels of LPS-induced inflammatory mediators and proinflammatory cytokine production in activated microglia, in part by way of inhibition of the MAPK and NF-κB signaling pathways. Our results *in vitro* and *in vivo* suggest that 3',4'-dihydroxyflavone has significant antineuroinflammatory activity and therefore might have the potential to treat microglia-mediated neuroinflammatory diseases.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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