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Anti-Inflammatory Mechanisms of Isoflavone Metabolites in Lipopolysaccharide-Stimulated Microglial Cells

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ABSTRACT

The microglial activation plays an important role in neurodegenerative diseases by producing several proinflammatory cytokines and nitric oxide (NO). We found that three types of isoflavones and their metabolites that are transformed by the human intestinal microflora suppress lipopolysaccharide (LPS)-induced release of NO and tumor necrosis factor (TNF)-α in primary cultured microglia and BV2 microglial cell lines. The inhibitory effect of the isoflavone metabolites (aglycon form) was more potent than that of isoflavones (glycoside form). The RNase protection assay showed that the isoflavone metabolites regulated inducible nitric oxide synthase (iNOS) and the cytokines at either the transcriptional or post-transcriptional level. A further molecular mechanism study was performed for irisolidone, a metabolite of kakkalide, which had the most potent anti-inflammatory effect among the six isoflavones tested. Irisolidone significantly inhibited the DNA binding and transcriptional activity of nuclear factor (NF)-κB and activator protein-1. Moreover, it repressed the LPS-induced extracellular signal-regulated kinase (ERK) phosphorylation without affecting the activity of c-Jun N-terminal kinase or p38 mitogen-activated protein kinase. The level of NF-κB inhibition by irisolidone correlated with the level of iNOS, TNF-α, and interleukin (IL)-1β suppression in LPS-stimulated microglia, whereas the level of ERK inhibition correlated with the level of TNF-α and IL-1β repression. Overall, the repression of proinflammatory cytokines and iNOS gene expression in activated microglia by isoflavones such as irisolidone might have therapeutic potential for various neurodegenerative diseases including ischemic cerebral disease.

Microglia are the primary immune cells of the brain that are activated in response to brain injury and release of various neurotrophic factors supporting neuronal cell survival or neurotoxic factors, including nitric oxide (NO) and proinflammatory cytokines such as TNF-α and IL-1β (Kreutzberg, 1996). There is accumulating evidence suggesting that microglial activation is not just a single phenotype but is also a complex array of responses (Town et al., 2005). It was suggested that once activated, microglial cells exist in at least two functionally distinct states, namely a phagocytic phenotype (innate activation) or an antigen-presenting phenotype (adaptive activation), as determined by their stimulatory environment. The imbalance of these states can render microglial activation either beneficial or detrimental to neurons.

A number of studies have reported that neuronal cell death causes microglial activation by releasing various signaling molecules (Aldskogius et al., 1999; Kim et al., 2005), which suggests that neuroinflammation is the result of an ongoing disease process. On the other hand, several studies have suggested that activated microglia play a role as a possible cause of Alzheimer's disease by secreting proinflammatory cytokines such as TNF-α and IL-1β, which have been shown to promote neuronal injury at high levels (Tan et al., 1999,

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ABBREVIATIONS: NO, nitric oxide; TNF, tumor necrosis factor; IL, interleukin; LPS, bacterial lipopolysaccharide; NF, nuclear factor; ERK, extracellular signal-regulated kinase; PD98059, 2′-amino-3′-methoxyflavone; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene; PDTC, pyrrolidone dithiocarbamate; MG132, carbobenzoxy-L-Leucyl-L-leucyl-L-lucinal; MEM, modified Eagle's medium; FCS, fetal calf serum; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; ELISA, enzyme-linked immunosorbent assay; TBST, Tris-buffered saline/Tween 20; MAPK, mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase; AP, activator protein; iNOS, inducible nitric-oxide synthase; EMSA, electrophoretic mobility shift assay.

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2002; Lim et al., 2000, 2001). In addition, the etiological role of microglial activation has been reported in other neurodegenerative diseases (McGeer et al., 1988; Gao et al., 2003). Although the relationship between microglial activation and neurodegenerative diseases is not completely understood, it is evident that microglial activation plays an important role in the progression of several neurodegenerative diseases (Wyss-Coray and Mucke, 2002; Wyss-Coray, 2006). Therefore, the inhibition of microglial activation would be an effective therapeutic approach to alleviating the progression of neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and multiple sclerosis.

Isoflavones are biologically active compounds that are found in a variety of plants, with relatively high levels being found in soybean. Recently, a series of isoflavonoids including kakkalide, tectoridin, and glycitin were isolated from the flowers of *Pueraria thunbergiana* (Leguminoseae), which are used in traditional Chinese medicine (Park et al., 1999). Because most traditional medicines are administered orally, their components inevitably come into contact with the intestinal microflora in the alimentary tract (Han et al., 2003). These intestinal bacteria transform most of the components before they are absorbed through the gastrointestinal tract. The human intestinal bacteria transform kakkalide, tectoridin, and glycitin into irisolidone, tectorigenin, and glycitein, respectively (Bae et al., 1999; Yamaki et al., 2002). The transformed metabolites have more potent hepatoprotective and anti-inflammatory activity than the glycoside form of isoflavones (Yamaki et al., 2002; Han et al., 2003). This suggests that kakkalide, tectoridin, and glycitin are prodrugs that can be transformed into the active compounds by human intestinal bacteria.

The isoflavones isolated from the rhizomes of *P. thunbergiana* have been used in traditional medicine as antipyretics and analgesics in treatment of the cold and whose flowers have been used to treat diabetes mellitus, ethanol-induced cell mortality, and hepatic injury (Kim, 1996). Recently, there have been several reports showing the anti-inflammatory effects of isoflavones in peripheral macrophages. Tectorigenin and tectoridin inhibited the production of prostaglandin E2 and the expression of COX-2 in rat peritoneal macrophages (Kim et al., 1999). It was reported that irisolidone, tectorigenin, genistein, and glycitein suppress 12-*O*-tetradecanoylphorbol-13-acetate-induced prostaglandin E2 production (Yamaki et al., 2002). Meanwhile, glycitein was shown to inhibit LPS-induced NO production in Raw264.7 macrophage cells (Sheu et al., 2001). These results have highlighted the therapeutic potential of these isoflavones in various inflammatory diseases.

Despite the anti-inflammatory effects in peripheral macrophage cells, the effects of the six isoflavones in microglial activation have not been reported. Moreover, the detailed molecular/signaling mechanisms underlying the anti-inflammatory effects of these isoflavones are not completely understood. Therefore, this study examined whether or not the three isoflavones isolated from the flowers of *P. thunbergiana* and their bacterial metabolites suppress microglial activation. It was found that the isoflavone metabolites (irisolidone, tectorigenin, glycitein) strongly suppressed the proinflammatory cytokines and NO production in LPS-stimulated microglial cells, whereas the glycoside isoflavones (kakkalide, tectoridin, glycitin) showed no significant inhibition. In addition, it was found that the NF-κB and ERK signaling pathways are involved in the anti-inflammatory effects of irisolidone, which had the most potent anti-inflammatory effect of the six isoflavones tested. Considering that isoflavones and their metabolites have minimal side effects in the body, the inhibition of microglial activation by isoflavone metabolites may be a good potential therapeutic modality for various neurodegenerative diseases.

Materials and Methods

Preparation of Three Isoflavones and Its Bacterial Metabolites. Glycitin, tectoridin, and kakkalide were isolated according to the previous methods (Bae et al., 1999; Lee et al., 2000; Yamaki et al., 2002; Han et al., 2003). The flowers of *P. thunbergiana* (500 g), which were produced in Korea, were extracted with 2.5 liters of boiling water, concentrated in a rotary evaporator, extracted three times with ethyl acetate, and evaporated. The resulting extract (28 g) was loaded on a silica-gel flash column chromatograph and eluted with $CHCl_3$:MeOH (20:1 $\rightarrow$ 4:1). We isolated glycitin (0.25 g), tectoridin (0.3 g), and kakkalide (2.3 g). Each isolated compound (0.2 g) was incubated with *Bacteriodes spercoris* HJ-15, a human intestinal bacterium, in a final volume of 500 ml of anaerobic dilution medium in an anaerobic glove box (Coy Laboratory Products Inc., Grass Lake, MI), extracted three times with ethyl acetate, and evaporated. The resulting extract (28 g) was loaded on a silica-gel flash column chromatograph and eluted with $CHCl_3$:MeOH (20:1 $\rightarrow$ 10:1). We isolated their metabolites, glycitein (32 mg), tectorigenin (25 mg), and irisolidone (36 mg): irisolidone [pale yellowish amorphous powder, m.p. 189 to 190°C; IR ν_{max} (KBr), 3447, 2991, 1658, and 1033 cm^{-1}; FAB-MS, 315 $[M + H]^+$]; tectorigenin [pale yellowish amorphous powder, m.p. 230 to 233°C; IR ν_{max} (KBr), 3447, 2921, 1648, and 1023 cm^{-1}; FAB-MS, 303 $[M + H]^+$]; and glycitein [pale yellowish amorphous powder, m.p. 178 to 180°C; IR ν_{max} (KBr), 3472, 1635, and 1178 cm^{-1}; FAB-MS, 315 $[M+H]^+$; FAB-MS, 290 $[M+H]^+$]. The chemical structures of isoflavones and their metabolites are shown in Fig. 1.

Reagents. LPS (*Escherichia coli* serotype 055:B5) was obtained from Sigma-Aldrich (St. Louis, MO). Two kinds of ERK inhibitors, PD98059 and U0126, and two kinds of NF-κB inhibitors, pyrrolidone dithiocarbamate (PDTC) and MG132, were purchased from Calbiochem (La Jolla, CA). All other chemicals were obtained from Sigma-Aldrich, unless stated otherwise.

Microglial Cell Cultures and Cell Viability. The immortalized murine BV2 microglial cell line was grown and maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, streptomycin (10 μg/ml), and penicillin (10 U/ml) at 37°C. Cultures of primary microglial cells were established based on the differential adherence of cells harvested from fetus rat cortex. The methods were modified from Frei et al. (1987). Mixed cell cultures were prepared from postnatal 1-day rat cerebral cortices. Cortices were dissociated by passing through a 130-μm nylon mesh and plated at 2×10^5 cells/cm^2 in T75 Falcon flasks. Cultures were fed every 3 to 4 days with modified Eagle's medium (MEM) supplemented with 10% fetal calf serum (FCS). On days 12 to 13, the culture plate was shaken on a rotatory shaker at 200 rpm for 30 min. The suspended cells were plated on 24- or 48-well culture plates. After 1-h incubation at 37°C, the medium containing suspended cells was discarded, and adherent cells were further incubated with 1% FCS-supplemented MEM for future experiments. The homogeneity of the culture was determined by immunostaining for Mac-1 cell surface antigen expression and was routinely found to be higher than 90%.

Cell viability was determined in microglial cells by MTT reduction assay. In brief, cells were added to MTT (5 mg/ml in phosphate-buffered saline) and incubated at 37°C for 2 to 3 h. The resulting dark blue crystals were dissolved with an equal volume of isopropa-

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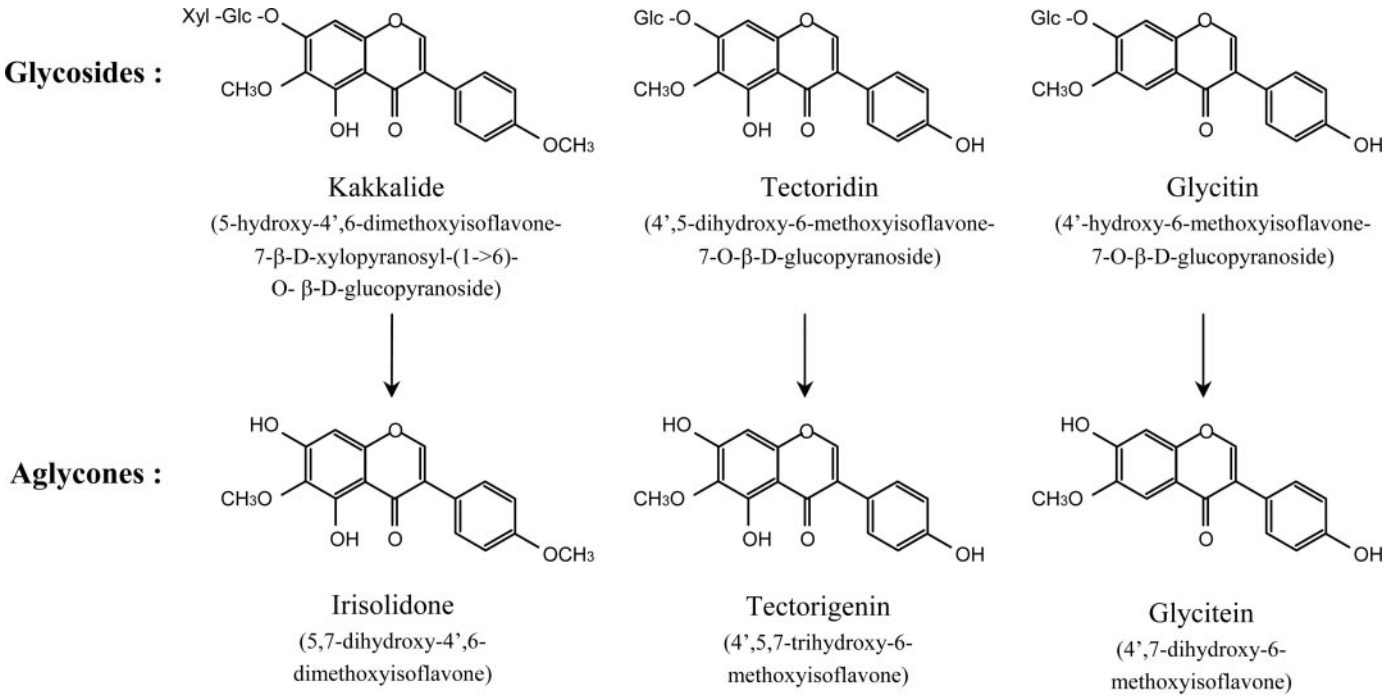

Fig. 1. The chemical structures of isoflavones used in this study. Glycosylated isoflavones and their aglycones (bacterial metabolites) are shown.

nol containing 40 mM HCl. Absorbance was determined at a test wavelength of 570 nm and a reference wavelength of 630 nm using a Microplate reader (SpectraMax 340pc; Molecular Devices, Sunnyvale, CA).

Measurement of Cytokine and Nitrite Levels. Microglial cells (1×10^5 cells per well in a 24-well plate) were pretreated with isoflavones for 30 min and stimulated with LPS (0.1 μg/ml) in the presence of serum. The supernatants of the cultured microglia were collected 6 or 24 h after LPS stimulation, and the concentrations of TNF-α and IL-1β were measured by enzyme-linked immunosorbent assay (ELISA) using monoclonal antibodies and the procedure recommended by the supplier (PharMingen, San Diego, CA). The serum in the media did not interfere with the assay. Accumulated nitrite was measured in the cell supernatant by the Griess reaction (Green et al., 1982). The conditions of cell culture and treatment were same with those in ELISA. In brief, 50-μl aliquots of cell supernatant from each well were mixed with 100 μl of Griess reagent (mixing equal volumes of 0.1% naphthylethylenediamine dihydrochloride and 1% sulfanilamide in 5% phosphoric acid) in a 96-well microtiter plate, and absorbance was read at 540 nm using a plate reader (Molecular Devices). Sodium nitrite, diluted in culture media at concentrations ranging from 10 to 100 μM, was used to prepare a standard curve.

RNase Protection Assay. BV2 cells (4×10^5 cells on a 35-mm dish) were treated with LPS in the presence or absence of isoflavone metabolites, and total RNA was extracted with TRIzol reagent (Sigma-Aldrich) according to the manufacturer's protocol. RNase protection assay was performed using a Riboquant multiprobe RNase protection assay kit (PharMingen). Protected fragments were resolved on 6% urea-polyacrylamide gel, and autography was performed after exposing the dried gel to X-ray film.

Western Blot. Cell extracts were prepared as described previously (Woo et al., 2003), and 50 μg of proteins was separated by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% bovine serum albumin in 10 mM Tris-HCl containing 150 mM NaCl and 0.5% Tween 20 (TBST) and then incubated with primary antibodies (1:1000) that recognize the phospho- or total forms of ERK1/2, p38 MAPK, and JNK (Cell Signaling Technology Inc., Beverly, MA). For Western blot of TNF-α, antibody that recognizes both the pro- and mature form of TNF-α (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used. After thoroughly washing with TBST, horseradish peroxidase-conjugated secondary antibodies (New England Biolabs, Beverly, MA; 1:3000 dilution in TBST) were applied, and the blots were developed using an enhanced chemiluminescence detection kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK).

Nuclear Extract Preparation and Electrophoretic Mobility Shift Assay. Nuclear extracts from treated microglia were prepared as described previously (Woo et al., 2003). The double-stranded DNA oligonucleotides containing the NF-κB or AP-1 consensus sequences (Promega) were end-labeled using T4 polynucleotide kinase (New England Biolabs) in the presence of [γ-^{32}P]ATP. Five micrograms of the nuclear proteins was incubated with ^{32}P-labeled NF-κB or AP-1 probe on ice for 30 min and resolved on a 5% acrylamide gel as described previously (Woo et al., 2003). For the supershift assay, antibodies to the p65 or p50 subunits of NF-κB (Santa Cruz Biotechnology, Inc.) were coincubated with the protein in the reaction mixture for 30 min at 4°C before adding the radiolabeled probe. For competition binding assays, binding reaction reagents and nuclear extracts were mixed with nonradioactive oligonucleotides in molar excess and incubated before adding ^{32}P-labeled probe.

Transient Transfection and Luciferase Assay. Transfection of the NF-κB or AP-1 reporter gene into BV2 cells was performed using Geneporter 2 transfection reagent (Gene Therapy Systems Inc., San Diego, CA). BV2 cells (1×10^5 per well in a 12-well plate) were transfected with 1 μg of the reporter construct mixed with Geneporter. After 48 h, cells were harvested, and a luciferase assay was performed as described previously (Woo et al., 2003). To determine the effect of isoflavones on LPS-induced NF-κB or AP-1 activity, cells were pretreated with the agents for 30 min before treating with LPS and incubated for 6 h before harvesting cells for luciferase assay. Transfection assays were performed three times in duplicate.

Statistical Analysis. The SAS program (SAS Institute, Cary, NC) was used for statistical analysis. The data are expressed as the mean ± S.E.M. and analyzed for statistical significance using analysis of variance, followed by Scheffe's test for multiple comparison. A p value < 0.05 was considered significant.

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Results

Effect of the Isoflavones on NO and Cytokine Production in LPS-Stimulated Microglial Cells. The BV2 microglial cells were stimulated with LPS (100 ng/ml) in the presence or absence of the isoflavones isolated from the flowers of *P. thunbergiana* to determine whether they reduce the level of NO, TNF-α, and IL-1β production, which play important roles in various inflammatory diseases. The stimulation of BV2 cells with LPS led to a significant increase in the NO, TNF-α, and IL-1β levels in the cell-conditioned media after 24 h. Pretreatment of BV2 cells with the isoflavone metabolites (irisolidone, tectorigenin, glycitein) significantly inhibited the LPS-induced NO, TNF-α, and IL-1β production in a dose-dependent manner (Fig. 2A). The potency of the inhibition of NO and TNF-α was in order of irisolidone > glycitein > tectorigenin, and the inhibition of IL-1β was in the order of glycitein > irisolidone > tectorigenin. In contrast, their proforms (glycoside isoflavones) showed no significant inhibition. The isoflavone metabolite-induced decrease in NO, TNF-α, and IL-1β was also observed in the rat primary microglia cultures (Fig. 2B). This suggests that the anti-inflammatory effect of isoflavone metabolites is not limited to a particular microglial cell line. To exclude the possibility that the decrease in the NO and cytokine levels was simply due to cell death, the cell viability was tested at various concentrations of isoflavones. The MTT assay showed that the isoflavones did not have any cytotoxicity at the concentrations (5–50 μM) used in this study in both the BV2 and primary microglial cells for at least 48 h (data not shown).

Isoflavone Metabolites Suppress mRNA Expressions of iNOS, TNF-α, and IL-1β. RNase protection assay was performed to determine whether the isoflavone metabolites regulate iNOS and cytokine expression at the transcriptional level. As shown in Fig. 3A, the three types of isoflavone metabolites at 50 μM significantly inhibited iNOS, TNF-α, and IL-1β mRNA expression. Despite the robust inhibition of TNF-α protein production by 50 μM of the isoflavone metabolites, the same concentration of metabolites decreased the TNF-α mRNA levels by only 20 to 30%. Therefore, these results suggest that three isoflavone metabolites regulate the expression of iNOS and IL-1β at the transcriptional level, whereas they regulate the expression of TNF-α at the transcriptional and/or post-transcriptional level. In support of this hypothesis, Western blot analysis revealed that the isoflavone metabolites significantly repressed both the pro- and mature form TNF-α at the protein level in the order of irisolidone > glycitein > tectorigenin, as shown in ELISA data (Fig. 3C). Based on these results, the molecular mechanism of irisolidone, which showed the most potent anti-inflammatory activity among three isoflavone metabolites tested, was further examined.

Irisolidone Inhibits the DNA Binding and Transcriptional Activities of NF-κB and AP-1 in LPS-Stimulated Microglial Cells. NF-κB is an important upstream modulator of cytokine and iNOS expression in microglia (Pahl, 1999), and the anti-inflammatory actions of a number of cytokines and chemicals have been explained in terms of the inhibition of NF-κB activity. Therefore, the involvement of NF-κB activity in irisolidone-induced suppression of NO and cytokines was examined. As shown in Fig. 4A, the stimulation of BV2 cells with LPS resulted in strong NF-κB binding, which was inhibited significantly by irisolidone. The compound also affected NF-κB-mediated transcription (Fig. 4B). The NF-κB transcriptional activity was assayed by transfecting the BV2 cells with a plasmid containing three NF-κB binding sites and a luciferase reporter gene. The luciferase assay showed that irisolidone repressed the LPS-stimulated NF-κB transcriptional activation in a dose-dependent man-

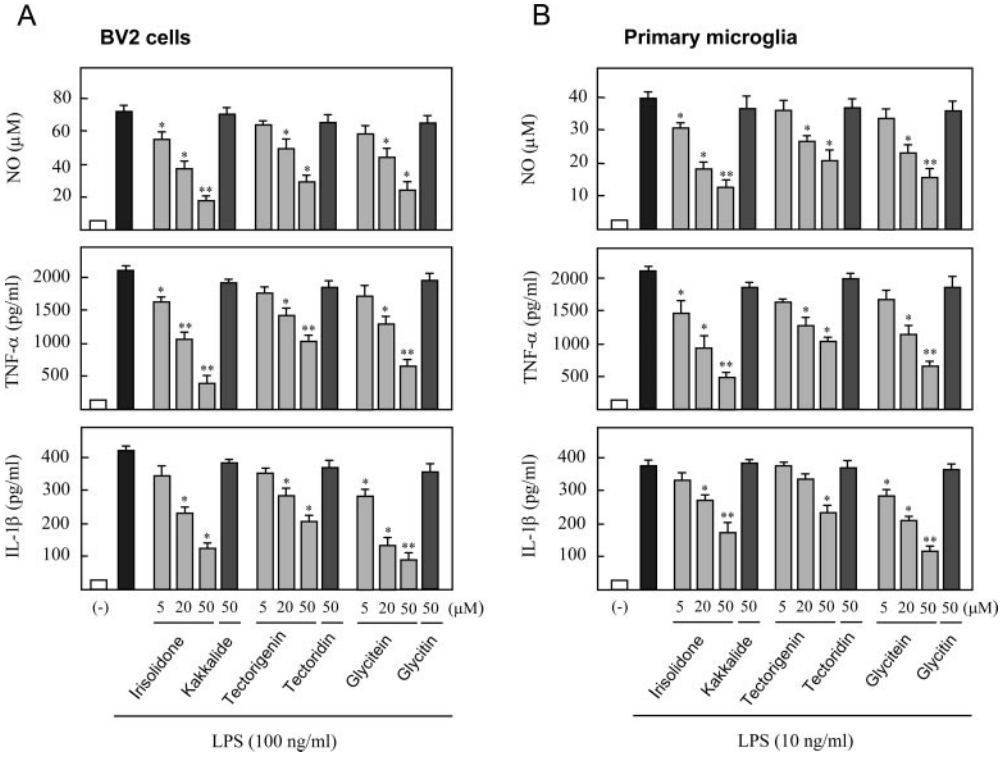

Fig. 2. Effect of isoflavones on NO and cytokine production in LPS-stimulated BV2 cells (A) and primary microglia (B). BV2 microglial cells (1×10^5 cells per well in a 24-well plate) were incubated with 0.1 μg/ml LPS and various concentrations of isoflavones in DMEM containing 10% fetal bovine serum for 24 h. Rat primary microglia were incubated with 10 ng/ml LPS and isoflavones in 1% FCS-supplemented MEM. The amounts of NO in the supernatants were measured using Griess reagent and TNF-α and IL-1β by ELISA. The data are expressed as the mean ± S.E.M. of four independent experiments. *, $p < 0.05$; **, $p < 0.01$; Scheffe's test; significantly different from the value in cells treated with LPS in the absence of isoflavones. The vehicle (0.2% dimethyl sulfoxide) where isoflavones were dissolved did not affect either basal or LPS-induced NO and cytokine levels.

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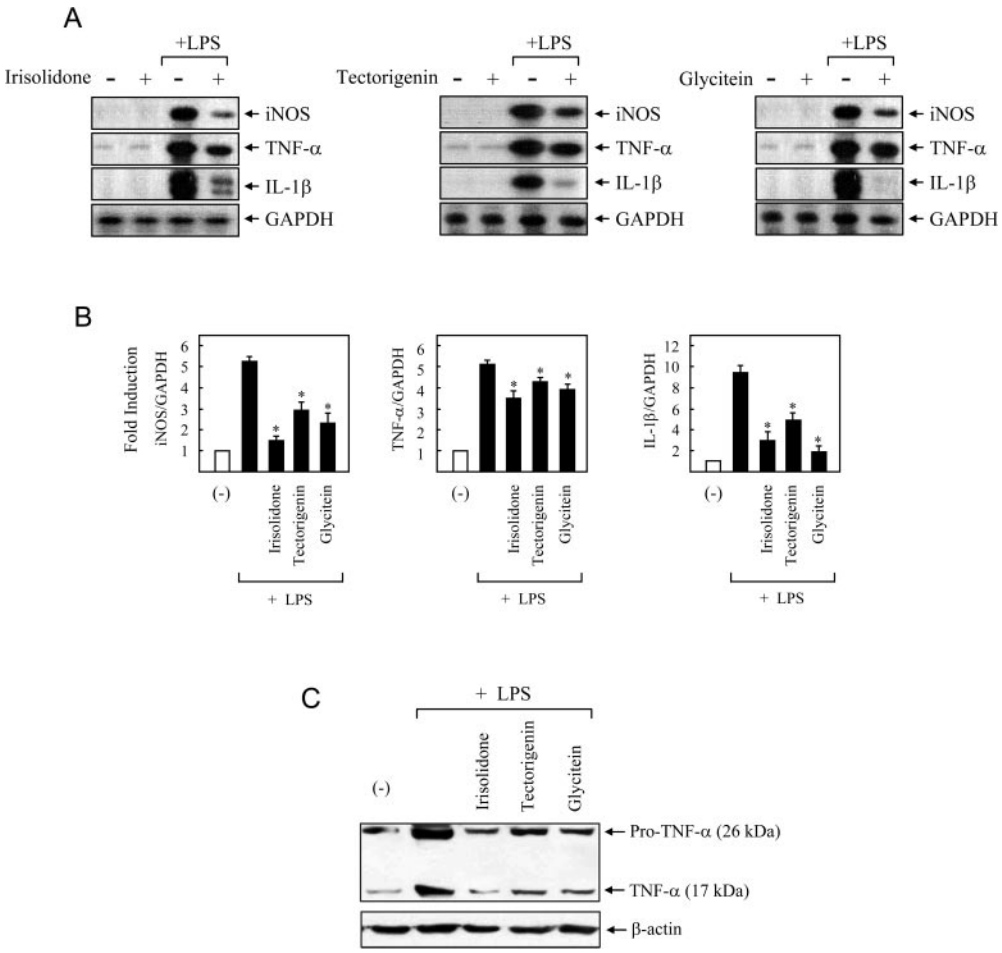

Fig. 3. Effect of isoflavone metabolites on iNOS and cytokine mRNA expressions in LPS-stimulated microglial cells. BV2 cells were treated with LPS (0.1 μg/ml) in the absence or presence of isoflavone metabolites (50 μM), and total RNA was isolated at 6 h after LPS treatment. A, iNOS, TNF-α, and IL-1β mRNA levels were determined by RNase protection assay. The band corresponding to each cytokine is indicated by an arrow. B, quantification of the mRNA data. Levels of iNOS and cytokines were normalized to GAPDH levels and expressed as relative -fold activation in comparison with LPS-treated samples. Values correspond to the mean ± S.E.M. of three independent experiments. *, $p < 0.05$, significantly different from LPS-treated samples. C, Western blot for TNF-α protein production. Cell extracts were prepared from BV2 microglial cultures treated with LPS (0.1 μg/ml) for 16 h in the absence or presence of isoflavone metabolites (50 μM) and were subjected to immunoblot analysis using antibodies that recognize both the pro- and mature form of TNF-α.

ner (Fig. 4B). Furthermore, irisolidone inhibited the DNA binding and transcriptional activity of AP-1, which is also an important modulator of inflammation (Fig. 4, C and D). Therefore, NF-κB and AP-1 may be important molecules that determine the anti-inflammatory activity of irisolidone in LPS-stimulated microglia.

Irisolidone Suppresses LPS-Induced ERK Phosphorylation in LPS-Stimulated Microglial Cells but Not JNK or p38 MAPK Phosphorylation. The effect of irisolidone on MAP kinases, which are upstream signaling molecules in inflammatory reactions, was examined. Western blot analysis was carried out using the phospho- or total forms of antibodies against the three MAP kinases, p38 MAPK, ERK1/2, and JNK. It was observed that irisolidone (50 μM) remarkably decreased the LPS-stimulated phosphorylation of ERK by 70 and 50% at 1 and 3 h, respectively, whereas it had no effect on either JNK or p38 MAPK activities (Fig. 5). This suggests that the ERK pathway may be involved in the anti-inflammatory activity of irisolidone.

Effect of ERK-Specific Inhibitor (PD98059) and NF-κB Inhibitor (PDTC) on Cytokine and iNOS Expression in LPS-Stimulated Microglial Cells. To determine whether the inhibition of ERK and NF-κB correlates with the anti-inflammatory mechanism of irisolidone, the BV2 cells were treated with either PD98059 (ERK-specific inhibitor) or PDTC (NF-κB inhibitor) before LPS stimulation, and iNOS and cytokine expression levels were examined. As shown in Fig. 6, A and B, PDTC decreased the LPS-induced mRNA and protein expression levels of iNOS, TNF-α, and IL-1β, whereas PD98059 repressed TNF-α and IL-1β without affecting the generation of NO. Although PD98059 dramatically inhibited TNF-α production, its effect on the mRNA level was minor. Instead, PD98059 inhibited the expression of the pro- and mature form of TNF-α protein, as shown in Western blot data (Fig. 6C), indicating that PD98059 downregulates TNF-α expression at the post-transcriptional level. Overall, these results suggest that the anti-inflammatory effect of irisolidone in LPS-stimulated microglial cells may be mediated through the inhibition of the NF-κB and ERK signaling pathways.

The Relationship between the ERK Pathway and NF-κB Activation in LPS-Stimulated Microglial Cells. The effect of an ERK-specific inhibitor on NF-κB or vice versa was examined to determine the relationship between ERK and NF-κB. As shown in Fig. 7, A and B, neither PD98059 nor U0126 had any significant effect on the LPS-induced DNA binding and transcriptional activities of NF-κB in BV2 cells. In contrast, two NF-κB inhibitors, PDTC and MG132, decreased the level of ERK-phosphorylation significantly (Fig. 7C). This suggests that at least in LPS-stimulated microglial cells, ERK is not upstream of NF-κB activation. Instead, it is believed that ERK and NF-κB modulate the cytokines independently. However, the inhibition of TNF-α by the NF-κB inhibitors appears to result in reduced level of ERK phosphorylation due to the autocrine/paracrine regulatory effect of TNF-α on ERK.

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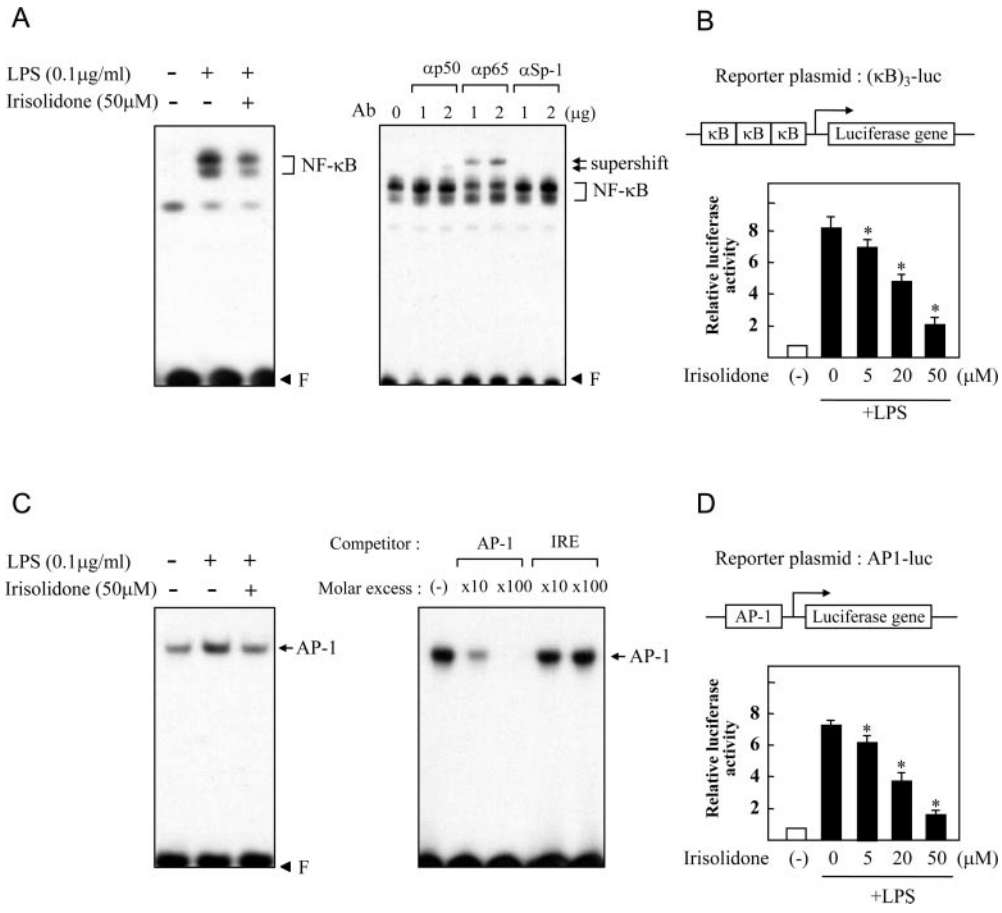

Fig. 4. Effect of irisolidone on DNA binding and transcriptional activities of NF-κB and AP-1. A, electrophoretic mobility shift assay (EMSA) for NF-κB DNA binding activity. Nuclear extracts were prepared from BV2 cells after treatment with LPS (0.1 μg/ml) for 3 h in the presence or absence of irisolidone (50 μM). The bracket indicates a DNA-protein complex of NF-κB. Supershift band was generated by incubation of the NF-κB complex and the antibody against p50 or p65 subunit of NF-κB. B, NF-κB reporter gene assay. Irisolidone repressed LPS-induced NF-κB promoter activity in a dose-dependent manner. C, EMSA for AP-1 DNA binding activity. Arrow, DNA-protein complex of AP-1. Competition assay revealed that the complex is AP-1-specific because it was competed by its own cold oligonucleotide but not by nonspecific IRE oligonucleotide. D, AP-1 reporter gene assay. Irisolidone repressed LPS-induced AP-1 promoter activity in a dose-dependent manner. *, significantly different from NF-κB or AP-1 promoter activity in cells treated with LPS alone.

Discussion

This article reports the inhibitory effects of the isoflavone metabolites against microglial activation. Three types of isoflavone metabolites (irisolidone, tectorigenin, glycitein) significantly suppressed the LPS-induced production of NO, TNF-α, and IL-1β in the BV2 microglial cells and rat primary cultured microglia. The RNase protection assay showed that the isoflavone metabolites repressed iNOS, TNF-α, and IL-1β expression at the transcriptional or post-transcriptional level. The potency of inhibition was in order of irisolidone > glycitein > tectorigenin in the case of NO and TNF-α generation and glycitein > irisolidone > tectorigenin in the case of IL-1β generation. In contrast, the glycosylated isoflavones did not significantly repress the inflammatory molecules, suggesting that glycosylation might decrease the biological activity. A comparison of the three isoflavone structures showed that they commonly posses a 6-methoxy residue, suggesting that the 6-methoxylation of isoflavones contributed to the anti-inflammatory activity of isoflavones in the microglia. Moreover, 4′-methoxylation appears to enhance the activity because irisolidone, which has the most potent anti-inflammatory effect, has this residue differently from other isoflavones (Fig. 1).

The molecular mechanisms underlying anti-inflammatory effect of irisolidone, which showed the most potent anti-inflammatory activity, were further studied. The results suggest that the NF-κB and ERK signaling pathways are largely involved in the modulation of iNOS, TNF-α, and IL-1β by irisolidone in LPS-stimulated microglial cells. The stimulation of microglial cells with LPS induced the activation of NF-κB and ERK, which subsequently led to the up-regulation of iNOS, TNF-α, and IL-1β expression. NF-κB appears to modulate iNOS, TNF-α, and IL-1β expression, whereas ERK regulate TNF-α and IL-1β without affecting iNOS. Overall, the possible target of irisolidone is believed to be NF-κB and ERK.

The fact that the ERK-specific inhibitors did not suppress the activity of NF-κB suggests that ERK is not upstream of NF-κB activation, at least in LPS-stimulated microglial cells. Instead, ERK and NF-κB appear to modulate cytokine expression independently. However, due to the autocrine/paracrine regulatory effect of TNF-α on ERK (Tortorella et al., 2004), the inhibition of TNF-α by the NF-κB inhibitors appears to decrease the level of ERK phosphorylation (Fig. 7C). Some investigations in peripheral cells have presented evidence showing that NF-κB is downstream of ERK (Kan et al., 1999; Jiang et al., 2001). However, other studies suggest that there are two independent signaling pathways (Doi et al., 2000; Chan and Riches, 2001). Moreover, our group previously reported that ERK plays a role as an upstream modulator of NF-κB in iNOS expression in interferon-γ-stimulated BV2 microglial cells (Park et al., 2005). Therefore, the interaction between the signaling molecules in the expression of certain genes differs according to the stimuli and cell types examined. More study will be needed to clarify the relationship between ERK and NF-κB in activated microglia.

Besides the anti-inflammatory effects of the isoflavones used in this study, a variety of biological effects of isoflavones

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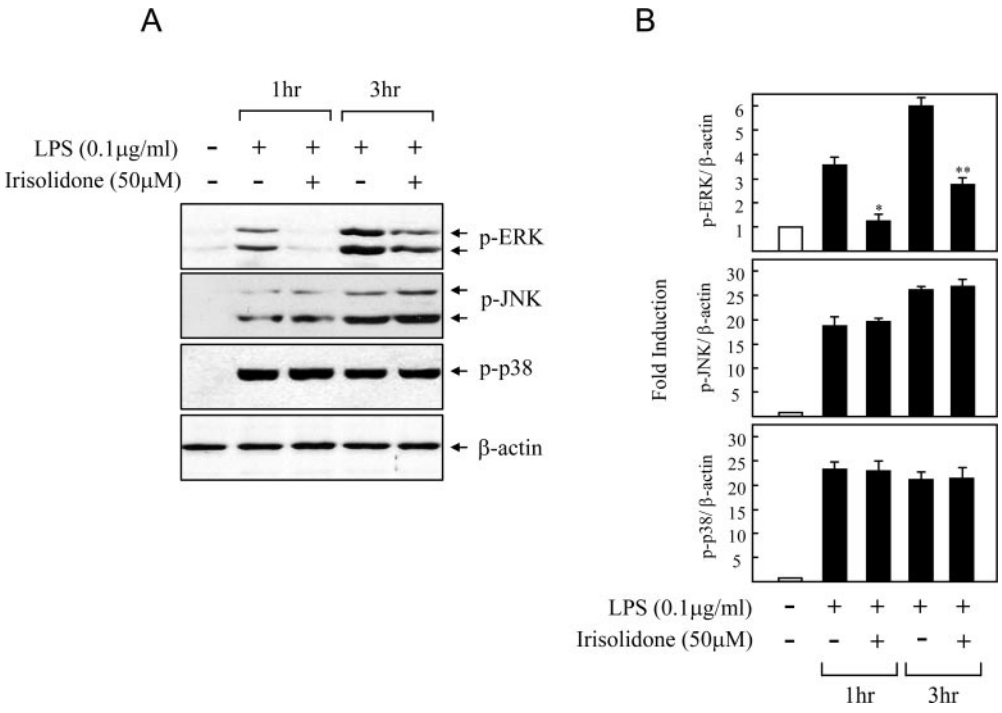

Fig. 5. Effect of irisolidone on LPS-induced MAP kinase activities. A, Western blots for MAP kinase activities. Cell extracts were prepared from microglial cultures treated with LPS (0.1 μg/ml) for the indicated times in the absence or presence of irisolidone (50 μM) and were subjected to immunoblot analysis using antibodies against phospho-form of the three MAP kinases (i.e., p38 MAPK, ERK1/2, and JNK). The autoradiograms are representative of three independent experiments. B, levels of MAP kinase were quantified by densitometer and expressed as -fold induction. Values correspond to the mean ± S.E.M. of three independent experiments. *, $p < 0.05$; **, $p < 0.01$; significantly different from LPS-treated samples.

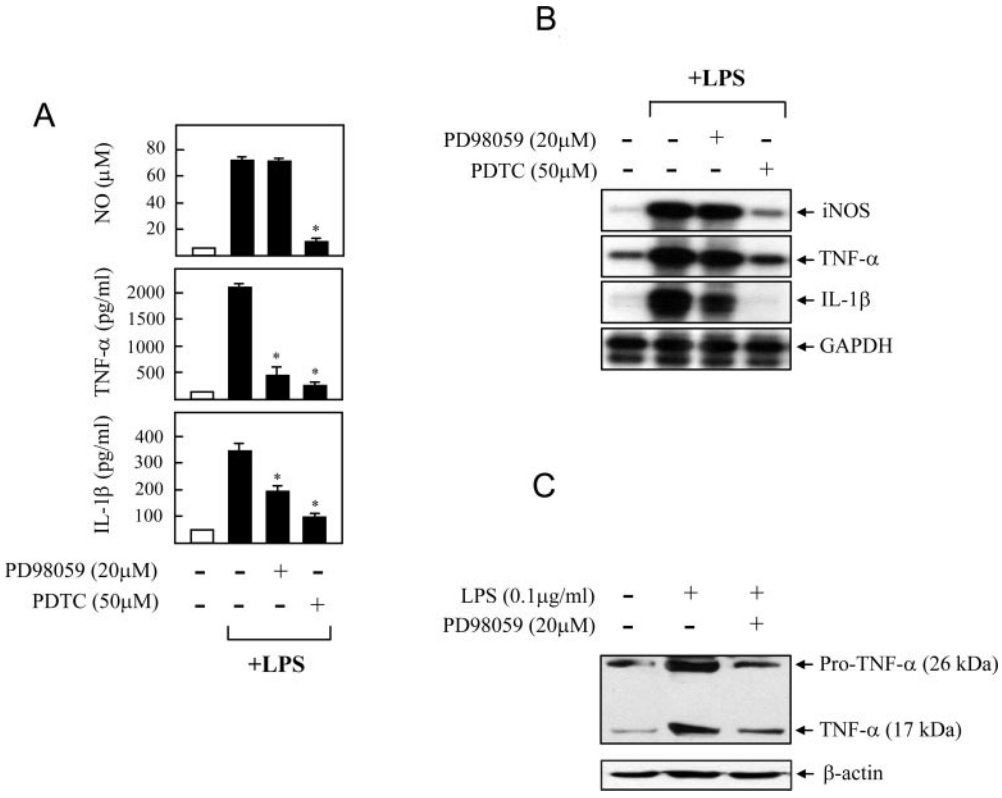

Fig. 6. Effect of ERK or NF-κB inhibitor on iNOS and cytokine expressions in LPS-stimulated microglial cells. A, BV2 cells were incubated with 0.1 μg/ml LPS in the presence or absence of PD98059 (20 μM) or PDTC (50 μM) for 24 h. The amounts of NO in the supernatants were measured using Griess reagent. TNF-α and IL-1β were measured by ELISA. The data are expressed as the mean ± S.E.M. of three independent experiments. *, $p < 0.05$, significantly different from the value in cells treated with LPS alone. B, RNase protection assay. Total RNA was isolated from the cells treated as in A and incubated for 6 h. PDTC significantly repressed iNOS, TNF-α, and IL-1β mRNA expression, whereas PD98059 repressed only IL-1β mRNA level. C, effect of PD98059 on pro- and active form of TNF-α production.

have been demonstrated until now. Genistein, daidzein, and glycitein have been found to have antioxidative ability, which can prevent atherosclerosis and stroke, and to reduce the risk of cancer (Hodgson et al., 1996; Cline and Hughes, 1998; Kopelovich et al., 2000). Tectorigenin and genistein also exhibited cytotoxicity against various human cancer cells (Hodgson et al., 1996; Lee et al., 2001). In addition, a recent study reported the cytoprotective effect of tectorigenin on oxidative stress induced by hydrogen peroxide (Kang et al., 2005). Genistein suppresses the expression of iNOS, TNF, intercellular adhesion molecule-1, and vascular cell adhesion molecule-1 through the inhibition of the tyrosine kinase activities (Weber et al., 1995). It also had a neuroprotective effect against β amyloid-induced neurotoxicity (Bang et al., 2004). The neuroprotective effects of isoflavones have been shown in animal models of Alzheimer's disease, Parkinson's

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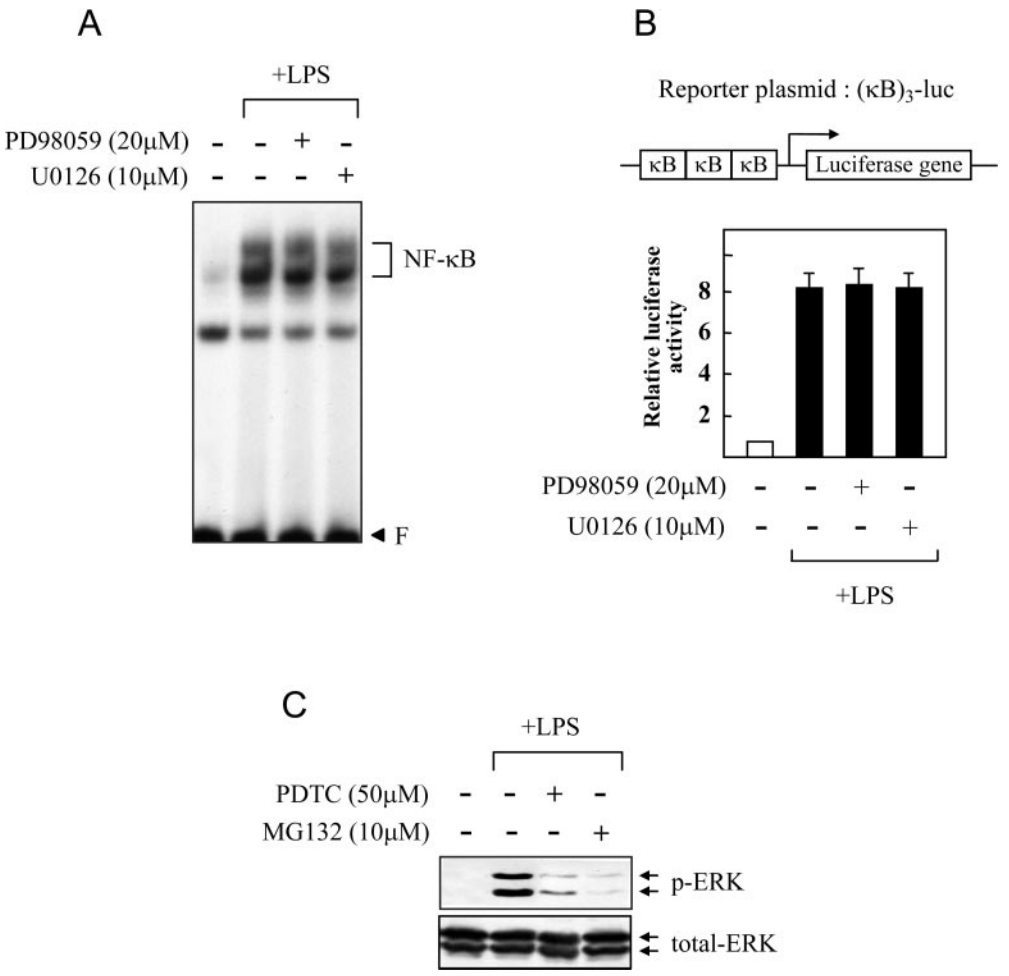

Fig. 7. Regulation of NF-κB and ERK in LPS-stimulated microglial cells. A, EMSA for NF-κB DNA binding activity. Nuclear extracts were prepared from BV2 cells after treatment with LPS (0.1 μg/ml) for 3 h in the presence or absence of ERK inhibitors, PD98059 (20 μM) and U0126 (10 μM). The bracket indicates a DNA-protein complex of NF-κB. B, NF-κB reporter gene assay. Neither PD98059 nor U0126 inhibited LPS-induced NF-κB promoter activity. C, Western blots for ERK activities. Cell extracts were prepared from BV2 microglial cultures treated with LPS (0.1 μg/ml) for 3 h in the absence or presence of NF-κB inhibitors, PDTC (50 μM) and MG132 (10 μM), and were subjected to immunoblot analysis using antibodies against phospho- or total form of ERK.

disease, and amyloid lateral sclerosis (Trieu and Uckun, 1999; Rezai-Zadeh et al., 2005; Wang et al., 2005). In the future, it will be necessary to search for more biologically active constituents in medicinal herbs, which can be applied to the development of pharmaceutical treatments for neurodegenerative diseases.

In conclusion, this article reports for the first time the anti-inflammatory activity of isoflavone metabolites (irisolidone, tectorigenin, glycitein) in microglial cells along with their underlying molecular mechanisms, with particular focus on irisolidone. Further research on the molecular mechanisms of the other two isoflavone metabolites (tectorigenin and glycitein) will be helpful to precisely correlate the structure-activity relationship of isoflavones. Considering that isoflavone metabolites are easily absorbed into the body and have a chemical structure that can readily penetrate the blood-brain barrier, the strong inhibition of microglial activation by these metabolites might have therapeutic potential for various neurodegenerative diseases.

References

Aldskogius H, Liu L, and Syensson M (1999) Glial responses to synaptic damage and plasticity. *J Neurosci Res* **58:**33–41.

Bae EA, Han MJ, Lee KT, Choi JW, Park HJ, and Kim DH (1999) Metabolism of 6″-*O*-xylosyltectordin and tectoridin by human intestinal bacteria and their hypoglycemic and in vitro cytotoxic activities. *Biol Pharm Bull* **22:**1314–1318.

Bang OY, Hong HS, Kim DH, Kim H, Boo JH, Huh K, and Mook-Jung I (2004) Neuroprotective effect of genistein against beta amyloid-induced neurotoxicity. *Neurobiol Dis* **16:**21–28.

Chan ED and Riches DW (2001) IFN-gamma + LPS induction of iNOS is modulated by ERK, JNK/SAPK, and p38(mapk) in a mouse macrophage cell line. *Am J Physiol* **280:**1077–1083.

Cline JM and Hughes CL Jr (1998) Phytochemicals for the prevention of breast and endometrial cancer. *Cancer Treat Res* **94:**107–134.

Doi M, Shichiri M, Katsuyama K, Marumo F, and Hirata Y (2000) Cytokine-activated p42/p44 MAP kinase is involved in inducible nitric oxide synthase gene expression independent from NF-kappa B activation in vascular smooth muscle cells. *Hypertens Res* **23:**659–667.

Frei K, Siepl C, Groscurth P, Bodmer S, Schwerdel C, and Fontana A (1987) Antigen presentation and tumor cytotoxicity by interferon-gamma-treated microglial cells. *Eur J Immunol* **17:**1271–1278.

Gao HM, Liu B, Zhang W, and Hong JS (2003) Critical role of microglial NADPH oxidase-derived free radicals in the in vitro MPTP model of Parkinson's disease. *FASEB J* **17:**1954–1956.

Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnock JS, and Tannenbaum SR (1982) Analysis of nitrate, nitrite, and [^{15}N] nitrate in biological fluids. *Ann Biochem* **126:**131–138.

Han YO, Han MJ, Park SH, and Kim DH (2003) Protective effects of kakkalide from *Flos puerariae* on ethanol-induced lethality and hepatic injury are dependent on its biotransformation by human intestinal microflora. *J Pharmacol Sci* **93:**331–336.

Hodgson JM, Croft KD, Puddey IB, Mori TA, and Beilin LJ (1996) Soybean isoflavonoids and their metabolic products inhibit in vitro lipoprotein oxidation in serum. *J Nutr Biochem* **7:**664–669.

Jiang B, Brecher P, and Cohen RA (2001) Persistent activation of nuclear factor-κB by interleukin-1β and subsequent inducible NO synthase expression requires extracellular signal-regulated kinase. *Arterioscler Thromb Vasc Biol* **21:**1915–1920.

Kan H, Xie Z, and Finkel MS (1999) TNF-alpha enhances cardiac myocyte NO production through MAP kinase-mediated NF-kappa B activation. *Am J Physiol* **277:**H1641–H1646.

Kang KA, Lee KH, Chae S, Zhang R, Jung MS, Kim SY, Kim HS, Kim DH, and Hyun JW (2005) Cytoprotective effect of tectorigenin, a metabolite formed by transformation of tectoridin by intestinal microflora, on oxidative stress induced by hydrogen peroxide. *Eur J Pharmacol* **519:**16–23.

Kim TJ (1996) *Korean Resources Plants*, Vol. 2, Seoul National University Press, Seoul, Korea.

Kim YP, Yamada M, Lim SS, Lee SH, Ryu N, Shin KH, and Ohuchi K (1999) Inhibition by tectorigenin and tectoridin of prostaglandin E2 production and

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cyclooxygenase-2 induction in rat peritoneal macrophages. *Biochim Biophys Acta* **1438:**399–407.
Kim YS, Kim SS, Cho JJ, Choi DH, Hwang O, Shin DH, Chun HS, Beal MF, and Joh TH (2005) Matrix metalloproteinase-3: a novel signaling proteinase from apoptotic neuronal cells that activates microglia. *J Neurosci* **25:**3701–3711.
Kopelovich L, Hawak ET, Lieberman R, Lawrence JA, Ali I, Viner JL, and Sigman CC (2000) Progress in cancer chemoprevention: development of diet-derived chemopreventive agents. *J Nutr* **130:**467S–471S.
Kreutzberg GW (1996) Microglia: a sensor for pathological events in the CNS. *Trends Neurosci* **19:**312–318.
Lee KT, Sohn IC, Kim DH, Choi JW, Kwon SH, and Park HJ (2000) Hypoglycemic and hypolipidemic effects of tectorigenin and kaikasaponin III in the streptozotocin-induced diabetic rat and their antioxidant activity in vitro. *Arch Pharm Res (NY)* **23:**461–466.
Lee KT, Sohn IC, Kim YK, Choi JH, Choi JW, Park HJ, Itoh Y, and Miyamoto K (2001) Tectorigenin, an isoflavone of *Pueraria thunbergiana* BENTH., induces differentiation and apoptosis in human promyelocytic leukemia HL-60 cells. *Biol Pharm Bull* **24:**1117–1121.
Lim GP, Chu T, Yang F, Beech W, Frautschy SA, and Cole GM (2001) The curry spice curcumin reduces oxidative damage and amyloid pathology in an Alzheimer transgenic mouse. *J Neurosci* **21:**8370–8377.
Lim GP, Yang F, Chu T, Chen P, Beech W, Teter B, Tran T, Ubeda O, Ashe KH, Frautschy SA, et al. (2000) Ibuprofen suppresses plaque pathology and inflammation in a mouse model for Alzheimer's disease. *J Neurosci* **20:**5709–5714.
McGeer PL, Itagaki S, Boyes BE, and McGeer EG (1988) Reactive microglia are positive for HLA-DR in the substantia nigra of Parkinson's and Alzheimer's disease brains. *Neurology* **38:**1285–1291.
Pahl HL (1999) Activators and target genes of Rel/NF-κB transcription factors. *Oncogene* **18:**6853–6866.
Park HJ, Park JH, Moon JO, Lee KT, Jung WT, Oh SR, and Lee HK (1999) Isoflavone glycosides from the flowers of *Pueraria thunbergiana*. *Phytochemistry* **51:**147–151.
Park JS, Woo MS, Kim SY, Kim WK, and Kim HS (2005) Repression of interferon-γ-induced inducible nitric oxide synthase (iNOS) gene expression in microglia by sodium butyrate is mediated through specific inhibition of ERK signaling pathways. *J Neuroimmunol* **168:**56–64.
Rezai-Zadeh K, Shytle D, Sun N, Mori T, Hou H, Jeanniton D, Ehrhart J, Townsend K, Zeng J, Morgan D, et al. (2005) Green tea epigallocatechin-3-gallate (EGCG) modulates amyloid precursor protein cleavage and reduces cerebral amyloidosis in Alzheimer transgenic mice. *J Neurosci* **25:**8807–8814.
Sheu F, Lai HH, and Yen GC (2001) Suppression effect of soy isoflavones on nitric oxide production in RAW264.7 macrophages. *J Agri Food Chem* **49:**1767–1772.
Tan J, Town T, Crawford F, Mori T, DelleDonne A, Crescentini R, Obregon D, Flavell RA, and Mullan MJ (2002) Role of CD40 ligand in amyloidosis in transgenic Alzheimer's disease. *Nat Neurosci* **5:**1288–1293.
Tan J, Town T, Paris D, Mori T, Suo Z, Crawford F, Mattson MP, Flavell RA, and Mullan MJ (1999) Microglial activation resulting from CD40-CD40L interaction after beta-amyloid stimulation. *Science (Wash DC)* **286:**2352–2355.
Tortorella C, Stella I, Piazzolla G, Simone O, Cappiello V, and Antonaci S (2004) Role of defective ERK phosphorylation in the impaired GM-CSF-induced oxidative response of neutrophils in elderly humans. *Mech Ageing Dev* **125:**539–546.
Town T, Nikolic V, and Tan J (2005) The microglial "activation" continuum: from innate to adaptive responses. *J Neuroinflammation* **2:**24–33.
Trieu VN and Uckun FM (1999) Genistein is neuroprotective in murine models of familial amyotrophic lateral sclerosis and stroke. *Biochem Biophys Res Commun* **258:**685–688.
Wang X, Chen S, Ma G, Ye M, and Lu G (2005) Genistein protects dopaminergic neurons by inhibiting microglial activation. *Neuroreport* **16:**267–270.
Weber C, Negrescu E, Erl W, Pietsch A, Frankenberger M, Ziegler-Heitbrock HW, Siess W, and Webber PC (1995) Inhibitors of protein tyrosine kinase suppress TNF-stimulated induction of endothelial cell adhesion molecules. *J Immunol* **155:**445–451.
Woo MS, Jang PG, Park JS, Kim WK, Joh TH, and Kim HS (2003) Selective modulation of lipopolysaccharide-stimulated cytokine expression and mitogen-activated protein kinase pathways by dibutyryl-cAMP in BV2 microglial cells. *Mol Brain Res* **113:**86–96.
Wyss-Coray T (2006) Inflammation in Alzheimer's disease: driving force, bystander or beneficial response? *Nat Med* **12:**1005–1015.
Wyss-Coray T and Mucke L (2002) Inflammation in neurodegenerative disease: a double-edged sword. *Neuron* **35:**419–432.
Yamaki K, Kim DH, Ryu N, Kim YP, Shin KH, and Ohuchi K (2002) Effects of naturally occurring isoflavones on prostaglandin E2 production. *Plant Med* **68:**97–100.

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