Available online at www.sciencedirect.com
ScienceDirect
Journal of Nutritional Biochemistry 22 (2011) 612–624
Journal of Nutritional Biochemistry

RESEARCH ARTICLES

Luteolin inhibits cytokine expression in endotoxin/cytokine-stimulated microglia

Tsung-Kuei Kao[a,1], Yen-Chuan Ou[b,1], Shih-Yi Lin[c], Hung-Chuan Pan[d], Pei-Jyuan Song[e], Shue-Ling Raung[f], Ching-Yi Lai[f], Su-Lan Liao[f], Hsi-Chi Lu[e], Chun-Jung Chen[f,g,h,i,*]

[a]Department of Nursing, Tajen University, Pingtung 907, Taiwan
[b]Division of Urology, Taichung Veterans General Hospital, Taichung 407, Taiwan
[c]Division of Endocrinology and Metabolism, Taichung Veterans General Hospital, Taichung 407, Taiwan
[d]Department of Neurosurgery, Taichung Veterans General Hospital, Taichung 407, Taiwan
[e]Department of Food Science, Tunghai University, Taichung 407, Taiwan
[f]Department of Education and Research, Taichung Veterans General Hospital, Taichung 407, Taiwan
[g]Center for General Education, Tunghai University, Taichung 407, Taiwan
[h]Institute of Medical and Molecular Toxicology, Chung-Shan Medical University, Taichung 402, Taiwan
[i]Institute of Biomedical Sciences, National Chung-Hsing University, Taichung 402, Taiwan

Received 16 July 2009; received in revised form 14 January 2010; accepted 25 January 2010

Abstract

Microglial activation plays a pivotal role in the pathogenesis of neurodegenerative disease by producing excessive proinflammatory cytokines and nitric oxide (NO). Luteolin, a naturally occurring polyphenolic flavonoid antioxidant, has potent anti-inflammatory and neuroprotective properties both *in vitro* and *in vivo*. However, the molecular mechanism of luteolin-mediated immune modulation in microglia is not fully understood. In the present study, we report the inhibitory effect of luteolin on lipopolysaccharide (LPS)/interferon γ (IFN-γ)-induced NO and proinflammatory cytokine production in rat primary microglia and BV-2 microglial cells. Luteolin concentration-dependently abolished LPS/IFN-γ-induced NO, tumor necrosis factor α (TNF-α) and interleukin 1β (IL-1β) production as well as inducible nitric oxide synthase (iNOS) protein and mRNA expression. Luteolin exerted an inhibitory effect on transcription factor activity including nuclear factor κB (NF-κB), signal transducer and activator of transcription 1 (STAT1) and interferon regulatory factor 1 (IRF-1) in LPS/IFN-γ-activated BV-2 microglial cells. Biochemical and pharmacological studies revealed that the anti-inflammatory effect of luteolin was accompanied by down-regulation of extracellular signal-regulated kinase (ERK), p38, c-Jun N-terminal kinase (JNK), Akt and Src. Further studies have demonstrated that the inhibitory effect of luteolin on intracellular signaling execution and proinflammatory cytokine expression is associated with resolution of oxidative stress and promotion of protein phosphatase activity. Together, these results suggest that luteolin suppresses NF-κB, STAT1 and IRF-1 signaling, thus attenuating inflammatory response of brain microglial cells.

Keywords: Cytokine; IRF; Luteolin; Microglia; NF-κB; STAT

1. Introduction

Inflammation is a beneficial host response to foreign challenge or tissue injury that leads ultimately to the restoration of tissue structure and function. Increasing evidence demonstrates that uncontrolled microglia-associated inflammation may have potentially damaging consequences and is actively involved in the pathogenesis of neurodegenerative diseases [1–6]. Microglia, representative of the resident macrophage population within the CNS, are considered to be the determinant cell type responsible for inflammation-mediated

Abbreviations: AP-1, activator protein-1; C/EBP, CCAAT/enhancer-binding protein; CNS, central nervous system; CREB, cyclic AMP response element-binding protein; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; DMTU, dimethylthiourea; ELISA, enzyme-linked immunosorbent assay; EMSA, electrophoretic mobility shift assay; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; GAS, γ-interferon-activated site; IFN-γ, interferon γ; IKK, IκB kinase; IL-1β, interleukin 1β; iNOS, inducible nitric oxide synthase; IRF-1, interferon regulatory factor 1; ISRE, interferon-stimulated responsive element; Jak, Janus kinase; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; NF-IL6, nuclear factor IL-6; NF-κB, nuclear factor κB; NO, nitric oxide; PBS, phosphate-buffered saline; PCNA, proliferating cell nuclear antigen; PIAS, protein inhibitors of activated STATs; ROS, reactive oxygen species; RT–PCR, reverse transcriptase–polymerase chain reaction; SHP, Src homology 2 domain containing phosphatase; SOCS-3, suppressor of cytokine signaling 3; STAT, signal transducers and activators of transcription; TBST, Tris-buffered saline–Tween 20; TLR4, toll-like receptor 4; TNF-α, tumor necrosis factor α.

* Corresponding author. Department of Education and Research, Taichung Veterans General Hospital, Taichung 407, Taiwan. Fax: +1 886 4 23592705.
E-mail address: cjchen@vghtc.gov.tw (C.-J. Chen).
[1] Equal contribution.

0955-2863/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.jnutbio.2010.01.011

neurotoxicity. Activation of microglia and its consequently released bioactive products are pathophysiological hallmarks observed in neurological disorders [1–6]. Activated microglia has the capability to release a wide range of soluble proinflammatory cytokines such as TNF-α and IL-1β and reactive nitrogen species such as NO, which impact on neurons to induce neurodegeneration [1–6]. Moreover, these individual factors can work in synergy to aggravate neurodegeneration. Due to its mediating role, microglia are an important target for therapeutic intervention against neurodegenerative diseases.

Of the various factors released by activated microglia, NO appears to play a critical role in stress-induced brain damage. NO, a product of the oxidation of L-arginine to L-citrulline, which is catalyzed by NOS, is a short-lived molecule and intracellular messenger that mediates a variety of biological functions, including neurotoxicity. Two forms of NOS are regulated by intracellular calcium and expressed constitutively in endothelial and neuronal cells [7]. iNOS, the high-output isoform, is rapidly transcribed and expressed in microglia after brain injury and contributes to neuronal dysfunction/destruction [6–9]. Thus, the inhibition of NO production by blocking iNOS expression may present a useful strategy for the treatment of various inflammatory diseases including neurological disorders.

Medicinal plants, plant extracts and isolated secondary metabolites have traditionally been used to treat several clinical diseases, including inflammation-associated diseases. Flavonoids, a group of well-known plant-derived secondary metabolites, are widely distributed in most plants and have been demonstrated to possess antioxidant and anti-inflammatory activities [10–19]. Luteolin (3′,4′,5′,7′-tetrahydroxyflavone), a polyphenolic compound found in plants such as celery, green peppers, perilla leaf and chamomile tea belongs to the flavone subclass of flavonoids. Recent studies have demonstrated remarkable beneficial actions of luteolin through antioxidant and anti-inflammatory activities. Luteolin protects mice against LPS-induced toxicity, alleviates bronchoconstriction and airway hyperreactivity in ovalbumin-sensitized mice and decreases *Chlamydia pneumoniae* infection-induced inflammatory reactions [20–22]. *In vitro*, luteolin inhibits NO and proinflammatory cytokine expression in primary bone marrow-derived macrophages, gingival fibroblast, alveolar macrophages, mast cells and RAW 264.7 cells [23–27]. Similar to suppressive action against peripheral immune cells, cell studies show that luteolin modulates glial activation and protects dopaminergic neurons against inflammation-induced injury [28–32]. These observations imply that luteolin may be useful for mitigating neuroinflammation. As proinflammatory cytokines derived from microglia are critical in brain damage, we extended this study by examining the effect of luteolin on NO, TNF-α and IL-1β production in primary microglia and murine BV-2 microglial cell lines and attempted to clarify the underlying molecular basis. The results will hopefully highlight the therapeutic potential of luteolin as a novel anti-inflammatory adjuvant in neurodegenerative diseases.

2. Materials and methods

2.1. Materials

Dimethyl sulfoxide and LPS and other reagents, unless specifically stated elsewhere, were purchased from Sigma-Aldrich (St. Louis, MO). 2′,7′-Dichlorofluorescein was obtained from Molecular Probes (Eugene, OR). Dulbecco's modified Eagle's medium and FBS were purchased from Invitrogen (Carlsbad, CA). U0126, SB203580, SP600125, LY294002, AG490, PP2 and DMTU were obtained from Tocris Cookson (Avonmouth, UK). Interferon γ was obtained from R&D Systems (Minneapolis, MN). Antibodies against phospho-ERK, ERK, p38, phospho-Akt, Akt, phospho-Src, Src, phospho-Jak1, Jak1, phospho-Jak2, Jak2, phospho-Tyk2, Tyk2, phospho-IκB-α, IκB-α, p50, p65, RelB, PCNA, SOCS-3 and IRF-1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); iNOS, phospho-JNK and phospho-p38 were obtained from BD Biosciences (San Diego, CA); β-actin was obtained from Sigma-Aldrich; JNK was obtained from R&D Systems; CD68 was obtained from Chemicon (Temecula, CA); phospho-STAT1 and STAT1 were purchased from Cell Signaling Technology (Beverly, MA).

2.2. Cell cultures

Rat primary glial cultures were prepared as described previously [14]. The protocol for animal studies was approved by the Animal Experimental Committee of Taichung Veterans General Hospital. Rat primary microglia were isolated from glial cultures. Briefly, glial cells were cultured in 75-mm^2 flasks for 10 to 14 days in DMEM supplemented with 10% FBS. To separate microglia, flasks were shaken for 3 h at 180 rpm in a rotary shaker at 37°C. Detached cells were replated on cultured plates. The purity of microglia cultures was assessed using CD68 antibody when more than 95% of cells were positively stained. Murine BV-2 microglial cells [14] were cultured in DMEM supplemented with 10% FBS. In all experiments, cells were treated with luteolin 30 min before the addition of LPS (100 ng/ml, *Escherichia coli*, serotype 0111:B4)/IFN-γ (10 U/ml) in serum-free DMEM. Pharmacological agents were dissolved in DMSO, and the final concentration of DMSO added to cells never exceeded 0.1%. Each assay was carried out at different appropriate time point after treatment according to preliminary evaluation.

2.3. Nitric oxide determination

For NO (nitrite/nitrate) determination, primary microglia and BV-2 (1×10^5 cells/well, 24-well cell culture plate) were allowed to adhere overnight. The final volume of reaction medium was 500 μl. The production of NO was determined based on the Griess reaction [14]. Briefly, 50 μl of culture supernatant was reacted with an equal volume of Griess reagent (1 part 0.1% naphthylethylenediamine, 1 part 1% sulfanilamide in 5% H_3PO_4) in 96-well plates for 10 min at room temperature in the dark. The absorbance at 550 nm was determined using a microplate reader (PowerWaveX 340, Bio-Tek Instruments). A standard nitrite curve was generated in the same fashion using $NaNO_2$.

2.4. Western blot analysis

Cells were washed twice with PBS and harvested in Laemmli SDS sample buffer. The protein concentration in the supernatant was determined by Bradford assay. Protein extracts were separated by SDS-PAGE and electrophoretically transferred to polyvinylidene difluoride membranes. Membranes were first incubated with 5% nonfat milk in PBS for 1 h at room temperature to reduce nonspecific binding. Membranes were washed with TBST and incubated for 1 h at room temperature with the indicated antibodies including iNOS, β-actin, p50, p65, RelB, PCNA, IRF-1 and SOCS-3 and phosphorylated and nonphosphorylated forms of ERK, JNK, p38, Akt, IκB-α, STAT1, Jak1, Jak2, Tyk2 and Src. After washing again with TBST buffer, a 1:10,000 (v/v) dilution of horseradish peroxidase-labeled IgG was added at room temperature for 1 h. The blots were developed using enhanced chemiluminescence Western blotting reagents. The intensity of each signal was determined by a computer image analysis system (Alpha Innotech Corporation, IS1000).

2.5. Isolation of RNA and RT–PCR

The isolation of RNA and synthesis of complement DNA were carried out as previously reported [14]. DNA fragments of specific genes and internal controls were coamplified in one tube containing *Taq* DNA polymerase (Promega, Madison, WI) and 0.8 μM of each sense and antisense primers. The PCR reaction was performed with a DNA thermal cycler under the following conditions: one cycle of 94°C for 3 min, 28 cycles of (94°C for 50 s, 58°C for 40 s and 72°C for 45 s) and then 72°C for 5 min. In preliminary experiments, we found that the PCR and product amplification was linear (r=.946–.977) under these PCR conditions. The amplified DNA fragments were resolved by 1.5% agarose gel electrophoresis and stained with ethidium bromide. DNA band intensity was determined by a computer image analysis system (Alpha Innotech Corporation, IS1000). Relative mRNA levels were expressed as the intensity ratio of each gene and internal control (β-actin). Oligonucleotides used in this study were as follows: 5′-ACAACGTGGAGAAAACCCCAGGTG and 5′-ACAGCTCCGGGCATCGAAGACC for iNOS; 5′-TCCTGTGGCATCCACGAAACT and 5′-GGAGCAATGATCTTGATCTTC for β-actin.

2.6. Enzyme-linked immunosorbent assay

The concentrations of TNF-α and IL-1β in the supernatants were measured with an enzyme immunoassay kit (R&D Systems), following the procedure provided by the manufacturer.

2.7. Preparation of nuclear extracts and EMSA

Nuclear extracts were prepared as described previously [14]. In brief, cells were washed twice with ice-cold PBS and pelleted. The cell pellet was resuspended in hypotonic buffer (10 mM HEPES, pH 8.0; 10 mM KCl; 1.5 mM $MgCl_2$; 5 mM dithiothreitol; 0.5 mM phenylmethylsulfonyl fluoride; 1 mM NaF; 1 mM Na_3VO_4) and incubated on ice for 15 min. The cells were then lysed by the addition of 0.5% Nonidet P-40 and vigorous vortexing for 30 s. The nuclei were pelleted by centrifugation at 12,000g for 1 min at 4°C and resuspended in extraction buffer (20 mM HEPES, pH 8.0; 420 mM NaCl; 1.5 mM $MgCl_2$; 0.2 mM EDTA; 1 mM dithiothreitol; 10% glycerol; 0.5 mM phenylmethylsulfonyl fluoride; 1 mM NaF; 1 mM Na_3VO_4). After 15 min on ice, lysates were centrifuged at 12,000g for 10 min at 4°C. Supernatants were obtained and stored at −70°C. The oligonucleotides, specific for each known transcription factor,

were synthesized and labeled with biotin including NF-κB (5′-AGTTGAGGGGACTTTCC-CAGGC) and STAT (5′-ATCGTTCATTTCCCGTAAATCCCTA). Nuclear extract (5 μg) was used for EMSA. The binding reaction mixture included 1 μg of poly (dI–dC), 0.1 μg poly L-lysine and 100 fmol biotin-labeled DNA probe in 20 μl binding buffer (10 mM HEPES, pH 7.6; 50 mM NaCl; 0.5 mM $MgCl_2$; 0.5 mM EDTA; 1 mM dithiothreitol; 5% glycerol). The DNA/protein complex was analyzed on 6% native polyacrylamide gels. The intensity of each signal was determined by a computer image analysis system (Alpha Innotech Corporation, IS1000).

2.8. Phosphatase assay

Cells were resuspended with PBS, subjected to three rounds of freeze/thaw and then sonicated for 10 s. Serine/threonine and tyrosine phosphatase activities were measured using a commercially available serine/threonine phosphatase assay kit and tyrosine phosphatase assay kit (Molecular Probes), respectively. Five micrograms of protein were added and reacted with preloaded substrates. The generated fluorescent product was determined using a fluorometer (E_x 358 nm and E_m 452 nm).

2.9. Free radical determination

Intracellular oxidative stress was assayed by measuring intracellular oxidation of dichlorofluorescein, as described previously [33]. Cultures were loaded with 10 μM 2′,7′-dichlorofluorescein at 37°C for 1 h, washed and subjected to the treatment. The fluorescence signal of oxidized 2′,7′-dichlorofluorescein was measured using a fluorometer (E_x 485 nm and E_m 510 nm).

2.10. Statistical analysis

Results are presented as means±S.D. A one-way analysis of variance was performed for multiple comparisons, and if there was significant variation between treatment groups, the mean values were compared with respective controls using Dunnett's *t* test. *P* values less than .05 were considered significant.

3. Results

3.1. Effect of luteolin on cytokine production in microglia

To elucidate potential suppressive effects against neuroinflammation, the effect of luteolin on NO production was investigated on primary microglia and BV-2 microglial cells. Treatment of primary microglia and BV-2 microglial cells with LPS/IFN-γ evoked NO production (Fig. 1A). The cells were pretreated with various concentrations of luteolin for 30 min, before being stimulated with LPS/IFN-γ for another 24 h. At concentrations used in this study, luteolin treatment was not toxic to either cell type according to the results of trypan blue dye exclusion assay (data not shown). Preincubation of primary microglia and BV-2 microglial cells with luteolin before LPS/IFN-γ treatment led to a concentration-dependent decrease in NO production (Fig. 1A). To determine whether the inhibitory ability of luteolin on NO production was due to a decrease in cytosolic iNOS protein levels, cells were treated with LPS/IFN-γ or LPS/IFN-γ plus luteolin for 16 h and levels of iNOS protein were detected by Western blot analysis. As shown in Fig. 1B, pretreatment with luteolin led to a decrease of iNOS protein levels in a concentration-dependent manner. As iNOS protein levels were down-regulated, RT–PCR analysis was performed to assess the effect of luteolin on the expression of iNOS mRNA. The appearance of iNOS mRNA was detected 8 h after exposure to LPS/IFN-γ (Fig. 1C). Luteolin did not affect the expression of the housekeeping gene β-actin. In contrast, it had an inhibitory effect upon the expression of iNOS mRNA (Fig. 1C). To further elicit the anti-inflammatory effect of luteolin, the production of IL-1β and TNF-α was measured.

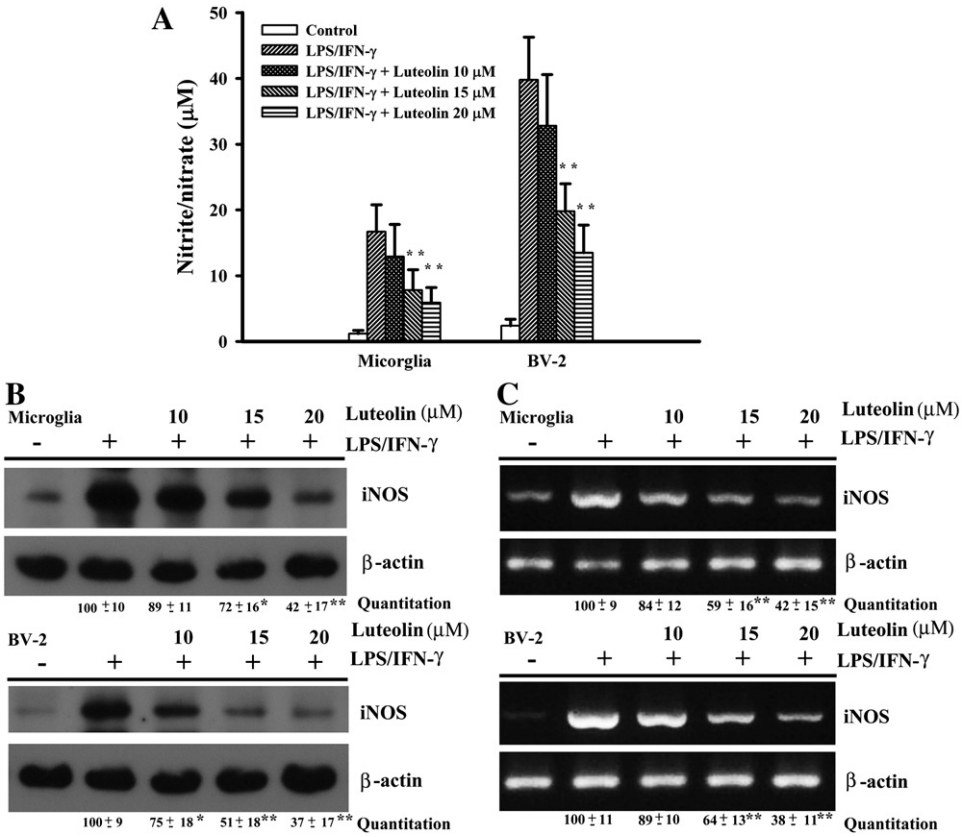

Fig. 1. Effect of luteolin on iNOS expression and NO production. Primary microglia and BV-2 microglial cells were pretreated with vehicle control or various concentrations of luteolin for 30 min before being incubated with LPS (100 ng/ml)/IFN-γ (10 U/ml). (A) The culture supernatants (24 h) were collected and analyzed for NO production. (B) The cell lysates (16 h) were isolated and subjected to Western blot for measurement of iNOS and β-actin. (C) Total RNAs (8 h) were isolated and subjected to RT–PCR measurement of expressions of iNOS and β-actin. (B and C) One of four independent results is shown. The quantitative results in panels B and C are depicted. The signal intensity of iNOS over β-actin in LPS/IFN-γ-treated group was defined as 100%. *P<.05 and **P<.01 vs. the LPS/IFN-γ-treated group, n=4.

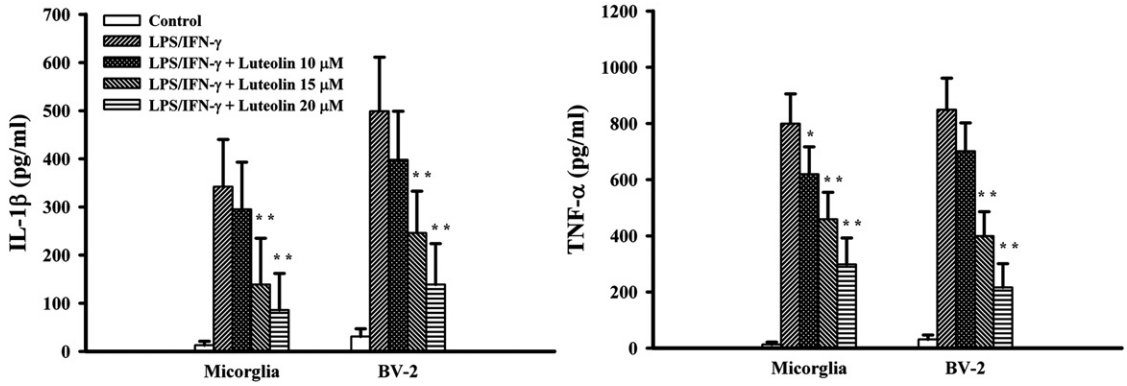

Fig. 2. Effect of luteolin on IL-1β and TNF-α production. Primary microglia and BV-2 microglial cells were pretreated with vehicle control or various concentrations of luteolin for 30 min before being incubated with LPS (100 ng/ml)/IFN-γ (10 U/ml). The cultured supernatants (24 h) were collected and analyzed for IL-1β and TNF-α production. *P<.05 and **P<.01 vs. the LPS/IFN-γ-treated group, n=4.

Incubation of primary microglia and BV-2 microglial cells with LPS/IFN-γ caused elevated release of IL-1β and TNF-α. Luteolin concentration-dependently reduced LPS/IFN-γ-induced IL-1β and TNF-α release (Fig. 2). These findings suggest that luteolin possesses an inhibitory effect against proinflammatory cytokine production from activated microglia. Due to feasibility, the experiments were done in BV-2 microglial cells.

3.2. Effect of luteolin on NF-κB signaling

Nuclear factor κB is one of the most important transcription factors for the inducibility of cytokines by LPS/IFN-γ [34]. Treatment of BV-2 microglial cells with LPS/IFN-γ caused a significant increase in the DNA-binding activity of NF-κB within 1 h. In the presence of luteolin, LPS/IFN-γ-induced NF-κB DNA binding was markedly suppressed (Fig. 3). The involvement of NF-κB signaling in proinflammatory cytokine expression by activated BV-2 microglial cells was supported by the suppressive effect of MG132, an inhibitor of NF-κB activation [35]. Treatment of BV-2 microglial cells with MG132 remarkably reduced LPS/IFN-γ-induced NO, IL-1β, and TNF-α release (Fig. 4). To further investigate the inactivation of NF-κB by luteolin, the activity of several NF-κB components was analyzed. Lipopolysaccharide/interferon γ caused an elevation in serine phosphorylation of IκB-α. Both basal and LPS/IFN-γ-increased serine phosphorylation of IκB-α were attenuated by luteolin (Fig. 5A). The consequently released NF-κB subunits including p50, p65 and RelB translocated into the nucleus. Intriguingly, LPS/IFN-γ-induced nuclear accumulation of p65 (Fig. 5C) and RelB (Fig. 5D) but not p50 (Fig. 5B) was decreased by luteolin. The results show that luteolin suppresses proinflammatory cytokine production in activated BV-2 microglial cells at least partly via an NF-κB-dependent mechanism.

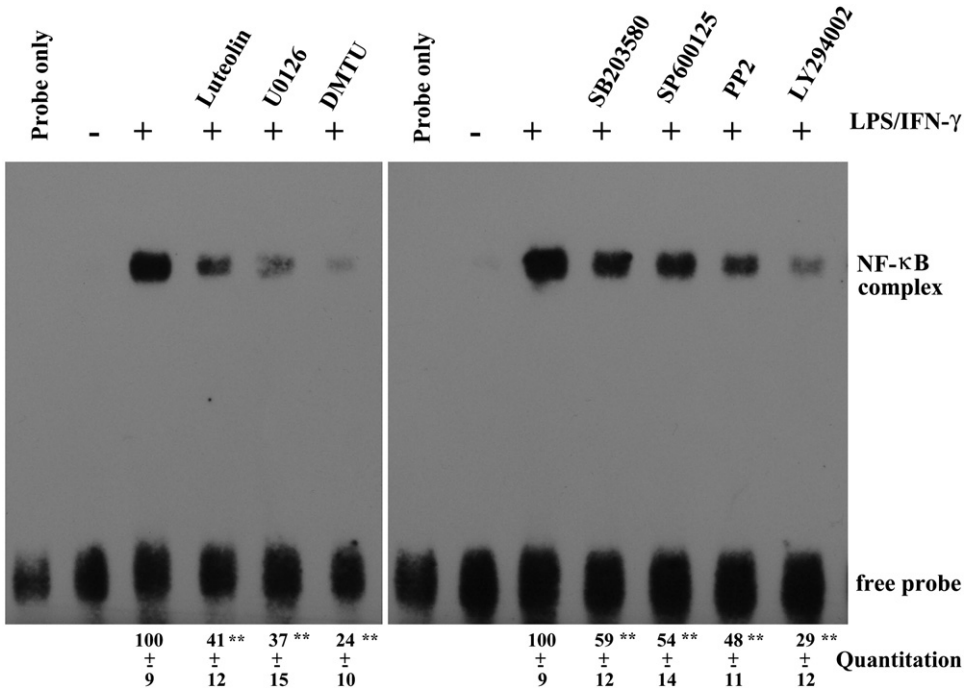

Fig. 3. Effect of luteolin on NF-κB DNA-binding activity. BV-2 microglial cells were pretreated with vehicle control or luteolin (20 μM), U0126 (10 μM), DMTU (1 mM), SB203580 (10 μM), SP600125 (10 μM), PP2 (10 μM) or LY294002 (10 μM) for 30 min before stimulation with LPS (100 ng/ml)/IFN-γ (10 U/ml). The nuclear extracts were prepared 1 h after treatment for analysis of NF-κB via EMSA. Similar results were obtained from three independent experiments. The quantitative results are depicted. The signal intensity in LPS/IFN-γ-treated group was defined as 100%. **P<.01 vs. the LPS/IFN-γ-treated group, n=3.

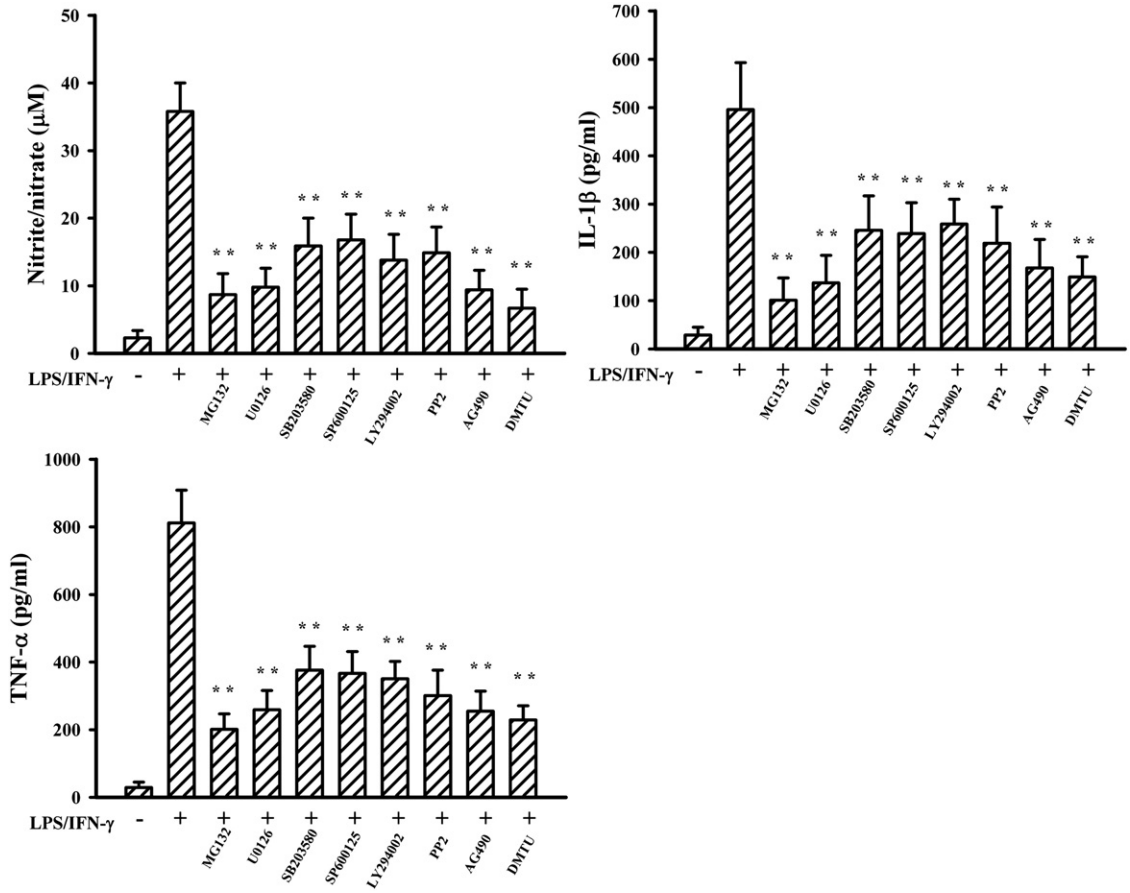

Fig. 4. Effect of pharmacological inhibitors on NO, IL-1β and TNF-α production. BV-2 microglial cells were pretreated with vehicle control or MG132 (0.5 μM), U0126 (10 μM), SB203580 (10 μM), SP600125 (10 μM), LY294002 (10 μM), PP2 (10 μM), AG490 (50 μM) or DMTU (1 mM) for 30 min before stimulation with LPS (100 ng/ml)/IFN-γ (10 U/ml). The cultured supernatants (24 h) were collected and analyzed for NO, IL-1β and TNF-α production. **$P<.01$ vs. the LPS/IFN-γ-treated group, n=4.

3.3. Effect of luteolin on MAPK and Akt signaling

The molecular mechanism by which luteolin attenuates LPS/IFN-γ-stimulated NF-κB leading to the inhibition of proinflammatory cytokine production was investigated by examining its effect on intracellular signaling molecules critical to NF-κB activation. The involvement of signaling molecules such as MAPKs and Akt was determined via pharmacological and biochemical approaches. Lipopolysaccharide/interferon γ stimulation increased phosphorylation of ERK (Fig. 6A), p38 (Fig. 6B), JNK (Fig. 6C) and Akt (Fig. 6D) in BV-2 microglial cells. Luteolin had a significant inhibitory effect on LPS/IFN-γ-increased phosphorylation in ERK, p38, JNK and Akt and in basal phosphorylation, particularly in ERK and Akt. Pharmacological inhibition of ERK, p38, JNK and Akt by U0126, SB203580, SP600125 and LY294002, respectively, led to the attenuation of LPS/IFN-γ-induced NO, IL-1β and TNF-α production (Fig. 4), indicating an association between ERK, p38, JNK and Akt inactivation and luteolin-mediated down-regulation of proinflammatory cytokine expression. An EMSA study further showed the importance of NF-κB in switching crosstalk because of inactivation of NF-κB activity by U0126, SB203580, SP600125 and LY294002 (Fig. 3). These results suggest that MAPKs and Akt inactivation are potential mechanisms contributing to luteolin-mediated down-regulation in NF-κB and proinflammatory cytokine expression.

3.4. Effect of luteolin on STAT signaling

The STAT family is another latent transcription factor important in regulating proinflammatory cytokine expression [36–39]. Treatment of BV-2 microglial cells with LPS/IFN-γ increased STAT DNA-binding activity (Fig. 7) and elevated phosphorylation in STAT1 (Fig. 8A). A Jak/STAT-selective tyrphostin pharmacological inhibitor AG490 [39] attenuated LPS/IFN-γ-induced STAT DNA-binding activity (Fig. 7), STAT1 phosphorylation (Fig. 8B), and NO, IL-1β and TNF-α production (Fig. 4), indicating a role of STAT1 signaling in activated BV-2 microglial cells for proinflammatory cytokine expression. In the presence of luteolin, there was a decline of STAT DNA-binding activity (Fig. 7) and STAT1 phosphorylation (Fig. 8A). Interferon regulatory factor 1 is a downstream effector molecule of STAT1 and also functions as a transcription factor regulating proinflammatory cytokine expression [40,41]. Inactivation of STAT1 by AG490 decreased LPS/IFN-γ-induced elevated IRF-1 expression (Fig. 8D). Luteolin attenuated both basal and LPS/IFN-γ-increased IRF-1 expression (Fig. 8C). The activation and functional execution of STAT are regulated at multiple steps including upstream stimulatory tyrosine kinases such as Jak and Src family kinases and negative regulators such as SHPs, SOCS and PIASs [37,42]. The phosphorylation of Jak1 (Fig. 9A), Jak2 (Fig. 9B), Tyk2 (Fig. 9C) and Src (Fig. 9D) were rapidly induced by LPS/IFN-γ in BV-2 microglial cells. Luteolin had no significant effect on LPS/IFN-γ-stimulated Jak1, Jak2 and Tyk2 phosphorylation. However, the elevated phosphorylation of Src was attenuated by luteolin. An Src-selective inhibitor PP2 [43] had inhibitory effects on LPS/IFN-γ-increased STAT DNA-binding activity (Fig. 7), STAT1 phosphorylation (Fig. 8B), IRF-1 expression (Fig. 8D), and NO, IL-1β and TNF-α production (Fig. 4). In addition to modulating STAT signaling, the inactivation of Src also remarkably alleviated NF-κB signaling. Treatment of BV-2 microglial cells with PP2 decreased LPS/IFN-γ-stimulated NF-κB DNA-binding

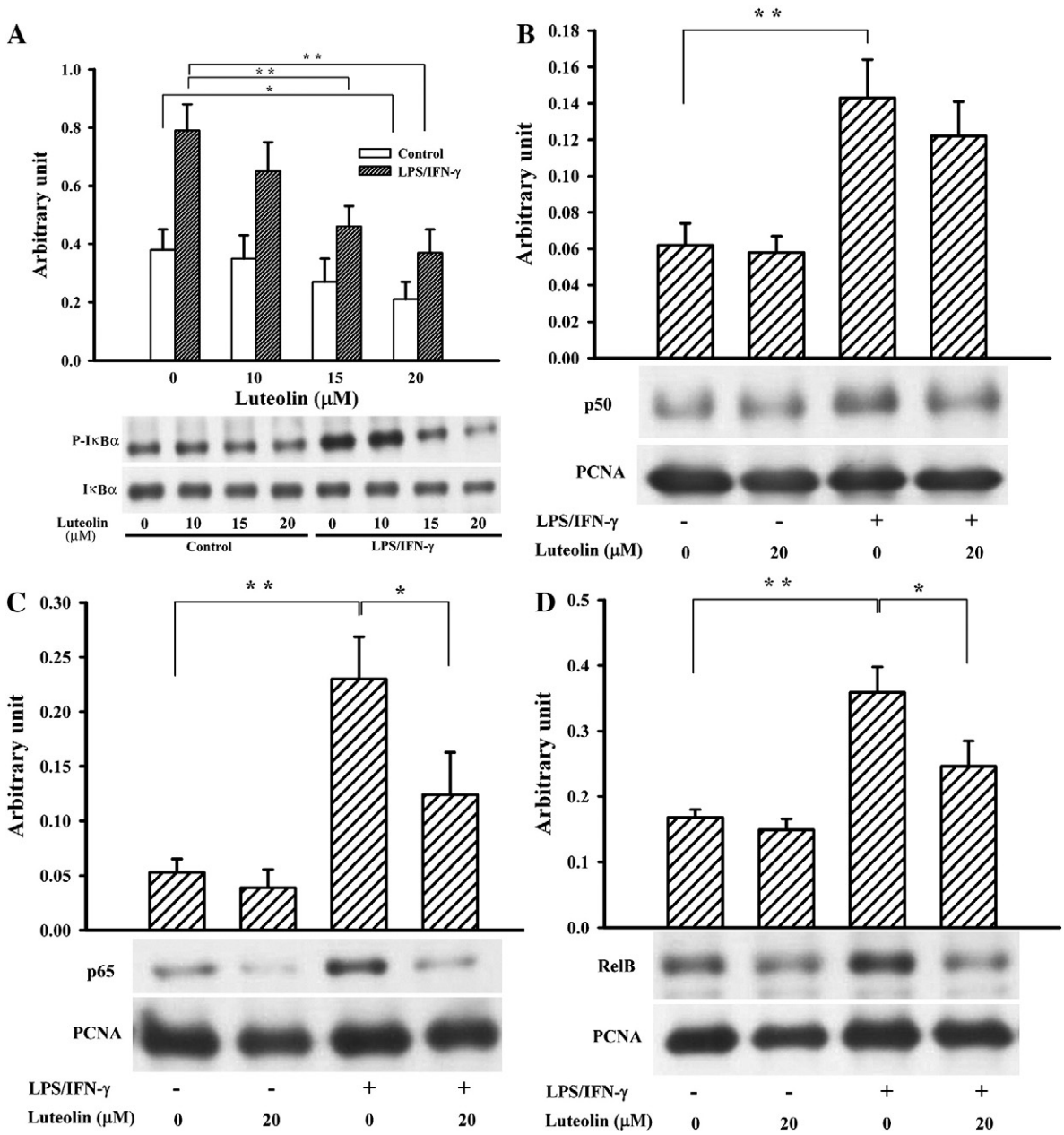

Fig. 5. Effect of luteolin on NF-κB components. BV-2 microglial cells were pretreated with vehicle control or various concentrations of luteolin for 30 min before stimulation with LPS (100 ng/ml)/IFN-γ (10 U/ml). (A) The total cell lysates (1 h) were isolated and subjected to Western blot for the detection of p-IκB-α and IκB-α. BV-2 microglial cells were pretreated with vehicle control or luteolin (20 μM) for 30 min before stimulation with LPS (100 ng/ml)/IFN-γ (10 U/ml). The nuclear extracts were prepared 1 h after treatment for analysis of (B) p50, (C) p65 and (D) RelB via Western blot. The content of IκB-α and PCNA was used for normalization. The quantitative results are depicted. *P<.05 and **P<.01, n=3.

activity (Fig. 3). On the other hand, treatment of BV-2 microglial cells with luteolin progressively increased protein expression of SOCS-3, independent of the presence of LPS/IFN-γ (Fig. 9E). This induction was undetectable within 1 h after treatment (data not shown), whereas an apparent induction was observed 5 h later (Fig. 9E). That is, the inactivation of STAT1 by luteolin in BV-2 microglial cells might act through attenuation of upstream stimulatory kinases and induction of negative regulators, leading to attenuation of proinflammatory cytokine production.

3.5. Effect of luteolin on ROS generation

The generation of free radicals plays a critical role in the activation of signaling molecules, transcription factors and proinflammatory cytokine expression [44]. To determine the antioxidative potential of luteolin in microglia, 2′,7′-dichlorofluorescein was applied in order to detect cell oxygen burst. Hydrogen peroxide exposure (Fig. 10A) and LPS/IFN-γ stimulation (Fig. 10B) increased intracellular ROS level and the elevated levels were repressed by luteolin treatment. Although the free radical scavenging and anti-inflammatory activities of luteolin were demonstrated, the relationship between these two biological activities is not known. Through the utilization of antioxidants, we found that DMTU decreased LPS/IFN-γ-induced NO, IL-1β and TNF-α production (Fig. 4). In parallel to this inhibitory effect, DMTU significantly attenuated LPS/IFN-γ-elevated NF-κB DNA-binding activity (Fig. 3), STAT DNA-binding activity (Fig. 7), STAT1 phosphorylation (Fig. 8B) and IRF-1 expression (Fig. 8D). The results suggest that the free radical scavenging potential might contribute to the anti-inflammatory activity of luteolin.

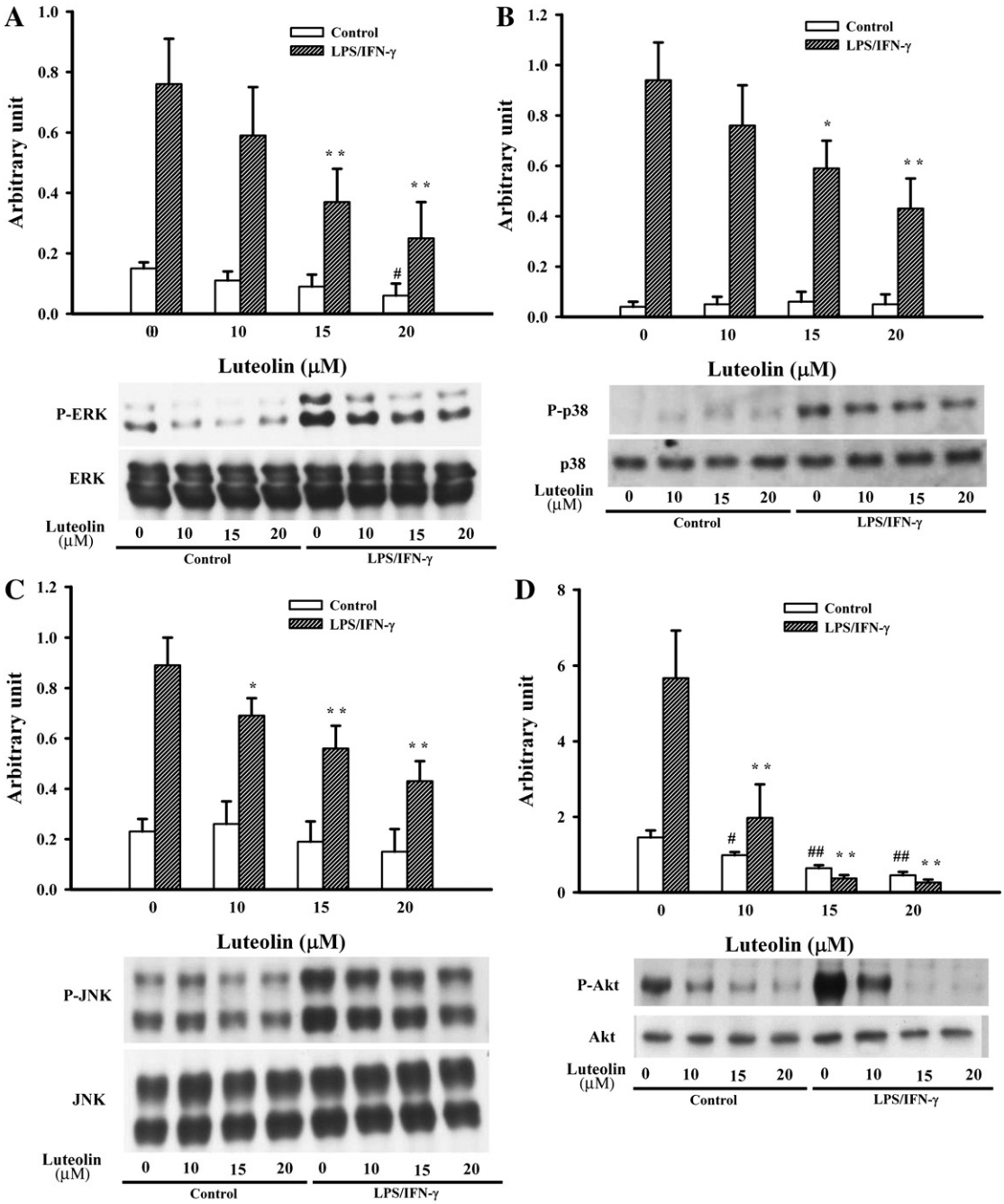

Fig. 6. Effect of luteolin on MAPK and Akt phosphorylation. BV-2 microglial cells were pretreated with vehicle control or various concentrations of luteolin for 30 min before stimulation with LPS (100 ng/ml)/IFN-γ (10 U/ml). The total cell lysates (1 h) were isolated and subjected to Western blot for detection of (A) p-ERK, ERK, (B) p-p38, p38, (C) p-JNK, JNK, and (D) p-Akt, Akt. The quantitative results of phosphorylation level are depicted. *P<.05 and **P<.01 vs. the LPS/IFN-γ-treated group and $^{\#}P$<.05 and $^{\#\#}P$<.01 vs. the control group, n=3.

3.6. Effect of luteolin on protein phosphatase activity

The homeostasis of protein phosphorylation events is governed by kinases and phosphatases. We found that luteolin treatment down-regulated protein phosphorylation in MAPKs, Akt, Src, IκB-α and STAT. It is not yet clear whether inactivation of kinases or activation of phosphatases orchestrates phosphorylation changes in response to luteolin treatment. Through an enzymatic assay, we found that luteolin increased cellular serine/threonine and tyrosine phosphatase activity and improved LPS/IFN-γ-induced inactivation (Fig. 11). These findings suggest that the down-regulation of protein phosphorylation levels in MAPKs, Akt, Src and STAT by luteolin might be partly attributed to the increased protein phosphatase activity.

4. Discussion

The flavonoids are a large group of naturally occurring polyphenolic compounds found in plants and are frequently consumed as part of the human diet. Luteolin is a typical flavone-type flavonoid ubiquitously present in plants and shows several beneficial effects [23–32]. In the present study, we addressed the roles of MAPKs, Akt, Src and Jak signaling molecules and transcription factors in luteolin-mediated anti-inflammation in one complete study. We showed that luteolin inhibits NO, TNF-α and IL-1β production in primary microglia and murine BV-2 microglial cells and that the effect was mostly mediated through the inhibition of NF-κB, STAT and IRF-1 transcription factor. The immunosuppressive effect of luteolin was

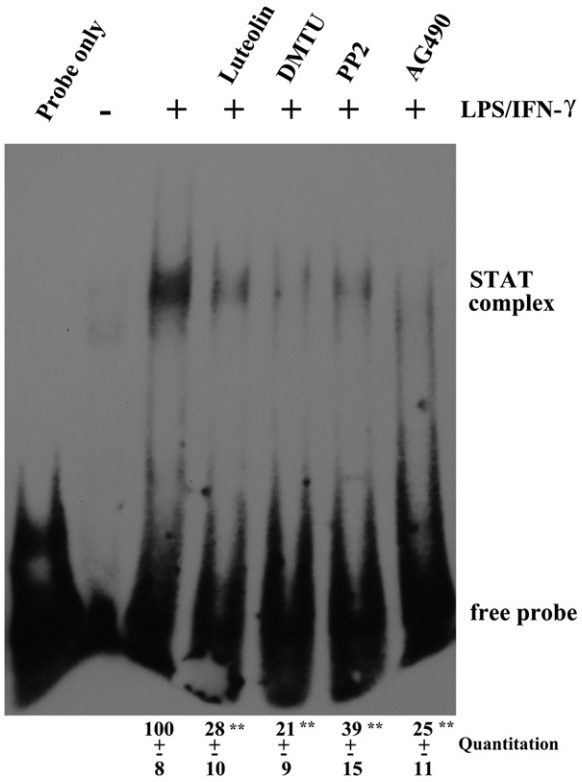

Fig. 7. Effect of luteolin on STAT DNA-binding activity. BV-2 microglial cells were pretreated with vehicle control or luteolin (20 μM), DMTU (1 mM), PP2 (10 μM) or AG490 (50 μM) for 30 min before stimulation with LPS (100 ng/ml)/IFN-γ (10 U/ml). The nuclear extracts were prepared 1 h after treatment for the analysis of STAT via EMSA. The quantitative results are depicted. The signal intensity in LPS/IFN-γ-treated group was defined as 100%. **P<.01 vs. the LPS/IFN-γ-treated group, n=3.

accompanied by the inhibition of free radical generation and activation of phosphatase activity. Therefore, the anti-inflammatory mechanism of luteolin against neuroinflammation is multifactorial, at least, involving the resolution of oxidative stress and enhancement of protein phosphatase activity.

The expression of proinflammatory cytokines is regulated mainly at the transcriptional level. A number of binding sites for transcription factors such as NF-κB, AP-1, IRF-1, C/EBP, NF-IL6, CREB and STAT have been identified in the iNOS promoter region in glial cells. These transcription factors are also key regulators of a variety of genes involved in inflammatory responses [8,14,15,26,31,34,45,46]. Increasing evidence suggests that transcription factors are the target for the immunosuppressive effect of flavonoids. For example, genistein, quercetin and baicalein differentially inactivated one or many types of transcription factors including NF-κB, AP-1, NF-IL6 and STAT1 [14,15,18]. Previous studies showed that luteolin inhibited NF-κB in LPS-stimulated gingival fibroblast [24] and NF-κB and AP-1 in LPS-treated alveolar macrophages [26]. In LPS-treated RAW 264.7 cells, luteolin inhibited NF-κB and IRF-3 activation [47]. The activation of STAT1 was inhibited by luteolin in IFN-γ-stimulated N9 microglial cells [32]. Inhibition of NF-κB by luteolin was demonstrated in LPS-activated BV-2 microglial cells by Kim et al. [28]. However, Jang et al. [31] showed that luteolin inhibited LPS-increased AP-1 but not NF-κB in BV-2 microglial cells. In this study, we showed that luteolin-mediated down-regulation of NO production through transcriptional modulation due to suppression of iNOS protein and mRNA expression. In the absence of stimulation, NF-κB is retained in the cytoplasm through the binding of inhibitory IκB protein. When dissociated from IκB, NF-κB subsequently translocates to the nucleus, binds to the specific DNA element and regulates transcription of target genes [48]. STATs are activated by tyrosine phosphorylation, which results in dimerization and translocation into the nucleus where they exert their effect on transcription of regulated target genes [37,42]. IRF-1 is an inducible transcription factor. The expressed IRF-1 dimerizes and binds to a regulatory DNA element for control of gene expression [41]. The results of biochemical and pharmacological studies showed that distinct transcription factors, including NF-κB, STAT1 and IRF-1, appear to be critical for luteolin-mediated suppression of NO, TNF-α and IL-1β expression in LPS/IFN-γ-stimulated BV-2 microglial cells. It has become increasingly evident that combinatory effects of transcription factors are important in gene expression. Transcription factors may interact for the cooperative induction of genes through cooperative binding of the two transcription factors or specific interactions between these proteins and the basal transcription machinery [49–51]. Therefore, the present results imply that luteolin possesses broadly inhibitory effects on distinct transcription factors in LPS/IFN-γ-stimulated microglia, leading to attenuation of proinflammatory cytokine expression.

Transcription factor NF-κB family consists of five members: RelA (RelA/p65), RelB, c-Rel, NF-κB1 (p105/p50) and NF-κB2 (p100/p52). The phosphorylation of IκB protein by IKK complex or other upstream kinases and IκB protein degradation trigger homodimeric and/or heterodimeric NF-κB activation [48]. Evidence indicates the crucial roles of MAPK members and Akt in the activation of NF-κB and other transcription factors. The phsophorylation of NF-κB upstream regulatory molecules is an inductive mechanism for MAPKs and Akt to trigger NF-κB activation [52,53]. Luteolin inhibited kinase activities of IKKβ, ERK, p38 and JNK in TNF-α-stimulated A549 cells [13]. Luteolin inhibited ERK, p38, Akt and IKK phosphorylation, MyD88-independent TBK1 kinase activity, p65 nuclear translocation and NF-κB activation in LPS-stimulated macrophages [26,47,54,55]. The phosphorylation of ERK, p38, and Akt and p50 nuclear translocation were suppressed by luteolin in LPS-activated gingival fibroblast [24]. The negative regulation of Akt and NF-κB by luteolin was observed in TNF-α-treated intestinal epithelial cells [17]. Previous studies showed that JNK phosphorylation and IκB-α degradation were inhibited by luteolin in LPS-stimulated BV-2 microglial cells [28,31]. Our data showed that inactivation of NF-κB activity by luteolin in BV-2 microglial cells was partly mediated through the inhibition of IκB-α phosphorylation resulting in interference in the dissociation of active NF-κB and nuclear translocation of NF-κB active subunits such as p65 and RelB. Potential upstream regulatory signaling molecules such as ERK, p38, JNK and Akt could be action targets for luteolin-mediated inactivation of NF-κB. In comparison with previous observations, it is reasonable to propose that intervention steps and targets by luteolin varied and is dependent on situation. However, the overall outcome results in immunosuppression. Taken together, the inactivation of NF-κB activity by luteolin in BV-2 microglial cells could be attributed to the inhibition of upstream regulators including ERK, p38, JNK and Akt. However, specific upstream regulators for NF-κB activation and NF-κB components in converging signals from ERK, p38, JNK and Akt after luteolin treatment are unknown.

The STAT pathway has been implicated in iNOS and proinflammatory cytokine expression in glial cells [56]. Phosphorylated STAT1 translocates to the nucleus and induces transcription of IRF-1. STAT1 and concomitant induced IRF-1 regulates gene expression at the transcriptional level [37,41,42]. Generally, STATs are activated by upstream stimulatory tyrosine kinases such as Jak and Src family kinases and inactivated by negative regulators such as SHPs, SOCS and PIASs [37,42]. Currently, the literature describing the effect of luteolin on STAT1 and IRF-1 activity is limited. Luteolin induced IRF-1 degradation in intestinal epithelial cells and inhibited IFN-γ-induced phosphorylation of STAT1 in N9 microglial cells [17,32]. Through AG490, we found that STAT1 and IRF-1 signaling pathways play a role in proinflammatory cytokine expression in BV-2 microglial cells. The present study showed that attenuation of upstream kinase Src at an

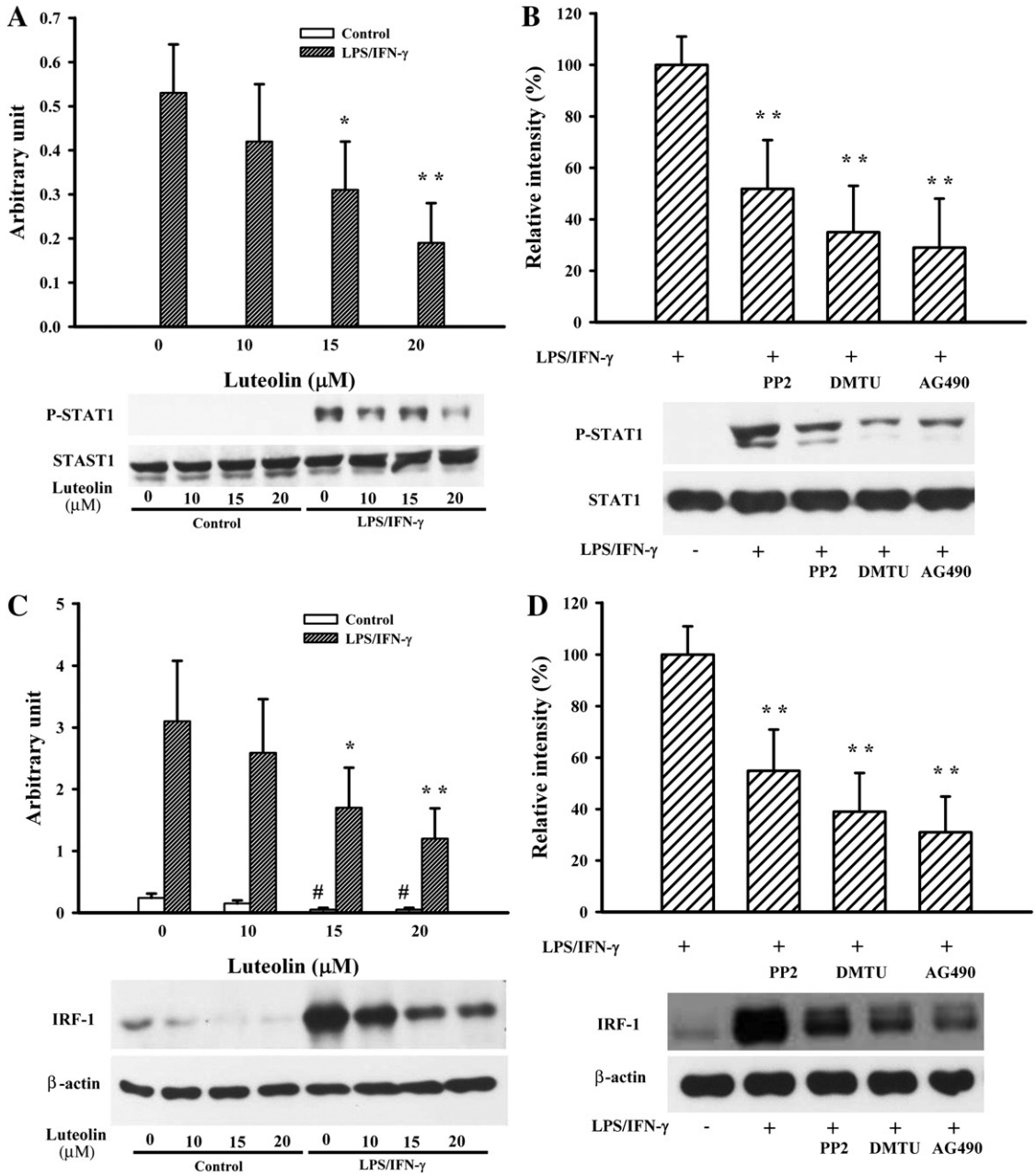

Fig. 8. Effect of luteolin and pharmacological inhibitors on STAT1 phosphorylation and IRF-1 expression. BV-2 microglial cells were pretreated with vehicle control or various concentrations of luteolin for 30 min before stimulation with LPS (100 ng/ml)/IFN-γ (10 U/ml). Total cell lysates (1 h) were isolated and subjected to Western blot for the detection of (A) p-STAT1, STAT1, and (C) IRF-1, β-actin. BV-2 microglial cells were pretreated with vehicle control or PP2 (10 μM), DMTU (1 mM) or AG490 (50 μM) for 30 min before stimulation with LPS (100 ng/ml)/IFN-γ (10 U/ml). Total cell lysates (1 h) were isolated and subjected to Western blot for the detection of (B) p-STAT1, STAT1, and (D) IRF-1, β-actin. The quantitative results of STAT1 phosphorylation level and IRF-1 content are depicted. *P<.05 and **P<.01 vs. the LPS/IFN-γ-treated group and $^{\#}P$<.05 vs. the control group, n=3.

early stage and induction of negative regulator SOCS-3 at a later course might contribute to luteolin-mediated inactivation of STAT1 in BV-2 microglial cells. The inactivation of STAT1 and IRF-1 signaling and inhibition of proinflammatory cytokine production by PP2 further supports the importance of Src signaling in mediating STAT1 and IRF-1 activation. That is, the inactivation of STAT1 by luteolin in BV-2 microglial cells might act through attenuation of upstream stimulatory kinases and induction of negative regulators.

Cumulative evidence concurs with the fact that a protein tyrosine kinase signaling cascade plays a pivotal role in the initiation of proinflammatory cytokine expression. Src is a nonreceptor type tyrosine kinase and possesses a diversity of biological activities [57]. Our results showed that Src could play a critical role in transmitting signals to activate STAT and NF-κB signaling in modulating proinflammatory cytokine expression in BV-2 microglial cells. Tyrosine phosphorylation of STAT1 was one of mechanism in which Src can trigger STAT1 signaling in BV-2 microglial cells. Evidence suggests that Src could regulate NF-κB activity through the activation of MAPKs, ERK in particular [43,58]. However, the linking bridge between Src and NF-κB signaling in BV-2 microglial cells is currently not known. These observations suggest that Src could be a critical target for luteolin-mediated anti-inflammatory effects.

Signals emanating from many cell-surface receptors and environmental cues converge to signaling molecules such as MAPKs, Akt, Src and Jak through modulation of protein phosphorylation events, which in turn phosphorylate and activate various transcription factors such as NF-κB and STAT. The induced proinflammatory cytokine production after LPS/IFN-γ treatment is mediated through distinct

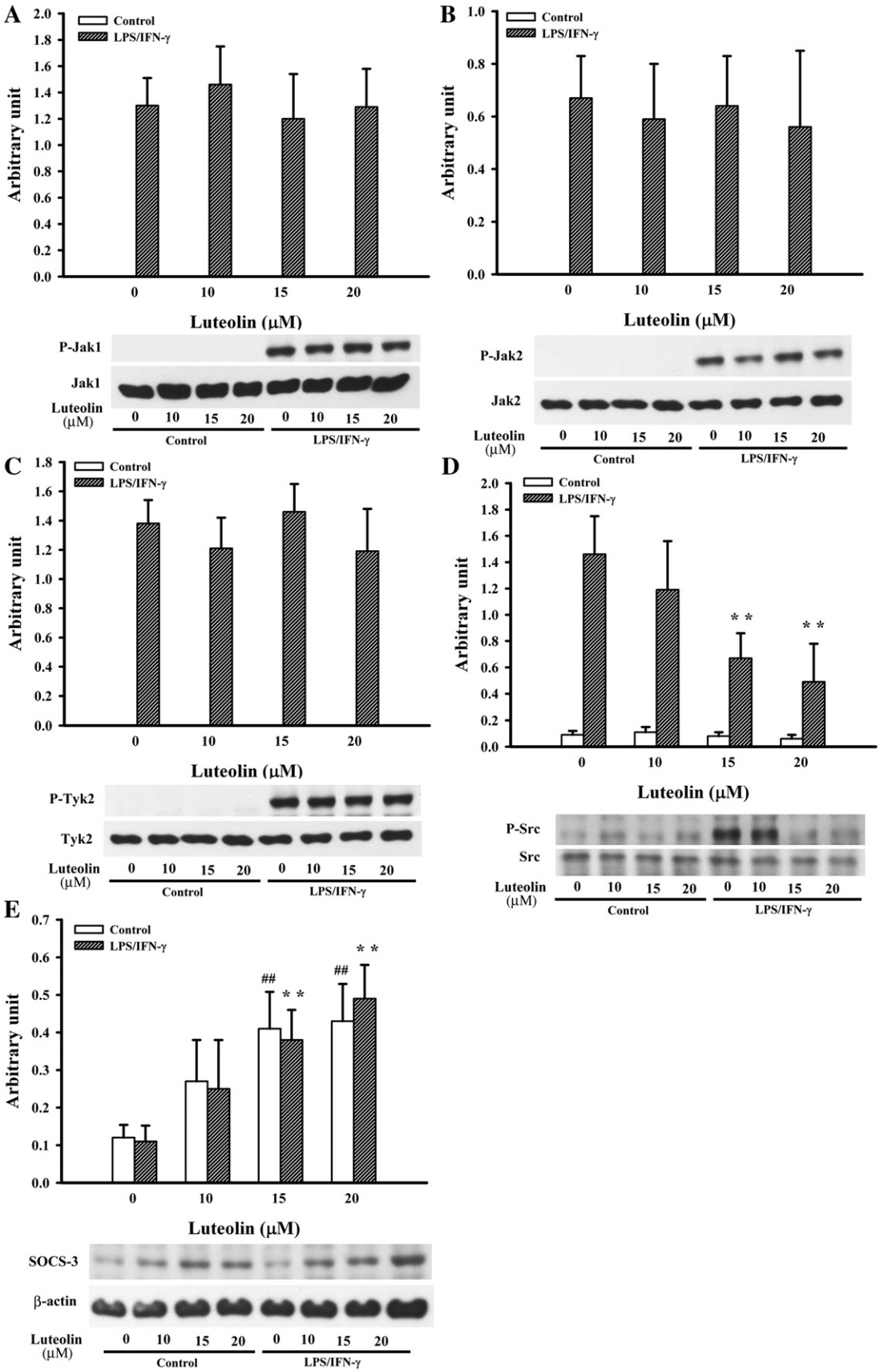

Fig. 9. Effect of luteolin on STAT signaling molecules. BV-2 microglial cells were pretreated with vehicle control or various concentrations of luteolin for 30 min before stimulation with LPS (100 ng/ml)/IFN-γ (10 U/ml). Total cell lysates (1 h) were isolated and subjected to Western blot for the detection of (A) p-Jak1, Jak1, (B) p-Jak2, Jak2, (C) p-Tyk2, Tyk2, and (D) p-Src, Src. (E)Another batch of cell lysates (5 h) were isolated and subjected to Western blot for detection of SOCS-3 and β-actin. The quantitative results of protein phosphorylation level and SOCS-3 content are depicted. $^{**}P<.01$ vs. the LPS/IFN-γ-treated group and $^{\#\#}P<.01$ vs. the control group, n=3.

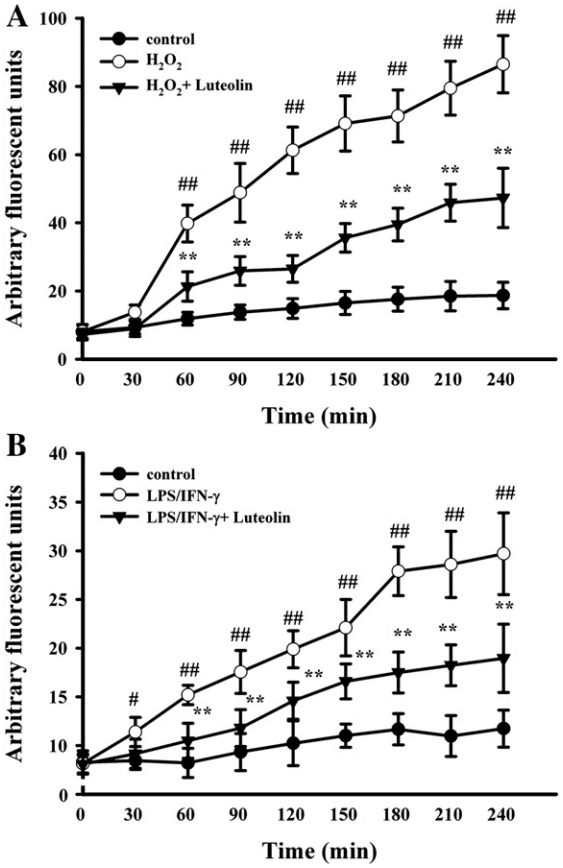

Fig. 10. Effect of luteolin on free radical generation. BV-2 microglial cells were preloaded with 10 μM 2′,7′-dichlorofluorescein for 1 h. The resultant cells were treated with (A) medium (control), 1 mM H_2O_2 and 1 mM H_2O_2 plus luteolin (20 μM) as well as (B) LPS (100 ng/ml)/IFN-γ (10 U/ml) and LPS (100 ng/ml)/IFN-γ (10 U/ml) plus luteolin (20 μM) over time. The fluorescent signals were recorded over the course of 240 min at 30-min intervals and the kinetics are depicted. $^{**}P<.01$ vs. each LPS/IFN-γ-treated group and $^{\#}P<.05$ and $^{\#\#}P<.01$ vs. each control group, n=6.

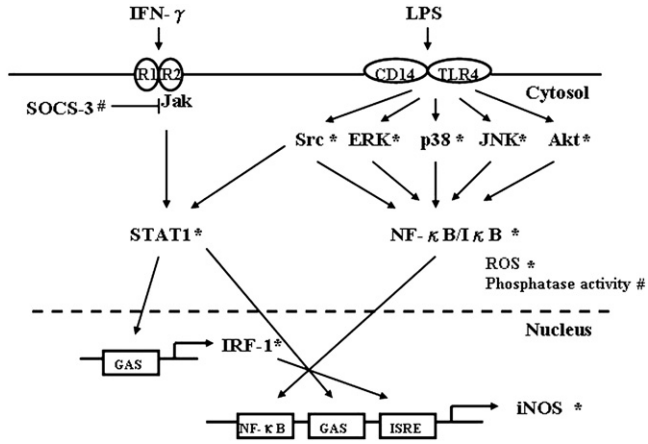

Fig. 12. Schematic diagram shows the affected molecules underlying the inhibition by luteolin. This schematic diagram indicates employed signaling molecules generally in mediating activation of transcription factors for iNOS expression in response to LPS/IFN-γ. Some additional signaling molecules and cascades have been omitted for the sake of clarity. *Its activity or expression was inhibited by luteolin; #Its activity or expression was stimulated by luteolin. The stimulatory action is marked by → and inhibitory route is indicated by ⊣.

complementary signaling pathways. Generally, LPS signaling involves a series of intracellular molecules such as MAPKs, Akt and Src after binding with CD14 and TLR4. However, the Jak/STAT pathway has been implicated in transducing signals stimulated by IFN-γ after its engagement with R1 and R2 receptor [56]. Protein phosphorylation is divided into serine/threonine and tyrosine categories, which rely on phosphorylated amino acid residues and is governed by kinases and phosphatases. Our data showed that several downstream effectors of LPS and IFN-γ signaling were inhibited by luteolin, manifested by decreased phosphorylation levels. Evidence indicates that flavonoids inactivate phosphorylation events by competing with the ATP binding site [59]. Enzymatic studies have shown that the suppressive effect of luteolin on the event of protein phosphorylation in BV-2 microglial cells was associated with the increased phosphatase activity, including serine/threonine and tyrosine phosphatase. That is, increased phosphatase activity might partly contribute to luteolin-mediated inactivation of intracellular signaling molecules. However, the identity of luteolin-altered phosphatases is not known. This unanswered question could be a potential explanation for the unresponsiveness of Jak family protein phosphorylation by luteolin.

Free radicals are commonly multiplied during inflammatory processes that involve signal transduction and gene activation and can contribute to host cell and organ damage. Evidence indicates that inhibition of free radical production inactivates intracellular signaling activation and decreases proinflammatory cytokine production,

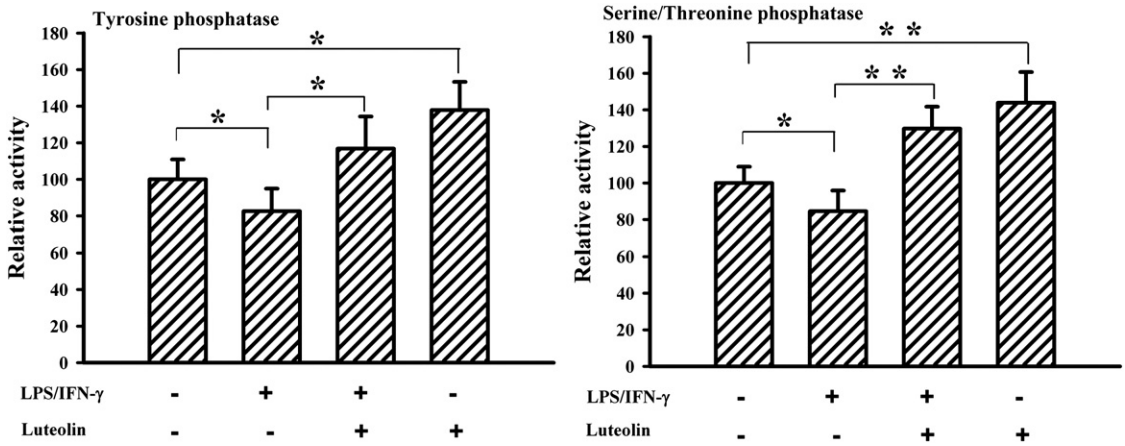

Fig. 11. Effect of luteolin on protein phosphatases. BV-2 microglial cells were pretreated with vehicle or luteolin (20 μM) for 30 min before stimulation with LPS (100 ng/ml)/IFN-γ (10 U/ml) for an additional 60 min. Whole-cell lysates were isolated and subjected to enzymatic assay for the measurement of serine/threonine phosphatase and tyrosine phosphatase activity. The activity in nontreated control group was defined as 100%. $^{*}P<.05$ and $^{**}P<.01$, n=3.

leading to a reduction in inflammatory responses [38,44,60]. The redox-sensitive signaling molecules and transcription factors such as MAPKs, Akt, Src, Jak/STAT and NF-κB are potential downstream effectors of free radicals. Luteolin showed obvious antioxidative capacity in scavenging H_2O_2 and LPS/IFN-induced free radical generation in BV-2 microglial cells. These data showed that one of the biological activities of luteolin is its antioxidant effect. The resolution of oxidative stress by luteolin might contribute partly to the inactivation of MAPKs, Akt, Src, STAT, IRF-1 and NF-κB leading to the reduction of proinflammatory cytokine production. Evidence shows that ROS induce activation of protein phosphatase activity [61]. However, antioxidant also possesses stimulatory effect on protein phosphatase activity through redox-dependent posttranslational modification [62]. Therefore, further research is needed to fully elucidate the action cascades involved and potential crosstalk among inhibition of oxidative stress, enhancement of phosphatase activity and these signaling pathways after luteolin treatment.

Plants are a good source of useful health-promoting agents. The health-promoting effects of natural substances originated from plants are attracting growing interest. The continuing search for novel health-promoting substances especially from plants with historically documented or pharmacological properties holds considerable nutraceutical and/or pharmaceutical promise. Among them, flavonoids are a group of polyphenolic compounds that are widely found in the plant kingdom. As intrinsic components of fruits, vegetables, beverages such as wine and tea and in some traditional herbal-containing medicines, many of the different flavonoids known to date are part of the regular human diet. Flavonoids are nonessential dietary factors, but their average daily consumption is estimated to be 1–2 g [18]. A potential advantage of plant-derived compounds in health care is that their utilization as food has a long history and their use has been accepted as safe. The possible utilization of plant-derived compounds and extracts as chemopreventive and health-promoting agents in the future has focused increasing attention on the understanding of their molecular mechanisms and targets of action. Flavonoids show several health benefits such as anticancer, antimicrobial, antiviral, anti-inflammatory, immunomodulatory, chemoprevention and antioxidant through diverse mechanisms [10–19,23–32]. This study provides an alternative target for the elucidation of potential physiological benefits of dietary flavonoids. Our results demonstrate that signaling actions of luteolin are involved in its anti-inflammatory action and that luteolin can act as a modulating molecule to regulate kinase/phosphatase-mediated signaling.

In summary, transcription factors represent a group of important effectors that could cause the convergence of multiple extrinsic and intrinsic signals resulting in the regulation of proinflammatory cytokine expression. We have showed that inhibition of LPS/IFN-γ-induced NO, TNF-α and IL-1β production by luteolin in microglia is attributed to down-regulation of NF-κB, STAT1 and IRF-1 activities probably via interference of ERK, p38, JNK and Src activation involving the promotion of phosphatase activity and antioxidant effects (Fig. 12). The immunosuppressive mechanism of luteolin seems to be multifactorial. It is reasonable to propose that inhibition of NF-κB, STAT1 and IRF-1 by luteolin represents a critical mechanism to attenuate microglial activation. However, the initial interacting targets of luteolin and additional anti-inflammatory mechanisms require further investigation.

Acknowledgments

This work was supported by grant from the Taichung Veterans General Hospital and Tunghai University (TCVGH-T967805), Taiwan.

References

[1] McGeer PL, Itagaki S, Boyes BE, McGeer EG. Reactive microglia and positive for HLA-DR in the substantia nigra of Parkinson's and Alzheimer's disease brain. Neurology 1988;38:1285–91.
[2] Dickson DW, Lee SC, Mattiace LA, Yen SH, Brosnan C. Microglia and cytokines in neurological disease, with special reference to AIDS and Alzheimer's disease. Glia 1993;7:75–83.
[3] Minghetti L, Levi G. Microglia as effector cells in brain damage and repair: focus on prostanoids and nitric oxide. Prog Neurobiol 1998;54:99–125.
[4] Gonzalez-Scarano F, Baltuch G. Microglia as mediators of inflammatory and degenerative diseases. Annu Rev Neurosci 1999;22:219–40.
[5] Hirsch EC. Glial cells and Parkinson's disease. J Neurol 2000;247:II58–62.
[6] Zhu DY, Deng Q, Yao HH, Wang DC, Deng Y, Liu GQ. Inducible nitric oxide synthase expression in the ischemic core and penumbra after transient focal cerebral ischemia in mice. Life Sci 2002;71:1985–96.
[7] Boucher JL, Moali C, Tenu JP. Nitric oxide biosynthesis, nitric oxide synthase inhibitors and arginase competition for L-arginine utilization. Cell Mol Life Sci 1999;55:1015–28.
[8] Simmons ML, Murphy S. Induction of nitric oxide synthase in glial cells. J Neurochem 1992;59:897–905.
[9] Liu B, Gao HM, Wang JY, Jeohn GH, Cooper CL, Hong JS. Role of nitric oxide in inflammation-mediated neurodegeneration. Ann NY Acad Sci 2002;962:700–15.
[10] Mora A, Paya M, Rios JL, Alcaraz MJ. Structure–activity relationships of polymethoxyflavones and other flavonoids as inhibitors of non-enzymatic lipid peroxidation. Biochem Pharmacol 1990;40:793–7.
[11] Lin CC, Shieh DE. The anti-inflammatory activity of *Scutellaria rivularis* extracts and its active components, baicalin, baicalein, and wogonin. Am J Chin Med 1996;24:31–6.
[12] Hwang YS, Shin CY, Huh Y, Ryu JH. Hwangryun-Hae-Dok-tang (Huanglian-Jie-Du-Tang) extract and its constitutes reduce ischemia–reperfusion brain injury and neutrophil infiltration in rats. Life Sci 2002;71:2105–17.
[13] Chen CC, Chow MP, Huang WC, Lin YC, Chang YJ. Flavonoids inhibit tumor necrosis factor-alpha-induced up-regulation of intracellular adhesion molecule-1 (ICAM-1) in respiratory epithelial cells through activator protein-1 and nuclear factor-kappaB: structure–activity relationships. Mol Pharmacol 2004;66:683–93.
[14] Chen CJ, Raung SL, Liao SL, Chen SY. Inhibition of inducible nitric oxide synthase expression by baicalein in endotoxin/cytokine-stimulated microglia. Biochem Pharmacol 2004;67:957–65.
[15] Chen JC, Ho FM, Lee Chao PD, Chen CP, Jeng KCG, Hsu HB, et al. Inhibition of iNOS gene expression by quercetin is mediated by the inhibition of IκB kinase, nuclear factor-kappa B and STAT1, and depends on heme oxygenase-1 induction in mouse BV-2 microglia. Eur J Pharmacol 2005;521:9–20.
[16] Cho JY, Kim IS, Jang YH, Kim AR, Lee SR. Protective effect of quercetin flavonoid against neuronal damage after transient global cerebral ischemia. Neurosci Lett 2006;404:330–5.
[17] Ruiz PA, Haller D. Functional diversity of flavonoids in the inhibition of the proinflammatory NF-κB, IRF, and Akt signaling pathways in murine intestinal epithelial cells. J Nutr 2006;136:664–71.
[18] Hämäläinen M, Nieminen R, Vuorela P, Heinonen M, Moilanen E. Anti-inflammatory effects of flavonoids: genistein, kaempferol, quercetin, and daidzein inhibit STAT-1 and NF-κB activations, whereas flavone, isorhamnetin, naringenin, and pelargonidin inhibit only NF-κB activation along with their inhibitory effect on iNOS expression and NO production in activated macrophages. Mediators Inflamm 2007;2007:45673.
[19] Bureau G, Longpré F, Martinoli MG. Resveratrol and quercetin, two natural polyphenols, reduce apoptotic neuronal cell death induced by neuroinflammation. J Neurosci Res 2008;86:403–10.
[20] Kotanidou A, Xagorari A, Bagli E, Kitsanta P, Fotsis T, Papapetropoulos A, et al. Luteolin reduces lipopolysaccharide-induced lethal toxicity and expression of proinflammatory molecules in mice. Am J Clin Microbiol Crit Care Med 2002;165:818–23.
[21] Das M, Ram A, Ghosh B. Luteolin alleviates bronchoconstriction and airway hyperreactivity in ovalbumin sensitized mice. Inflamm Res 2003;52:101–6.
[22] Tormakangas L, Vuorela P, Saario E, Leinonen M, Saikku P, Vuorela H. In vivo treatment of acute *Chlamydia pneumoniae* infection with the flavonoids quercetin and luteolin and an alkyl gallate, octyl gallate, in a mouse model. Biochem Pharmacol 2005;70:1222–30.
[23] Comalada M, Ballester I, Bailon E, Sierra S, Xaus J, Galvez J, et al. Inhibition of pro-inflammatory markers in primary bone marrow-derived mouse macrophages by naturally occurring flavonoids: analysis of the structure–activity relationship. Biochem Pharmacol 2006;72:1010–21.
[24] Gutiérrez-Venegas G, Kawasaki-Cárdenas P, Arroyo-Cruz SR, Maldonado-Frías S. Luteolin inhibits lipopolysaccharide actions on human gingival fibroblasts. Eur J Pharmacol 2006;541:95–105.
[25] Harris GK, Qian Y, Leonard SS, Sbarra DC, Shi X. Luteolin and chrysin differentially inhibit cyclooxygenase-2 expression and scavenge reactive oxygen species but similarly inhibit prostaglandin-E2 formation in RAW 264.7 cells. J Nutr 2006;136:1517–21.
[26] Chen CY, Peng WH, Tsai KD, Hsu SL. Luteolin suppresses inflammation-associated gene expression by blocking NF-κB and AP-1 activation pathway in mouse alveolar macrophages. Life Sci 2007;81:1602–14.
[27] Kempuraj D, Tagen M, Iliopoulou BP, Clemons A, Vasiadi M, Boucher W, et al. Luteolin inhibits myelin basic protein-induced human mast cell activation

and mast cell-dependent stimulation of Jurkat T cells. Br J Pharmacol 2008;155: 1076–84.
[28] Kim JS, Lee HJ, Lee MH, Kim J, Jin C, Ryu JH. Luteolin inhibits LPS-stimulated inducible nitric oxide synthase expression in BV-2 microglial cells. Planta Med 2006;72:65–8.
[29] Sharma V, Mishra M, Ghosh S, Tewari R, Basu A, Seth P, et al. Modulation of interleukin-1β mediated inflammatory response in human astrocytes by flavonoids: implications in neuroprotection. Brain Res Bull 2007;73:55–63.
[30] Chen HQ, Jin ZY, Wang XJ, Xu XM, Deng L, Zhao JW. Luteolin protects dopaminergic neurons from inflammation-induced injury through inhibition of microglial activation. Neurosci Lett 2008;448:175–9.
[31] Jang S, Kelley KW, Johnson RW. Luteolin reduces IL-6 production in microglia by inhibiting JNK phosphorylation and activation of AP-1. Proc Natl Acad Sci USA 2008;105:7534–9.
[32] Rezai-Zadeh K, Ehrhart J, Bai Y, Sanberg PR, Bickford P, Tan J, et al. Apigenin and luteolin modulate microglial activation via inhibition of STAT1-induced CD40 expression. J Neuroinflammation 2008;5:41.
[33] Chen CJ, Liao SL. Oxidative stress involves in astrocytic alterations induced by manganese. Exp Neurol 2002;175:216–25.
[34] Xie QW, Whisnant R, Nathan C. Promoter of the mouse gene encoding calcium-independent nitric oxide synthase confers inducibility by interferon γ and bacterial lipopolysaccharide. J Exp Med 1993;177:1779–84.
[35] Sitcheran R, Comb WC, Cogswell PC, Baldwin AS. Essential role for epidermal growth factor receptor in glutamate receptor signaling to NF-κB. Mol Cell Biol 2008;28:5061–70.
[36] Kim HY, Park EJ, Joe EH, Jou I. Curcumin suppresses Janus kinase-STAT inflammatory signaling through activation of Src homology 2 domain-containing tyrosine phosphatase 2 in brain microglia. J Immunol 2003;171:6072–9.
[37] Simon AR, Rai U, Fanburg BL, Cochran BH. Activation of the JAK-STAT pathway by reactive oxygen species. Am J Physiol 1998;275:C1640–52.
[38] McCormick J, Barry SP, Sivarajah A, Stefanutti G, Townscend PA, Lawrence KM, et al. Free radical scavenging inhibits STAT phosphorylation following in vivo ischemia/reperfusion injury. FASEB J 2006;20:1404–10.
[39] Huang C, Ma R, Sun S, Wei G, Fang Y, Liu R, et al. Jak2-STAT3 signaling pathway mediates thrombin-induced proinflammatory actions of microglia in vitro. J Neuroimmunol 2008;204:118–25.
[40] Boehm U, Klamp T, Groot M, Howard JC. Cellular responses to interferon-γ. Annu Rev Immunol 1997;15:749–95.
[41] Taniguchi T, Ogasawara K, Takaoka A, Tanaka N. IRF family of transcription factors as regulators of host defense. Annu Rev Immunol 2001;19:623–55.
[42] Kile BT, Nicola NA, Alexander WS. Negative regulators of cytokine signaling. Int J Hematol 2001;73:292–8.
[43] Raung SL, Chen SY, Liao SL, Chen JH, Chen CJ. Japanese encephalitis virus infection stimulates Src tyrosine kinase in neuron/glia. Neurosci Lett 2007;419:263–8.
[44] Halliwell B, Gutteridge JMC. Oxygen toxicity, oxygen radicals, transition metals and disease. Biomed J 1984;219:1–4.
[45] Lee J, Hur J, Lee P, Kim JY, Cho N, Kim SY, et al. Dual role of inflammatory stimuli in activation-induced cell death of mouse microglial cells: initiation of two separate apoptotic pathways via induction of interferon regulatory factor-1 and caspase-11. J Biol Chem 2001;276:32956–65.
[46] Chang LC, Tsao LT, Chang CS, Chen CJ, Huang LJ, Kuo SC, et al. Inhibition of nitric oxide production by the carbazole compound LCY-2-CHO via blockade of activator protein-1 and CCAAT/enhancer-binding protein activation in microglia. Biochem Pharmacol 2008;76:507–19.
[47] Lee JK, Kim SY, Kim YS, Lee WH, Hwang DH, Lee JY. Suppression of the TRIF-dependent signaling pathway of Toll-like receptors by luteolin. Biochem Pharmacol 2009;77:1391–400.
[48] Ghosh S, May MJ, Kopp EB. NF-κB and Rel proteins: evolutionarily conserved mediators of immune responses. Annu Rev Immunol 1998;16:225–60.
[49] Matsusaka T, Fujikawa K, Nishio Y, Mukaida N, Matsushima K, Kishimoto T, et al. Transcription factors NF-IL6 and NF-kappa B synergistically activate transcription of the inflammatory cytokines, interleukin 6 and interleukin 8. Proc Natl Acad Sci USA 1993;90:10193–7.
[50] Nerlov C, Ziff EB. CCAAT/enhancer binding protein-alpha amino acid motifs with dual TBP and TFIIB binding ability co-operate to activate transcription in both yeast and mammalian cells. EMBO J 1995;14:4318–28.
[51] Saura M, Zaragoza C, Bao C, McMillan A, Lowenstein CJ. Interaction of interferon regulatory factor-1 and nuclear factor kappaB during activation of inducible nitric oxide synthase transcription. J Mol Biol 1999;289:459–71.
[52] Zhao Q, Lee FS. Mitogen-activated protein kinase/ERK kinase kinase 2 and 3 activate nuclear factor-κB through IκB kinase-α and IκB kinase-β. J Biol Chem 1999;274:8355–8.
[53] Madrid LV, Mayo MW, Reuther JY, Baldwin Jr AS. Akt stimulates the transactivation potential of the RelA/p65 subunit of NF-kappa B through utilization of the Ikappa B kinase and activation of the mitogen-activated protein kinase p38. J Biol Chem 2001;276:18934–40.
[54] Xagorari A, Papapetropoulos A, Mauromatis A, Economou M, Fotsis T, Roussos C. Luteolin inhibits an endotoxin-stimulated phosphorylation cascade and proinflammatory cytokine production in macrophages. J Pharmacol Exp Ther 2001;296:181–7.
[55] Xagorari A, Roussos C, Papapetropoulos A. Inhibition of LPS-stimulated pathways in macrophages by the flavonoid luteolin. Br J Pharmacol 2002;136:1058–64.
[56] Kitamura Y, Takahashi H, Normura Y, Tangiguchi T. Possible involvement of Janus kinase Jak2 in interferon-γ induction of nitric oxide synthase in rat glial cells. Eur J Pharmacol 1996;306:297–306.
[57] Lowell CA, Soriano P. Knockputs of Src-family kinases: stiff bones, wimpy T cells, and bad memories. Genes Dev 1996;10:1845–57.
[58] Reich H, Tritchler D, Herzenberg AM, Kassiri Z, Zhou X, Gao W, et al. Albumin activates ERK via EGF receptor in human renal epithelial cells. J Am Soc Nephrol 2005;16:1266–78.
[59] Ferriola PC, Cody V, Middleton Jr E, Protein kinase C. inhibition by plant flavonoids. Kinetic mechanisms and structure activity relationships. Biochem Pharmacol 1989;38:1617–24.
[60] Wang HK, Park UJ, Kim SY, Lee JH, Kim SU, Gwag BJ, et al. Free radical production in CA1 neurons induces MIP-1α expression, microglia recruitment, and delayed neuronal death after transient forebrain ischemia. J Neurosci 2008; 28:1721–7.
[61] Park SJ, Kim HY, Kim H, Park SM, Joe E, Jou I, et al. Oxidative stress induces lipid-raft-mediated activation of Src homology 2 domain-containing protein-tyrosine phosphatase 2 in astrocytes. Free Radic Biol Med 2009;46: 1694–702.
[62] Haridas V, Nishimura G, Xu ZX, Connolly F, Hanausek M, Walaszek Z, et al. Avicin D: a protein reactive plant isoprenoid dephosphorylates Stat 3 by regulating both kinase and phosphatase activities. PLoS One 2009;4:e5578.