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Inhibition of inducible nitric oxide synthase expression by baicalein in endotoxin/cytokine-stimulated microglia

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Abstract

Excessive production of nitric oxide (NO) in the central nervous system (CNS) mediated by activation of microglia has been implicated in neurotoxicity after stresses such as ischemia. Baicalein, a polyphenolic flavonoid antioxidant, is known to have anti-inflammatory, anticarcinogenic, and neuroprotective effects. In the present study, we report the inhibitory effect of baicalein on endotoxin/cytokine-induced NO production and inducible nitric oxide synthase (iNOS) gene expression in microglia. Baicalein abolished the endotoxin/cytokine-induced expression of iNOS protein, iNOS mRNA, and iNOS promoter activity in a parallel concentration-dependent manner. The suppression of iNOS expression was not mediated through the down-regulation of tumor necrosis factor-alpha (TNF-α) by baicalein because TNF-α failed to enhance endotoxin/cytokine-induced NO production in microglia. From the electrophoretic mobility shift assay (EMSA), we found that baicalein exerted a distinct inhibitory effect on the DNA binding activity of transcription factors, and this was significantly greater in nuclear factor IL-6 (NF-IL6) than in nuclear factor kappa B (NF-κB) and activated protein 1 (AP-1). Although extracellular signal-regulated kinase (ERK) is critical to iNOS expression, endotoxin/cytokine-stimulated phosphorylation of ERK1/2 was not significantly inhibited by baicalein. These results indicate that NF-IL6 inactivation could be the major determinant for the suppression of NO production by baicalein in microglia. Furthermore, it suggests that the inhibitory effect of baicalein on microglia activation and neurotoxic factor production is responsible for its neuroprotective action.

Keywords: Baicalein; BV-2; Inducible nitric oxide synthase; Microglia; Nitric oxide; NF-IL6

1. Introduction

Microglia represent the resident macrophage population within the CNS, where they perform a similar range of functions to macrophages found elsewhere in the body [1]. Activation of microglia has been increasingly associated with the pathogenesis of several neurodegenerative disorders including Parkinson's disease, Alzheimer's disease, multiple sclerosis, the AIDS dementia complex, and ischemia [2–6]. Activated microglia produce a wide range of cytotoxic factors, including TNF-α, IL-1β, eicosanoids, reactive oxygen species (ROS), and NO, that impact on neurons to induce neurodegeneration [7–9]. Of the various factors released by activated microglia, NO appears to play a critical role in stress-induced brain damage.

NO is a short-lived free radical and intracellular messenger that mediates a variety of biological functions, including vascular homeostasis, neurotransmission, antimicrobial defense, antitumor activity, and cytotoxicity [10]. Three major types of NO synthase (NOS) isoenzymes that catalyze the conversion of L-arginine to NO and L-citrulline have been identified. Two forms are regulated by intracellular calcium and expressed constitutively in endothelial and neuronal cells [10]. The iNOS, the high-output isoform, is rapidly transcribed and expressed in microglia after stimulation with immunogens such as LPS, cytokines, and amyloid β [10–12]. Excessive production of

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Abbreviations: NO, nitric oxide; iNOS, inducible nitric oxide synthase; CNS, central nervous system; TNF-α, tumor necrosis factor-alpha; EMSA, electrophoretic mobility shift assay; NF-κB, nuclear factor kappa B; AP-1, activated protein 1; NF-IL6, nuclear factor IL-6; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; PPAR-γ, peroxisome proliferator-activated receptor gamma; LPS, lipopolysaccharide; IFN-γ, interferon gamma; JNK, c-Jun N-terminal kinase; IL-1β, interleukin-1 beta.

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NO in the CNS could be toxic to many cell types, including neurons [13]. 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.

Scutellaria baicalensis Georgi (Huang Qui) is one of the important medicinal herbs widely used for the treatment of various inflammatory diseases, hepatitis, tumors, and diarrhea in East Asian countries such as China, Korea, Taiwan, and Japan [14]. The plant contains a large number of flavonoids such as baicalein, which possess anti-inflammatory activity [15]. Recently, the extract of *S. baicalensis* Georgi and its flavonoid constituent baicalein have been demonstrated to attenuate ischemic injury by inhibiting neutrophil-mediated inflammatory reactions in the rat brain [16]. However, the actual role of baicalein in the inflammatory process in the transient cerebral ischemic model in rats remains unclear. It is well known that the brain is subject to inflammatory reactions in response to ischemic damage [17]. The activation of iNOS in the brain is critical to the delayed neuronal death induced by ischemia and the suppression of its expression reduces brain infarction [6]. Inhibition of iNOS expression by baicalein was demonstrated in LPS-stimulated peripheral macrophages through either activation of PPAR-γ, inhibition of cyclooxygenase/lipoxygenase pathways, or free radical scavenging effect [18–20]. Recently, Suk *et al.* [21] had also reported that baicalein could attenuate LPS-activated microglia death through the suppression of cytotoxic NO production. However, its role in NO production by central microglia has not been reported. We investigated the effect of baicalein on the endotoxin/cytokine-mediated induction of NO production and iNOS gene expression in primary microglia and murine BV-2 microglia cell line. Our results show that baicalein inhibits endotoxin/cytokine-induced increase of NO production and activation of iNOS gene expression, through the downregulation of transcription factor, is mainly induced by the NF-IL6.

2. Materials and methods

2.1. Cell cultures

Rat primary glial cultures were prepared as described previously [22]. Rat primary microglia were isolated from the glial cultures. Briefly, glial cells were cultured in 75-mm^2 flasks for 10–14 days in Dulbecco's modified Eagle's medium (DMEM, Gibco Life Technologies) supplemented with 10% fetal bovine serum (FBS, Gibco Life Technologies), 100 U/mL penicillin (Gibco Life Technologies) and 100 μg/mL streptomycin (Gibco Life Technologies). To separate microglia, flasks were shaken for 3 hr at 180 rpm in a rotary shaker at 37°. Detached cells were plated on 24-well plates at a density of 1×10^5 cells per well. The purity of microglia cultures was assessed using ED-1 antibody (Biosource) and more than 95% of cells were stained positively.

The murine BV-2 cell line [23] was kindly donated by Dr. Hong JS (Research Triangle Park, NIEH, NIH). RAW 264.7 cells, a murine macrophage cell line, were obtained from the ATCC (American Type Culture Collection). Both cells were cultured in DMEM supplemented with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin, and were maintained in a humidified incubator with 5% CO_2. In all experiments, cells were treated with baicalein 60 min before the addition of LPS (100 ng/mL, *Escherichia coli*, serotype 0111:B4, Sigma Chemical Co.)/IFN-γ (10 U/mL, Sigma Chemical Co.) in serum-free DMEM. Baicalein (Sigma Chemical Co.) was dissolved in DMSO and the final concentration of DMSO added to cells never exceeded 0.1%.

2.2. Nitrite determination

For nitrite determination, primary microglia (1×10^5 cells/well, 24-well), BV-2 and RAW 264.7 (1×10^4 cells/well, 96-well) were allowed to adhere overnight. The final volume of reaction medium was 500 μL for primary microglia and 200 μL for BV-2 and RAW 264.6, respectively. The production of NO was determined based on the Griess reaction [24]. 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, Inc.). A standard nitrite curve was generated in the same fashion using $NaNO_2$.

2.3. Immunoblot analysis

Cells were washed twice with phosphate-buffered saline (PBS) and harvested in Laemmli SDS sample buffer. The protein concentration in the supernatant was determined by Bradford assay (Bio-Rad). Protein extracts were separated by SDS–PAGE and electrophoretically transferred to polyvinylidene difluoride membranes (Amersham Pharmacia Biotech.). Membranes were first incubated with 5% nonfat milk in PBS for 1 hr at room temperature to reduce nonspecific binding. Membranes were washed with PBS containing 0.1% Tween-20 (PBST), and then incubated for 1 hr at room temperature with the indicated antibodies including iNOS (1:5000, Upstate Biotechnology), phosphorylated and non-phosphorylated forms of ERK1/2, JNK, and p38 (1:5000, Santa Cruz Biotechnology). After washing again with PBST buffer, a 1:10,000 (v/v) dilution of horseradish peroxidase-labeled IgG was added at room temperature for 1 hr. The blots were developed using ECL Western blotting reagents (Amersham Pharmacia Biotech.).

2.4. Isolation of RNA and reverse transcriptase-polymerase chain reaction (RT-PCR)

The isolation of RNA and synthesis of cDNA were carried out as previously reported [25]. DNA fragments of specific genes and internal controls were co-amplified in one tube containing *Taq* DNA polymerase (Promega) and 0.8 μM of each sense and antisense primers. The PCR reaction was performed with a DNA thermal cycler (Perkin-Elmer-Cetus) under the following conditions: one cycle of 94° for 3 min, 28 cycles of (94° for 50 s, 58° for 40 s, and 72° for 45 s), and then 72° for 5 min. In preliminary experiments, we found that the PCR and product amplification was linear ($r = 0.946$–0.977) under this PCR condition. The amplified DNA fragments were resolved by 1.5% agarose gel electrophoresis and stained with EtBr. The DNA band's intensity was determined by a computer image analysis system (Alpha Innotech Corporation, IS1000). Relative mRNA levels are expressed as the intensity ratio of each gene and internal control (β-actin). Oligonucleotides (synthesized by Gibco Life Technologies) used in this study were as follows: 5′-CCCTCACACTCAGATCATCTTCTCAA and 5′-TCTAAGTACTTGGGCAGGTTGACCTC for TNF-α; 5′-ATGGCAACTGTCCCTGAACTC and 5′-GTCGTTGCTTGTCTCTCCTTG for IL-1β; 5′-ACAACGTGGAGAAAACCCCAGGTG and 5′-ACAGCTCCGGGCATCGAAGACC for iNOS; 5′-TCCTGTGGCATCCACGAAACT and 5′-GGAGCAATGATCTTGATCTTC for β-actin.

2.5. Plasmids, transient transfection, and reporter assay

Firefly luciferase gene under the control of the iNOS promoter, piNOS-LUC (provided by Dr. Wang JP, Taichung Veterans General Hospital, Taiwan) [26], was used to quantify iNOS promoter activity. A *Renilla* luciferase reporter under the control of the herpes simplex virus thymidine kinase promoter, pRL-TK (provided by Dr. Wang JP, Taichung Veterans General Hospital, Taiwan) [26], was used as an internal control to normalize the reporter gene activity. BV-2 cells were transfected by lipofectAMINE (Gibco Life Technologies) liposomal transfection reagent according to the instructions of the manufacturer. Twenty-four hours later, the culture medium was replaced, and cells were pretreated with vehicle or baicalein for 1 hr followed by stimulation with LPS/IFN-γ. After 36 hr, the cells were lysed, and the luciferase activity was determined. Luciferase activities were determined by a luminometer using a Dual-luciferase reporter assay according to the instructions of the manufacturer (Promega Co.).

2.6. Preparation of nuclear extracts and EMSA

Nuclear extracts were prepared as described previously [26]. 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. Then the cells were lysed by the addition of 0.5% Nonidet P-40 and vigorous vortexing for 30 s. The nuclei were pelleted by centrifugation at 12,000 *g* for 1 min at 4° 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 12,000 *g* for 10 min at 4°. Supernatants were obtained and stored at −70°. The oligonucleotides, specific for each known transcription factor, were synthesized and labeled with biotin including NF-κB (5′-AGTTGAGGGGACTTTCCCAGGC from Promega), AP-1 (5′-CGCTTGATGAGTCAGCCGGAA from Promega), and NF-IL6 (5′-CCACAGAGTGATGTAATCA, corresponding to the NF-IL6 site (−153 to −142) of the mouse iNOS promoter) [27]. Nuclear extract (10 μg) was used for EMSA. The binding reaction mixture included 1 μg of poly(dI-dC), 0.1 μg poly-L-lysine, and 100 fmole 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.

2.7. Statistical analysis

Results are presented as means ± SEM. 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 the respective control using Student's *t*-test. *P* values less than 0.05 were considered significant. Analysis of the regression line test was used to calculate IC_{50} values.

3. Results

3.1. Effect of baicalein on NO production in primary microglia and BV-2 cells

Incubation of primary microglia and BV-2 cells with LPS/IFN-γ-induced iNOS expression as indicated by the accumulation of nitrite, the stable metabolite of NO, in the cell culture media (Fig. 1A). The induction feature of iNOS expression was further characterized by the inhibitory action of NOS inhibitors such as N^G-nitro-L-arginine methyl ester (L-NAME) and diphenyleneiodonium chloride (DPI). The cells were pretreated with various concentrations of baicalein for 60 min, then stimulated with LPS/IFN-γ for another 16 hr. At concentrations used in this study, baicalein treatment was not toxic to either cell type according to the results of

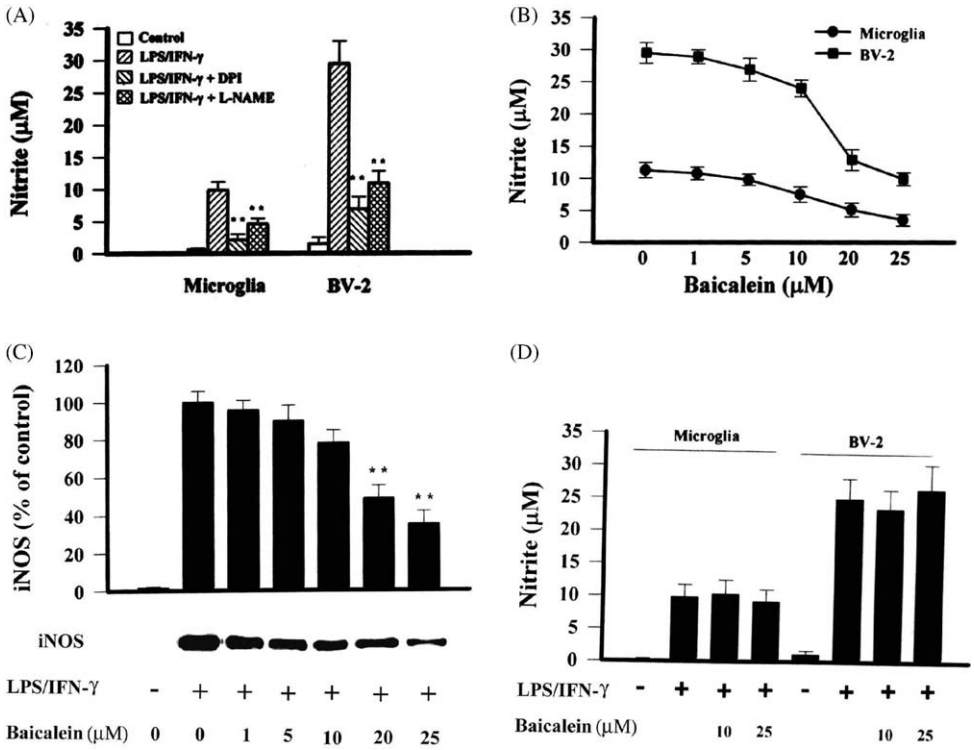

Fig. 1. Inhibition of nitrite production by baicalein. (A) Primary microglia and BV-2 cells were pretreated with vehicle, DPI (0.5 μM), or L-NAME (1 mM) for 60 min before being incubated with LPS (100 ng/mL)/IFN-γ (10 U/mL) for 16 hr. The culture supernatants were isolated and analyzed for nitrite production. (B) Primary microglia and BV-2 cells were pretreated with the indicated concentrations of baicalein for 60 min before being incubated with LPS (100 ng/mL)/IFN-γ (10 U/mL) for 16 hr. The culture supernatants were isolated and analyzed for nitrite production. (C) Primary microglia were pretreated with vehicle or various concentrations of baicalein for 60 min before being incubated with LPS (100 ng/mL)/IFN-γ (10 U/mL) for 16 hr. The cell lysates were isolated and subjected to SDS–PAGE and immunoblot analysis using anti-iNOS antibody. (D) Primary microglia and BV-2 cells were treated with vehicle or LPS (100 ng/mL)/IFN-γ (10 U/mL) for 12 hr. The cultured media were switched to fresh medium containing various concentrations of baicalein for another 16 hr. The culture supernatants were isolated and analyzed for nitrite production. $^{**}P < 0.01$ vs. LPS/IFN-γ-treated group (100%), N = 3.

the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) assay (data not shown). Preincubation of primary microglia with baicalein before LPS/IFN-γ treatment led to a concentration-dependent decrease in nitrite accumulation, with an IC_{50} of about 22.5 ± 1.3 μM. Similar effects were observed in LPS/IFN-γ-treated BV-2 cells, with an IC_{50} of about 19.6 ± 1.1 μM (Fig. 1B). On the other hand, iNOS enzymatic activity was not inhibited by baicalein (Fig. 1D). To determine whether the inhibitory ability of baicalein on NO production was due to a decrease in the cytosolic iNOS protein level, primary microglia were treated with LPS/IFN-γ or LPS/IFN-γ plus baicalein for 16 hr and the levels of iNOS protein were detected by immunoblot analysis. As shown in Fig. 1C, pretreatment with baicalein led to a decrease of iNOS protein levels in a concentration-dependent manner, with an IC_{50} of about 20.6 ± 2.1 μM.

Effect of baicalein on iNOS mRNA expression. Since iNOS protein levels were down regulated, RT-PCR analysis was performed to assess the effect of baicalein on the expression of iNOS mRNA. The expression of iNOS mRNA was hardly detectable in unstimulated primary microglia. However, the appearance of iNOS mRNA occurred gradually until 8 hr after exposure to LPS/IFN-γ (Fig. 2A). Baicalein 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. 2B). Baicalein did not enhance the degradation of iNOS mRNA, which was assessed by measuring the half-life of the expressed iNOS mRNA induced by LPS/IFN-γ (Fig. 2C).

3.2. Role of TNF-α in baicalein-mediated suppression of NO production

Proinflammatory cytokines such as TNF-α and IL-1β are strong stimulators of iNOS gene expression in certain cell types [28,29]. Hence, the expression kinetics of their respective mRNAs in microglia was assessed by RT-PCR. TNF-α mRNA quickly appeared after 1-hr of LPS/IFN-γ treatment and levels were maintained for 8 hr followed by a gradual decrease. Levels of IL-1β mRNA increased markedly until 8 hr after stimulation (Fig. 2A). Both peak expressions of mRNAs were attenuated by baicalein (Fig. 2B). Due to the induction kinetics, the potential involvement of IL-1β in the action of baicalein was ruled out.

To analyze the inducibility of iNOS expression in microglia by TNF-α and its role in the action of baicalein,

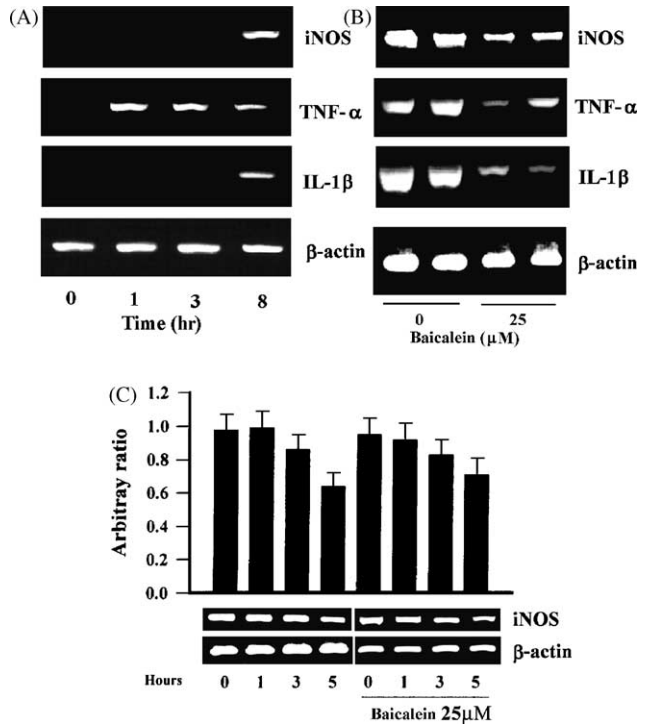

Fig. 2. Inhibition of gene expression by baicalein. (A) Primary microglia were treated with LPS (100 ng/mL)/IFN-γ (10 U/mL) over time. Total RNAs were isolated and subjected to RT-PCR measuring the expressions of iNOS, TNF-α, IL-1β, and housekeeping gene, β-actin. (B) Primary microglia were pretreated with vehicle or baicalein (25 μM) for 60 min before being incubated with LPS (100 ng/mL)/IFN-γ (10 U/mL). Total RNAs were isolated 1 hr after treatment for the analysis of TNF-α, and 8 hr after treatment for the analysis of iNOS and IL-1β. One of two independent results is shown. (C) Primary microglia were treated with LPS (100 ng/mL)/IFN-γ (10 U/mL) for 8 hr. Then, the cultured media were switched to fresh medium containing baicalein (0, 25 μM) for various times (0, 1, 3, and 5 hr). Total RNAs were isolated for the analysis of iNOS and β-actin (N = 3). Arbitrary unit is the ratio of DNA intensity between iNOS and β-actin.

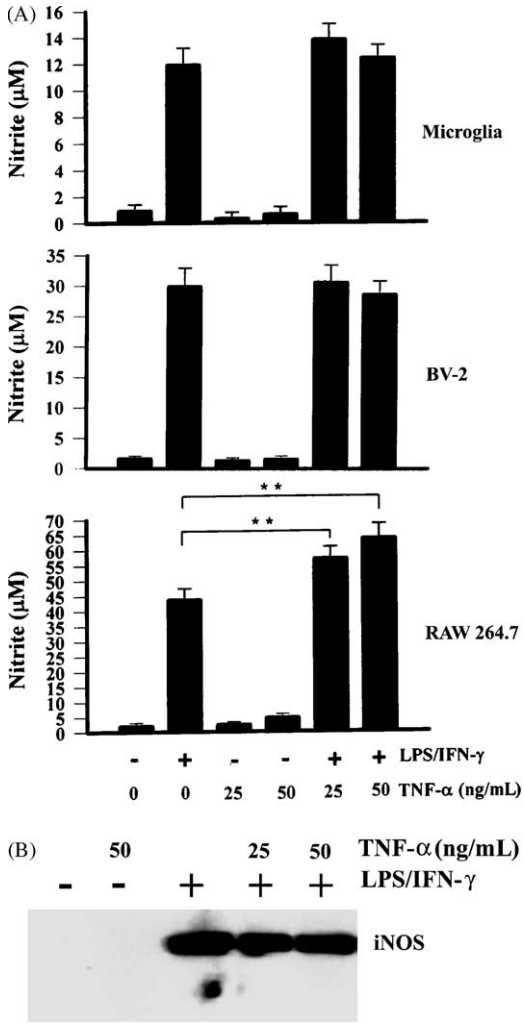

Fig. 3. Effect of TNF-α on nitrite production. (A) Primary microglia, BV-2, and RAW 264.7 cells were treated with vehicle, LPS (100 ng/mL)/IFN-γ (10 U/mL), various concentrations of TNF-α alone, or in combinations for 16 hr. The culture supernatants were isolated and analyzed for nitrite production. $^{**}P < 0.01$, N = 3. (B) BV-2 cells were treated with vehicle, LPS (100 ng/mL)/IFN-γ (10 U/mL), various concentrations of TNF-α alone, or in combinations for 16 hr. The cell lysates were isolated and subjected to SDS–PAGE and immunoblot analysis using anti-iNOS antibody. One of two independent results is shown.

the accumulation of nitrite was measured 16 hr after TNF-α or LPS/IFN-γ plus TNF-α treatments. TNF-α alone was unable to induce iNOS expression by both primary microglia and BV-2 cells. Addition of TNF-α failed to increase LPS/IFN-γ-induced NO production (Fig. 3A) and iNOS protein expression (Fig. 3B). However, TNF-α further stimulated LPS/IFN-γ-induced NO production by RAW 264.7 monocyte/macrophage cells (Fig. 3A).

3.3. Effect of baicalein on iNOS promoter activity

To understand the effect of baicalein on the expression of the iNOS gene, BV-2 cells were transiently transfected with a piNOS-LUC reporter construct containing the iNOS promoter sequence ($-1749 \sim +1$) linked to the luciferase gene. Exposure of the cells to LPS/IFN-γ significantly increased the luciferase activity (Fig. 4). The LPS/IFN-γ-stimulated iNOS promoter activity was attenuated by baicalein in a concentration-dependent manner. Baicalein alone had no effect on iNOS promoter activity.

3.4. Effect of baicalein on NF-κB, AP-1, and NF-IL6 DNA binding activity

It is well known that NF-κB, AP-1 and NF-IL6 are important transcription factors for the inducibility of iNOS gene by LPS/IFN-γ [27–29]. To further investigate whether these transcription factors are important targets for the action of baicalein in primary microglia and BV-2 cells, we performed an electrophoretic mobility shift assay. Treatment of BV-2 cells with LPS/IFN-γ caused a significant increase in the DNA binding activity of NF-κB and AP-1 within 1 hr and NF-IL6 within 2–3 hr. In the presence of baicalein, LPS/IFN-γ-induced NF-IL6 binding was markedly suppressed (by $73 \pm 5\%$) whereas, NF-κB and AP-1 binding were only slightly

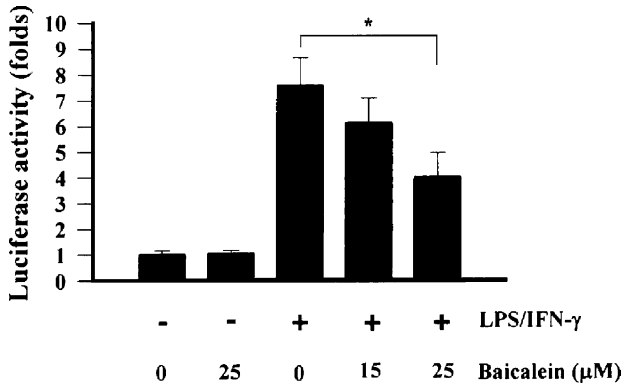

Fig. 4. Effect of baicalein on iNOS promoter activity. BV-2 cells were transiently co-transfected with the piNOS-LUC reporter gene and *Renilla* luciferase reporter gene. Twenty-four hours later, cells were pretreated with vehicle or the indicated concentrations of baicalein for 60 min before stimulation with LPS (100 ng/mL)/IFN-γ (10 U/mL) for another 36 hr. Cells were then lysed, and the lysates were harvested. The *Firefly* and *Renilla* luciferase activities were detected by the Dual-luciferase reporter assay system. The *Firefly* luciferase activity was normalized to the respective *Renilla* luciferase activity. Untreated group was the control ($^{*}P < 0.05$, N = 4).

inhibited (by $31 \pm 4\%$ and $21 \pm 5\%$, respectively) (Fig. 5). The specificity of each DNA binding was assessed by competition of non-labeled oligonucleotide (data not shown).

3.5. Effect of baicalein on LPS/IFN-γ-induced phosphorylation of MAPK

Evidence has accumulated that the MAPK pathway is important in the activation of iNOS expression following stress [30]. To investigate whether the inhibition of iNOS expression by baicalein is mediated through the modulation of the MAPK pathway, we examined the effect of baicalein on the LPS/IFN-γ-stimulated phosphorylation of ERK1/2, JNK, and p38 in BV-2 cells using immunoblot analysis. Pretreatment of cells with baicalein did not significantly suppress the phosphorylation of ERK1/2 in LPS/IFN-γ-stimulated cells (Fig. 6). The phosphorylation of JNK and p38 MAP kinase was also not suppressed by baicalein (Fig. 6).

3.6. Role of PPAR-α in baicalein-mediated suppression of NO production

Baicalein-mediated suppression of NO production in RAW 264.7 macrophages could be reverted by PPAR-α agonist [19]. The effect of a synthetic PPAR-α agonist WY 14643 on baicalein-mediated suppression of NO production in microglia was examined. We found that WY 14643 failed to suppress LPS/IFN-γ-induced NO production in BV-2 cells. Furthermore, the suppressive effect of baicalein on LPS/IFN-γ-induced NO production in BV-2 cells was not abolished by the treatment of WY 14643 (Fig. 7).

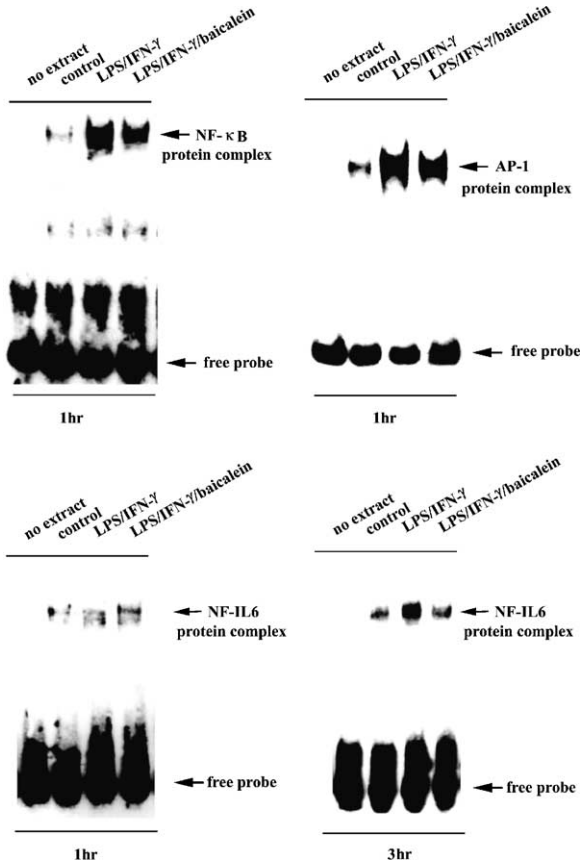

Fig. 5. Effect of baicalein on NF-κB, AP-1, and NF-IL6 DNA binding activities. BV-2 cells were pretreated with vehicle or baicalein (25 μM) for 60 min before stimulation with LPS (100 ng/mL)/IFN-γ (10 U/mL). The nuclear extracts were prepared 1 and 3 hr after treatment for the analysis of NF-κB, AP-1, and NF-IL6. Similar results were obtained from three independent experiments.

4. Discussion

NO plays a pivotal role in the normal function of the host defense system, but because the cytotoxic effect of NO is nonspecific, the consequence of overproduction of NO can be detrimental to the host. Therefore, precise regulation of NO production under pathophysiological conditions is critical for the survival of host cells. Some approaches have been proved to effectively reduce NO production, including inhibition of iNOS enzyme activity [31], depletion of arginine substrate by arginase [32], and transcriptional down-regulation of iNOS gene expression by endogenous or exogenous manipulations. Baicalein is known to have anti-inflammatory and antioxidant effects, and it is a well-known inhibitor of 12-lipoxygenase [15]. In the present study, we demonstrated that baicalein inhibits NO production and iNOS gene expression in primary microglia and murine BV-2 microglia cell line (Fig. 1), and that the effect is mostly mediated through the inhibition of NF-IL6 transcription factor (Fig. 5). As stated earlier, NO plays an important role in the pathogenesis of ischemic injury [6] and baicalein reduces ischemia/reperfusion brain injury [16]. Therefore, the neuroprotec-

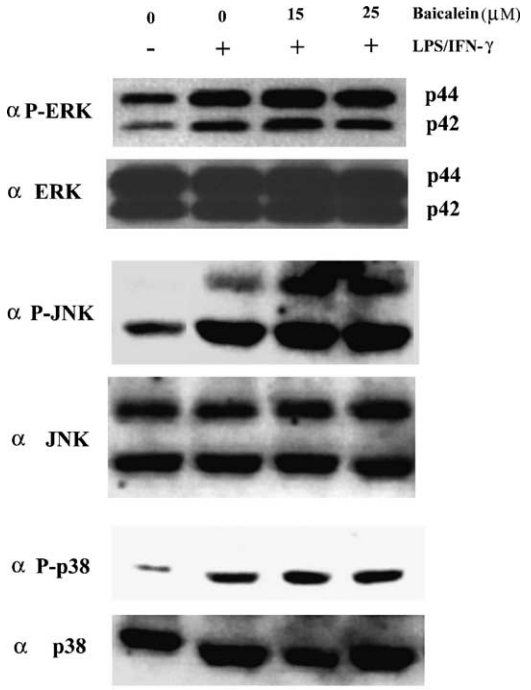

Fig. 6. Effect of baicalein on MAPK phosphorylation. BV-2 cells were pretreated with vehicle or indicated concentrations of baicalein for 60 min before stimulation with LPS (100 ng/mL)/IFN-γ (10 U/mL) for 30 min. The whole-cell lysates were analyzed by immunoblot analysis using antibody against phospho- and total-ERK1/2, JNK, and p38. The results presented are representative of three independent experiments.

tive action of baicalein appears to involve inhibition of iNOS gene expression.

The iNOS gene expression is regulated mainly at the transcriptional level, and the major transcriptional regulators of iNOS gene are NF-κB, AP-1, and NF-IL6, which are also key regulators of a variety of genes involved in immune and inflammatory responses [27,28]. In this study, we showed that baicalein-mediated down-regulation of NO production was through transcriptional modulation because of the suppression of iNOS mRNA expression (Fig. 2) and iNOS promoter activity (Fig. 4). The 5′-flanking region of the murine iNOS gene is known to

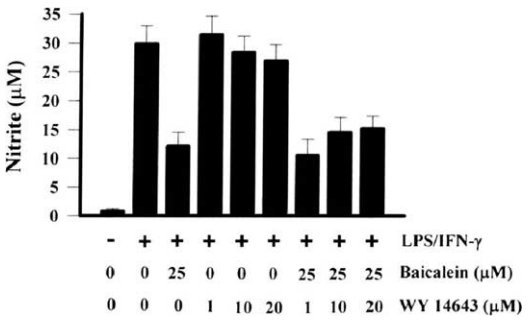

Fig. 7. Effect of WY 14643 on nitrite production. BV-2 cells were pretreated with vehicle, baicalein (25 μM), various concentrations of WY 14643 or in combinations for 60 min before being incubated with LPS (100 ng/mL)/IFN-γ (10 U/mL) for 16 hr. The culture supernatants were isolated and analyzed for nitrite production (N = 3).

contain two transcriptional regulatory regions, an enhancer and a basal promoter. The promoter contains numerous consensus sequences for known transcription factors, including sequences specified as IFN-γ response element (γ-IRE), γ-activated site (GAS), NF-κB, AP-1, TNF response element (TNF-RE), CREB/ATF, and NF-IL-6 [27,28]. NF-IL6, a member of the CCAAT/enhancer binding protein (C/EBP) family of transcription factors, belongs to a class of DNA binding proteins called basic leucine zipper protein family, which includes C/EBPα, C/EBPβ (NF-IL6), C/EBPγ, and C/EBPδ (NF-IL6β) [33]. Treatment with baicalein only caused slight inhibition in LPS/IFN-γ-induced increases of NF-κB and AP-1 DNA binding activities (Fig. 5). In contrast, NF-IL6 appears to be critical for LPS/IFN-γ-mediated induction of iNOS in microglia, since LPS/IFN-γ-induced NF-IL6 binding was markedly suppressed by baicalein (Fig. 5).

Interestingly, the data presented here demonstrated that both LPS/IFN-γ-induced NF-κB and AP-1 DNA binding were slightly affected by baicalein. However, the iNOS expression was suppressed (Fig. 5). Although NF-κB and AP-1 were suggested to be essential for the LPS/IFN-γ-mediated induction of iNOS, they were likely not sufficient in microglia. This implied that other transcription factors must be activated in addition to gain full induction of iNOS expression. Our findings suggest that NF-IL6 could be a suitable candidate for this. Therefore, NF-IL6 may complement NF-κB and AP-1 to maintain full iNOS induction. Previous reports had demonstrated that NF-IL6 maintained a high transcription rate of iNOS but did not trigger the initial induction [27,34]. This finding was in agreement with our observation that after LPS/IFN-γ stimulation of microglia activation of NF-IL6 occurred later than that of NF-κB and AP-1 (Fig. 5). Therefore, NF-κB and AP-1 probably acts as first inducer of iNOS transcription, and this stimulus is preserved by the action of NF-IL6. It has become increasingly evident that combinatory effects of transcription factors are very important in gene expression. Evidence has been provided for a functional and physical interaction between NF-IL6 and NF-κB through cooperative binding of the two transcription factors or specific interactions between these proteins and the basal transcription machinery [35–37]. In addition to conventional NF-κB and AP-1, our findings suggest that NF-IL6 binding is of importance in the suppression of iNOS expression by baicalein in microglia.

In investigating the signal transduction pathways responsible for iNOS induction by endotoxin and cytokines, the MAPK pathway has been well established. The results strongly indicate the crucial roles of ERK, JNK, and p38 MAP kinase in AP-1 and NF-κB activation and in iNOS gene expression [38,39]. The activation of the MAPK cascade appears to be necessary for the induction of iNOS by IL-1β and IFN-γ, especially in cardiac myocytes, skeletal muscle, and mouse astrocytes [29,40,41]. Our results indicate that no significant changes in the

LPS/IFN-γ-induced phosphorylation of ERK1/2, JNK or p38 MAP kinase after baicalein treatment within assayed concentrations were observed (Fig. 6). In parallel, the AP-1 and NF-κB DNA binding were also not significantly suppressed by baicalein treatment (Fig. 5). Although baicalein is shown to inhibit histamine- or A23187-induced phosphorylation of ERK1/2 in C6 glioma cells, angiotensin II-induced phosphorylation of p38 in adrenocortical cells, and norepinephrine-induced phosphorylation of p38 in vascular smooth muscle cells [42–44], our results suggest that MAPKs are not involved in the inhibitory effect of baicalein on LPS/IFN-γ-stimulated iNOS expression in microglia. These results imply that baicalein inhibits the MAPK cascade and iNOS expression, but the extent of the effect depends on stimulating stress and cell types.

Inflammation is characterized by the activation of immune competent cells, including macrophages and microglia and secretion of pro-inflammatory mediators such as TNF-α, IL-1β, or NO. The anti-inflammatory effect is one of the most important properties of baicalein [15]. Thus, the suppression of TNF-α, IL-1β, and NO production by baicalein might partially explain the anti-inflammatory action (Figs. 1 and 2). The DNA binding activities of NF-κB, AP-1, and NF-IL6 play important roles in the induction of TNF-α, IL-1β, and iNOS [27,28]. Therefore, attenuation of pro-inflammatory mediator expression by baicalein could result from the distinct reduction of the DNA binding activities of NF-κB, AP-1, and NF-IL6.

Pro-inflammatory cytokines such as TNF-α and IL-1β are strong stimulators and/or co-stimulators for iNOS gene expression in certain cell types [28,29]. To monitor the mRNA expression following stimulation, we found that the time frame of iNOS expression was parallel to that of IL-1β but, up to 6 hr later than that of TNF-α (Fig. 2). Thus, we speculated that the suppressive effect of baicalein on iNOS expression could also result secondarily from the low level of TNF-α co-stimulator since baicalein effectively inhibited TNF-α expression (Fig. 2). Unfortunately, we failed to show the stimulating effect of TNF-α either alone or in combination with LPS/IFN-γ on NO production in both primary microglia and BV-2 cells (Fig. 3). In contrast, the synergistic effect of TNF-α on LPS/IFN-γ-induced NO production was shown in RAW 264.7 macrophages (Fig. 3) and other previous reports [45,46]. Those findings suggest the existence of a distinct signaling pathway underlying LPS/IFN-γ-induced NO production in microglia and macrophages. This notion was partially supported by the kinetics of baicalein-mediated suppression of NO production (Fig. 1). Baicalein effectively inhibited LPS/IFN-γ-induced NO production in macrophages (IC_{50} 6.3 ± 1.4 μM) and microglia (IC_{50} 22.5 ± 1.3 μM for primary microglia and IC_{50} 19.6 ± 1.1 μM for BV-2), but the effect was more pronounced in the former cells. Taken together, these findings suggest that TNF-α plays a distinct role in the induction of iNOS expression by central microglia and peripheral monocyte/macrophage cells following LPS/IFN-γ stimulation and is not involved in the suppressive action of baicalein against NO production, although its expression was attenuated by baicalein preceding the onset of iNOS gene expression. TNF-α has a profound stimulatory influence on MAPKs and NF-κB activation and iNOS expression [47]. In this study, we showed that MAPKs and NF-κB activation were not the major determinants for the suppression of NO production by baicalein in microglia (Figs. 5 and 6). These findings might explain, in part, the un-responsiveness of microglia to TNF-α in NO production. Liang *et al.* [20] demonstrated that flavonoids could suppress iNOS expression in macrophages by activation of the immunosuppressant PPAR-β when transfection with PPAR-β expression plasmid. Furthermore, baicalein-mediated suppression of NO production in RAW 264.7 macrophages could be reverted by PPAR-α agonist [19]. Thus, PPARs might be involved in LPS-induced NO production in macrophages. In this study, we found that the suppressive effect of baicalein on LPS/IFN-γ-induced NO production in microglia was not abolished by the treatment of PPAR-γ antagonist or PPAR-γ agonist (Fig. 7). The findings suggest that the PPARs were not essential to the suppressive effect of baicalein on NO production in microglia.

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