Flavonoid Baicalein Attenuates Activation-Induced Cell Death of Brain Microglia

Kyoungho Suk, Heasuk Lee, Sang Soo Kang, Gyeong Jae Cho, and Wan Sung Choi

Department of Anatomy & Neurobiology and Gyeongsang Institute of Health Science,
College of Medicine, Gyeongsang National University, Jinju, Korea
Running title: Inhibition of microglial apoptosis by baicalein

Corresponding author: Wan Sung Choi, Department of Anatomy and Neurobiology, College of Medicine, Gyeongsang National University, 92 Chilam-dong, Jinju, Kyungnam 660-751, Korea. (Tel) 82-55-751-8716; (Fax) 82-55-759-0779; (E-mail) choiws@nongae.gsnu.ac.kr

Number of text pages: 33
Number of tables: 1
Number of figures: 8
Number of references: 42
Number of words in the Abstract: 231
Number of words in the Introduction: 708
Number of words in the Discussion: 1170

Abbreviations used: NO, nitric oxide; LPS, lipopolysaccharide; IFN, interferon; iNOS, inducible nitric oxide synthase; NF-κB, nuclear factor-κB; IRF, interferon regulatory factor; STAT, signal transducer and activator of transcription; AICD, activation-induced cell death; CNS, central nervous system; TNF, tumor necrosis factor; NMMA, N-monomethyl L-arginine; SNAP, S-nitroso-N-acetylpenicillamine; NAC, N-acetyl cysteine; z-VAD-fmk, z-Val-Ala-Asp(OMe)-CH2F; 12(S)-HETE, 12-hydroxyeicosatetraenoic acid; MTT, 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; PMSF, phenylmethylsulfonyl fluoride; HEPES, N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid.

Recommended section assignment: Cellular & Molecular
Abstract

Baicalein (5,6,7-trihydroxyflavone), a flavonoid originated from the root of Chinese medicinal herb *Scutellaria baicalensis*, has been shown to exert anti-inflammatory and anti-oxidant effects, and it is a well-known inhibitor of 12-lipoxygenase. We have previously reported that neuroglia undergo nitric oxide (NO)-dependent and NO-independent apoptosis upon inflammatory activation. In the current work, we asked how anti-inflammatory baicalein influences auto-regulatory apoptosis of activated microglia and their NO production. Baicalein attenuated NO production and apoptosis of lipopolysaccharide (LPS)-activated, but not interferon (IFN)γ-activated, BV-2 mouse microglial cells as well as rat primary microglia cultures. The inhibition of NO production by baicalein was due to the suppression of inducible NO synthase (iNOS) induction. Moreover, baicalein inhibited LPS-induced NF-κB activity in BV-2 cells without affecting caspase-11 activation, IRF-1 induction, or STAT1 phosphorylation. Transfection of BV-2 cells with a p65 subunit of NF-κB abolished the apoptosis-attenuating effects of baicalein, indicating that the inhibition of NF-κB is a major mechanism of action. Baicalein, however, did not significantly affect NO donor-mediated cytotoxicity, and the apoptosis-attenuating effects of baicalein were independent of 12-lipoxygenase inhibition. Based on our previous findings that activation-induced cell death (AICD) of microglia occurs through two separate pathways (NO-dependent pathway and caspase-11-dependent pathway), our current results suggest that baicalein selectively inhibits the NO-dependent apoptotic pathway of activated microglia by suppressing cytotoxic NO production. Also, the AICD-inhibiting effects of baicalein were specific for the inflammatory stimulus that activated microglia.
Flavonoids are a group of low molecular weight polyphenolic compounds of plant origin. They exhibit a variety of biological activities such as anti-inflammatory, anti-oxidant, anti-viral, and anti-tumor actions (Middleton 1988). Baicalein (5,6,7-trihydroxyflavone) is a flavonoid derived from the root of *Scutellaria baicalensis* Georgi, a medicinal plant traditionally used in Oriental medicine (Kim et al., 2001b). This flavonoid has been shown to exert anti-inflammatory effects; it inhibited lipopolysaccharide (LPS)-induced production of nitric oxide (NO) in RAW264.7 mouse macrophages (Wakabayashi 1999), and inhibited adhesion molecule expression induced by thrombin in cultured human umbilical vein endothelial cells (Kimura et al., 2001). In vivo anti-inflammatory effects of baicalein have been also demonstrated; baicalein ameliorated all the considered inflammatory symptoms in dextran sulfate sodium-induced colitis in mice (Hong et al., 2002). Baicalein also showed free radical scavenging and anti-oxidant activities (Gao et al., 1999; Hamada et al., 1993; Shieh et al., 2000), and cytoprotective effects that are related to these activities (Choi et al., 2002; Gao et al., 2001; Ishige et al., 2001; Lebeau et al., 2001). The flavonoid was protective against hydrogen peroxide-induced oxidative stress in HS-SY5Y human neuroblastoma cells (Gao et al., 2001). Baicalein protected rat cortical neurons from β-amyloid (25-35)-induced toxicity (Lebeau et al., 2001) and HT-22 mouse hippocampal cells from various types of oxidative injuries (Ishige et al., 2001), respectively. Baicalein was also protective against benzo[a]pyrene- and aflatoxin B1-induced genotoxicities (Ueng et al., 2001). In vivo neuroprotective effects of baicalein against cerebral ischemia was demonstrated in gerbils (Hamada et al., 1993). In contrast to these cytoprotective effects, numerous reports also demonstrated apoptosis-
inducing and anti-proliferative effects of baicalein. As a 12-lipoxygenase inhibitor, baicalein induced apoptosis of a variety of human cancer cells such as breast cancer cells (Tong et al., 2002), hepatoblastoma cells (Chang et al., 2002), prostate cancer cells (Pidgeon et al., 2002), and gastric cancer cells (Wong et al., 2001). In many cases, the apoptogenic action of baicalein was associated with caspase activation, mitochondrial dysfunction, and modulation of Bcl-2 family proteins. Compared to well-known effects of baicalein on human cancer cells and its anti-inflammatory actions in peripheral inflammatory cells, little information is available about its effects on the inflammatory activation of microglia, their NO production, and apoptosis in central nervous system (CNS).

Microglia are a type of neuroglia that support, nurture, and protect the neurons maintaining homeostasis of the fluid that bathes neurons. Microglia function as macrophages in CNS; they migrate to area of injured nervous tissue, and they engulf and destroy microbes and cellular debris (Gehrmann et al., 1995). Stimulated microglia produce diverse inflammatory mediators such as NO and TNFα. There is growing evidence that toxic mediators produced by activated microglial cells might be involved in the pathogenesis of various neurodegenerative diseases (Gonzalez-Scarano and Baltuch 1999; McGeer and McGeer 1995; Minghetti and Levi 1998). Thus, in CNS, the production of toxic inflammatory mediators by activated microglial cells must be strictly regulated to avoid harmful effects. Potential mechanisms for down-regulation of activated microglia may include the deactivation or elimination of activated cells. We and others have previously shown that activated microglial cells (Lee et al., 2001b; Liu et al., 2001) and astrocytes (Suk et al., 2001c) undergo apoptosis.
for the regulation of their own activation states, which is similar to activation-induced
cell death (AICD) of lymphocytes. Because the process of auto-regulatory apoptosis
of neuroglial cells heavily depends on autocrine NO production (Lee et al., 2001b; Suk
et al., 2001c), this type of programmed cell death may be either positively or negatively
influenced by exogenous factors that stimulate or inhibit NO production. Wogonin,
another flavonoid found in Scutellaria baicalensis Georgi, interfered with astrocyte
AICD by inhibiting NO production (Kim et al., 2001a). Based on the known anti-
inflammatory activities of baicalein, we hypothesized that baicalein may modulate NO
production in microglial cells, which in turn may regulate apoptosis of activated
microglial cells. In order to test this hypothesis we utilized BV-2 mouse microglial
cells and rat primary microglia cultures which were activated with LPS and IFNγ in the
presence or absence of baicalein, and evaluated the effects of the flavonoid on NO
production as well as microglial apoptosis. We also investigated the mechanism(s) by
which baicalein modulates auto-regulatory apoptosis of activated microglia with a focus
on the apoptotic signaling pathways.
Methods

Reagents. Lipopolysaccharide (LPS), $N$-monomethyl L-arginine (NMMA), S-nitroso-$N$-acetylpenicillamine (SNAP), and $N$-acetyl cysteine (NAC) were obtained from Sigma Chemical Co. (St. Louis, MO). Recombinant mouse and rat interferon ($\gamma$) was purchased from R&D Systems (Minneapolis, MN). A caspase inhibitor $z$-Val-Ala-Asp(OMe)-CH$_2$F ($z$-VAD-fmk) and a fluorogenic caspase substrate Ac-Leu-Glu-His-Asp-7-amino-4-trifluoromethyl coumarin (Ac-LEHD-AFC) were from Calbiochem (La Jolla, CA). 5,6,7-trihydroxyflavone (baicalein), 12-hydroxyeicosatetraenoic acid (12(S)-HETE), and 5,8,11-eicosatriynoic acid were obtained from Cayman Chemicals (Ann Arbor, MI). All other chemicals were obtained from Sigma Chemical Co., unless stated otherwise.

Microglial cells. BV-2 mouse microglial cell line originally developed by Dr. V. Bocchini at University of Perugia (Perugia, Italy) (Blasi et al., 1990) was generously provided by Dr. E. Choi at Korea University (Seoul, Korea). The cell line was maintained in DMEM supplemented with 5% FBS, 2 mM glutamine, and penicillin-streptomycin (Gibco-BRL, Gaithersburg, MD). Rat primary microglial cells were prepared as previously described with minor modifications (Aloisi et al., 1999; Lee et al., 2001a). In brief, forebrains of newborn Sprague Dawley rats were chopped and dissociated by trypsinization and mechanical disruption. The cells were seeded into poly-L-lysine-coated flasks. After in vitro culture for 10 days, microglial cells were detached by rapid and gentle shaking of the culture flasks and seeded into plastic surfaces. After an additional 1 hr incubation, non-adherent cells were removed by replacing culture
medium. The purity of microglial cultures was greater than 92% as determined by OX-42 immunocytochemical staining (data not shown).

**Assessment of cytotoxicity by MTT assay or trypan blue exclusion assay.** Cells (3 \( \times 10^4 \) cells in 200 \( \mu l \)/well for BV-2 cells, 2 \( \times 10^4 \) cells in 200 \( \mu l \)/well for rat primary microglial cells) were seeded in 96-well plates and treated with LPS and IFN\( \gamma \) for the indicated time periods. The optimal concentrations for the cytotoxic action were 100 ng/ml for LPS and 100 units/ml for IFN\( \gamma \) (Lee et al., 2001a; Lee et al., 2001b). In some experiments, cells were pretreated with a caspase inhibitor or NAC for 1 hr before LPS/IFN\( \gamma \) treatment. After the treatment, the medium was removed and 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, 0.5 mg/ml) was added, followed by incubation at 37\(^\circ\)C for 2 hr in CO\(_2\) incubator. After a brief centrifugation, supernatants were carefully removed and DMSO was added to the cells. After insoluble crystals were completely dissolved, absorbance at 540 nm was measured using Thermomax microplate reader (Molecular Devices, Sunnyvale, CA). For trypan blue exclusion assay, cell suspension was mixed with the same volume of 0.4% trypan blue solution (Gibco-BRL). Afterwards, the number of stained cells and the total number of cells were counted using hemocytometer (Marienfeld, Germany).

**Nitrite quantification.** After cells (3 \( \times 10^4 \) cells in 200 \( \mu l \)/well for BV-2 cells, 2 \( \times 10^4 \) cells in 200 \( \mu l \)/well for rat primary microglial cells) were treated with activating agents in 96-well plates, NO\(_2^-\) in culture supernatants was measured to assess NO production in microglial cells. Fifty \( \mu l \) of sample aliquots were mixed with 50 \( \mu l \) of Griess reagent (1% sulfanilamide/0.1% naphthylethylene diamine dihydrochloride/2% phosphoric acid/1% sodium nitrite) and incubated at room temperature for 10 min. The absorbance at 540 nm was measured using a spectrophotometer (Molecular Devices, Sunnyvale, CA).
acid) in 96-well plate and incubated at 25°C for 10 min. The absorbance at 550 nm was measured on a microplate reader. NaNO₂ was used as the standard to calculate NO₂⁻ concentrations.

**DNA ploidy analysis.** Cells were suspended in PBS-5 mM EDTA, and fixed by adding 100% ethanol dropwise. RNase A (40 µg/ml) was added to resuspended cells, and incubation was carried out at room temperature for 30 min. Propidium iodide (50 µg/ml) was then added for flow cytometric analyses (FACS Vantage; Becton Dickinson, Franklin Lakes, NJ).

**Western blot analysis.** Cells were lysed in triple-detergent lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.02% sodium azide, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate, 1 mM PMSF). Protein concentration in cell lysates was determined using Bio-Rad protein assay kit (Hercules, CA). An equal amount of protein for each sample was separated by 8 - 12% SDS-PAGE and transferred to Hybond ECL nitrocellulose membranes (Amersham, Buckinghamshire, UK). The membranes were blocked with 5% skim milk and sequentially incubated with primary antibodies (polyclonal rabbit anti-mouse/rat iNOS antibody, Transduction Laboratories; polyclonal rabbit anti-mouse IRF-1 antibody, Santa Cruz Biotech; polyclonal rabbit anti-mouse phospho-STAT1 antibody, New England Biolabs) and HRP-conjugated secondary antibodies (anti-rabbit IgG, Amersham), followed by ECL detection (Amersham).

**Gel shift assays.** Nuclear extracts were prepared from BV-2 cells as previously described (Schreiber et al., 1989). Synthetic double-strand oligonucleotides of
consensus NF-κB binding sequence, GAT CCC AAC GGC AGG GGA (Promega, Madison, WI), were end-labeled with $[^\gamma-\text{32P}]$ATP using T4 polynucleotide kinase. Nuclear extract was incubated with the labeled probe in the presence of poly(dI-dC) in a binding buffer containing 20 mM HEPES at room temperature for 30 min. For supershift assays, a total of 0.2 µg of antibodies against p65 subunit of NF-κB (Santa Cruz Biotech) were included in the reaction. DNA-protein complexes were resolved by electrophoresis in a 5% non-denaturing polyacrylamide gel, dried, and visualized by autoradiography.

**NF-κB reporter assays.** NF-κB reporter activity was measured using Dual-luciferase reporter assay system (Promega). In brief, BV-2 cells in 12-well plates were co-transfected with 0.5 µg of NF-κB-responsive reporter gene construct carrying two copies of κB sequences linked to luciferase gene (IgGκ NF-κB-luciferase, generously provided by Dr. G. D. Rosen, Stanford University, Stanford, CA) (Lee et al., 1999) together with 0.1 µg of Renilla luciferase gene under HSV thymidine kinase promoter (pRL-TK, Promega) using LipofectAMINE reagent (GibcoBRL). At 24 hr after the transfection, cells were treated with stimuli. After 6 hr, activities of firefly luciferase and Renilla luciferase in transfected cells were measured sequentially from a single sample using Dual-luciferase reporter assay system. Results were presented as firefly luciferase activity normalized to Renilla luciferase activity. In some experiments, cells were co-transfected with NF-κB p65 expression plasmid (0.5 µg, kindly provided by Dr. D. W. Ballard at Vanderbilt University, Nashville, TN) (Ballard et al., 1992) along with NF-κB-responsive reporter plasmid (0.5 µg) and pRL-TK (0.1 µg) before luciferase assays.
**Transient transfection.** BV-2 cells in 6-well plates were co-transfected with 1 µg of NF-κB p65 expression plasmid (or empty vector pcDNA3) together with 0.2 µg of lacZ gene (pCH110, Pharmacia) using lipofectAMINE reagent (GibcoBRL). At 48 hr after the transfection, the cells were treated with LPS and baicalein. After another 48 hr, the cells were fixed with 0.5% glutaraldehyde for 10 min at room temperature and stained with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal, 1 mg/ml) in 4 mM potassium ferricyanide/4 mM potassium ferrocyanide/2 mM magnesium chloride at 37°C for detection of blue cells. At least 250 blue cells were counted for each experiment, and transfection efficiency was 19 - 24%.

**Reverse transcription-polymerase chain reaction (RT-PCR).** Total RNA was extracted from cells by a sequential addition of 4 M guanidinium thiocyanate, 2 M sodium acetate, and acid phenol/chloroform. Reverse transcription was carried out using Superscript (Gibco-BRL) and oligo(dT) primer. PCR amplification using primer sets specific for caspase-11 or β-actin was carried out at 55°C annealing temperature for 25 cycles. Nucleotide sequences of the primers were based on published cDNA sequences of mouse caspase-11 or β-actin: caspase-11 forward, CTT CAC AGT GCG AAA GAA CT; caspase-11 reverse, GGT CCA CAC TGA AGA A TG TCT GGA GAA GCA TTT CA; β-actin forward, ATC CTG AAA GAC CTC TAT GC; β-actin reverse, AAC GCA GCT CAG TAA CAG TC.

**Assessment of caspase activity.** Caspase-11-like activity was measured using a Caspase assay kit (Pharmingen) according to the supplier’s instruction with slight
modifications. In brief, caspase-11 fluorogenic substrates (Ac-LEHD-AFC) were incubated with cell lysates for 1 hr at 37°C, then AFC liberated from Ac-LEHD-AFC was measured using a fluorometric plate reader (FLx800TB; Bio-Tek Instruments, Winooski, VT) with an excitation wavelength of 400 nm and an emission wavelength of 480 - 520 nm. Ac-LEHD-AFC has been previously used as a substrate for caspase-11 (Kang et al., 2000; Lee et al., 2001a).

**Statistical analysis.** All data were presented as mean ± SD from three or more independent experiments. Statistical comparison between different treatments was done by either Student’s *t*-test or one-way ANOVA with Dunnett’s multiple comparison test using GraphPad Prism program (GraphPad Software Inc., San Diego, CA). Differences with *p* value less than 0.05 were considered statistically significant.
Results

Baicalein selectively inhibits NO production and AICD of microglia following LPS stimulation. We have previously demonstrated that microglia undergo AICD upon inflammatory stimulation; inflammatory activation of microglia triggers auto-regulatory apoptosis by two separate signaling pathways, one being NO-dependent and the other being caspase-11-dependent, but NO-independent (Lee et al., 2001a). In order to determine how a plant flavonoid baicalein affects this auto-regulatory apoptosis of activated microglia, we first evaluated the effects of baicalein on microglial NO production and cell viability following inflammatory activation. Treatment of BV-2 mouse microglial cells with inflammatory stimuli such as LPS and IFNγ induced NO production as well as a decrease in the cell viability (Fig. 1 and 2). LPS dose-dependently induced NO production, which was inversely correlated with viability of microglia (Fig. 1A, B). Treatment of BV-2 cells with baicalein before inflammatory activation resulted in a marked decrease in NO production (Fig. 1C). Baicalein pretreatment also attenuated AICD of BV-2 cells (Fig. 1D) at 10 and 50 µM. The AICD-attenuating effect of baicalein at 10 µM was greater than that at 50 µM because of the cytotoxicity of baicalein itself. A slight cytotoxicity was observed upon treatment with 50 µM of baicalein alone (91.3 ± 2.5% viability compared to untreated control set to 100%). No significant cytotoxicity of baicalein was detected by concentrations ranging from 0.1 to 10 µM (data not shown). The inhibitory effect of 10 µM baicalein on BV-2 cell death was comparable to 0.5 mM NMMA, a NOS inhibitor (data not shown). The same pattern of microglial cell death following inflammatory activation and the inhibitory effect of baicalein was obtained by trypan
blue exclusion assays (100 ng/ml LPS treatment for 48 hr, 55.1 ± 2.3% cytotoxicity; 100 ng/ml LPS plus 10 µM baicalein treatment for 48 hr, 23.6 ± 4.1% cytotoxicity).

Treatment of BV-2 cells with either IFNγ or LPS plus IFNγ also induced NO production as well as cell death (Fig. 2A, B). However, baicalein did not affect NO production or microglial cell death following the treatment with IFNγ alone, indicating that the effects of baicalein were specific for the inflammatory stimulus that activated microglia. Microglial cell death caused by the LPS treatment was due to apoptosis as determined by DNA ploidy analysis, and the reduction of cell death by baicalein was due to its anti-apoptotic effects (Fig. 2C). Apoptotic nature of LPS-induced microglial cell death has been well characterized in our previous report (including LPS time and dose responses, appearance of DNA ladder, and nuclear condensation, etc.) (Lee et al., 2001b). The inhibitory effects of baicalein on LPS/IFNγ-induced NO production and subsequent cell death were similarly observed in rat primary microglia cultures, indicating that the AICD-attenuating effect of baicalein is not limited to a microglial cell line (Fig. 3).

Because NO production in microglia is mostly controlled by the level of iNOS expression, we next examined the effects of baicalein on iNOS induction. Baicalein at 10 and 50 µM significantly inhibited LPS-induced iNOS protein levels indicating that the inhibitory effect of baicalein on microglial NO production was due to inhibition of iNOS induction (Fig. 4).

**Baicalein targets NF-κB to inhibit NO-dependent pathway of microglial AICD.**

As the inflammatory induction of iNOS in neuroglial cells is known to be mediated mainly through NF-κB activation (Nomura 2001), we speculated that baicalein may inhibit iNOS induction by down-regulating NF-κB activity in BV-2 cells. Gel shift
assays indicated that LPS treatment of BV-2 cells strongly induced NF-κB activity, and this was inhibited by baicalein co-treatment suggesting that the inhibition of NF-κB may be an underlying mechanism of baicalein action on iNOS induction (Fig. 5A). NF-κB-inhibiting action of baicalein was confirmed by NF-κB reporter assays (Fig. 5B). A strong anti-oxidant NAC also inhibited LPS-induced NF-κB reporter activity. Inhibition of NF-κB by baicalein was mainly due to the inhibition of nuclear translocation of NF-κB as demonstrated by Western blot analysis of nuclear fraction of microglial cells (Fig. 5C). To further demonstrate the involvement of NF-κB in the AICD-inhibiting action of baicalein, BV-2 cells were transiently transfected with p65 subunit of NF-κB to enhance NF-κB activity, and then the effects of baicalein on microglial AICD were assessed. Forced expression of p65 increased NF-κB activity in BV-2 cells (Fig. 5D), and this resulted in a complete abrogation of AICD-inhibiting activity of baicalein (Fig. 5E). These results suggest that baicalein inhibits microglial AICD by inhibiting NF-κB, which is responsible for iNOS induction and subsequent NO production. Baicalein, however, did not significantly protect microglia against the exogenous NO donor (SNAP)-induced cytotoxicity (at 1 mM), although modest protective effects of baicalein were observed at low concentrations of SNAP (0.2 - 0.5 mM) (Fig. 6A). In contrast, a broad-spectrum caspase inhibitor, z-VAD-fmk, significantly inhibited SNAP-induced cytotoxicity at all concentration ranges tested, suggesting that NO induced caspase-dependent apoptotic pathway in microglia. Taken together, our results indicate that the inhibitory effect of baicalein on the apoptosis of activated microglia appears to be mediated mainly through the suppression of cytotoxic NO production rather than the inhibition of apoptogenic action of NO. Besides NF-κB, IRF-1 and STAT1 are also known to be involved in the inflammatory induction of NO,
and the expression of IRF-1 and phosphorylation of STAT1 is induced by LPS or IFNγ (Lee et al., 2001a; Suk et al., 2001b). Baicalein, however, did not influence IRF-1 induction or STAT1 phosphorylation after the inflammatory stimulation further supporting that NF-κB is the main target of baicalein (Fig. 6B).

**Baicalein does not influence NO-independent microglial AICD pathway.** We next asked whether baicalein affects NO-independent apoptotic pathways. Inflammatory activation of microglia has been shown to induce caspase-11 expression followed by auto-activation of this caspase, which in turn activates downstream executioner caspases such as caspases-1 or -3 (Lee et al., 2001a). This constitutes NO-independent apoptotic pathway of activated microglia. We sought to determine whether baicalein modulates this part of microglial AICD pathways by evaluating the effects of the flavonoid on caspase-11 expression and its activity. Pretreatment of BV-2 cells with baicalein did not alter inflammatory induction of caspase-11 expression (Fig. 7A) or activity (Fig. 7B). A caspase inhibitor, z-VAD-fmk, which has been used as a control, inhibited an increase in caspase-11 activity.

**The AICD-inhibiting effect of baicalein is not dependent on 12-lipoxygenase inhibition.** Baicalein has been widely used as a 12-lipoxygenase inhibitor (Dailey and Imming 1999). However, the AICD-inhibiting effect of baicalein was independent of 12-lipoxygenase inhibition, because: 1) another 12-lipoxygenase inhibitor 5,8,11-eicosatriynoic acid did not exert the same inhibitory effects; and 2) the product of arachidonic acid metabolism of 12-lipoxygenase, 12(S)-hydroxyeicosatetraenoic acid (HETE), did not reverse the inhibitory effect of baicalein (Table 1). The 5,8,11-
eicosatriynoic acid at 25 μM and 12(S)-HETE at 1 μM have been previously shown to inhibit 12-lipoxygenase (Hammarstrom 1977) and abolish 12-lipoxygenase-inhibiting effects of baicalein (Pidgeon et al., 2002), respectively.
Discussion

In the current work, we have demonstrated that baicalein, an anti-inflammatory flavonoid derived from a Chinese medicinal herb, suppressed auto-regulatory apoptosis of activated microglial cells by inhibiting NO production. The effects of baicalein were found not only in BV-2 mouse microglial cell line but also in rat primary microglia cultures. Previous works suggested that the elimination of activated microglial cells by apoptosis could be an important mechanism whereby undesirable effects of long-term activation of microglial cells can be minimized (Lee et al., 2001b; Liu et al., 2001). Inflammatory mediators such as TNFα and NO that are produced by activated glial cells in CNS may have harmful effects on neurons. Inflammatory responses and their mediators may play a central role in the pathogenesis of various neurodegenerative diseases that involve chronic activation of microglial cells such as Alzheimer disease, Parkinson disease, and AIDS-related dementia (Gonzalez-Scarano and Baltuch 1999). Therefore, one can speculate that the auto-regulatory apoptosis mechanisms that control microglial activation may exist in vivo, and the failure of these auto-regulatory mechanisms may be responsible for the deleterious effects of microglial activation. A pathophysiological importance of the regulation of microglial activation by AICD is supported by previous reports: up-regulated Bcl-xL expression has been detected in reactive microglia of patients with neurodegenerative diseases (Drache et al., 1997); and the expression of anti-apoptotic Bcl-2 protein has been associated with aged brain and neurodegenerative diseases (Migheli et al., 1994). Authors proposed that high level of Bcl-xL or Bcl-2 protein might render microglia more resistant to cytotoxic environment such as areas of neurodegeneration. Anti-inflammatory action of baicalein could be
considered beneficial for the treatment of such neurodegenerative diseases associated with chronic inflammatory responses. However, it should be kept in mind that NO-inhibiting action of baicalein might also block auto-regulatory apoptosis of activated microglia.

We have shown that AICD-inhibiting action of baicalein is mediated through its inhibitory effects on iNOS induction and the ensuing NO production. This NO-inhibiting effect of baicalein was based on the specific inhibition of NF-κB activity without affecting other known AICD pathways in microglia (Fig. 8). The AICD of neuroglia (microglia and astrocytes) occurs by two separate apoptotic pathways; inflammatory stimuli induce production of the autocrine toxic mediator (NO) in the IRF-1/NF-κB-dependent manner, and concurrently they initiate NO-independent apoptotic pathway through caspase-11 induction (Lee et al., 2001a; Suk et al., 2002). Increased expression of caspase-11 is known to auto-activate the protease, and then activated caspase-11 is mobilized to cleave downstream caspases to initiate the apoptotic cascade (Kang et al., 2000). Meanwhile, NO seems to directly activate downstream executioner caspases (Lee et al., 2001a). Among these many players involved in the microglial AICD, NF-κB appears to be a unique target of baicalein action. Transcriptional regulation of many inflammatory genes is under control of NF-κB (Christman et al., 2000). Microglial iNOS is one of them. We have previously shown that iNOS expression is regulated by NF-κB in BV-2 microglial cells. Inhibition of NF-κB by the transfection of BV-2 cells with a super-repressor IκB mutant or anti-oxidants treatment led to a reduced NO production and the inhibition of AICD (Lee et al., 2001a). These results indicated that NF-κB in the process of microglial
AICD does not play a cytoprotective role in contrast to previous studies conducted under different conditions (Beg and Baltimore 1996; Suk et al., 2001a; Wang et al., 1998; Wang et al., 1996). With respect to the AICD of microglia, the main function of NF-κB appears to be the production of NO that acts as an autocrine mediator of apoptosis. Baicalein seems to selectively target this NF-κB activity in order to attenuate AICD of microglia. However, the AICD-inhibiting effect of baicalein was only partial due to the presence of NO-independent apoptotic pathways that are not affected by baicalein (Fig. 1D).

The AICD-attenuating effect of baicalein in microglia was not dependent on 12-lipoxygenase inhibition. This was supported by the following evidence: a 12-lipoxygenase inhibitor without a known anti-oxidant activity, eicosatriynoic acid (Hammarstrom 1977), did not reproduce the AICD-inhibitory effect of baicalein; and the predominant product of arachidonic acid metabolism of 12-lipoxygenase, 12(S)-HETE, did not reverse the inhibitory effect of baicalein. Although baicalein has been widely used as a 12-lipoxygenase inhibitor (Dailey and Imming 1999), many reports also demonstrated strong anti-oxidant and free radical scavenging effects of baicalein (Gao et al., 1999; Hamada et al., 1993; Shieh et al., 2000). Anti-oxidants are known to inhibit NF-κB activity in a variety of cell types (Christman et al., 2000), and BV-2 was not an exception (Fig. 5B). This led us to speculate that the anti-oxidant activity of baicalein might be responsible for the inhibition of NF-κB (and subsequent inhibition of NO production and AICD of microglia). Regardless of the relevance of 12-lipoxygenase inhibition to microglial AICD, baicalein might have inhibited 12-lipoxygenase activity in microglia under our experimental conditions. As opposed to
apoptosis-inducing effects of baicalein as a 12-lipoxygenase inhibitor in many cancer cell types, baicalein up to 10 µM did not induce cell death by itself in our study. In fact, baicalein at similar concentrations previously protected neurons under various conditions by acting as an anti-oxidant (Gao et al., 2001; Ishige et al., 2001; Lebeau et al., 2001). This suggests that the effect of 12-lipoxygenase inhibition on cell viability depends on the cell types studied; while a variety of human cancer cells in periphery undergo apoptosis upon exposure to 12-lipoxygenase inhibitor (Dailey and Imming 1999), neurons and neuroglia in CNS may not be affected by 12-lipoxygenase inhibition. Moreover, because baicalein has dual effects as a 12-lipoxygenase inhibitor and anti-oxidant, the flavonoid was able to protect microglia from AICD.

In the current study, baicalein showed a modest protective effect against SNAP-induced cytotoxicity. NO donors produce a range of reactive nitrogen intermediates (RNI), which may be related to cytotoxic effects of NO. Free radical scavenging activity of baicalein may be responsible for the protective effects against NO donors. However, the protective effect of baicalein was observed only at low concentrations of SNAP, and the degree of protection was modest compared to the protective effects of baicalein on LPS-induced microglial cell death (Fig. 6A). This further indicates that the AICD-attenuating effect of baicalein may be primarily due to the inhibition of NO-producing NF-κB rather than the blockade of cytotoxic action of NO produced. To obtain further evidence that NF-κB is the main target of baicalein action, we employed transient transfection of BV-2 cells with p65 subunit of NF-κB. The transfection led to an increase in NF-κB activity, which then abrogated the AICD-attenuating effect of baicalein (Fig. 5D and E).
In conclusion, the plant flavonoid baicalein attenuated microglial AICD by selectively inhibiting NO-producing NF-κB activity. These effects of the flavonoid were not related to 12-lipoxygenase inhibition. The AICD-inhibiting effect of baicalein was specific for the inflammatory stimulus in that the flavonoid blocked microglial NO production and cell death following the treatment with LPS, but not IFNγ. Our results suggest that baicalein could be a key template for the development of therapeutic agents to selectively modulate inflammatory responses and cellular apoptosis in CNS.
References


Lee P, Lee J, Kim S, Yagita H, Lee MS, Kim SY, Kim H and Suk K (2001b) NO as an


Footnotes

This work was supported by grants to K. S. from the Korea Health 21 R&D Project, Ministry of Health & Welfare, Republic of Korea (01-PJ8-PG3-21304-0005).

Name and address of person to receive reprint requests: Wan Sung Choi or Kyoungho Suk, Department of Anatomy & Neurobiology, College of Medicine, Gyeongsang National University, 92 Chilam-dong, Jinju, Kyungnam 660-751, Korea. (Tel) 82-55-751-8716; (Fax) 82-55-759-0779; (E-mail) choiws@nongae.gsnu.ac.kr (W. S. C.) or ksuk@nongae.gsnu.ac.kr (K. S.)
Legends for Figures

Fig. 1. **Inverse correlation between NO production and microglial cell viability upon inflammatory activation, and the effects of baicalein on microglial AICD.** BV-2 cells were treated with indicated concentrations of LPS for 48 hr (A) or treated with 100 ng/ml of LPS for the indicated time periods (B), and then NO production or cell viability was assessed. Alternatively, BV-2 cells were treated with 100 ng/ml of LPS in the presence or absence of baicalein (0.1 - 50 µM) for 48 hr, and then NO production (C) or cell viability (D) was evaluated. Viability of untreated cells was set to 100%. The results are mean ± SD of three independent experiments. Asterisks indicate significant differences from treatment with LPS alone ($p < 0.05$) (C, D).

Fig. 2. **Specific effects of baicalein on microglial AICD.** BV-2 cells were treated with LPS (100 ng/ml), IFNγ (100 units/ml), or both in the presence of absence of baicalein (10 µM) for 24 hr, and then NO production or cell viability was assessed (A, B). Viability of untreated cells was set to 100%. The results are mean ± SD of four independent experiments. Asterisks indicate significant differences between two treatments ($p < 0.05$). (C) BV-2 cells treated with LPS in the presence or absence of baicalein (10 µM) for 24 hr were subjected to DNA ploidy analysis. Numbers in histograms indicate the percentage of sub-diploidy cells undergoing apoptosis.

Fig. 3. **Inhibition of NO production and AICD by baicalein in rat primary microglial cultures.** Primary microglial cultures prepared from neonatal rat brain were treated with LPS (100 ng/ml) and IFNγ (100 units/ml) for 72 hr with or without
baicalein (10 µM), then NO production (A) or cell viability (B) was similarly evaluated. Viability of untreated cells was set to 100%. The results are mean ± SD of three independent experiments. Asterisks indicate significant differences between two treatments (p < 0.05).

Fig. 4. Suppression of LPS-induced iNOS induction by baicalein. After treatment of BV-2 cells with LPS (100 ng/ml) for 16 hr in the absence or presence of baicalein (0.1 - 50 µM), iNOS expression was evaluated by Western blot analysis using antibodies specific for iNOS or β-tubulin (upper). The result of iNOS Western blot was subjected to densitometric analysis (lower). Densitometric units were normalized to β-tubulin.

Fig. 5. NF-κB as the major target of baicalein action. (A) Treatment of BV-2 cells with LPS (100 ng/ml) for 1 hr induced NF-κB activation as evidenced by gel shift assays. Co-treatment of BV-2 cells with baicalein (10 µM) significantly reduced NF-κB activity. The identity of DNA-complexed proteins was confirmed by supershift assays using antibodies against p65 (p65 Ab). (B) Inhibition of LPS-induced NF-κB activity by baicalein was confirmed by NF-κB reporter assays. NF-κB activity was also inhibited by anti-oxidant NAC (10 mM) treatment. Values are fold increases in firefly luciferase activity normalized to Renilla luciferase activity after treatment with indicated stimuli for 6 hr (see Materials and Methods). (C) Inhibition of LPS-induced NF-κB activity by baicalein was accompanied by the inhibition of nuclear translocation of p65 subunit of NF-κB as demonstrated by Western blot analysis of nuclear fraction of BV-2 cells (1 hr treatment, 100 ng/ml LPS, 10 µM baicalein). (D, E) Transient
transfection of p65 subunit of NF-κB increased NF-κB reporter activity (D) and abolished AICD-inhibiting activity of baicalein (E). The number of blue cells expressing lacZ in untreated wells was set to 100% viability. The results are mean ± SD of three independent experiments. Asterisks indicate significant differences between two treatments (p < 0.05).

**Fig. 6.** No significant effects of baicalein on NO donor-induced cytotoxicity or STAT1/IRF-1 signaling. (A) BV-2 cells were treated with SNAP (0.05 - 1 mM) for 24 hr with or without baicalein (10 µM) or z-VAD-fmk (50 µM), and then cell viability was evaluated. Viability of untreated cells was set to 100%. The results are mean ± SD of three independent experiments. (B) After treatment of BV-2 cells with LPS (100 ng/ml) or IFNγ (100 units/ml) with or without baicalein (10 µM) for 4 hr (IRF-1) or 1 hr (phospho-STAT1), the levels of IRF-1 protein or phosphorylated STAT1 protein were measured by Western blot analysis. Total proteins were stained with Ponceau-S to confirm an equal loading of the samples (bottom).

**Fig. 7.** No effects of baicalein on caspase-11 induction or activation. (A) After 6 hr treatment of BV-2 cells with LPS (100 ng/ml), IFNγ (100 units/ml), and baicalein (10 µM) as indicated, caspase-11 expression was assessed by RT-PCR. (B) Following treatment of BV-2 cells with LPS (100 ng/ml) in the presence of absence of baicalein (10 µM) or z-VAD-fmk (50 µM) for the indicated time period, caspase-11 activity was measured using fluorogenic substrate.

**Fig. 8.** Inhibition of microglial AICD signaling by baicalein. Upon inflammatory
activation, microglia undergo self-regulatory apoptosis by two separate signaling pathways. One is NO-dependent, that is, iNOS is induced by IRF-1 or NF-κB to give rise to massive NO production followed by apoptosis. The other is NO-independent pathway in that inflammatory stimuli induce activation of caspase-11, which in turn activates downstream executioner caspases for the induction of apoptosis. In this scheme, baicalein blocks AICD of microglia by targeting NF-κB. While the inhibition of NO-producing NF-κB is the main mechanism of action (solid line with ‘-’), apoptogenic action of NO may also be hindered by baicalein due to its anti-oxidant and free radical scavenging activity (broken line with ‘-’).
Tables

Table 1. The AICD-attenuating effect of baicalein is not dependent on 12-lipoxygenase inhibition.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Viability (%)^a</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>LPS</td>
<td>43.4 ± 1.5 ^b,^c</td>
</tr>
<tr>
<td>LPS + Baicalein (10 µM)</td>
<td>78.5 ± 2.3 ^b</td>
</tr>
<tr>
<td>LPS + Eicosatriynoic acid (25 µM)</td>
<td>44.6 ± 2.1</td>
</tr>
<tr>
<td>LPS + Baicalein (10 µM) + 12(S)-HETE (1 µM)</td>
<td>76.5 ± 3.8 ^c</td>
</tr>
</tbody>
</table>

^a BV-2 cells were treated with LPS (100 ng/ml) and other reagents as indicated for 48 hr, and then cell viability was evaluated by MTT assays. Viability of untreated cells was set to 100%. The results are mean ± SD of four independent experiments.

^b Significantly different between the two values (p < 0.05)

^c Significantly different between the two values (p < 0.05)
Figure 1

A

LPS concentrations (ng/ml) vs. Nitrite (µM)

B

Time (hr) vs. Nitrite (µM)

C

Nitrite (µM) vs. Viability

D

% viability vs. LPS and Baicalein concentrations
Figure 2

A

B

C

Nitrite (µM)

% viability

Cell number

Fluorescence intensity (DNA content)

None

LPS

IFNγ

LPS+IFNγ

None

LPS+IFNγ

None

IFNγ

LPS+IFNγ

None

LPS

LPS + baicalein

2.8 %

33.4 %

18.8 %
Figure 3

A

Nitrite (µM)

None

LPS+IFNγ

Baicalein

B

% viability

None

LPS+IFNγ

None

LPS+IFNγ

Baicalein

*
Figure 5

Panel A: Western blot showing NF-κB supershift with LPS + Baicalein treatment.

Panel B: Graph depicting fold increase in p65 of NF-κB with different treatments.

Panel C: Western blot showing p65 with LPS + Baicalein treatment.

Panel D: Graph illustrating fold increase in lacZ with empty vector and p65 of NF-κB.

Panel E: Graph showing percentage of blue cells with different treatments.
Figure 6

A

% viability

SNAP (mM)

SNAP
SNAP + Baicalein
SNAP + z-VAD

B

Baicalein

None LPS IFNγ None LPS IFNγ

IRF-1

phospho-STAT1

Ponceau S
Figure 7

A

<table>
<thead>
<tr>
<th></th>
<th>None</th>
<th>LPS</th>
<th>IFNγ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baicalein</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- Caspase-11
- β-actin

B

- LPS
- LPS + Baicalein
- LPS + z-VAD

Relative fluorescence vs. Time (hr)

0 3 6 9 12 15 18 21 24