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Flavonoid wogonin from medicinal herb is neuroprotective by inhibiting inflammatory activation of microglia

Heasuk Lee,* Young Ok Kim,† Hocheol Kim,† Sun Yeou Kim,† Hae Sook Noh,* Sang Soo Kang,* Gyeong Jae Cho,* Wan Sung Choi,* and Kyoungho Suk*

*Department of Anatomy and Neurobiology, Research Institute of Natural Science, and Institute of Health Sciences, Gyeongsang National University College of Medicine, Jinju, 660-751 Korea; and †Department of Herbal Pharmacology, Graduate School of East-West Medical Science, Kyunghee University, Seoul, 130-701 Korea

Corresponding author: Kyoungho Suk, Department of Anatomy and Neurobiology, Gyeongsang National University College of Medicine, 92 Chilam-dong, Jinju, Kyungnam 660-751, Korea. E-mail: ksuk@nongae.gsnu.ac.kr

ABSTRACT

Wogonin (5,7-dihydroxy-8-methoxyflavone), a flavonoid originated from the root of a medicinal herb *Scutellaria baicalensis* Georgi, has been previously shown to have anti-inflammatory activities in various cell types including macrophages. In this work, we have found that wogonin is a potent neuroprotector from natural source. Wogonin inhibited inflammatory activation of cultured brain microglia by diminishing lipopolysaccharide-induced tumor necrosis factor-α (TNF-α), interleukin-1β, and nitric oxide (NO) production. Wogonin inhibited NO production by suppressing inducible NO synthase (iNOS) induction and NF-κB activation in microglia. Inhibition of inflammatory activation of microglia by wogonin led to the reduction in microglial cytotoxicity toward cocultured PC12 cells, supporting a neuroprotective role for wogonin in vitro. The neuroprotective effect of wogonin was further demonstrated in vivo using two experimental brain injury models; transient global ischemia by four-vessel occlusion and excitotoxic injury by systemic kainate injection. In both animal models, wogonin conferred neuroprotection by attenuating the death of hippocampal neurons, and the neuroprotective effect was associated with inhibition of the inflammatory activation of microglia. Hippocampal induction of inflammatory mediators such as iNOS and TNF-α was reduced by wogonin in the global ischemia model, and microglial activation was markedly down-regulated by wogonin in the kainate injection model as judged by microglia-specific isolectin B4 staining. Taken together, our results indicate that wogonin exerts its neuroprotective effect by inhibiting microglial activation, which is a critical component of pathogenic inflammatory responses in neurodegenerative diseases. The current study emphasizes the importance of medicinal herbs and their constituents as an invaluable source for the development of novel neuroprotective drugs.

Key words: nitric oxide • *Scutellaria baicalensis* • inflammation • neurodegenerative diseases

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 (1). Wogonin (5,7-dihydroxy-8-methoxyflavone) is a flavonoid derived from the root of *Scutellaria baicalensis* Georgi, a medicinal plant traditionally

used in Oriental medicine (2). This flavonoid has been shown to exert various anti-inflammatory activities in vitro as well as in vivo. It inhibited lipopolysaccharide (LPS)-induced production of nitric oxide (NO; refs 3, 4) and prostaglandin E_2 (5) in macrophages. Wogonin inhibited monocyte chemotactic protein-1 gene expression in human endothelial cells (6). It also inhibited TPA-induced cyclooxygenase-2 expression and skin inflammation in mice (7). Moreover, wogonin showed free radical scavenging and anti-oxidant activities (8–10). In the central nervous system (CNS), however, little information is available about its effects on glial cells and neurons. Gao et al. (8, 11) demonstrated neuroprotective effects of four flavonoids from *S. baicalensis*, including wogonin, in cultured human neuroblastoma cells. Recently, we have shown that wogonin inhibits NO production and inducible NO synthase (iNOS) induction in cultured rat astrocytes (12), suggesting that the flavonoid may act as an anti-inflammatory agent in CNS as well.

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 (13). Stimulated microglia produce diverse inflammatory mediators such as NO and TNF-α. Now, there is growing evidence that toxic mediators produced by activated microglial cells might be involved in the pathogenesis of various neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, and HIV-associated dementia (14–16). Thus, it is of great interest to find a means to modulate microglial activation and CNS inflammatory responses for the therapeutic interventions against these neurodegenerative diseases. Based on the anti-inflammatory activity of wogonin in macrophages, which are closely related with brain microglia, we hypothesized that wogonin may exert a similar anti-inflammatory effect in microglia and may be neuroprotective against brain injury where microglia-mediated inflammatory responses play an important pathogenic role. The anti-inflammatory effect of wogonin was first demonstrated using cultured microglia in vitro, and the neuroprotective effect of wogonin was further investigated in vivo using two brain injury models (global ischemia and excitotoxic injury). Our results indicate that wogonin is neuroprotective against experimental brain injury by inhibiting inflammatory activation of microglia.

MATERIALS AND METHODS

Reagents and cells

Wogonin was purchased from Wako Pure Chemicals (Japan) and dissolved in 0.2 M NaOH to make 50 mM or 10 mg/ml stock solution. 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). Kainate, (2*S*, 3*S*, 4*R*)-carboxy-4-(1-methylethenyl)-3-pyrrolidineacetic acid, was purchased from Tocris Cookson Inc. (Ellisville, MO). All other chemicals were obtained from Sigma Chemical Co., unless stated otherwise. The BV-2 mouse microglial cell line originally developed by Dr. V. Bocchini at University of Perugia (Perugia, Italy; ref 17) was generously provided by Dr. E. Choi at Korea University (Seoul, Korea). PC12 rat pheochromocytoma cells were obtained from American Type Culture Collection (Manassas, VA). The cell lines were grown in DMEM containing 10% FBS, 2 mM glutamine, and penicillin-streptomycin (Gibco-BRL, Gaithersburg, MD). For PC12 cells, 5% horse serum was added to the culture medium and the cells were grown on culture dishes precoated with poly-L-lysine (50 μg/ml). PC12 cells were differentiated to neuronal phenotype by treatment with 50

ng/ml NGF in DMEM containing 1% horse serum for 7 days as described previously (18). Rat primary microglia cultures were prepared as described previously with minor modifications (19, 20). 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 h incubation, nonadherent cells were removed by replacing culture medium. The purity of microglial cultures was >92% as determined by OX-42 immunocytochemical staining (data not shown). The experimental protocols for the laboratory animals were in accordance with the National Institutes of Health guidelines for the use of live animals and were approved by the institutional review committee of Gyeongsang National University. All efforts were made to minimize the number of animals used and their suffering.

Nitrite quantification

After cells (3×10^4 cells in 200 μl/well for BV-2 cells, 2×10^4 cells in 200 μl/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 microliters of sample aliquots were mixed with 50 μl of Griess reagent (1% sulfanilamide/0.1% naphthylethylene diamine dihydrochloride/2% phosphoric acid) in 96-well plate and incubated at 25°C for 10 min. The absorbance at 550 nm was measured on a microplate reader. $NaNO_2$ was used as the standard to calculate NO_2^- concentrations.

TNF-α and IL-1β ELISA

TNF-α or IL-1β secreted in microglial culture supernatants was measured as described (21) by specific ELISA using rat monoclonal anti-mouse TNF-α or IL-1β antibody as capture antibody and goat biotinylated polyclonal anti-mouse TNF-α or IL-1β antibody as detection antibody (ELISA development reagents; R&D Systems, Minneapolis, MN). The biotinylated anti-TNF-α or -IL-1β antibody was detected by sequential incubation with streptavidin-horseradish peroxidase (HRP) conjugate and chromogenic substrates.

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 phenylmethylsulfonyl fluoride) as described previously (22). 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% SDS-PAGE and transferred to Hybond ECL nitrocellulose membranes (Amersham, Buckinghamshire, UK). The membranes were blocked with 5% skim milk and sequentially incubated with rabbit polyclonal anti-mouse/rat iNOS antibody (Transduction Laboratories, Lexington, KY) and HRP-conjugated anti-rabbit IgG antibody (Amersham) followed by ECL detection (Amersham).

Gel shift assays

Nuclear extracts were prepared from BV-2 cells as described previously (23–25). Synthetic double-strand oligonucleotides of consensus NF-κB binding sequence, GAT CCC AAC GGC

AGG GGA (Promega, Madison, WI), were end-labeled with [γ-^{32}P]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 *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid (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, Santa Cruz, CA) was included in the reaction. DNA-protein complexes were resolved by electrophoresis in a 5% nondenaturing polyacrylamide gel, dried, and visualized by autoradiography.

NF-κB reporter assays

NF-κB reporter activity was measured using dual-luciferase reporter assay system (Promega) as described previously (24, 25). In brief, BV-2 cells in 12-well plates were cotransfected 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; ref 26) together with 0.1 μg of *Renilla* luciferase gene under HSV thymidine kinase promoter (pRL-TK, Promega) using LipofectAMINE reagent (Gibco-BRL). At 24 h after the transfection, cells were treated with stimuli. After 6 h, 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. Transfection efficiency was 15-30%, as determined in our previous study (25).

Coculture of BV-2 cells and PC12 cells and assessment of cytotoxicity by MTT assay

BV-2 cells were seeded onto cell culture inserts (pore size of 0.2 μm; Nunc, Roskilde, Denmark). After overnight incubation, BV-2 cells were left untreated or stimulated with 100 ng/ml of LPS (with or without 50 μM of wogonin) for 6 h and then were washed with PBS three times. These BV-2 cells were cocultured for 24 h with PC12 cells by transferring the culture inserts containing BV-2 cells onto PC12 cell monolayers. For MTT assay of PC12 cells, BV-2 cell culture inserts was removed after the coculture for the indicated time period. Afterwards, the culture medium was removed and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, 0.5 mg/ml) was added to the remaining PC12 cells, followed by incubation at 37°C for 2 h 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). Lactate dehydrogenase (LDH) release assay was performed as described previously (27). Medium from control cells was used as a blank, and a standard curve was constructed with known amounts of LDH added to culture medium.

Transient global ischemia by four-vessel occlusion

Male Wistar rats weighing between 160 and 180 g were used. Before the experiment, food was withheld overnight but water was freely available. The animals were anesthetized with isoflurane (initiated with 5% and maintained with 1.5% of isoflurane). Surgery for four-vessel occlusion and induction of ischemia were done as originally described by Pulsinelli and Brierley (28). Body temperature was monitored and maintained at 37 ± 0.5°C with a rectal thermistor coupled to a heating blanket (Homeothermic Blanket Control Unit, Harvard apparatus, Edenbridge, UK). It has been previously demonstrated that the rectal temperature measured with a probe inserted at

least 6 cm into the rectum adequately reflects the brain temperature for animals with intact skulls (29), and the body temperature was measured in this manner. Sham-operated animals that underwent the surgery were used for nonischemic control. Histological analysis was performed as described previously (30) at 7 days after ischemia. Neuronal cell density was measured by counting viable cells in the total 6 frames (1.0 mm x 1.0 mm) of left and right CA1 regions of three coronal sections (~3.3, 3.5, and 3.7 mm caudal to the Bregma) for each animal. Neuronal cell density is equivalent to the average number of viable cells in one frame. Cell counting was done by three technicians blinded to the experimental conditions.

Hippocampal RNA analysis

Total RNA was extracted from dissected hippocampus by a sequential addition of 4 M guanidinium thiocyanate, 2 M sodium acetate, and acid phenol/chloroform as described previously (31). Reverse transcription was carried out using Superscript (Gibco-BRL) and oligo(dT) primer. PCR amplification using primer sets specific for iNOS, TNF-α, or D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was carried out at 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, and repeated 35 cycles followed by 72°C for 7 min. Nucleotide sequences of the primers were based on published cDNA sequences of rat iNOS, TNF-α, or GAPDH: iNOS forward, ACA ACG TGG AGA AAA CCC CAG GTG; iNOS reverse, ACA GCT CCG GGC ATC GAA GAC C; TNF-α forward, CCC TCA CAC TCA GAT CAT CTT CTC AA; TNF-α reverse, TCT AAG TAC TTG GGC AGG TTG ACC TC; GAPDH forward, CTG CCA CTC AGA AGA CTG TGG; GAPDH reverse, CTT GAT GTC ATC ATA CTT GGC. The expected size of RT-PCR products is 557 bp for iNOS, 433 bp for TNF-α, and 234 bp for GAPDH, respectively. The RT-PCR products were electrophoresed on 1.5% agarose gel, which was then denatured with 0.4 N NaOH followed by neutralization in 0.2 M Tris (pH 7.5)-2x SSC-0.1% SDS for Southern blot analysis. DNA was then transferred to Hybond-N+ membrane (Amersham). The membranes were UV-crosslinked, prehybridized, and hybridized at 65°C for 18 h with an oligonucleotide probe labeled with [α-^{32}P]dCTP (Amersham) specific for the sequences of iNOS, TNF-α, or GAPDH RT-PCR products. The membranes were washed with 2x SSC-0.1% SDS at room temperature and then with 0.1x SSC-0.1% SDS at 65°C to be exposed to X-ray films.

Kainate-induced excitotoxic brain injury

Male ICR mice were injected intraperitoneally (ip) with a single dose of kainate (30 mg/kg) in 0.9% NaCl as described (32). The experiments were done between 10:00 and 16:00 to minimize possible complications due to circadian rhythms (33). Two days after kainate-treatment, animals were anesthetized with ketamine (30 mg/kg, ip) and transcardially perfused with 4% paraformaldehyde in 0.1 M PBS. Brains were removed and postfixed overnight in the same fixative at 4°C. Serial sections were cut at 30 μm thickness using a freezing microtome and then were stained with Cresyl violet for histological examination of neuronal damage. Neuronal loss in the pyramidal layers of each section was scored on a 0-4 scale as described previously (34) by observers blinded to the treatment conditions: 0, no lesion; 1, occasional injured neurons; 2, minimal lesion <10%; 3, cellular loss between 10% and 50%; 4, extended neuronal loss >50%. For the histochemical detection of microglia, tissue sections were stained with HRP-labeled isolectin B4 (from *Griffonia simplicifolia*, Sigma) of 10 μg/ml at 4°C overnight followed by diaminobenzidine detection. OX-42 antibody was not used, because the antibody was raised against rat microglia antigens.

Statistical analysis

All data were presented as means ±SE 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 $P<0.05$ were considered statistically significant.

RESULTS

Wogonin inhibited inflammatory activation of cultured microglia

Because wogonin has been previously shown to exert anti-inflammatory activities in macrophages and other immune cells in periphery, we hypothesized that the flavonoid may be also anti-inflammatory in CNS microglia, and this anti-inflammatory action of wogonin may provide neuroprotection under the conditions where the inflammatory activation of brain microglia plays a pathogenic role in neuronal injury. First, potential anti-inflammatory activity of wogonin in brain microglia was tested by evaluating the production of inflammatory mediators from cultured microglial cells. When BV-2 mouse microglial cells were stimulated with LPS, a canonical activator of microglia/macrophages, a strong induction of TNF-α, IL-1β, and NO production was observed, which was then markedly inhibited by wogonin cotreatment (Fig. 1*A* and *C*). In rat primary microglia cultures, a similar pattern of NO production and wogonin inhibition was observed (Fig. 1*B*), confirming the results obtained with the BV-2 cells. These results indicated that wogonin inhibited inflammatory activation of cultured microglia. To further study the inhibitory effect of wogonin on microglial NO production, we next examined whether wogonin affects iNOS induction and NF-κB activation in cultured microglial cells. Production of NO in microglia is mainly controlled by iNOS gene expression, which is in turn regulated by NF-κB (20, 25). Wogonin cotreatment inhibited LPS-induced iNOS protein induction in a concentration-dependent manner (Fig. 2*A*) and NF-κB activation (Fig. 2*B* and *C*). Wogonin inhibition of microglial NF-κB activation was demonstrated by gel shift assay (Fig. 2*B*) as well as NF-κB-dependent reporter assay (Fig. 2*C*). The inhibitory effect of wogonin on LPS-induced iNOS protein expression was not observed when microglia were treated with wogonin (50 μM) 2 h after LPS (100 ng/ml) stimulation (data not shown).

Wogonin protected PC12 cells against microglial cytotoxicity

We next employed coculture of microglia and neurons to determine whether wogonin inhibition of inflammatory activation of microglia could confer neuroprotection in vitro. As the inflammatory products of activated microglia are thought to cause neuronal injury (35, 36), inhibition of microglial activation may protect cocultured neurons against cytotoxic effect of activated microglia. We tested this possibility using coculture of BV-2 microglia cells and PC12 cells. BV-2 cells that have been activated with LPS in the presence or absence of wogonin were cocultured with PC12 cells using cell culture inserts, and then viability of PC12 cells was measured. Wogonin significantly attenuated the cytotoxicity of activated BV-2 microglial cells toward cocultured PC12 cells as determined by MTT assay (Fig. 3*A*), suggesting that wogonin is cytoprotective by inhibiting microglial activation in vitro. A similar result was obtained by LDH release assay (data not shown). Wogonin, however, did not protect PC12 cells against NO donor-induced cytotoxicity (Fig. 3*B*), suggesting that the cytoprotective effect of wogonin observed in the coculture experiments is not due to its direct protective effect on neurons against toxic

inflammatory mediators. Rather, the cytoprotection afforded by wogonin appears to be due to the inhibition of microglial activation.

Wogonin was protective against ischemic brain injury in vivo

To evaluate the potential neuroprotective effect of wogonin in vivo, two experimental models have been employed; transient forebrain ischemia by the four-vessel occlusion and excitotoxic brain injury by kainate injection. First of all, the neuroprotective effect of wogonin against ischemic brain injury was assessed. The 10 min transient ischemia caused selective and delayed neuronal cell loss in the hippocampal CA1 region (Fig. 4*B* and *E*) as judged by Cresyl violet staining. Treatment of experimental animals with wogonin (10 mg/kg, ip, 0 and 90 min right after 10 min ischemia and reperfusion) conferred neuroprotection by markedly reducing the number of damaged pyramidal cells in the CA1 subfield (Fig. 4*C* and *F*). The neuroprotective effect of wogonin was dose dependent. Compared with saline-treated ischemic animals, injection of wogonin at 0.5, 1, and 10 mg/kg resulted in 9.9, 35.1, and 60.9% inhibition of CA1 cell death, respectively (Fig. 4*G*). Based on the anti-inflammatory activities of wogonin in vitro, we next analyzed the effect of wogonin on the production of inflammatory mediators in hippocampus. RT-PCR of hippocampal tissues revealed that the expression of iNOS and TNF-α message was enhanced by the transient ischemia, which was then inhibited by wogonin injection (Fig. 5). These results suggest that the neuroprotection provided by wogonin may be due to its anti-inflammatory activities.

Wogonin was protective against excitotoxic brain injury

To further examine the neuroprotective effect of wogonin in vivo, excitotoxic neuronal injury was induced by systemic administration of kainate (30 mg/kg, ip). Injection of kainate induced a severe neuronal cell death in CA1 and CA3 of hippocampus. Pretreatment with wogonin (10 mg/kg, ip, 60 min before kainate injection) significantly attenuated the hippocampal cell death both in CA1 and CA3 as determined by histological scoring (Table 1). Representative results of Nissl staining of hippocampus performed at 2 days after kainate administration are shown in Fig. 6. Wogonin at 1 mg/kg, however, did not provide a significant neuroprotection in this model (Table 1). Considering the known anti-inflammatory activity of wogonin in macrophages and microglia in vitro and the inhibition of ischemic induction of iNOS and TNF-α expression (Fig. 5), we hypothesized that the neuroprotective effect of wogonin demonstrated in vivo might be due to the inhibition of microglial activation, which is a critical component of CNS inflammation. In the next set of experiments, we evaluated the effect of wogonin on the activation of microglia, a functional equivalent of macrophages in CNS. The excitotoxic injury of hippocampal neurons by kainate administration was accompanied by strong microglial activation as determined by isolectin B4 histochemistry, which specifically stained brain microglia (Fig. 7*B*, *E*, *G*, *H*, and *I*). Microglial activation was evident in both CA1 (Fig. 7*E*) and CA3 (Fig. 7*G*, *H*, and *I*) regions in kainate-injected animals. The number of isolectin B4-positive microglia and the intensity of the staining in hippocampus were significantly reduced by wogonin (10 mg/kg; Fig. 7*C* and *F*), indicating the suppression of microglial activation by wogonin. A typical morphology of activated microglia with poorly ramified, short and thick processes was seen in hippocampus of the kainate-injected animals (Fig. 7*G*, *H*, and *I*).

DISCUSSION

In the current work, we have demonstrated that wogonin, an anti-inflammatory flavonoid, suppresses inflammatory activation of microglia in vitro as well as in vivo, and this anti-inflammatory action of wogonin provides neuroprotection against ischemic and excitotoxic brain injury. Wogonin is one of the major constituents of *Scutellaria baicalensis*, a plant that has been widely used in traditional Oriental medicine to treat various inflammatory and neurological symptoms (2). The root of *S. baicalensis* contains a number of flavone derivatives (37). Although wogonin is the first flavone isolated from the root of *S. baicalensis*, it is present only in small amounts in the root; the flavone glycoside named baicalin predominates by far. Acid hydrolysis of baicalin yields glucuronic acid and a flavone aglycone named baicalein. Anti-inflammatory effects of these three major constituents–wogonin, baicalin, and baicalein–are well documented (38). Recently, we have demonstrated that methanol extract of *S. baicalensis* is neuroprotective against global ischemia, and the extract inhibited microglial TNF-α and NO production in vitro (39). Now, our current results indicate that wogonin may be a critical component of *S. baicalensis* extract that mediates the neuroprotective effects, although contribution of other components cannot be excluded. In fact, a significant neuroprotective effect of baicalein has been demonstrated against transient global ischemia in rats (Kim, Y., et al., unpublished observations) as well as in gerbils (40). Baicalein at 1 mg/kg conferred ~31% inhibition of hippocampal CA1 cell death after 10 min ischemia by four-vessel occlusion. The neuroprotective effect of baicalein was slightly lower than that of wogonin at the same dose. It should be noted that the neuroprotective effect of wogonin is partial. Wogonin may act together with other components of *S. baicalensis* extract in a synergistic or additive manner.

In the four-vessel occlusion model, hippocampal CA1 neurons are selectively killed by ischemia (28). In the kainate model, however, both CA1 and CA3 regions are damaged (32). Therefore, only the CA1 region for the four-vessel occlusion model (Fig. 4) and both the CA1 and CA3 regions for kainate model (Table 1 and Figs. 6 and 7) have been analyzed, respectively. In the current study, the dose of wogonin was chosen based on its content (2. 68%) in the crude extract of *S. baicalensis* and the amount of wogonin that can be attained by consuming the herbal extract. Wogonin of 10 mg/kg, which did not show an apparent toxicity in animal experiments, can be easily attained by a daily consumption of the crude drug for adults (200-500 mg/kg). Stability of wogonin in blood has been recently determined by liquid chromatography-tandem mass spectrometry (41). After a single oral administration of 5 mg/kg wogonin to rats, plasma concentrations of free wogonin were detectable for at least 24 h. After the administration of 5 mg/kg wogonin, initial plasma concentration of wogonin and its major metabolite, wogonin-7β-D-glucuronide, was around 10 ng/ml and 1000 ng/ml, respectively. At 24 after the administration, plasma concentration of wogonin and wogonin-7β-D-glucuronide was around 1 ng/ml and 10 ng/ml, respectively.

In spite of wide use of *S. baicalensis* as an herbal medicine against bacterial infections, various inflammatory diseases, and stroke (2), scientific evidence related to its effectiveness or precise modes of action has not been available. Moreover, herbal remedies have been thought to contain toxic heavy metals, and their indications and dosage are not well defined, because the concentrations of the active components for most herbal medicines are not precisely determined. Now, our identification of wogonin as one of the active components of *S. baicalensis* that exert neuroprotective activities via inhibition of microglial activation suggests the possibility of

clinical applications of *S. baicalensis* and its constituents as neuroprotective agents on the basis of scientific evidence.

We have previously shown that wogonin inhibits NO production in rat astrocytes through the suppression of iNOS induction and NF-κB activation (12). Based on the similar mechanism of wogonin action in microglia and astrocytes, it is speculated that microglia may not be the only target of wogonin action in vivo. As stimulated astrocytes are also an important cellular source of inflammatory mediators in CNS and the proliferation of astrocytes is frequently observed upon brain injury (42), neuroprotection afforded by wogonin in the current study may be in part due to its effect on astrocytes. This possibility is being investigated in our laboratory.

NF-κB appears to be an important intracellular target of wogonin. NF-κB is a key transcription factor activated by several cellular signal transduction pathways that are associated with host defense, inflammation, and apoptosis (43, 44). Particularly, in inflammatory responses, NF-κB plays a central role by regulating genes encoding proinflammatory cytokines, chemokines, and adhesion molecules (45). The molecular mechanisms underlying NF-κB activation have been well studied and involve a sequential activation of cytoplasmic protein kinases and the ultimate nuclear translocation of active subunit of NF-κB (46, 47). As NF-κB has been considered as a redox-sensitive transcription factor (48), NF-κB-inhibitory action of wogonin may result from its anti-oxidant activities. Also, the inhibition of NF-κB may be a molecular basis of wogonin inhibition of microglial production of inflammatory mediators in vitro and in vivo. Besides the inhibition of NF-κB in microglia, it is possible that wogonin may also directly act on neurons to exert its protective effects as an anti-oxidant; wogonin may protect neurons from cytotoxic action of reactive oxygen or nitrogen species, which are also believed to be important neurotoxic mediators of neuroglial origin. However, our findings that neurotoxic action of exogenous NO donor was not alleviated by wogonin (Fig. 3*B*) argue against this possibility. Our results indicate that at least in vitro cultured neuronal cells are not protected by wogonin against cytotoxic action of NO, suggesting that the target of wogonin in vivo may be microglia rather than neurons.

In neurodegenerative diseases, a pathogenic role of uncontrolled microglial activation is widely accepted at present (16). In search of neuroprotective agents, now it is time to focus on killer cells (microglia) instead of killed cells (neurons); eliminating or at least suppressing killer microglial activation will provide a better chance for neuroprotection compared with just salvaging dying neurons. Now, our current work identified a potent neuroprotector from natural source that inhibits the killer cell activity. Our work will certainly instigate further investigations in the related areas, which will ultimately lead to the successful development of novel neuroprotective drugs based on wogonin or other constituents of the medicinal herb *S. baicalensis*.

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Table 1

The effect of wogonin on the histological changes in hippocampus after kainate injection

Experimental animal groups	Histology scores [1]
Saline (*n*=3)	0
Kainate (*n*=5)	3.11 ± 0.45 [2]
Kainate+Wogonin (1 mg/kg) (*n*=5)	2.67 ± 0.37
Kainate+Wogonin (10 mg/kg) (*n*=6)	2.25 ± 0.32 [2]

Values are means ± SE. [1]Damage or loss of hippocampal neurons was assessed by Nissl staining at 2 days after kainate administration with or without wogonin pretreatment. Histological damage was scored as follows (31): 0, no damage; 1, occasional injured neurons in CA1 or CA3; 2, small area (<10%) with neuronal damage or loss in CA1 or CA3; 3, greater area (10-50%) with neuronal damage of loss in CA1 or CA3; 4, extended (>50%) neuronal damage of loss in both in CA1 and CA3. [2] Statistically significant difference among each other ($P < 0.05$).

Fig. 1

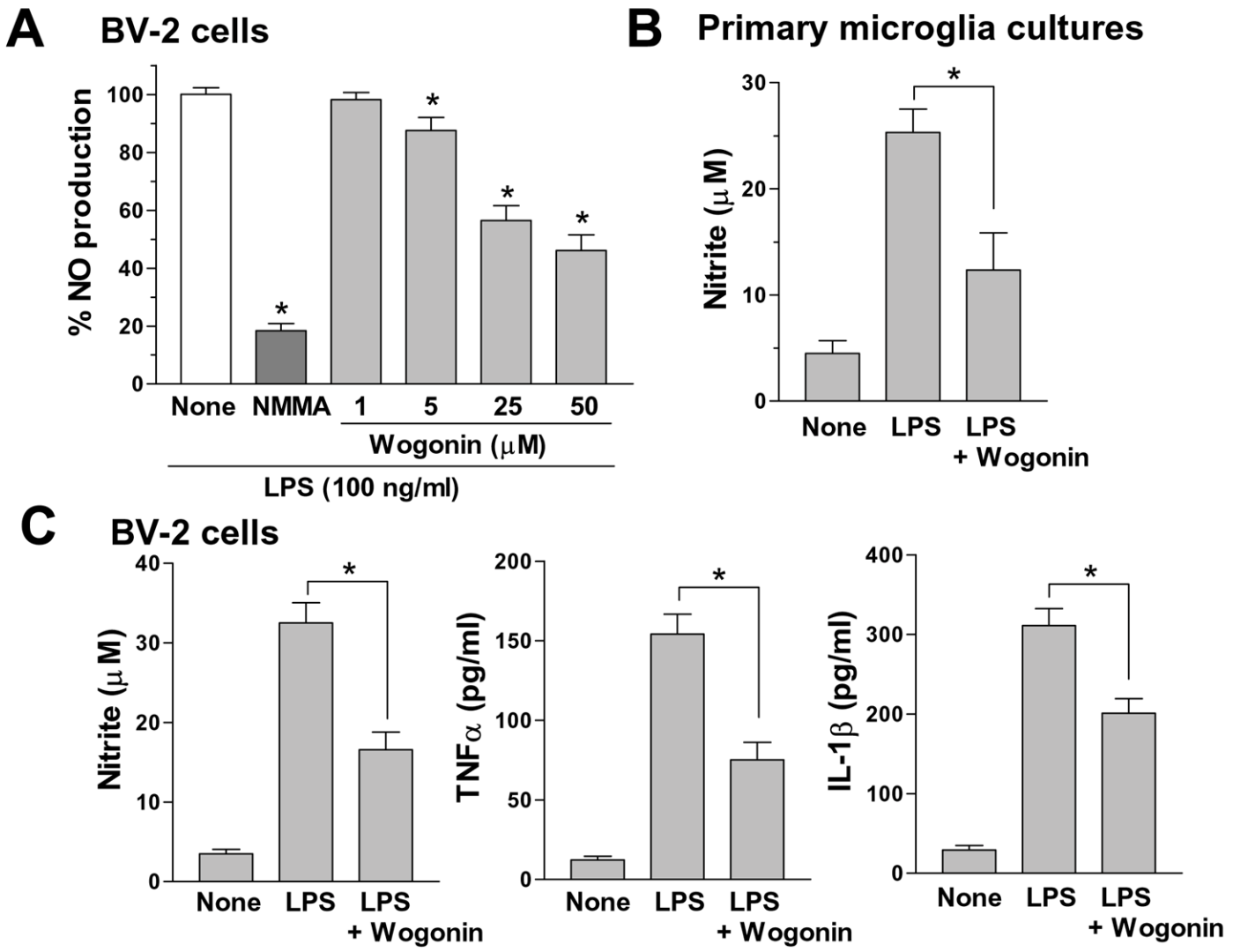

Figure 1. Wogonin inhibited inflammatory activation of cultured microglia. ***A)*** BV-2 cells were treated for 24 h with LPS (100 ng/ml) in the presence of NMMA (0.5 mM) or wogonin (1, 5, 25, 50 μM), and then NO production was assessed by Griess reaction. NMMA (NOS inhibitor) was used for comparison. NO production by LPS alone was set to 100%. Results are means ± SE of 4 independent experiments. *Significant differences from treatment with LPS alone (P<0.05). Either rat primary microglia cultures (***B***) or BV-2 cells (***C***) were treated for 72 h (***B***) or 24 h (***C***) with LPS (100 ng/ml) in the absence or presence of wogonin (50 μM), and then production of NO or cytokines was evaluated by Griess reaction or specific ELISA. Cells were pretreated with wogonin for 1 h before LPS stimulation. *Significant differences ($p < 0.05$).

Fig. 2

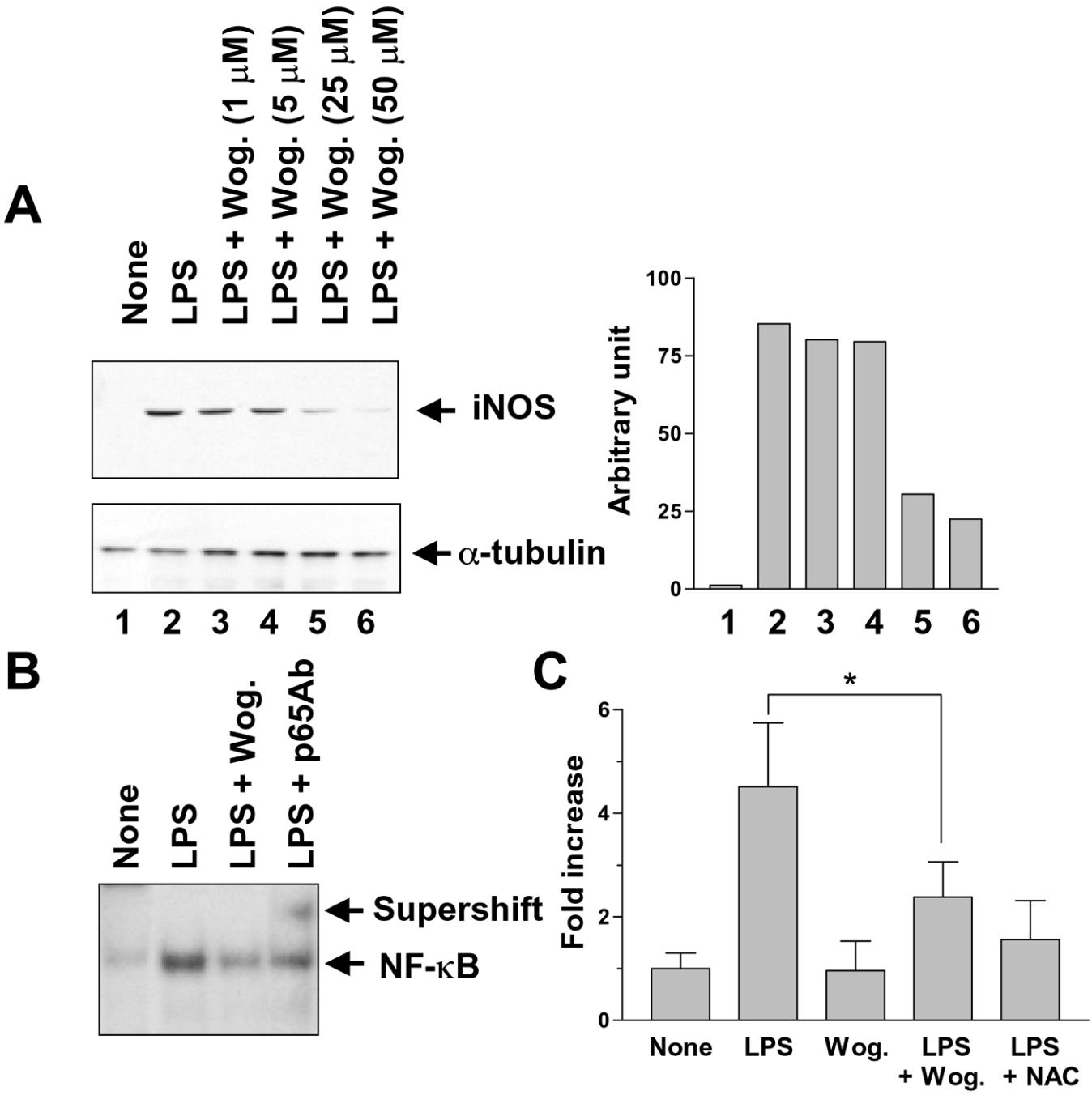

Figure 2. **Wogonin inhibited LPS-induced iNOS expression and NF-κB activation. *A*)** After treatment of BV-2 cells with LPS (100 ng/ml) for 16 h in the absence or presence of wogonin (1 - 50 μM), iNOS expression was evaluated by Western blot analysis using antibodies specific for iNOS or α-tubulin (*left*). The result of iNOS Western blot was subjected to densitometric analysis (*right*). Densitometric units were normalized to α-tubulin. ***B*)** Treatment of BV-2 cells with LPS (100 ng/ml) for 1 h induced NF-κB activation as evidenced by gel shift assays. Cotreatment of BV-2 cells with wogonin (50 μM) significantly reduced NF-κB activity. The identity of DNA-complexed proteins was confirmed by supershift assays using antibodies against p65 (p65 Ab). ***C*)** Inhibition of LPS-induced NF-κB activity by wogonin was confirmed by NF-κB-dependent reporter assays. NF-κB activity was also inhibited by anti-oxidant NAC (10 mM) treatment, which was used for comparison. Values are fold increases in firefly luciferase activity normalized to *Renilla* luciferase activity after treatment with indicated stimuli for 6 h (see Materials and Methods). Results are either representative or means ± SE of 3 independent experiments. *Significant differences between the 2 treatments ($P<0.05$).

Fig. 3

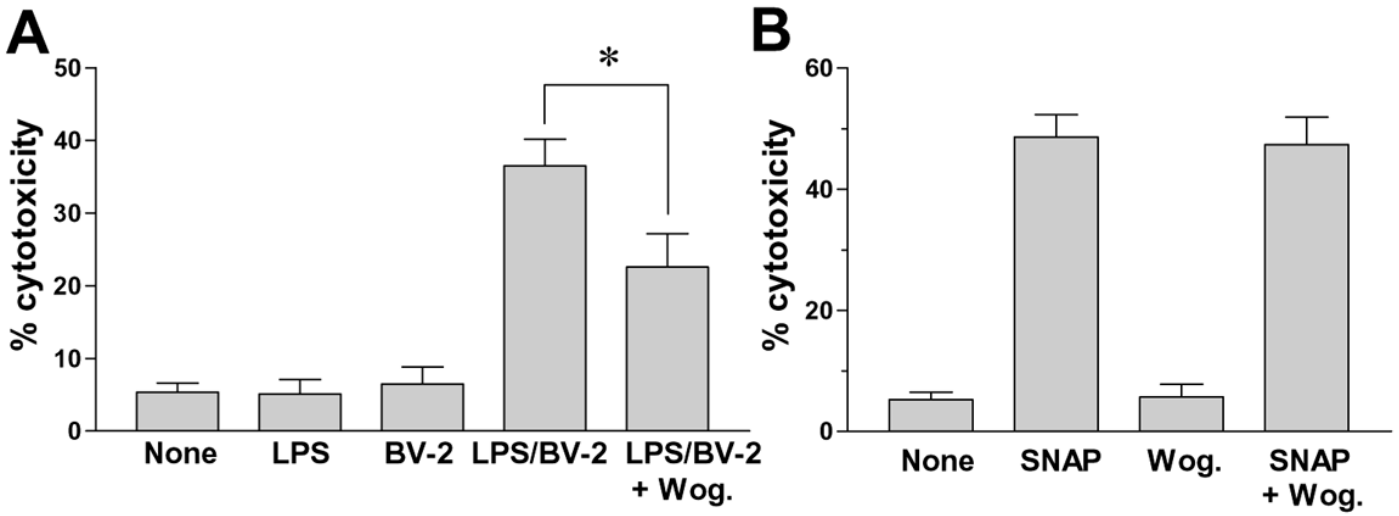

Figure 3. Wogonin protected PC12 cells from co-cultured BV-2 cell cytotoxicity. ***A*)** PC12 cells were co-cultured for 24 h with either unstimulated ("BV-2") or LPS (100 ng/ml)-stimulated BV-2 cells ("LPS/BV-2") with or without wogonin (50 μM), and then PC12 cell death was assessed by MTT assay. PC12 cells were also treated with LPS alone to assess the direct effect of LPS on the cell viability. *Significant differences between the 2 treatments (P<0.05). ***B*)** PC12 cells were also treated with a NO donor SNAP (0.5 mM) for 24 h with or without wogonin (50 μM), and then cytotoxicity was similarly evaluated. Wogonin did not significantly affect SNAP cytotoxicity. Results are means ±SE of 4 independent experiments.

Fig. 4

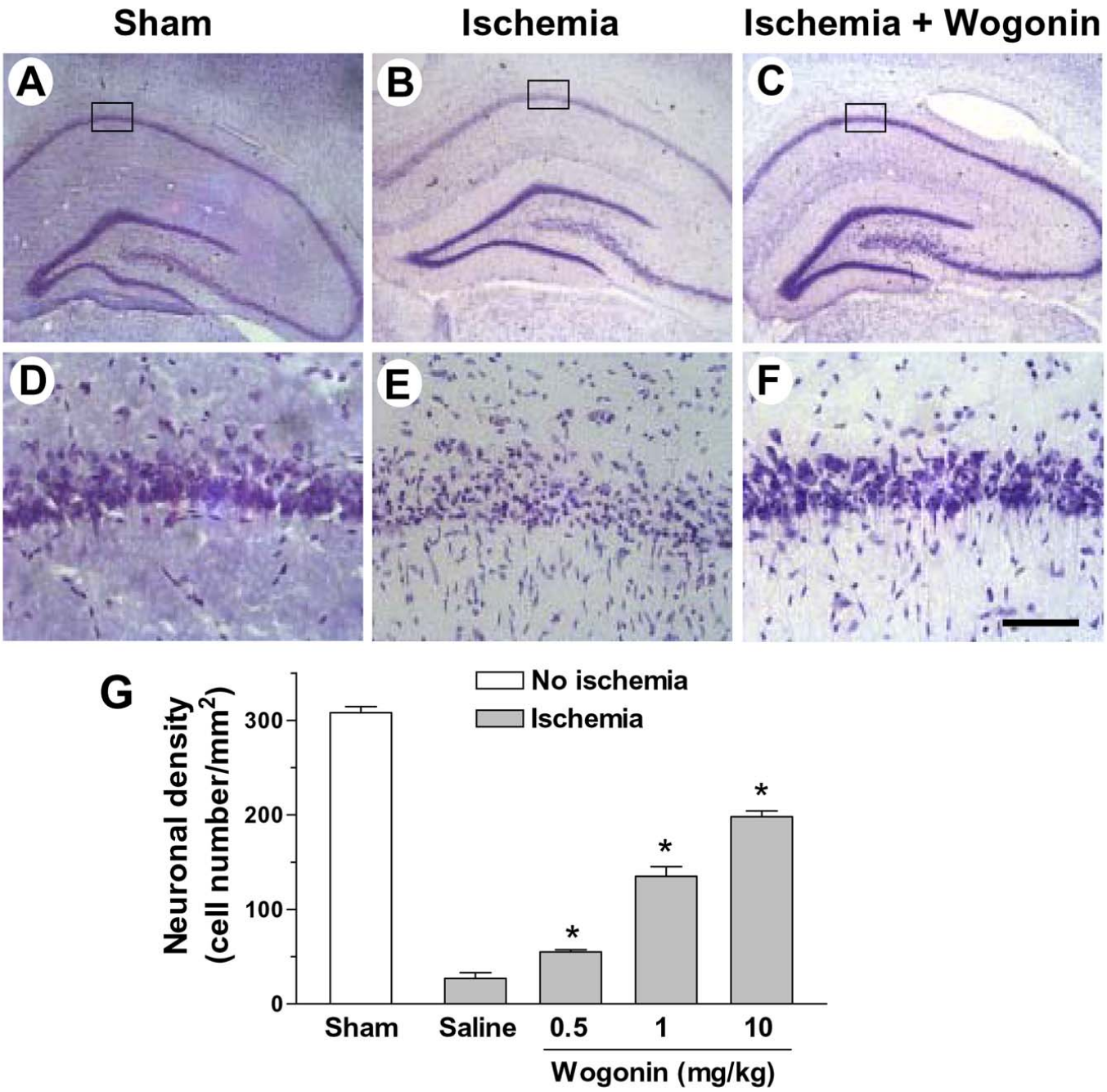

Figure 4. Neuroprotective effects of wogonin against ischemic brain injury. *A*-*F*) Representative photomicrographs of Cresyl violet-stained hippocampal regions of sham-operated animals (***A, D***) or animals that had been subjected to 10 min ischemia followed by treatment with either saline (***B, E***) or 10 mg/kg of wogonin (***C, F***). Boxed regions in ***A***, ***B***, and ***C*** (x40) are shown in ***D***, ***E***, and ***F*** (x200), respectively. Scale bar is 100 μm. ***G)*** Either saline or wogonin (0.5, 1, and 10 mg/kg) was intraperitoneally administered into animals after 10 min ischemia. Seven days later, neuronal cell density in CA1 region was measured by Nissl staining and cell counting. *Statistically significant differences from saline-treated ischemic group ($P < 0.05$). Sham, sham-operated animals (n=7); Saline, saline-treated animals after ischemia (n=7); wogonin, wogonin-treated animals after ischemia (n=3).

Fig. 5

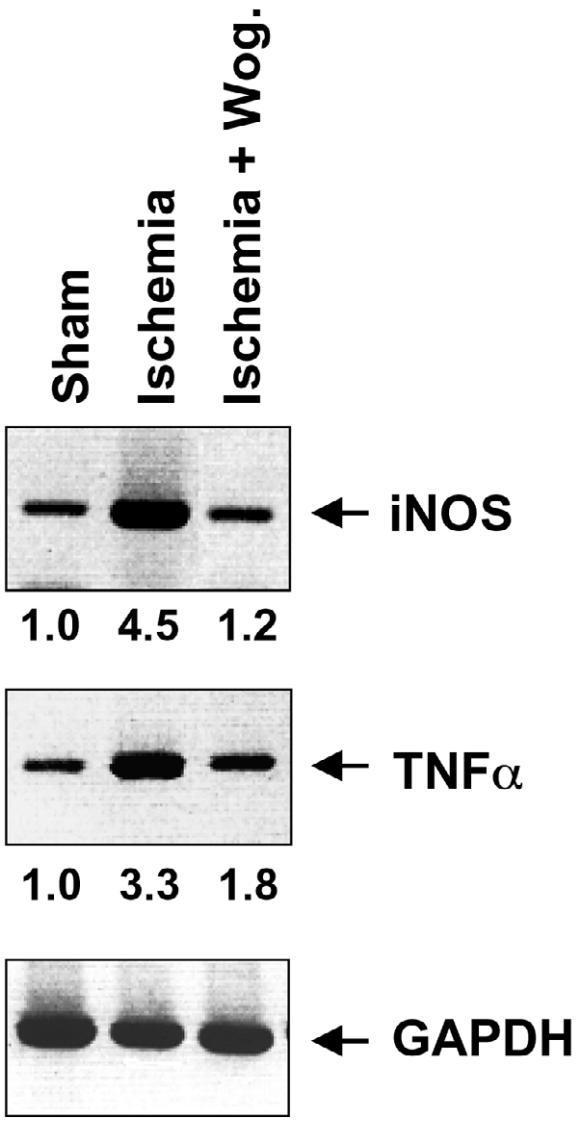

Figure 5. Wogonin inhibited expression of inflammatory mediators after ischemic brain injury. At 4 days after forebrain ischemia, the expression of iNOS and TNFα was assessed by RT-PCR analysis of hippocampal tissue followed by Southern blot analysis using sequence-specific oligonucleotide probes. Wogonin (10 mg/kg) markedly reduced the ischemic induction of iNOS and TNFα. Results are 3 independent experiments. The numbers are a fold induction of the gene expression normalized to GAPDH as determined by densitometric analysis of Southern blot of RT-PCR products.

Fig. 6

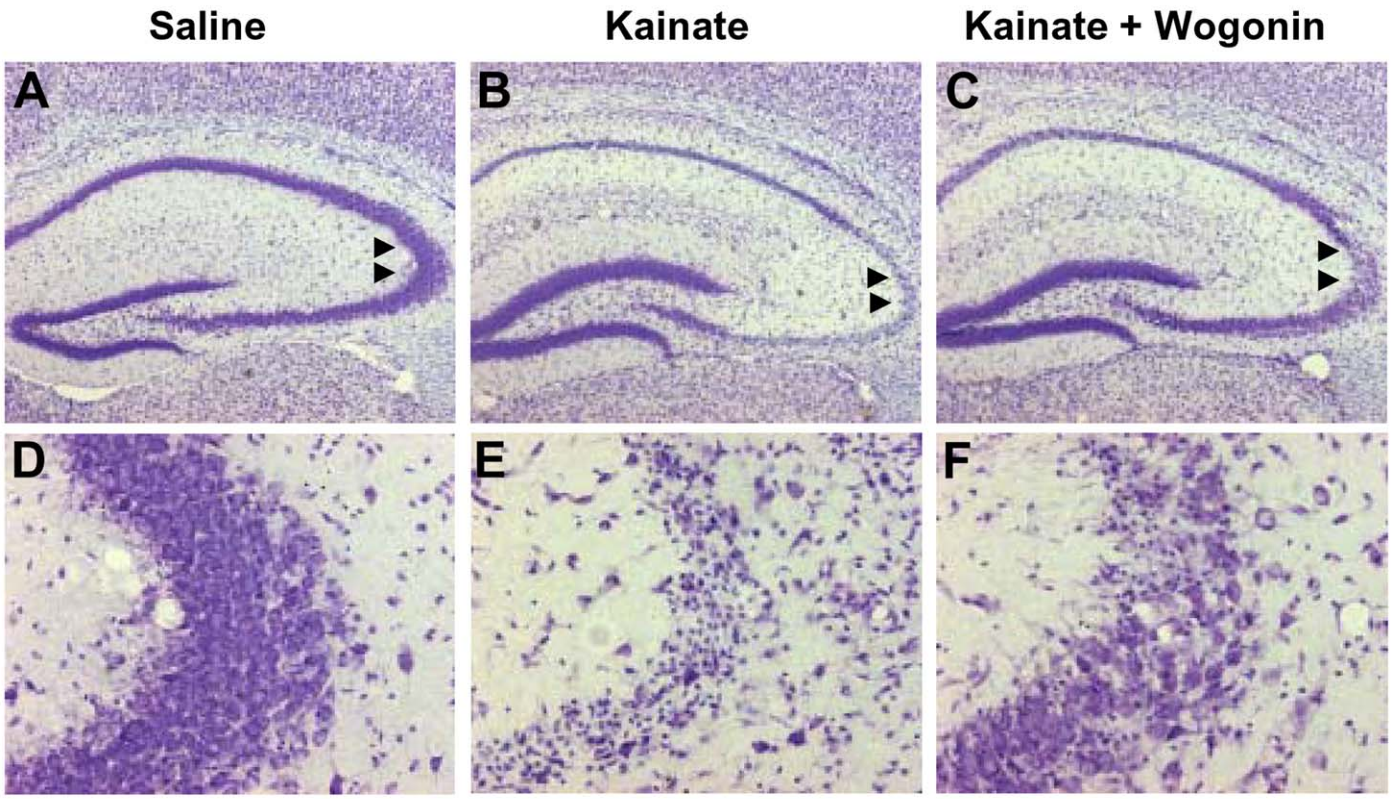

Figure 6. Neuroprotection by wogonin against kainate-induced excitotoxic brain injury. Compared with extensive hippocampal cell death by administration of kainate (***B, E***), pretreatment of wogonin (10 mg/kg) before kainate injection (***C, F***) afforded neuroprotection by attenuating hippocampal cell death both in CA1 and CA3. Neuronal loss or damage is not visible in saline-administered control animals (***A, D***). ***D***, ***E***, and ***F*** (x 200) are CA3 regions of ***A***, ***B***, and ***C*** (x 40) as indicated by arrowheads, respectively. Photomicrographs are representative results of Cresyl violet staining performed at 2 days after kainate injection.

Fig. 7

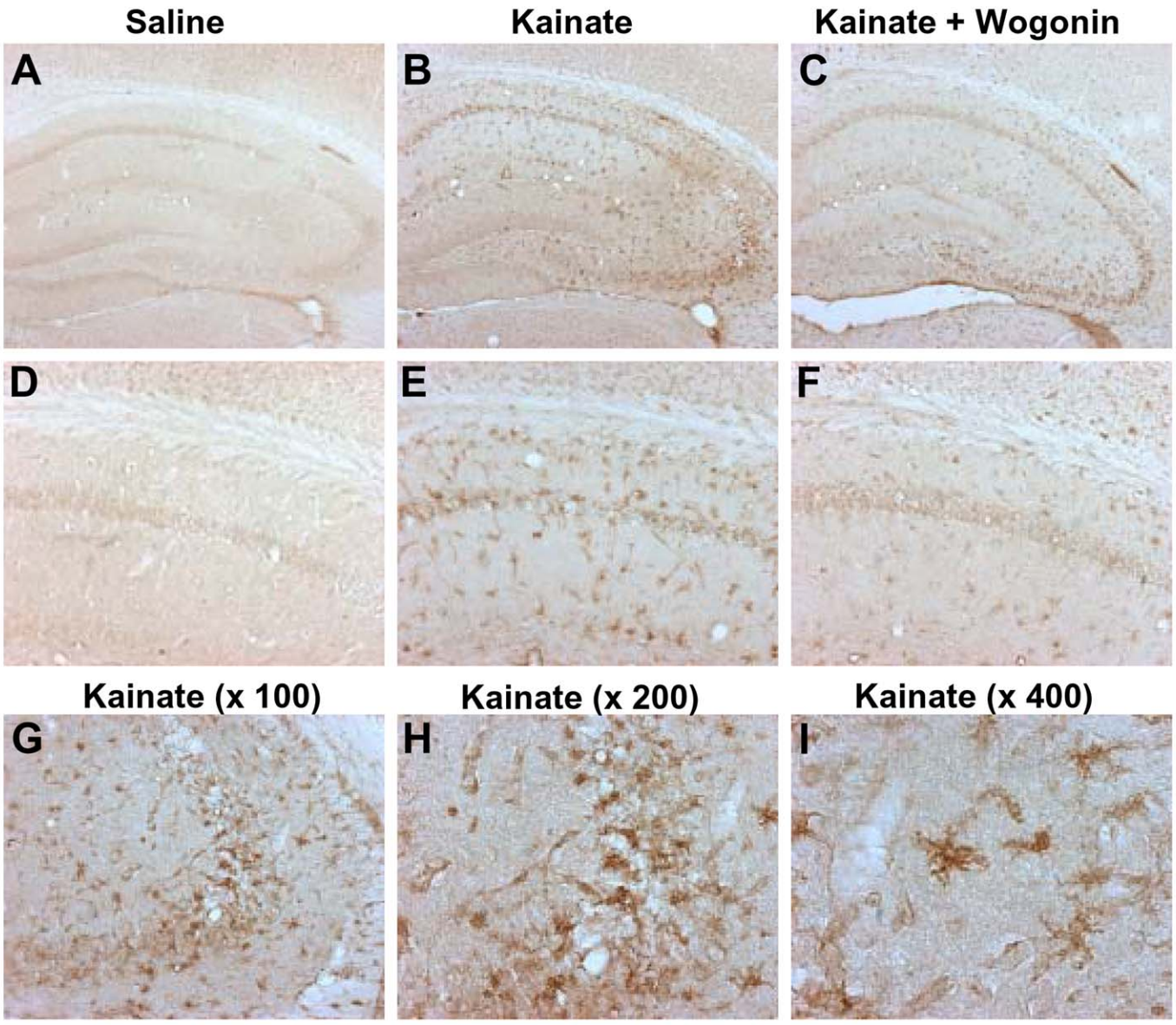

Figure 7. Wogonin inhibited activation of microglia after kainate injection. ***A-F)*** Kainate injection induced a strong activation of microglia as determined by microglia-specific isolectin B4 staining (***B, E***), which was inhibited by wogonin (10 mg/kg) pretreatment (**C,** ***F***). Isolectin B4 histochemistry was done at 2 days after kainate injection. Activation of microglia is not seen in control animals injected with saline alone (***A, D***). ***D***, ***E***, and ***F*** (x100) are CA1 regions of ***A***, ***B***, and ***C*** (x40), respectively. ***G-I***) Identity of isolectin-positive cells as microglia in CA3 region of kainate-injected animals was confirmed morphologically (***G***, x100; ***H***, x200; ***I***, x400). Numerous activated microglia with a typical morphology (large cell body, short and thick processes, and poorly ramified) are seen (***G, H, I***).