Arch Pharm Res Vol 27, No 9, 930-936, 2004

Archives of Pharmacal Research
http://apr.psk.or.kr

Neuroprotective Effect of Wogonin: Potential Roles of Inflammatory Cytokines

Hua Zi Piao[1,2], Shun Ai Jin[3], Hyang Sook Chun[4], Jae-Chul Lee[1], and Won-Ki Kim[1]

[1]Department of Pharmacology, College of Medicine, Ewha Institute of Neuroscience, Ewha Womens University, Seoul, Korea, [2]Department of pharmacology, College of Medicine, Yanbian University, Yanji, China, [3]Yan-Ji Hospital, Yanji, China, and [4]Korea Food Research Institute, Sungnam, Kyoungki-do, Korea

(Received May 10, 2004)

Wogonin (5,7-dihydroxy-8-methoxyflavone), an active component originated from the root of Scutellaria baicalensis Georgi, has been reported to possess antioxidant and anti-inflammatory properties. In this study, we investigated the neuroprotective effect of wogonin in a focal cerebral ischemia rat model. Wogonin markedly reduced the infarct volume after 2 h middle cerebral artery occlusion followed by 22 h reperfusion. Wogonin decreased the production of nitric oxide and inflammatory cytokines such as TNF-α and IL-6 in lipopolisaccharide-stimulated microglial cells. While wogonin reduced the activity of NF-κB, it did not change the activity of mitogen-activated protein kinases family members, p38, ERK and JNK. The lipopolisaccharide-stimulated production of NO and cytokines was significantly blocked by various kinds of NF-κB inhibitors such as *N*-acetyl cysteine, pyrrolidinedithiocarbamate and MG-132. The data may indicate that wogonin has neuroprotective effect by preventing the overactivation of microglial cells, possibly by inactivating NF-κB signaling pathway

Key words: Wogonin, NF-κB, Microglia, Cytokine, Neuroprotection

INTRODUCTION

Microglial cells, which are considered as brain resident macrophages, play an active role in various neurodegenerative diseases such as cerebral stroke (Kim *et al.*, 2000; Liu and Hong, 2003). Many of the effects of activated microglial cells are mediated by their numerous secreted products, of which are nitric oxide (NO) (Liu *et al.*, 2002), inflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6), and anti-inflammatory cytokines such as TGF-β1 (Kim *et al.*, 2000). When the magnitude of the microglial cells reaction surpasses a critical threshold level, these biologically active molecules may contribute to the establishment and maintenance of brain damage.

Wogonin is an active component isolated from Scutellaria baicalensis radix and has been widely used in clinical treatment of inflammatory diseases, including atopic dermatitis, hyperlipemia, and atherosclerosis in previous study (Nakamura *et al.*, 2003; Park *et al.*, 2001; Suk *et al.*, 2003; Wakabayashi and Yasui, 2000). Wogonin was reported to reduce the production of NO, COX-2 and cytokines in activated macrophages (Chen *et al.*, 2001). However, little information is available of the effect of wogonin in activated microglial cells.

One of the most potent stimuli for microglial cells is bacterial endotoxin lipopolysaccharide (LPS), which is derived from the outer cell wall of gram-negative bacteria. In response to LPS, microglial cells release large quantities of proinflammatory cytokines including TNF-α, interleukin-1 (IL-1) and IL-6. Previously, we reported that LPS-stimulated microglial cells enhanced the *N*-methyl-D-aspartate-induced or hypoxia/ischemia-evoked neurotoxicity (Kim and Ko, 1998; Kim *et al.*, 1999a, b; Lee *et al.*, 2003; Kim *et al.*, 2003). The enhanced neurotoxicity by LPS-stimulated microglial cells were mediated through the production of NO and other proinflammatory cytokines.

In the present study, we examined whether wogonin protected rat brain tissue from hypoxic/ischemic insults. We further studied the suppressive effect of wogonin on LPS-stimulated production of NO and cytokines in microglia. We found that wogonin markedly reduced the cerebral hypoxic/ischemic injury in a rat focal ischemic model.

Correspondence to: Won-Ki Kim, Department of Pharmacology, College of Medicine, Ewha Women's University, 70 Chongro-6-ga, Chongro-ku, Seoul 110-783, Korea
Tel: 82-2-760-5501, Fax: 82-2-760-5524
E-mail: wonki@mm.ewha.ac.kr

Wogonin also decreased the production of NO and inflammatory cytokines such as TNF-a and IL-6 in LPS-stimulated microglial cells. The LPS-stimulated production of NO and cytokines was regulated by NF-κB. Wogonin reduced the activity of NF-κB, implying that wogonin decreased the production of NO and cytokines by inactivating NF-κB activites.

MATERIALS AND METHODS

Materials

Lipoplysaccharide (LPS), nitrite, naphthylethylene diamine, sulfanilamide were purchased from Sigma Chemical Co (St.Louis, MO, USA). Dulbeccos modified Eagles media (DMEM:Hams F12 (1:1 mixed)), MEM, trypsin/EDTA, penicillin/streptomycin and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY, USA). TNF-α and IL-6 ELISA kits were purchased from R&D Systems (Minneapolis, USA). Wogonin was generously provided by DaeWoong pharm.co.LtD. (Seoul, Korea). The purity of wogonin was >95% (w/w).

Animals

Male Sprague-Dawley rats weighing between 240 and 250 g were purchased from Charles River Laboratories (Seoul, Korea) and kept on a 12 h light/dark cycle with *ad-libitum* access to food and water. Rats were acclimated to their environment for 5 d before use for experiments.

Focal cerebral ischemia model

Rats were anesthetized with 3.0% isoflurane in a 70% N_2O and 30% O_2 (v/v) mixture *via* face mask. Anesthesia was maintained with 2% isoflurane. A rectal temperature probe was introduced, and a heating pad maintained the body temperature at 37°C during whole surgery period. Focal cerebral ischemia was achieved by right-sided endovascular middle cerebral artery occlusion (MCAO) (Belayev *et al.*, 1996; Takano *et al.*, 1997). Briefly, the right carotid arteries were exposed through a midline cervical incision. The right external carotid artery (ECA) was dissected free and isolated distally by coagulating its branches and placing a distal ligation prior to transection. A piece of 3-0-monofilament nylon suture (Ethicon, Johnson-Johnson, Brussels, Belgium), with its tip rounded by gentle heating and coated by 0.1% (w/v) poly-L-lysine, was inserted into the lumen of right ECA stump and gently advanced 17.5 mm into the internal carotid artery (ICA) from the bifurcation to occlude the ostium of MCAO. After 2 h of ischemia, the suture was pulled back and the animal was allowed to recover. The total volume of infarction was determined by the integration of the distance of the eight brain sections. The percentage of infarct volume was obtained by calculating the portion of the corrected infarct size in the total size of the normal hemisphere.

Cell cultures

Primary culture of mixed glial and microglial cells were prepared as previously described (Kim and Ko, 1998). In brief, cells were cultured from the prefrontal cortices of 1- to 2 day-old Sprague-Dawley rat pups. Mixed glial cells were dissociated by mild trypsinization (for 8 min at 37°C, with 0.1% trypsin-containing HBSS) and passed through sterile nylon sieves (130 μm, pore size) into MEM (Minimum Essential Medium) containing 10% heat-inactivated FBS, penicillin and streptomycin 10 mL. They were then plated onto poly-D-lysine (2 μg/mL) coated 75-cm^2 culture bottles and maintained in MEM supplemented with 10% FBS (7-8 days in culture). The mixed glial cells were then trypsinized, washed, and plated in growth medium onto poly-L-lysine (2 μg/mL)-coated 48-well plates. Microglia-enriched or astrocytes-enriched cultures were prepared from primary mixed glial cell cultures as described (Kong *et al.*, 1997). Briefly, on day 9-12 of the mixed glial cultures, microglial cells were shaken off by plating shake incubation at 37°C for 3 min at 200 rpm and plating the dissociated cells onto the 48-well plates at 3.5×10^5 /mL. Cells were used for experiments 12 h after plating.

BV2 microglial cell line (generously provided by Dr Tong H. Jon at Burke Institute, Cornell University, NY) was cultured in DMEM containing 5% heat-inactivated fetal bovine serum, 100 units/mL penicillin and 0.1 μg/mL streptomycin. Cells were activated by treatment with 100 ng/mL of LPS for the indicated time period.

Measurement of NO

Release of NO was measured with Greiss reagent (mixing equal volumes of 0.1% napthylethylenediamine and 1% sulfanilamide in 5% phosphoric acid). The absorbance at 550 nm was determined using a microtiter plate reader (Molecular Devices, USA). Sodium nitrite, diluted in culture media at concentration of 10-100 μM, was used to make a standard curve.

RT-PCR (reverse transcriptase-PCR) for detection of iNOS, IL-6 and TNF-α mRNA expression

BV2 cells (4×10^5 cells on a 35 mm dish) were stimulated with LPS (500 ng/mL) in the presence or absence of wogonin and pridnisolone for 6 h. Cells were collected and total RNA was isolated using TRIZOL (Gibco, Grand Island, NY) according to the manufacturer's protocol. For reverse transcription-polymerase chain reaction (RT-PCR) analysis, 2 mg of total RNA were reverse transcribed for 1 h at 37°C in a reaction mixture containing RNA, 5 units RNase inhibitor (Amersham, Piscataway, NJ), 0.5 mM deoxynucleotide triphosphate (Boehringer Mannheim, Indianapolis, IN), 2 μM random hexamer (Stratagene, La Jolla,

CA), 1×reverse transcription buffer and 5 units MuMLV reverse transcriptase (Qiagen). PCR was performed using the above-prepared cDNA as a template. The following primers were used for PCR: iNOS (forward, 5'-CAA GAG TTT GAC CAG AGG ACC-3'; reverse, 5'-TGG AAC CAC TCG TAC TTG GGA-3'), TNF-α (forward, 5'-CAC GCT CTT CTG TCT ACT GA-3'; reverse, 5'-GGA CTC CGT GAT GTC TAA GT-3'), IL-6 (forward, 5'-TGT TCT CAG GGA GAT CTT GG-3'; reverse, 5'-TCT GAC CAC AGT GAG GAA TG-3').

Determination of amounts of TNF-α and IL-6

Microglial cells were plated in 48 well culture plates. Microglial cells were incubated with complete medium and stimulated with 100 ng/mL LPS in the presence or absence of wogonin (20, 40 μM) or prednisolone (10 μM) for 6 h and 12 h. Cell-free supernatants were harvested at indicated time points and kept frozen (-20°C) until TNF-a detection by using ELISA Kit.

Gel mobility shift assays

Microglial cells were plated at $5x10^6$ cells/well of a 100 mm dish. The Gel Mobility Shift Assays was performed as described previously (Ma *et al.*, 2001). In brief, the cells were collected in lysis buffer (pH 7.9) containing 10 mM Tris, 10 mM NaCl, 0.5% NP-40, and 0.5 mM phenylmethylsulfonyl fluoride (PMSF). The pellet was collected and resuspended in 30 μL of a ice-cold Nuclear extraction buffer (20 mM HEPES pH 7.9, 20% glycerol, 300 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT) containing the anti-protease mixture (1 mM PMSF, 2 μg/mL of pepstatin A, leupeptin, aprotinin) by pipitting gently and incubate on ice for 60 min at 4°C. After incubation, lysates were microcentrifuged at 14,000 rpm for 15 min at 4°C. The supernatant containing the nuclear proteins was collected and frozen at -70°C. Protein concentration was determined by the Bradford method. Nuclear proteins (5 μg) were mixed for 30 min at 4°C with ^{32}P-labeled oligonucleotide probe Sp1, and the complexes were subjected to nondenaturing 5% polyacrylamide gel electrophoresis for 90 min. The gel was dried and exposed to X-ray film. The oligonucleotide sequences for Sp1 are as follows: 5'-d (AGT TGA GGG GAC TTT CCC AGG C)-3' and 3'-d (GCC TGG GAA AGT CCC CTC AAC T)-5' (Promega Corp., Madison, WI).

Statistical analysis

Data are expressed as the mean ± standard error of mean (S.E.M.) and analyzed for statistical significance by using Student *t*-test or one-way ANOVA followed by Scheffe's test for multiple comparison. A p value <0.05 was considered significant.

RESULTS

Neuroprotective effect of wogonin in cerebral ischemic injury

A marked ischemic injury was induced after 2 h middle cerebral artery occlusion (MCAO) followed by 22 h reperfusion (Fig. 1). Intraperitoneal administration of wogonin (50 mg/kg) at the end of MCAO significantly reduced the infarct volume (Fig. 1). We further found that wogonin significantly prevented the reduction of neuronal cell counts in CA1 regions after global ischemic insult in gerbils (data not shown).

Wogonin inhibited the NO production in LPS-stimulated microglial cells

In agreement of our previous report (Kim and Ko,

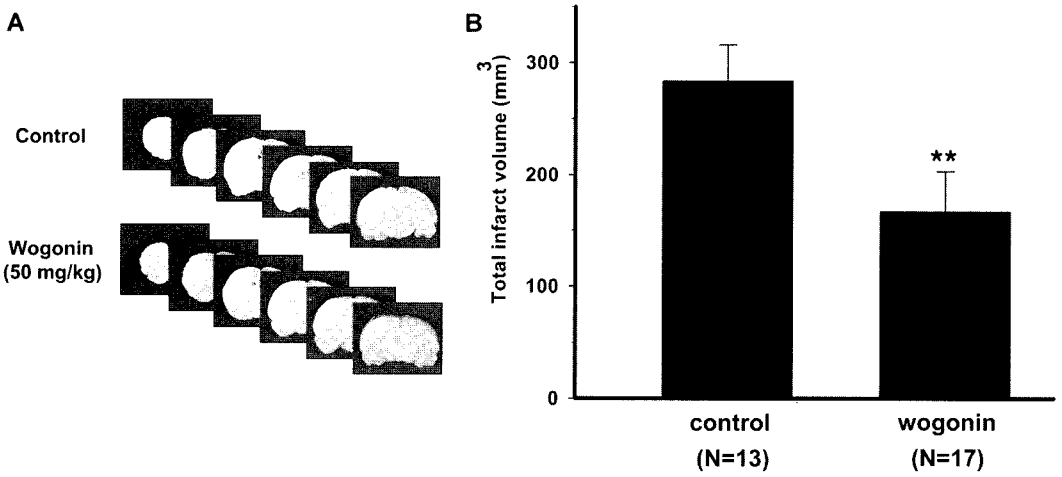

Fig. 1. Wogonin reduces infarct volume. Wogonin (50 mg/kg) was ip administered after 2-h middle cerebral artery occlusion right before reperfusion. (A) Representative TTC-stained brain coronal sections from control and wogonin treatment group. (B) Averaged total infarct volume. Total infarct area was expressed as mean ±standard deviation. **p<0.01 (paired *t*-test).

1998), microglial cells markedly produced NO in response to LPS (data not shown). Wogonin reduced the production of NO in a dose-dependent manner (Fig. 2A). Wogonin also reduced the mRNA expression of iNOS in BV2 mouse microglial cells (Fig. 2B).

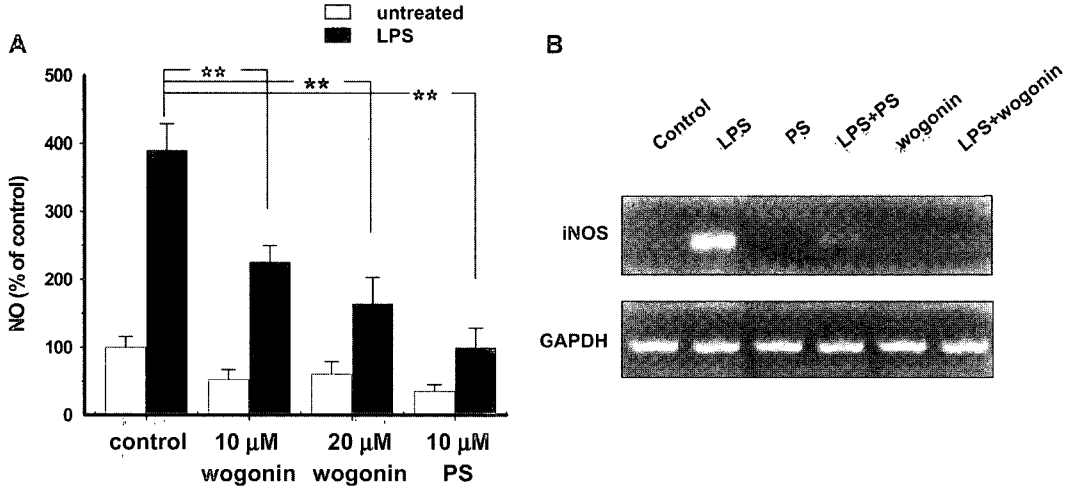

Fig. 2. Wogonin suppresses LPS-stimulated iNOS expression and NO production. (A) Microglial cells in primary cultures were treated with LPS 100 ng/mL in the absence or presence of indicated amounts of wogonin or prednisolone for 24 h. The amount of NO was determined by Greiss reaction. (B) BV2 cells (2×10^5 cells per well) were pre-treated with prednisolone (10 μM) or wogonin (50 μM) for 30 min, and treated with LPS (100 ng/ml) for 8 h. mRNA levels for iNOS and GAPDH were determined by RT-PCR from total RNA extracts. Values were mean ± S.E. of three experiments. N=4. **p<0.01 (Sheffes test).

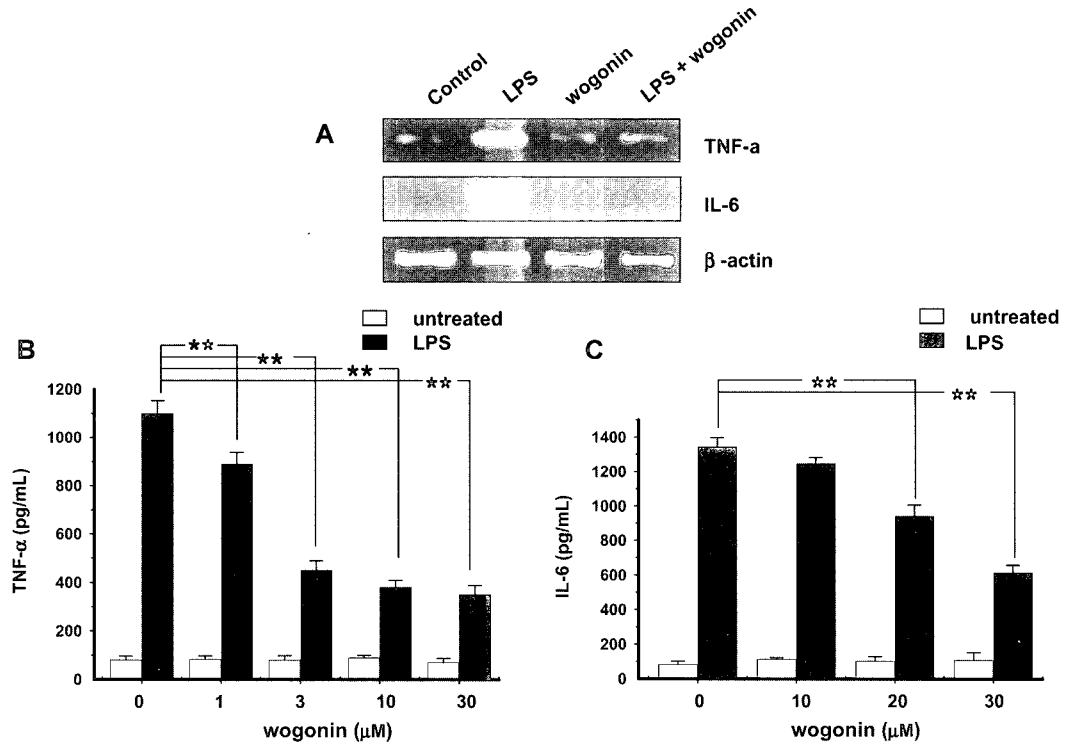

Fig. 3. Wogonin represses LPS-stimulated mRNA expression and production of TNF-a and IL-6. (A) BV2 cells were pre-treated with wogonin (50 μM) for 30 min, and treated with LPS (100 ng/mL) for 6 h. The mRNA levels for TNF-a and IL-6 were determined by RT-PCR from total RNA extracts. Line 1. control, 2. LPS, 3. wogonin, 4. LPS + wogonin. (B and C) Microglial cells were treated with LPS 100 ng/ml in the absence or presence of indicated amounts of wogonin for 6 h. The amount of TNF-α (B) and IL-6 (C) were determined by ELISA. Each point represents mean ±sem. N=4. *p<0.05, **p<0.01 (Sheffes test).

Wogonin inhibited the production of TNF-α and IL-6 in LPS-stimulated microglial cells

We next analyzed whether wogonin reduced the expression of pro-inflammatory cytokines in microglial cells. Wogonin reduced the expression of mRNA of TNF-α and IL-6 in BV2 mouse microglial cells (Fig. 3A). LPS (100 ng/mL) markedly increased the production of TNF-α and IL-6 in primary cultures of microglial cells (Fig. 3B and C). The LPS-stimulated production of TNF-α and IL-6 was significantly inhibited by wogonin (Fig. 3B and C).

Inhibition of NO and IL-6 production by wogonin via suppression of NF-κB

Several lines of studies showed that LPS modulated the production of NO and cytokines via activation of nuclear factor-κB (NF-κB) and/or activation of members of the mitogen-activated protein kinases (MAPKs) family (Vander *et al.*, 1999). In the present study, LPS treatment (500 ng/mL) of microglial cells induced significant NF-κB DNA binding activity within 1 h (Fig. 1). Wogonin significantly reduced LPS-induced increases in NF-κB activity (Fig. 4). N-acetyl cysteine (NAC), a well-known NF-κB inhibitor, reduced the production of NO (Fig. 5A) and IL-6 (Fig. 5B) in LPS-treated microglial cells. Similar results were also obtained with other NF-κB inhibitors including pyrrolidine-dithiocarbamate and MG-132 (data not shown). The data may indicate that the anti-inflammatory activity of wogonin

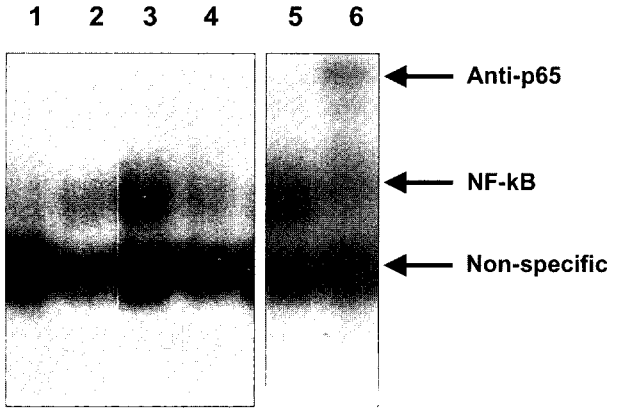

Fig. 4. Wogonin reduces NF-κB activities in primary microglial cells. Primary microglial cells were pre-treated with wogonin (50 μM) for 30 min, and further treated with 500 ng/ml LPS in the absence and presence of wogonin (50 μM). NF-κB activity was determined 1 h after LPS treatment. Lane: 1. control, 2. wogonin 40 M, 3. LPS 500 ng/mL, 4. LPS+wogonin, Supershift assay: 5. LPS, 6. LPS+ anti-p65

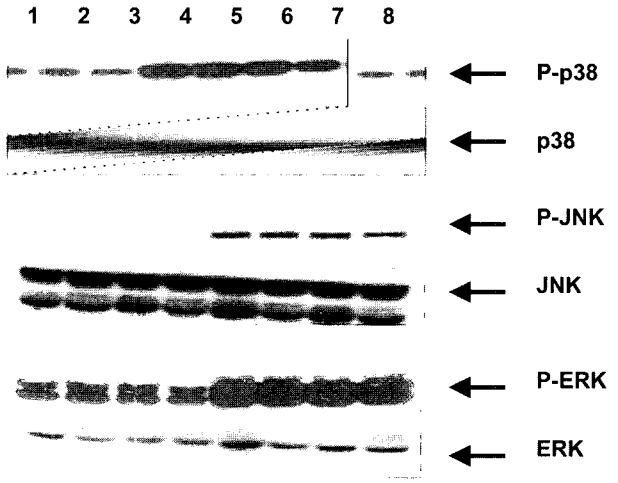

Fig. 6. Wogonin does not affect MAPKs activities in primary microglia. Primary microglia were stimulated with LPS (500 ng/mL) in the absence or presence of wogonin (20, 40 μM) or prednisolone (10 μM) for 1 h. The activities of MAPKs were detected by Western blot (p-p38/p38, p-ERK1/2/ERK, p-JNK/JNK): Lane: 1, control; 2, wogonin 20 μM; 3, wogonin 40 μM; 4, prednisolone 10 μM; 5, LPS 500 ng/mL; 6. LPS+ wogonin 20 μM; 7, LPS+ wogonin 20 μM; 8, LPS+ prednisolone 10 μM.

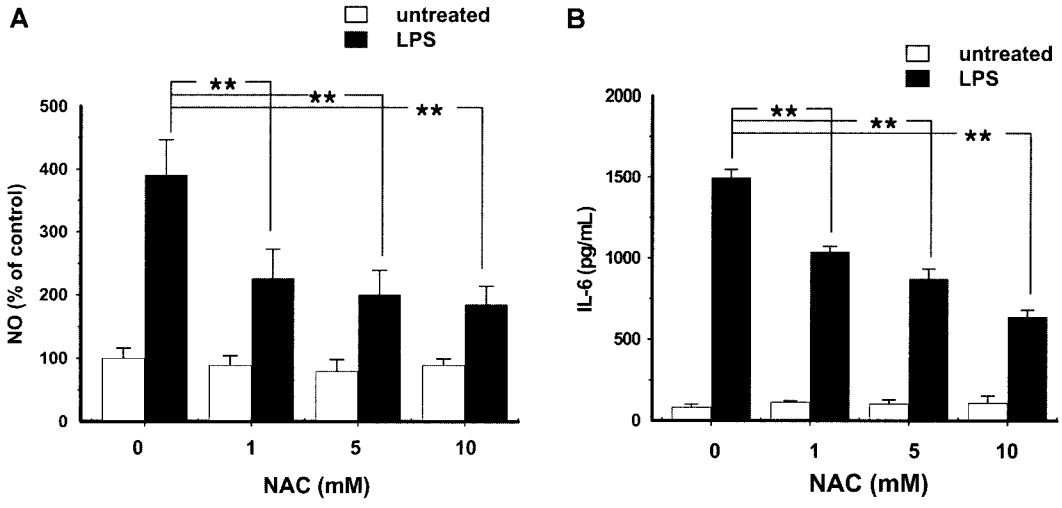

Fig. 5. NAC inhibited LPS-stimulated NO and IL-6 production. Microglial cells were incubated with LPS 100 ng/mL at indicated concentrations of NAC. The amount of NO (A) was determined by Griess reaction and IL-6 by ELISA. N=4. ** $p<0.01$ (Sheffes test).

be associated with its inactivation of NF-κB activity. To test whether the inhibitory effect of wogonin on activation of microglial cells was also mediated by MAPKs, we further tested the effect of wogonin on the activity of MAPK families by LPS. Unlike NF-κB, however, the activation of MAPK families was not altered by wogonin (Fig. 6).

DISCUSSION

Previously, we have reported that activated microglial cells played important roles in cerebral ischemic injury, (Kim and Ko, 1998; Choi *et al.*, 1999a,b; Lee *et al.*, 2003; Kim *et al.*, 2003). In the present study, we demonstrated that wogonin reduced cerebral ischemic injury in a focal ischemia model. Because wogonin remarkably suppressed inflammatory activation of microglial cells (e.g., production of NO and proinflammatory cytokines), although speculative, wogonin might reduce the infarct volume in part *via* inhibition of activated microglial cells.

Nitric oxide has been recognized to be an important mediator of cellular communication in several cell types such as neurons, microglia and infiltrating immune cells such as macrophages and neutrophils in the central nervous system. Over-production of NO is in general detrimental to neuronal cells. NO and its degradation products (nitrogen oxides, such as peroxynitrite ($ONOO^-$), NO_2, NO_2^-) are reactive molecules causing DNA deamination (Wink *et al.*, 1991), formation of iron-NO complexes of respiratory enzymes (Drapier and Hibbs, 1998), and oxidation of protein sulfhydryls (Minami *et al.*, 1992). Therefore, drugs that inhibit iNOS expression and/or enzyme activity may have beneficial therapeutic effects in the treatment of neuro-inflammatory diseases. Cytokines serve cellular communication in the immune system. Cytokines constitute a significant portion of the immuno- and neuromodulatory messengers that can be released by activated microglial cells. Increased levels of TNF-α in the brain have been observed especially in the injury caused by ischemia. The prominent source of TNF-α may be activated microglial cells. IL-6 is also thought to be a major mediator of microglial cells activation (Ye and Johnson, 1999). IL-6 is considered as a proinflammatory cytokine in the initiation and coordination of inflammatory responses. Therefore, the neuroprotective effect of wogonin observed in focal cerebral ischemia may be derived from its inhibition of NO, TNF-α and IL-6 production in microglial cells activated after hypoxic/ischemic injury.

NF-κB, a redox-sensitive transcriptional factor, is known to be involved in the regulation of cytokines expression. Like NF-κB inhibitors NAC, PDTC and MG-132, wogonin significantly inhibited NF-κB DNA-binding activity. Inactivation of NF-κB resulted in a reduction of NO and cytokines, indicating that wogonin suppressed NO and cytokine production *via* inhibition of NF-κB signaling pathway. Because none of commercially available NF-κB inhibitors are specifically inhibiting NF-κB, in our study we used three different chemicals containing the inhibitory activity against NF-κB, such as the antioxidants *N*-acetyl cysteine and pyrrolidinedithiocarbamate (PDTC), which inhibits IKB degradation (Lauzurica *et al.*, 1999) and the proteasome inhibitor MG-132 (Tsubuki *et al.*, 1996).

Previously, Chang and her colleagues showed that wogonin did not inhibit TNF-α-induced NF-κB activation in human endothelial cells (Chang *et al.*, 2001). They further showed that wogonin inhibited the phorbol ester-induced expression of monocyte chemotactic protein-1 and this inhibition was not mediated by NF-κB activation. NF-κB activation is known to be highly cell type- and stimulus-dependent, and occurs through divergent mechanisms of activation. Thus, the difference between our and other researchers findings could be due to the different cell types and different stimulants. In our study, wogonin did not alter the activity of MAPKs. In human endothelial cells, however, wogonin greatly attenuated the activities of ERK1/2 kinase and JNK kinase. Thus, we think that like NF-κB MAPKs are also differentially regulated by wogonin in cell type- and stimulant-dependent manners.

In summary, our study suggests that inhibition by wogonin of LPS-stimulated expression and production of NO and cytokines in microglial cells is mediated *via* the down-regulation of NF-κB activity. Our results provide the first evidence of the role of NF-κB in NO and cytokine production in activated microglial cells. This study may suggest that wogonin is a therapeutically useful to attenuate inflammatory responses during cerebral ischemic insults.

ACKNOWELDGEMENTS

This study was supported by a grant from the Agriculture R&D Promotion Center, the Ministry of Agriculture and Forestry, and a research project on Functional Food Development from the Office for Government Policy Coordination, Republic of Korea.

REFERENCES

Chang, Y. L., Shen, J. J., Wung, B. S., Cheng, J. J., and Wang, D. L., Chinese herbal remedy wogonin inhibits monocyte chemotactic protein-1 gene expression in human endothelial cells. *Mol. Pharmacol.*, 60, 507-513 (2001).

Chen, Y. C., Shen, S. C., Chen, L. G., Lee, T. J., and Yang L. L., Wogonin, baicalin, and baicalein inhibition of inducible nitric oxide synthase and cyclooxygenase-2 gene expressions induced by nitric oxide synthase inhibitors and lipopolysaccharide. *Biochem Pharmacol.*, 61, 1417-1427 (2001).

Choi, J. J., Oh, Y. K., Kim, H. S., Kim, H. C., Ko, K. H., and Kim,

W. K., Mimosine prevents the death of glucose-deprived immunostimulated astrocytes by scavenging peroxynitrite. *Glia.*, 39, 37-46 (2002).

Drapier, J. C. and Hibbs, J. B. Jr., Differentiation of murine macrophages to express nonspecific cytotoxicity for tumor cells results in L-arginine-dependent inhibition of mitochondrial iron-sulfur enzymes in the macrophage effector cells. *J. Immunol.*, 140, 2829-2838 (1988).

Kim, W. G., Mohney, R. P., Wilson, B., Jeohn, G. H., Liu, B., and Hong, J. S., Regional difference in susceptibility to lipopolysaccharide-induced neurotoxicity in the rat brain: role of microglia. *J. Neurosci.*, 20, 6309-6316 (2000).

Kim, W.-K., Chung, J.-H., Kim, H.-C., and Ko, K. H., Nitric oxide-enhanced excitotoxicity-independent apoptosis of glucose-deprived neurons. *Neurosci. Res.*, 33, 281-289 (1999a).

Kim, W.-K. and Ko, K. H., Potentiation of *N*-methyl-D-aspartate-mediated neurotoxicity by immunostimulated murine microglia. *J. Neurosci Res.*, 54, 17-26 (1998).

Kim, W.-K., Lee, J.-C., Cho, G.-S., Kim, H. J., Choi, and K., Choi, C., Time-dependent modulation of cerebral ischemic injury after lipopolysaccharide microinjection into rat corpus callosum. *Glia*, S2, 61 (2003).

Kim, W.-K., Seo, D. O., Choi, J.-J., and Ko, K.-H., Immunostimulated glial cells potentiate glucose deprivation-induced death of cultured rat cerebellar granule cells. *J. Neurotrauma.*, 16, 415-424 (1999b).

Kumar, S. M., Porterfield, D. M., Muller, K. J., Smith, P. J., and Sahley, C. L., Nerve injury induces a rapid efflux of nitric oxide (NO) detected with a novel NO microsensor. *J. Neurosci.*, 21, 215-220 (2001).

Lauzurica, P, Martinez-Martinez, S., Marazuela, M., Gomez, del Arco P., Martinez, C., Sanchez-Madrid, F., and Redondo, J. M., Pyrrolidine dithiocarbamate protects mice from lethal shock induced by LPS or TNF-alpha. *Eur. J. Immunol.*, 29,1890-900 (1999).

Lee, J.-C., Cho, G.-S., Kim, H. J., and Kim, W.-K.., Augmented cerebral ischemic injury by activated macrophages/microglia: Role of nitric oxide. *Glia*, S2, 61 (2003).

Lieb, K., Engels, S., and Fiebich, B. L., Inhibition of LPS-induced iNOS and NO synthesis in primary rat microglial cells. *Neurochem Int.*, 42, 131-137 (2003).

Liu, B., Gao, H. M., Wang, J. Y., Jeohn, G. H., Cooper, C. L., and Hong, J. S., Role of nitric oxide in inflammation-mediated neurodegeneration. *Ann. N. Y. Acad. Sci.*, 962, 318-331 (2002).

Liu, B. and Hong, J. S., Role of microglia in inflammation-mediated neurodegenerative diseases: mechanisms and strategies for therapeutic intervention. *J. Pharmacol. Exp. Ther.*, 304, 1-7 (2003).

Ma, W., Lim, W., Gee, K., Aucoin, S., Nandan, D., Kozlowski, M., Diaz-Mitoma, F., and Kumar, A., The p38 mitogen-activated kinase pathway regulates the human interleukin-10 promoter *via* the activation of Sp1 transcription factor in lipopolysaccharide-stimulated human macrophages. *J. Biol. Chem.*, 276, 13664-13674 (2001).

Minami, M., Kuraishi, Y., Yabuuchi, K., Yamazaki, A., and Satoh, M., Induction of interleukine-1beta mRNA in rat brain after transient forebrain ischemia. *J. Neurochem.*, 58, 390-392 (1992).

Nakamura, N., Hayasaka, S., Zhang, X. Y., Nagaki, Y., Matsumoto, M., Hayasaka, Y., and Terasawa, K., Effects of baicalin, baicalein, and wogonin on interleukin-6 and interleukin-8 expression, and nuclear factor-kappab binding activities induced by interleukin-1beta in human retinal pigment epithelial cell line. *Exp. Eye Res.*, 77, 195-202 (2003).

Park, B. K., Heo, M. Y., Park, H., and Kim, H. P., Inhibition of TPA-induced cyclooxygenase-2 expression and skin inflammation in mice by wogonin, a plant flavone from Scutellaria radix. *Eur. J. Pharmacol.*, 425, 153-157 (2001).

Polazzi, E., Gianni, T., and Contestabile, A., Microglial cells protect cerebellar granule neurons from apoptosis: evidence for reciprocal signaling. *Glia*, 36, 271-280 (2001).

Suk, K., Lee, H., Kang, S. S., Cho, G. J., and Choi, W. S., Flavonoid baicalein attenuates activation-induced cell death of brain microglia. *J. Pharmacol. Exp. Ther.*, 305, 638-645 (2003).

Tsubuki, S., Saito, Y., Tomioka, M., Ito, H., and Kawashima, S., Differential inhibition of calpain and proteasome activities by peptidyl aldehydes of di-leucine and tri-leucine. *J. Biochem.*, 119, 572-576 (1996).

van der Bruggen, T., Nijenhuis, S., van Raaij, E., Verhoef, J., and van Asbeck. B. S., Lipopolysaccharide-induced tumor necrosis factor alpha production by human monocytes involves the raf-1/MEK1-MEK2/ERK1-ERK2 pathway. *Infect Immun.*, 67, 3824-3829 (1999).

Wakabayashi, I. and Yasui, K., Wogonin inhibits inducible prostaglandin E (2) production in macrophages. *Eur. J. Pharmacol.*, 406, 477-481 (2000).

Wink, D. A., Kasprzak, K. S., Maragos, C. M., Elespuru, R. K., Misra, M., Dunams, T. M., Cebula, T. A., Koch, W. H., Andrews, A. W., and Allen, J. S., *et al.*, DNA deaminating ability and genotoxicity of nitric oxide and its progenitors. *Science*, 254, 1001-1003 (1991).

Yang, Y., Li, Q., Wang, C. X., Jeerakathil, T., and Shuaib, A., Dose-dependent neuroprotection with tiagabine in a focal cerebral ischemia model in rat. *Neuroreport*, 11, 2307-2311 (2000).