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Apigenin inhibits the production of NO and PGE_2 in microglia and inhibits neuronal cell death in a middle cerebral artery occlusion-induced focal ischemia mice model

Sang Keun Ha [a], Pyeongjae Lee [b], Jeong A Park [a], Hye Rim Oh [c], Sang Yeon Lee [a], Ji-Ho Park [a], Eunjoo H. Lee [a], Jong Hoon Ryu [c], Kang Ro Lee [d], Sun Yeou Kim [a,*]

[a] *Graduate School of East-West Medical Science, Kyung Hee University, #1 Seocheon-dong, Kihung-ku, Yongin-City, Kyungki-Do 449-701, Republic of Korea*
[b] *Department of Natural Medicine Resource, Semyung University San 21-1, Sinwoul-dong, Jecheon-City, Chungbuk 390-711, Republic of Korea*
[c] *Department of Oriental Pharmaceutical Science, Kyung Hee University, Seoul, Republic of Korea*
[d] *College of Pharmacy, SungKyunKwan University, Suwon 440-746, Republic of Korea*

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Abstract

Flavonoids have been intensively studied on their pharmacological activities such as anti-cancer, anti-oxidant and anti-inflammation. However, little is known about their neuroprotective effects. Recent studies suggest that inflammation mediated by microglia may play a role in neurodegenerative diseases. In this study, we evaluated the anti-inflammatory effect of various flavonoid compounds by using BV-2, a murine microglia cell line. Of the compounds that were evaluated, apigenin inhibited the production of nitric oxide and prostaglandin E_2 by suppressing the expression of inducible nitric oxide synthase and cyclooxygenase-2 protein, respectively. Moreover, apigenin suppressed p38 mitogen-activated protein kinase (MAPK), c-Jun N-terminal kinase (JNK) phosphorylation without affecting the activity of extracellular signal-regulated kinase (ERK). Apigenin was also found to protect neuronal cells from injury in middle cerebral artery occlusion.

Keywords: Flavonoid; Nitric oxide; Apigenin; Inflammation; Prostaglandin E_2; Mitogen-activated protein kinase; Middle cerebral artery occlusion

1. Introduction

Although there are various inducing factors in diverse neurodegenerative diseases, neuronal cell death is the final result in the diseases. Recent studies demonstrate that neuroinflammation mediated by microglia contributes to neuronal cell death, which is not restricted to a specific disease but implicated in various diseases such as ischemia, Parkinson's disease and Alzheimer's disease (Gehrmann et al., 1992; Le et al., 2001; Ryu et al., 2002; Stoll and Jander, 1999). It has been proposed that microglia, a resident macrophage-like population of brain cells, plays a role in host defense and tissue repair in the central nervous system (CNS) (Perry and Gordon, 1988). However, overactivated microglia cells produce excessive inflammatory substances such as nitric oxide, various cytokines, and prostaglandins. Accumulation of these proinflammatory and cytotoxic factors is deleterious to neurons (Chao et al., 1992).

In brain hypoxia/ischemia, expression of inducible nitric oxide synthase (iNOS) increases in infiltrating macrophages and activated microglia (Giulian and Lachman, 1985; Nakashima et al., 1995). Some researchers report that iNOS is in part responsible for ischemic injury (del Zoppo et al., 2000; Eliasson et al., 1999), and inhibition of nitric oxide (NO) production protects neuronal cells from damage caused by oxygen and glucose deficiency (Cardenas et al., 1998; Jiang et al., 2005). Therefore, anti-inflammatory agents are thought to be potentially potent neuroprotective drugs.

Flavonoids are abundant in vegetables and fruits and have been extensively studied for their biological activities. It has

* Corresponding author. Tel.: +82 31 201 2177; fax: +82 31 205 8962.
E-mail address: sunnykim@khu.ac.kr (S.Y. Kim).

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been reported that flavonoids have anti-oxidant, anti-inflammation and anti-cancer activities (Ishige et al., 2001; Middleton et al., 2000). Furthermore, baicalein was recently found to inhibit LPS and cytokine-stimulated microglia cells and wogonin was reported to protect neuronal cells from ischemic damage by blocking microglial activation (Chen et al., 2004; Lee et al., 2003), thus, suggesting that flavonoids may be developed as neuroprotective agents. However, each compound included in flavonoid family shows different biological activity.

Therefore, it is vital to study the connection between structural features of the flavonoids and the corresponding biological activities. In this study, we investigated the relationship between flavonoid structure and inhibitory activity on NO production in microglia. It has been shown that apigenin (5,7,4′-trihydroxyflavone) possesses anti-inflammatory, anti-carcinogen and free radical scavenging effect (Kim et al., 1998), but little is known about any neuroprotective effects that it may possess. We investigated the neuroprotective effect of apigenin using a murine microglia cell line and middle cerebral arterial occlusion (MCAO).

2. Materials and methods

2.1. Cell culture

BV-2 cells, a murine microglia cell line, were maintained in 10 ml of Dulbecco's Modified Eagle's Medium (DMEM, Gibco BRL) supplemented with 5% fetal bovine serum and 1% penicillin-streptomycin (Gibco BRL). Primary microglia cells were cultured from the whole brains of neonatal Sprague–Dawley rats (1 day old). The whole brains were triturated into single cells in DMEM containing 10% FBS and plated into a 75 cm^2 T-flask for 2 weeks. The microglia were detached by mild shaking and applied to a nylon mesh (40 μm, Spectrum, California, USA) in order to remove astrocytes and cell clumps that detached with the microglia. The purity of the microglial cultures was over 95% as judged by immunostaining with an anti-OX-42 antibody (Chemicon, Temecula, CA).

2.2. Measurement of nitrite production and cytotoxicity

In order to measure NO production, BV-2 cells were plated into 96-well plate (3×10^4 cells/well) and treated with 100 ng/ml of LPS (Sigma, St. Louis, MO, USA) in the presence or absence of flavonoids (1, 5, and 10 μM) for 24 h. Nitrite, a soluble oxidation product of NO, in the culture media, was determined using the Griess reaction. The supernatant (50 μl) was harvested and mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% *N*-1-napthylethylenediamine dihydrochloride in 5% phosphoric acid). After 10 min, the absorbance at 540 nm was measured using a microplate reader. Sodium nitrite was used as a standard to calculate NO_2^- concentration. Cell viability was measured using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) assay.

2.3. Scavenging activity against nitric oxide

Sodium nitroprusside (SNP) was dissolved in phosphate buffered saline (PBS) at 100 mM concentration. This SNP solution (50 μl) was mixed with 950 μl PBS containing various concentrations of apigenin (1, 5, and 10 μM). These mixtures were incubated at 25 °C for 2.5 h and nitrite formed through a combination of oxygen and NO released from SNP was measured by Griess reaction.

2.4. Measurement of iNOS enzyme activity

For a measurement of iNOS enzyme activity, we modified the Yen-Chou Chen's method (Chen et al., 2001). BV-2 cells were disseminated onto 96-well plates at a density of 3×10^4 cells/well and allowed to stabilize for 24 h. The cells were treated with 100 ng/ml LPS for 12 h and washed twice with fresh DMEM media. BV-2 cells were then treated with increasing concentrations of apigenin (1, 5, and 10 μM) for 12 h. The supernatant was removed, and the Griess reaction was performed.

2.5. Western blot analysis

The protein sample (80 μg for each) from the BV-2 cells extract was separated by 8% SDS-PAGE and transferred to a nitrocellulose membrane (Amersham pharmacia biotech, Buckinghamshire, UK). The membrane was blocked with 5% skim milk and incubated with the primary antibodies (rabbit anti-iNOS; Transduction Laboratories, San Diego, CA, USA, goat anti-COX-1, COX-2; Santa Cruz, CA, USA, rabbit anti p-p38; stressgen, Victoria, Canada, rabbit anti-p38, ERK, p-ERK, JNK, p-JNK; Cell Signaling, MA, USA) and secondary antibodies (goat anti-rabbit IgG; Amersham pharmacia biotech, Buckinghamshire, UK, donkey anti-goat IgG; Santa Cruz, CA, USA). The blots were developed using ECL Western Blotting Detection Reagents (Amersham pharmacia biotech, Buckinghamshire, UK). Densitometry analysis of bands was performed with the ImageMaster™ 2D Elite software, version 3.1 (Amersham pharmacia biotech, Buckinghamshire, UK).

2.6. PGE_2 measurement

Media were collected and centrifuged 24 h after treatment with LPS (100 ng/ml) in the presence or absence of apigenin. Supernatant from culture medium was harvested and used for measuring level of PGE_2. PGE_2 was measured by competitive enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI, USA) according to manufacturer's direction.

2.7. Middle cerebral arterial occlusion (MCAO)

All experimental procedures were performed according to the Principles of Laboratory Animal Care (NIH publication, #85-23, revised 1985) and the Animal Care and Use Guidelines of Kyung Hee University, Korea. Adult male ICR mice weighing 24–32 g were used for the study. The animals were anesthetized in a chamber with a mixture of N_2O and O_2 (70:30) containing 2.5% isoflurane, and were maintained by the inhalation of 1.5% isoflurane. A middle cerebral artery occlusion (MCAO) was induced using the intraluminal filament method, as described previously (Kilic et al., 2001) with the following modifications. A mid-line incision was made on the ventral surface of the neck, and the right common carotid arteries and external carotid artery were isolated and ligated with an 8.0 silk suture. A polyamide monofilament (Ethilon, Johnson and Johnson Intl, Belgium) coated to a round tip with silicone resin (Xantopren, Byer Dental, Germany) (thread thickness) was introduced into the intracranial internal carotid artery through an incision in the common carotid artery. The filament was carefully advanced approximately 10 mm distal to the carotid bifurcation, which was beyond the origin of the middle cerebral artery. After 90 min of MCAO, the mice were again anesthetized and the occluding filament was withdrawn gently back into the common carotid artery to allow reperfusion to take place. The sham-operated mice underwent the same surgical procedure, except that the filament was not advanced far enough to occlude the middle cerebral artery. The rectal temperature was maintained at 37 ± 0.5 °C throughout the surgery using a heating pad (Biomed S.L, Spain). The heart rate, arterial blood oxygen saturation of arterial hemoglobin and ECG were monitored throughout the procedure (SurgiVet, USA). The mice subjected to MCAO were randomly assigned to receive either drug or the vehicle treatment. Apigenin was dissolved in either saline administered orally (20 mg/kg) 30 min after the MCAO. 3-Methyl-1-phenyl-2-pyrazolium-5-one (MCI-186, Sigma, USA) was used as a positive control. In the case of the MCI-186 treatment, MCI-186 (5 mg/kg) dissolved in saline was injected intravenously at the same time schedule. The mice in the vehicle group underwent the same experimental protocol, only that they received the same volume/weight of the vehicle. After 22.5 h reperfusion, the animals were anesthetized with pentobarbital sodium (60 mg/kg, i.p.) and sacrificed by decapitation. Then their brains were carefully removed. The area of the cerebral infarction was identified using 2,3,5-triphenyltetrazolium chloride (TTC) staining. TTC marks

mitochondrial function and can reliably indicate ischemic areas for up to 3 days after ischemia (Bederson et al., 1986). The area of the infarction (mm^2) in each section was determined using a computerized image analysis system (TDI Scope Eye 3.0).

2.8. Immunohistochemistry

For immunohistochemistry, the brain was perfused *in situ* through the abdominal aorta with 4% paraformaldehyde in phosphate buffer solution (PBS) and removed. The brain was further immersion-fixed for 48 h at room temperature, cryoprotected overnight in 20% sucrose–PBS and rapidly frozen in liquid nitrogen-chilled isopentane. Coronal brain sections, 20 μm thick, were cut using a cryotome. For detection of microglial cells, tissue sections were incubated with the mouse anti-rat CD11b (OX-42, Serotec Ltd., UK) diluted 1:50 in Tris buffer solution (TBS) add 0.2% Triton-x for 30 h at 4 °C in a humid atmosphere. To remove unbound primary antibodies, it was washed three times for 10 min each with TBS add 0.2% Triton-x. Subsequently, the secondary antibody, Cy3-conjugated donkey anti-mouse IgG (Jackson immunoresearch Inc., USA) diluted 1:200 in TBS add 0.2% Triton-x. It was applied for 1 h at room temperature and unbound antibodies were removed as described above. All images were collected using a LSM 510 Laser Scanning Confocal Microscope (Carl Zeiss Inc., Germany). Immunofluorescence images were obtained by excitation at 514 nm with observation between 560 and 615 nm to detect Cy-3.

2.9. Statistical analysis

The data was analyzed using the Statistical Analysis System (PRISM). All the data is expressed as a mean ± standard deviation except for the *in vivo* experiment. Statistical comparisons between the different treatments were performed using one-way ANOVA with Tukey's multiple comparison post-test. p values <0.05, 0.01 and 0.001 were considered significantly.

Table 1
Structures, names of tested flavonoids

Structure	Name	Substituents		
		3′	4′	5′
Flavones	Chrysin	H	H	H
	Apigenin	H	OH	H
	Luteolin	OH	OH	H
Flavonols	Galangin	H	H	H
	Kaempferol	H	OH	H
	Quercetin	OH	OH	H
	Myricetin	OH	OH	OH
Flavanones	Pinocembrin	H	H	H
	Naringenin	H	OH	H

3. Results

3.1. Effect of flavonoids on nitric oxide production in LPS-activated BV-2 cells

To assess the effect of 9 flavonoids (Table 1) on LPS-activated NO production, BV-2 cells were treated with flavonoids with 100 ng/ml LPS. Three groups of flavonoids including flavones (chrysin, apigenin, and luteolin), flavonols (galangin, kaempferol, quercetin, and myricetin) and flavanones (pinocembrin and naringenin) were tested. Five flavonoids (chrysin, apigenin, luteolin, kaempferol, and quercetin) showed inhibitory effects on NO production in dose-dependent manners (Fig. 1). Among tested flavonoids, apigenin is more effective on inhibition of NO production than the others.

3.2. The inhibitory effect of apigenin on NO production

Apigenin inhibited NO production at 5 and 10 μM in a dose dependent manner and showed significant inhibitory effect at 5 and 10 μM (Fig. 1). Also, apigenin similarly reduced NO production in primary microglia cells (Fig. 2A).

NO in LPS-induced BV-2 cells was measured by the accumulation of nitrite, the stable metabolite of NO, in the culture media. At concentrations used in this study, apigenin did not show cytotoxicity according to the results of the MTT assay (data not shown).

The NO scavenging activity of apigenin was examined using sodium nitroprusside (SNP) as a NO donor. NO released from SNP reacts with oxygen to produce nitrite. NO scavenger competes with oxygen in reacting to NO released from SNP solution in PBS. NO scavenging activity of apigenin was not significant (Fig. 2B). Inhibitory effect of apigenin on iNOS enzyme activity was indirectly measured. After 12 h we gave LPS-treatment to BV-2 cells. Apigenin was then added to the cells. Nitrite was measured 12 h later. We found that apigenin at 10 μM reduced the NO production (Fig. 2C).

Western blot analysis was carried out to investigate the effect of apigenin on iNOS protein level induced by LPS in BV-2 cells. LPS-induced iNOS protein expressions were suppressed in dose-dependent manners (Fig. 2D).

3.3. The inhibitory effect of apigenin on PGE_2 production

Prostaglandin E_2 (PGE_2) may be one of the most potent substances in terms of the initiation and propagation of brain inflammation. PGE_2 is sequentially synthesized from arachidonic acid by cyclooxygenase (COX). There are two isoforms of COX. COX-1 is recognized as a constitutive form and COX-2 as an inducible form (Nakayama et al., 1998; Smith et al., 2000). COX-2 mediates inflammatory injury. Stimulation of BV-2 cells with LPS led to the increased production of PGE_2 through COX-2 induction.

To test whether apigenin reduces the production of PGE_2, BV-2 cells were stimulated with LPS in the presence or absence

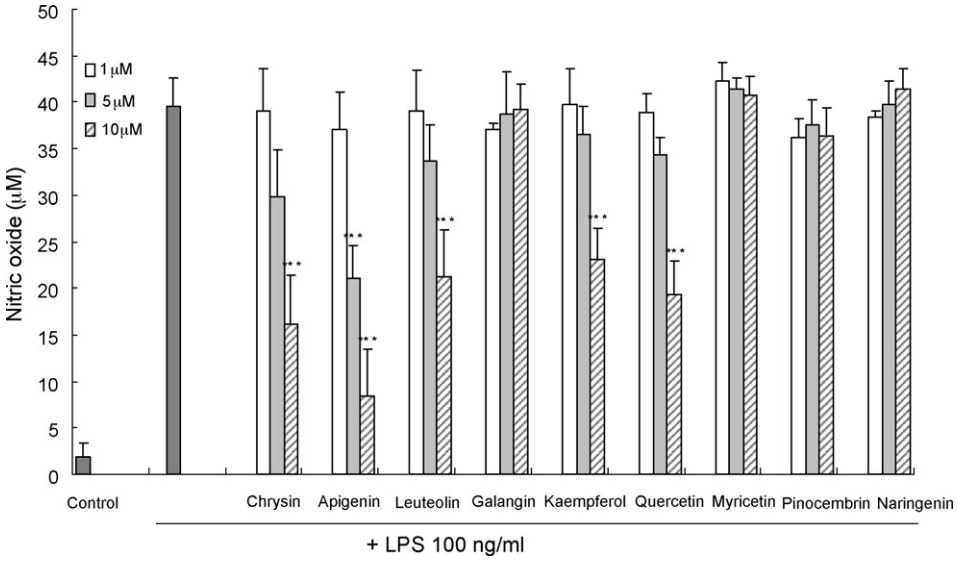

Fig. 1. Effect of flavonoids on LPS-induced NO production in BV-2 cells. Nitrite was measured using Griess reaction at 24 h after treatment with LPS (100 ng/ml) in the presence or absence of flavonoids (1, 5, and 10 μM). All data were presented as the mean ± S.D. of four independent experiments. ***$p < 0.001$ indicate statistically significant differences from treatment with LPS alone.

of apigenin. As shown in Fig. 3A, PGE_2 production was inhibited by apigenin in LPS-activated BV-2 cells.

Western blot was performed to determine whether the inhibitory effect of apigenin on PGE_2 is related to the modulation of the expression of COX-2 enzyme. LPS-induced expression of COX-2 was significantly reduced in 10 μM concentration (Fig. 3B). On the other hand, 10 μM apigenin slightly inhibited COX-1 expression (Fig. 3B).

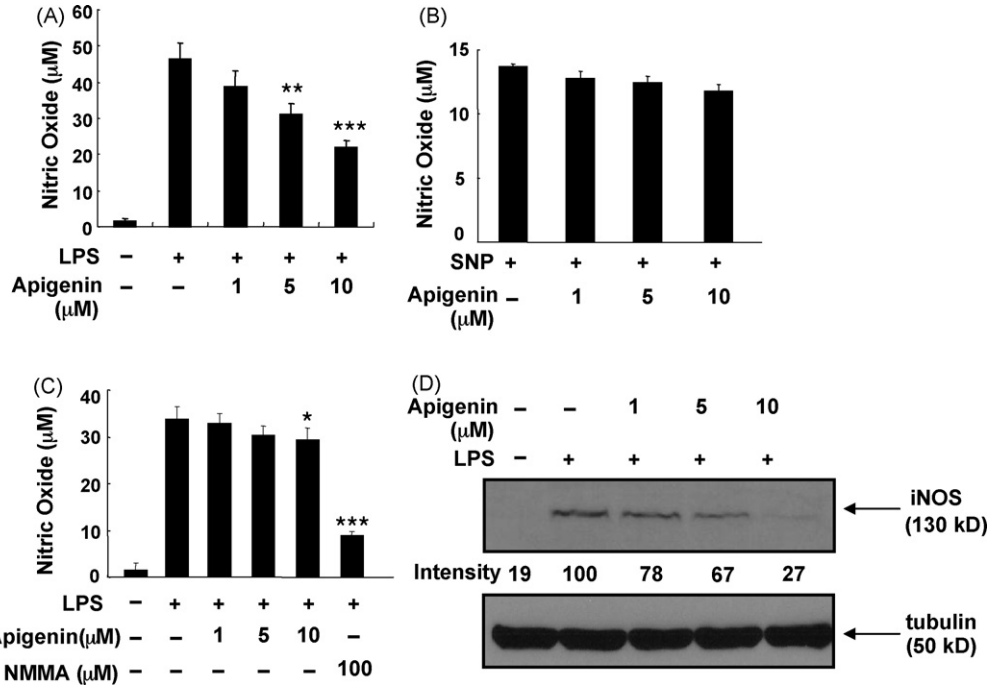

Fig. 2. The inhibitory effect of apigenin on NO production. (A) Effect of apigenin on LPS-induced NO production in primary microglia cells. Nitrite was measured using Griess reaction at 24 h after treatment with LPS (100 ng/ml) in the presence or absence of apigenin (1, 5, and 10 μM). (B) Effect of apigenin on NO scavenging activity. Nitrite was measured after addition of SNP (final 5 mM) to each sample using Griess reaction. (C) Effect of apigenin on iNOS enzyme activity in BV-2 cells. NMMA 100 μM was treated for positive control. (D) Effect of apigenin on iNOS expression in BV-2 cells. iNOS protein was detected using Western blot analysis at 6 h after treatment with LPS (100 ng/ml) in the presence or absence of apigenin (1, 5, and 10 μM). Equal protein loading was confirmed by blotting with anti-tubulin. All data were presented as the mean ± S.D. of three independent experiments. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ indicate statistically significant differences from treatment with LPS or SNP alone.

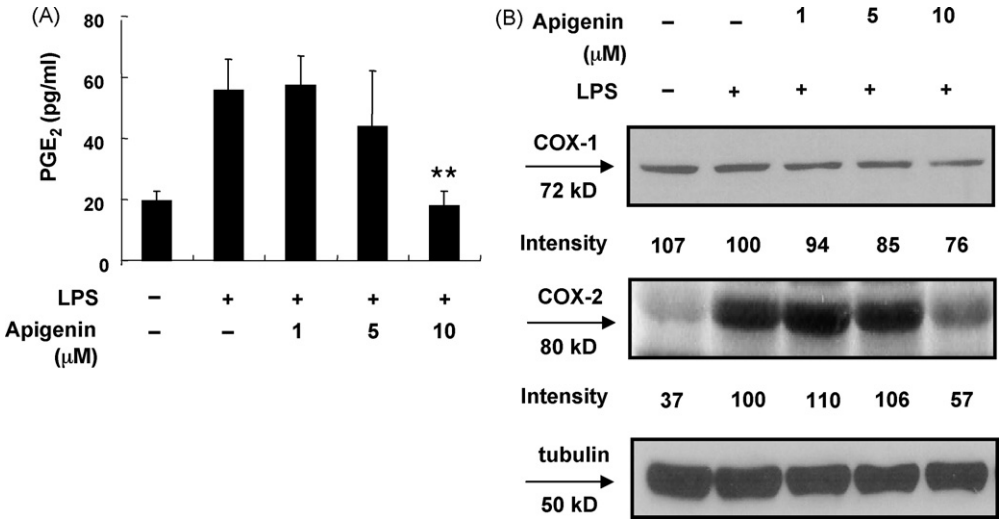

Fig. 3. (A) Effect of apigenin on PGE_2 production in LPS-induced BV-2 cells. PGE_2 was assessed by using competitive enzyme immunoassay kit after treatment with LPS (100 ng/ml) in the presence or absence of apigenin (1, 5, and 10 μM). (B) Effect of apigenin on COX-1 and COX-2 expression in BV-2 cells. COX-1 and COX-2 protein was detected using Western blot analysis at 6 h after treatment with LPS (100 ng/ml) in the presence or absence of apigenin (1, 5, and 10 μM). Equal protein loading was confirmed by blotting with anti-tubulin. All data were presented as the mean ± S.D. of three independent experiments. $^{**}p < 0.01$ indicate statistically significant differences from treatment with LPS alone.

3.4. Effect of apigenin on MAPK activation

To understand the NO inhibitory mechanism by apigenin in BV-2 cells, the effect of apigenin on MAPK, which are upstream signaling molecules in inflammatory reactions, was examined. Western blot analysis was carried out using the phospho- or total forms of antibodies against the MAPK (p38 MAPK, ERK and JNK). BV-2 cells were pretreated with apigenin (10 μM) for 30 min, and then stimulated with LPS for 30 min. As shown in Fig. 4, treatment of BV-2 cells with LPS for 30 min resulted in the strong phosphorylation of p38 MAPK, ERK and JNK. Apigenin did not affect ERK activation

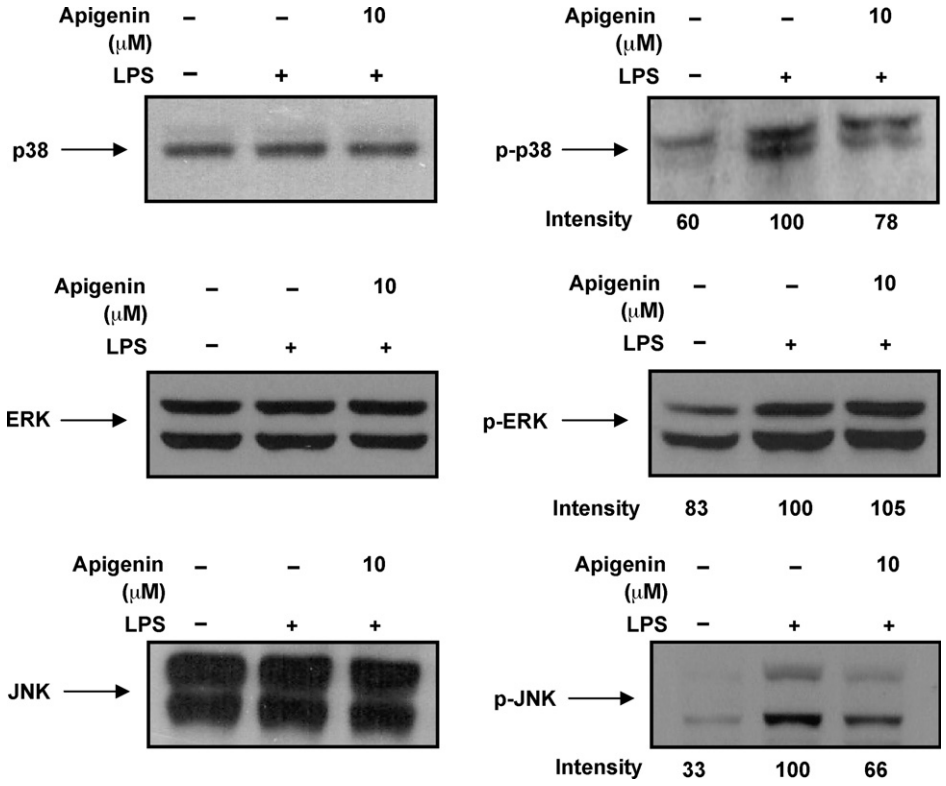

Fig. 4. The effect of apigenin on LPS-induced MAPK activation. BV-2 cells were treated with LPS (100 ng/ml) for 30 min in the presence or absence of apigenin (10 μM), and then p38 MAPK, JNK and ERK activation were assessed by Western blot analysis using antibodies specific for either total kinases or phosphorylated forms of kinases. The results are representatives of three or more independent experiments.

but it inhibited JNK and p38 MAPK phosphorylation in response to LPS.

3.5. Effect of apigenin on infarct volume in MCAO mice

Potential neuroprotective effects of apigenin were examined in adult mice subjected to transient focal cerebral ischemia caused by middle cerebral artery occlusion (MCAO). The area of the cerebral infarction was identified using 2,3,5-triphenyltetrazolium chloride (TTC) staining.

Ninety minutes of MCAO and 22.5 h of reperfusion caused a significant infarction as shown in TTC stained brain sections (Fig. 5A). As shown in Fig. 5B, apigenin (20 mg/kg) reduced the infarct volume significantly ($129.7 \pm 12.6\ mm^3$) as compared to the "MCAO + Vehicle" group ($162.3 \pm 7.1\ mm^3$). 3-Methyl-1-phenyl-2-pyrazolium-5-one (MCI-186) was used as the positive control. The antioxidant MCI-186 was reported to decrease cerebral infarction in the transient MCAO model (Nakashima et al., 1999).

Also, the effect of apigenin on microglial activation was assessed through immunohistochemistry using the OX-42 antibody in the cerebral cortex. To quantify OX-42 positive cells, each cell was counted under a confocal microscope. Apigenin decreased (about 30.4%) the number of microglia as compared to the vehicle treated group (Fig. 6).

4. Discussion

Microglia are believed to play a major role in host defense and tissue repair in the central nervous system (Kreutzberg, 1996). Nevertheless, activation of microglia is observed in brain injuries (Giulian et al., 1994) and is also induced after the exposure of lipopolysaccharide, interferon-γ or β-amyloid (Zielasek and Hartung, 1996). Microglia activation has been considered as a result of neuronal damage. Also, recently it becomes to recognize as a possible cause of the damage in various neurodegenerative diseases. Previous studies have also demonstrated that a decrease of proinflammatory mediators in microglia could attenuate the severity of these disorders (Liu and Hong, 2003; Gao et al., 2003; Eikelenboom and van Gool, 2004). Therefore, modulation of activated microglia is important to the protection of neuronal cells from exposure to inflammatory substances and may have therapeutic potential in neurodegenerative diseases accompanied by microglial activation.

There have been many reports on various drugs having inhibitory effects on neuroinflammation. Minocycline, derivative of tetracycline, exerts neuroprotective effect by inhibiting microglial activation *in vitro* and *in vivo* neurodegenerative disease model (Yrjanheikki et al., 1998, 1999; Wu et al., 2002). Naloxone reduces the neurotoxicity mediated by cytotoxic agents released from microglia *in vitro* and *in vivo* experiment (Liu et al., 2000a,b). Wogonin, one of the flavonoid compounds, was reported to protect neuronal cells by suppressing the activation of microglia cells in the 4-vessel occlusion model (Lee et al., 2003). This suggested that other flavonoid compounds might have anti-neuroinflammatory

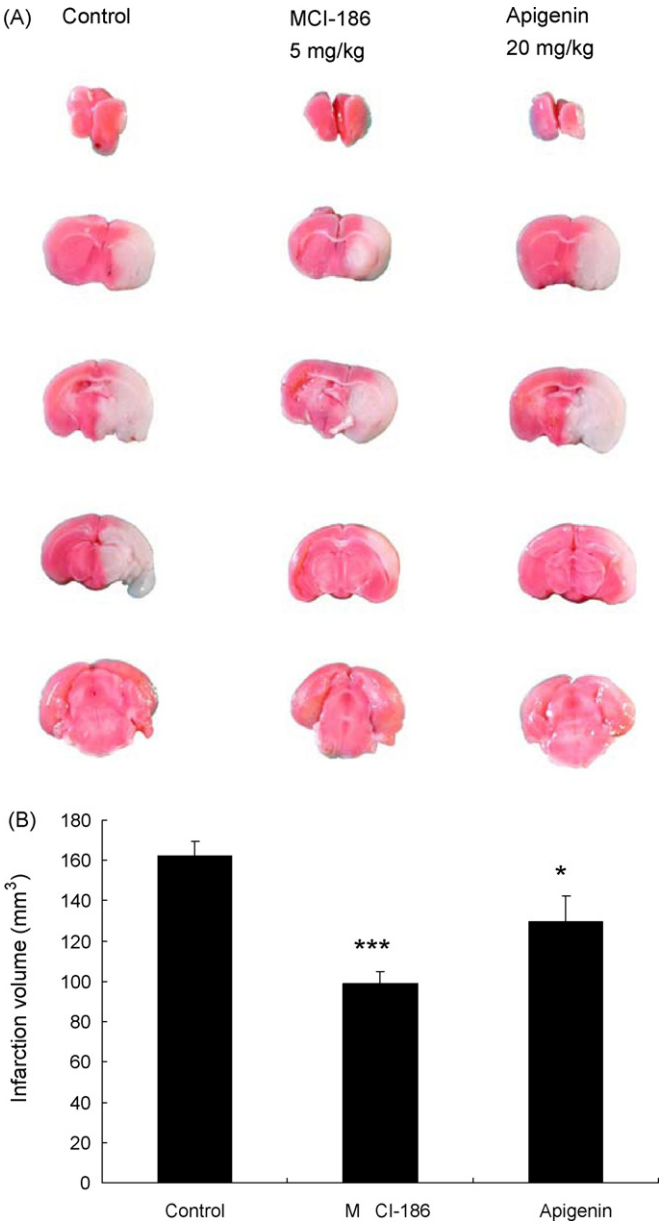

Fig. 5. (A) Representative coronal slices of the brain after MCAO in mice. The slices were stained with TTC after 90 min of MCAO and 22.5 h of reperfusion to show the area of infarct. The pale white area represents infarcted brain tissue. (B) Effect of apigenin on infarct volume after 24 h of transient MCAO. The control group treated with the vehicle; sample group treated with apigenin 20 mg/kg by P.O. MCI-186 (5 mg/kg) was used as the positive control. The data is represented as a mean ± S.E.M. of three independent experiments. Significantly different from the control group (*$p < 0.05$, ***$p < 0.001$).

effects. Therefore we evaluated the effect of the 9 flavonoids on NO production in LPS-treated BV-2 cells.

In terms of chemical structure, flavone showed stronger inhibitory effect on NO production than flavanone, suggesting that 2, 3 of double bond were crucial to prevent NO production. We also observed that the flavonoids that have 4′-hydroxyl groups (apigenin in flavone and kaempferol in flavonol) are more effective at the inhibition of NO production than flavonoids that do not have 4′-hydroxyl group (chrysin in flavone and galangin in flavonol). Therefore, it is reasoned that

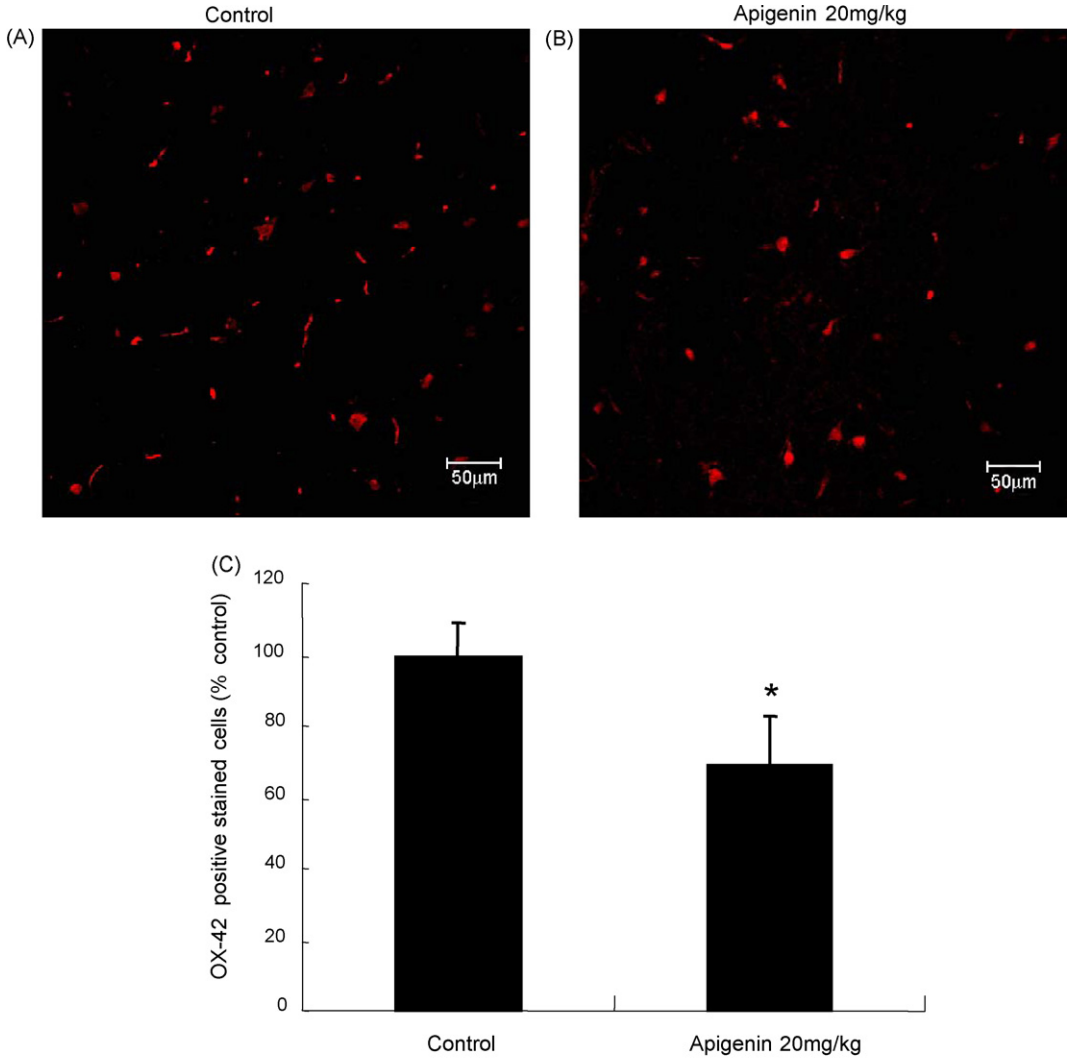

Fig. 6. Effect of apigenin on microglial activation in MCAO mice. Representative photomicrographs of OX-42 positive cells using confocal microscope in the cerebral cortex. Microglia cell activation was identified using the OX-42 antibody. Apigenin treated group (A) decreased (about 32.6%) the number of microglia as compared with vehicle treated group (B). Microglia activation was examined by immunostaining with OX-42 antibody followed by quantification of the positively stained cells (C). The bar represents 50 μm. The data is represented as a mean ± S.E.M. of the three independent experiments. Significantly different from the control group ($*p < 0.05$).

2, 3 of double bond and 4′-hydroxyl group play an important role in inhibiting NO production. Interestingly, naringenin with a 4′-hydroxoyl group did not affect the amount of NO in LPS-treated BV-2 cells. This result indicated that 4′-hydroxyl group linked to 2, 3 of double bond can contribute to the inhibitory effect. Luteolin and myricetin were less effective than apigenin and kaempferol, respectively. The decreased inhibitory activity on NO production has been attributed to the increased hydrophilicity, which may decrease absorption into cells. Moreover, the case of myricetin, which has six OH moieties, did not show inhibitory effect on NO production.

We investigated how apigenin is involved in the inhibition of NO production. There are several mechanisms inhibiting NO production induced by LPS in microglia: suppression of iNOS expression, inhibition of iNOS activity and NO scavenging. Apigenin was observed to have some inhibitory effects on iNOS enzyme activity. However this result is not the main mechanism of the inhibitory effect of apigenin on the NO production. Apigenin strongly inhibited expression of iNOS responsible for the production of NO. Therefore, inhibition of NO by apigenin was due to the reduction of iNOS enzyme expression. In addition, apigenin inhibited NO production in primary microglia cells.

PGE_2, a lipid mediator produced by most mammalian tissues, regulates multiple biological processes under both normal and pathological conditions. PGE_2 is an important mediator associated with inflammation. COX-2 enzyme catalyzes the rate-limiting step in PGE_2 biosynthesis. In this study, apigenin decreased PGE_2 in LPS-stimulated BV-2 cells. Also, LPS did not enhanced COX-1 expression and apigenin slightly suppressed COX-1 expression in 10 μM concentration (Fig. 3B). A previous study showed that apigenin did not affect COX enzyme activity in the cell free system (Al-Fayez et al., 2006). Therefore, reduction of PGE_2 amount by apigenin is

caused by inhibition of COX-2 expression, mediating inflammatory injury.

MAPKs such as p38 MAPK, JNK and ERK play a critical role in the regulation of cell growth and differentiation, and in the control of cellular responses to cytokines and stresses. Also, MAPK is among the most important molecules in the signaling pathways that control the synthesis and release of proinflammatory substances by activated microglia (Koistinaho and Koistinaho, 2002). p38 MAPK is thought to mediate inflammatory responses in various cell types (Chen and Wang, 1999), including microglia (Bhat et al., 1998). p38 MAPK can be predominantly activated by LPS and inflammatory cytokines such as TNF and IL-1, and can play an important role in the expression of a number of proinflammatory molecules (Lee and Young, 1996). JNKs are essential mediators of relevant proinflammatory functions in microglia (Waetzig et al., 2005). ERK signaling pathways appear to play a key role in the down-regulation of iNOS in the IFN-gamma-stimulated microglia (Park et al., 2005). On the other hand, ERK pathway is not essential in NO production induced by LPS (Watters et al., 2002). It has been reported that apigenin is an inhibitor of MAPK but more specifically, an inhibitor of ERK activity (Kuo and Yang, 1995). However, other studies also showed that apigenin-mediated modulation of MAPK activity varies depending on the induction method and cell type (O'Prey et al., 2003; Van Dross et al., 2003; Llorens et al., 2002; Yin et al., 2001). LPS is known to activate a series of MAPK in immune cells (Guha and Mackman, 2001; DeFranco et al., 1995). In the present study, we found that LPS-induced p38 MAPK, JNK phosphorylation were inhibited by apigenin in BV-2 microglia cells, suggesting that apigenin shows anti-inflammatory effect through p38 MAPK, JNK. But apigenin had no significant effect on ERK activation. The present study indicates that apigenin is an inhibitor of p38 MAPK and JNK induced by LPS stimulation in BV-2 microglia.

Based on the anti-neuroinflammatory effect *in vitro*, we applied apigenin into a MCAO model and it attenuated the infarction area. To clarify the relationship between brain injury and microglia cells, activated microglia cells were detected using OX-42 antibody immunohistochemistry. This result proves that apigenin reduced neuronal cell death through inhibition of microglia cell activation. Overall, apigenin has the potential to be developed as a neuroprotective agent, indicating that compounds included in the flavonoid family as well as their derivatives must be considered as candidates of neuroprotective compounds. As for the development of flavonoids as neuroprotective agents, there is one important consideration. Unlike macrophage, microglia cells are in the brain, which means that the flavonoid must penetrate the brain blood barrier (BBB) for attenuating the neuroinflammatory reaction. Although a recent report demonstrated that naringenin is able to traverse the BBB (Youdim et al., 2004), little is known about the relationship between flavonoid structures and the penetration of BBB. In general, low molecular weight and hydrophobic molecules can easily enter the brain through BBB. The hydroxyl group of flavonoids may interfere with the penetration of BBB. Therefore, structural modifications that give flavonoids enhanced hydrophobicity and pharmacological effect would be vital to their development as neuroprotective agents. We are currently studying the relationship between the structure of flavonoids and their neuroprotective effects.

In conclusion, apigenin may act as a useful candidate in the treatment of neurodegenerative diseases.

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