

Anti-inflammatory effects of saponins derived from the roots of *Platycodon grandiflorus* in lipopolysaccharide-stimulated BV2 microglial cells

KYUNG-JUN JANG¹, HONG KI KIM¹, MIN HO HAN², YOU NA OH³, HYUN-MIN YOON¹, YOON HO CHUNG³, GI YOUNG KIM⁴, HYE JIN HWANG^{2,5}, BYUNG WOO KIM^{2,6} and YUNG HYUN CHOI^{2,7}

¹Department of Acupuncture and Moxibustion, Dongeui University College of Oriental Medicine, Busan 614-052;

²Blue-Bio Industry RIC and Anti-Aging Research Center, Dongeui University, Busan 614-714; ³Duksan B&F Co., Ltd., Busan 614-853; ⁴Laboratory of Immunobiology, Department of Marine Life Sciences, Jeju National University, Jeju 690-756;

⁵Department of Food and Nutrition, College of Human Ecology, ⁶Department of Life Science and Biotechnology, College of Natural Science, Dongeui University, Busan 614-714; ⁷Department of Biochemistry, Dongeui University College of Oriental Medicine, Busan 614-052, Republic of Korea

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Abstract. *Radix platycodi* is the root of *Platycodon grandiflorus* A. DC, which has been widely used as a food material and for the treatment of a number of chronic inflammatory diseases in traditional oriental medicine. In this study, the anti-inflammatory effects of the saponins isolated from *radix platycodi* (PGS) on the production of inflammatory mediators and cytokines in lipopolysaccharide (LPS)-stimulated BV2 murine microglial cells were examined. We also investigated the effects of PGS on LPS-induced nuclear factor- κ B (NF- κ B) activation and phosphoinositide 3-kinase (PI3K)/AKT and mitogen-activated protein kinase (MAPK) signaling pathways. Following stimulation with LPS, elevated nitric oxide (NO), prostaglandin E₂ (PGE₂) and pro-inflammatory cytokine production was detected in the BV2 microglial cells. However, PGS significantly inhibited the excessive production of NO, PGE₂ and pro-inflammatory cytokines, including interleukin-1 β (IL-1 β) and tumor necrosis factor- α (TNF- α) in a concentration-dependent manner without causing any cytotoxic effects. In addition, PGS suppressed NF- κ B translocation and inhibited the LPS-induced phosphorylation of AKT and MAPKs. Our results indicate that the inhibitory effect of PGS on LPS-stimulated inflammatory response in BV2 microglial cells is associated with the suppression of NF- κ B activation and the PI3K/AKT and MAPK signaling pathways. Therefore,

these findings suggest that PGS may be useful in the treatment of neurodegenerative diseases by inhibiting inflammatory responses in activated microglial cells.

Introduction

Inflammation is the biological response to harmful stimuli, such as autoimmune diseases, pathogenic infections, damaged cells and irritants; inflammation initiates the healing process to prevent injury. Inflammation increases the expression of inflammatory mediators, such as nitric oxide (NO) and prostaglandin E₂ (PGE₂), which are regulated by NO synthase (NOS) and cyclooxygenases (COXs), respectively, as well as inflammatory cytokines, such as interleukin (IL)-1 β , IL-6 and tumor necrosis factor- α (TNF- α) (1,2). These inflammatory regulators are required to regulate the cellular pathways involved in protecting the organs (3-5). However, excessive inflammatory response can lead to an overexpression of pro-inflammatory factors, which can result in severe inflammatory disorders (5-7).

Microglial cells are the resident macrophage-like cells in the brain and have been proposed to play a major role in host defense and tissue repair in the central nervous system (CNS). In pathological conditions, activated microglial cells release neurotoxic and pro-inflammatory mediators, including NO, PGE₂, reactive oxygen species (ROS) and pro-inflammatory cytokines (8,9). Overproduction of these inflammatory mediators and cytokines causes severe forms of various neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD) and cerebral ischemia (10,11). Not surprisingly then, activated microglial cells have been shown to be a major cellular source of pro-inflammatory and/or cytotoxic factors that cause neuronal damage in the CNS (10,12). Previous studies have also demonstrated that a decrease in the number of pro-inflammatory factors in microglial cells may attenuate the severity of these disorders (9-12). Therefore, agents that attenuate pro-inflammatory mediators and cyto-

Correspondence to: Dr Yung Hyun Choi, Department of Biochemistry, Dongeui University College of Oriental Medicine, Yangjung-dong San 45, Busanjin-gu, Busan 614-052, Republic of Korea
E-mail: choiyh@deu.ac.kr

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kines in microglial cells may represent promising strategies to tackle brain injury and neurodegenerative diseases.

Platycodon grandiflorus (*P. grandiflorus*) A. DC., known as bellflower or balloon flower, belongs to the species in the genus *Platycodon* L. of the family Campanulaceae. This plant is an herbaceous perennial native to Northeastern Asia and widely distributed in South Korea. The root of *P. grandiflorus*, *radix platycodi*, has been consumed as a food and has been used as a folk remedy for conditions such as coughing, the common cold, bronchitis, asthma, pulmonary tuberculosis and inflammation (13,14). *Radix platycodi* is abundant in saponins (15,16). Several studies have reported that various platycodon saponins exhibit strong anti-inflammatory activity by blocking the generation of pro-inflammatory mediators and cytokines through the inhibition of nuclear factor- κ B (NF- κ B) and/or mitogen-activated protein kinase (MAPK) activation in 12-*O*-tetradecanoylphorbol 13-acetate (TPA) and lipopolysaccharide (LPS)-treated macrophages (17-21). Although Jang *et al.* (22) have demonstrated the anti-inflammatory effects of an aqueous extract of *P. grandiflorus* on LPS-stimulated PGE₂ synthesis, NO generation and IL-8 production in BV2 microglial cells, the precise anti-inflammatory mechanisms of action of saponins isolated from *radix platycodi* (PGS) have not yet been elucidated in microglial cells.

In the current study, we investigated the inhibitory effects of PGS and the mechanisms by which PGS induces anti-inflammatory effects by assessing the suppression of anti-inflammatory mediator and cytokine production and expression in an LPS-stimulated BV2 microglial cell model. The results indicated that PGS inhibited the release of NO, PGE₂, IL- β and TNF- α , as well as their regulatory genes, which was associated with the suppression of NF- κ B translocation from the cytosol to the nucleus. The regulation of NF- κ B activity by PGS was also associated with the inhibition of the phosphorylation of AKT and MAPKs in an LPS-induced anti-inflammatory reaction. The data from our study suggest that PGS may be a candidate for use in the treatment of various neurodegenerative brain disorders.

Materials and methods

Cell culture and treatment with PGS. The BV2 murine cell line was maintained in Dulbecco's modified Eagle's medium (Gibco-BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/ml of penicillin and 100 mg/ml of streptomycin (Gibco-BRL) at 37°C in a humidified incubator with 5% CO₂. Confluent cultures were passed by trypsinization. For the preparation of PGS, dried samples of *radix platycodi* were purchased from Dongeui University Hospital (Busan, Korea). The samples were extracted twice with methanol by refluxing at 80°C for 2 h, and then the methanol extract was suspended in water and partitioned sequentially with *n*-hexane, chloroform, ethyl acetate and *n*-butanol. Subsequently, the water-saturated *n*-butanol fraction was evaporated to dryness in a vacuum. The recovered crude saponins were loaded onto Diaion® HP-20 MCI gel (Sigma-Aldrich Chemical Co., St. Louis, MO, USA), and the sugar residues were then removed with 40% CH₃OH. The fractions were eluted with 60-80% CH₃OH, collected, and then dried to obtain PGS. Cells used in the experiments were washed

twice with warm DMEM and treated in serum-free medium for at least 4 h prior to the treatments. In all the experiments, the cells were treated with various concentrations of PGS for the indicated periods of time prior to exposure to 500 ng/ml LPS (*Escherichia coli* 026:B6, Sigma-Aldrich Chemical Co.).

Cell viability assay. Cell viability was measured based on the formation of blue formazan that was metabolized from colorless 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT; Sigma-Aldrich) by mitochondrial dehydrogenases, which are active only in live cells. For this study, BV2 cells were plated in 24-well plates at a density of 2x10⁵ cells/well for 24 h, and then washed. The cells were incubated with various concentrations of PGS in the presence or absence of LPS (500 ng/ml) for 24 h, and then incubated in 0.5 mg/ml of MTT solution. Three hours later, the supernatant was removed, and the formation of formazan was measured at 540 nm using an enzyme-linked immunosorbent assay (ELISA) plate reader (Dynatech MR-7000; Dynatech Laboratories, Chantilly, VA, USA).

NO production. Concentrations of NO in the culture supernatants were determined by measuring the levels of nitrite, which is a major stable product of NO, using Griess reagent (Sigma-Aldrich). For this study, cells (5x10⁵ cells/ml) were treated with PGS in the presence or absence of LPS in 24-well plates for 24 h, and then 100 μ l of each culture medium were mixed with an equal volume of Griess reagent [1% sulfanilamide/0.1% *N*-(1-naphthyl)-ethylenediamine dihydrochloride/2.5% H₃PO₄]. Nitrite levels were determined using an ELISA plate reader at 540 nm, and nitrite concentrations were calculated by referencing a standard curve generated by known concentrations of sodium nitrite.

RNA isolation and reverse transcription-polymerase chain reaction (PCR). Total RNA was isolated using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). Total RNA (1.0 μ g) obtained from the cells was reverse transcribed using Moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega, Madison, WI, USA) to produce DNA. The inducible NO synthase (iNOS), COX-2, IL-1 β and TNF- α genes were amplified from the cDNA by PCR. PCR amplification was carried out for 26 cycles under the following cycling conditions: denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec and extension at 72°C for 1 min. A final extension cycle was then performed at 72°C for 10 min. A sample of each amplified product was subjected to 1.0% agarose gel electrophoresis and stained with ethidium bromide (EtBr; Sigma-Aldrich) and visualized using ultra violet (UV) illumination. The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) housekeeping gene transcript was used as the control. Primer sequences used in the RT-PCR analysis are presented in Table I.

Protein extraction and western blot analysis. Cells were washed 3 times with PBS, and total cell lysates were lysed in extraction buffer [25 mM of Tris-Cl (pH 7.5), 250 mM of NaCl, 5 mM of ethylenediaminetetra acetic acid (EDTA), 1% Nonidet P-40 (NP-40), 0.1 mM of sodium orthovanadate, 2 μ g/ml of leupeptin and 100 μ g/ml of phenylmethylsulfonyl fluoride (PMSF)] containing protease inhibitor cocktail tablets

Table I. Details of the primer pairs used in this study.

Gene	Primer sequence
iNOS	F: 5'-ATG TCC GAA GCA AAC ATC AC-3' R: 5'-TAA TGT CCA GGA AGT AGG TG-3'
COX-2	F: 5'-CAG CAA ATC CTT GCT GTT CC-3' R: 5'-TGG GCA AAG AAT GCA AAC ATC-3'
IL-1 β	F: 5'-ATG GCA ACT GTT CCT GAA CTC AAC T-3' R: 5'-TTT CCT TTC TTA GAT ATG GAC AGG AC-3'
TNF- α	F: 5'-ATG AGC ACA GAA AGC ATG ATC-3' R: 5'-TAC AGG CTT GTC ACT CGA ATT-3'
GAPDH	F: 5'-CGT CTT CAC CAC CAT GGA GA-3' R: 5'-CGG CCA TCA CGC CAC AGT TT-3'

iNOS, inducible nitric oxide synthase; F, forward; R, reverse; COX-2, cyclooxygenase-2; IL-1 β , interleukin-1 β ; TNF- α , tumor necrosis factor- α ; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

(Roche Diagnostics, Mannheim, Germany). In a parallel experiment, cytoplasmic and nuclear proteins were extracted using NE-PER[®] Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer's instructions. Protein concentration was determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA). For western blot analysis, an equal amount of protein was subjected to electrophoresis on sodium dodecyl

sulfate (SDS)-polyacrylamide gels and transferred onto nitrocellulose membranes (Schleicher & Schuell Bioscience, Inc., Keene, NH, USA) by electroblotting. The blots were probed with the desired primary antibodies for 1 h, incubated with the diluted enzyme-linked secondary antibodies (Amersham Co., Arlington Heights, IL, USA), and visualized using the enhanced chemiluminescence (ECL) method according to the recommended procedure (Amersham Co.). The primary antibodies were purchased from BD Biosciences (San Jose, CA, USA), Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA), Cell Signaling Technology, Inc. (Danvers, MA, USA) and Oncogene Science (Cambridge, MA, USA) (Table II). Actin and lamin B were used as the internal controls for cytosolic and nuclear fractions, respectively.

Cytokine assays. The concentrations of TNF- α and IL-1 β were determined using commercially available ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Briefly, BV2 cells (5×10^5 cells/ml) were plated in 24-well plates and pre-treated with the indicated concentrations of PGS for 1 h prior to treatment with 500 ng/ml of LPS. After 24 h of treatment, fluid samples were diluted 1:10,000 in a Tris-buffered saline solution (pH 8.0) containing 1% bovine serum albumin (BSA) and 0.5% Tween-20. They were then incubated overnight in plates at 4°C, after which the plates were washed 5 times. Standard TNF- α and IL-1 β were added to each plate in serial dilutions, and a standard curve was constructed from which the concentrations of TNF- α and IL-1 β were obtained. The absorbance values were determined

Table II. List of antibodies used in this study.

Antibody	Dilution	Product no.	Type of antibody, supplier
iNOS	1:1,000	610333	Rabbit polyclonal, BD Biosciences
COX-2	1:500	SC-1999	Mouse monoclonal, Santa Cruz Biotechnology
TNF- α	1:1,000	3707S	Rabbit polyclonal, Cell Signaling Technology, Inc.
IL-1 β	1:1,000	SC-7884	Rabbit polyclonal, Santa Cruz Biotechnology
NF- κ B p65	1:500	SC-109	Mouse monoclonal, Santa Cruz Biotechnology
AKT	1:500	SC-8312	Rabbit polyclonal, Santa Cruz Biotechnology
p38 MAPK	1:500	SC-728	Rabbit polyclonal, Santa Cruz Biotechnology
ERK	1:2,000	SC-535	Rabbit polyclonal, Santa Cruz Biotechnology
JNK	1:500	9252S	Rabbit polyclonal, Cell Signaling Technology, Inc.
p-AKT	1:500	9271S	Rabbit polyclonal, Cell Signaling Technology, Inc.
AKT	1:500	SC-8312	Rabbit polyclonal, Santa Cruz Biotechnology
p-p38 MAPK	1:500	9211S	Rabbit polyclonal, Cell Signaling Technology, Inc.
p38 MAPK	1:500	sc-535	Rabbit polyclonal, Santa Cruz Biotechnology
p-ERK	1:500	9106S	Mouse monoclonal, Cell Signaling Technology, Inc.
ERK	1:500	SC-154	Rabbit polyclonal, Santa Cruz Biotechnology
p-JNK	1:500	9255S	Mouse monoclonal, Cell Signaling Technology, Inc.
JNK	1:500	9252S	Rabbit polyclonal, Cell Signaling Technology, Inc.
Lamin B	1:1,000	NA12	Mouse monoclonal, Oncogene Science
Actin	1:1,000	SC-1616	Goat polyclonal, Cell Signaling Technology Inc.

iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2; TNF- α , tumor necrosis factor- α ; IL-1 β , interleukin-1 β ; NF- κ B, nuclear factor- κ B; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase.

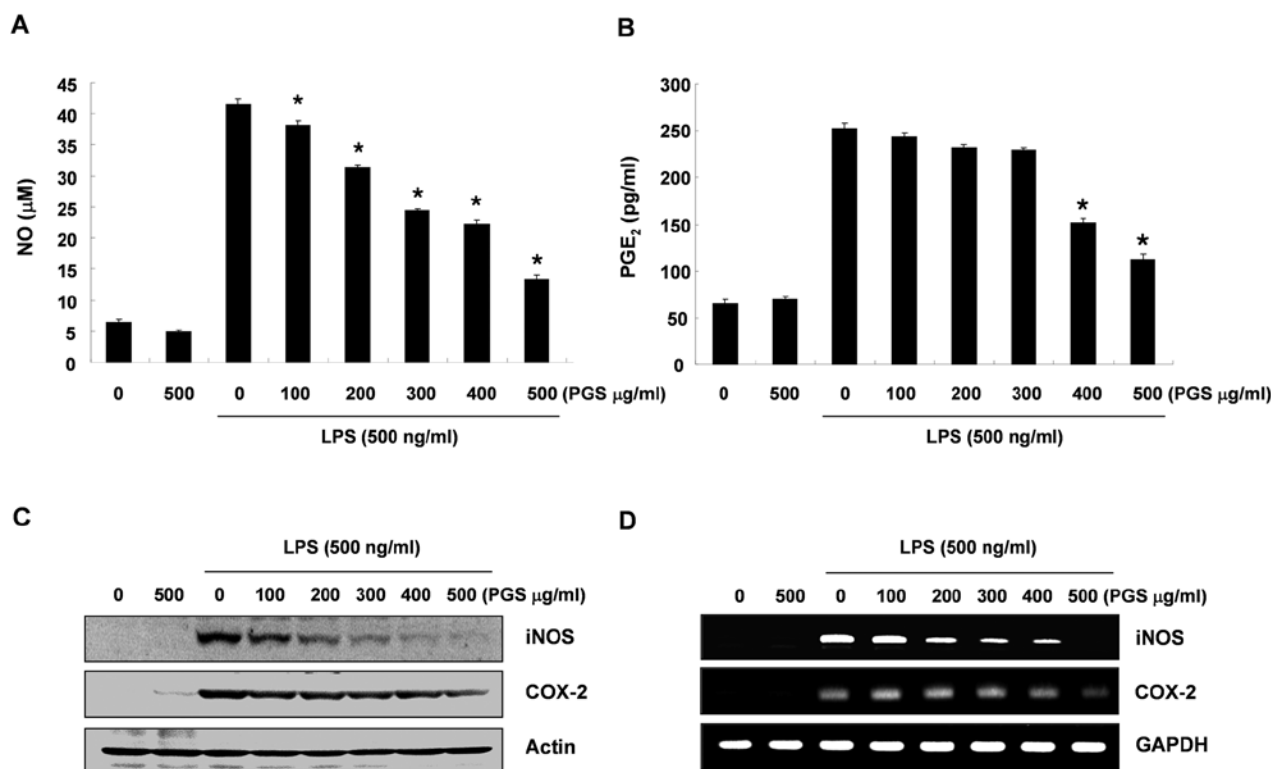


Figure 1. Inhibition of nitric oxide (NO) and prostaglandin E₂ (PGE₂) production by saponins isolated from *radix platycodi* (PGS) in lipopolysaccharide (LPS)-stimulated BV2 microglial cells. BV2 cells were pre-treated with various concentrations of PGS for 1 h prior to incubation with LPS (500 ng/ml) for 24 h. (A and B) Nitrite content was measured using Griess reaction (A), and PGE₂ concentration was measured in the culture medium using a commercial ELISA kit (B). Each value represents the mean \pm SD of 3 independent experiments. * $p < 0.05$, significant difference from the value obtained for the cells treated with LPS in the absence of PGS. (C) Cell lysates were then prepared, and western blot analysis was performed using antibodies specific for murine inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2). (D) Following treatment with LPS for 6 h, total RNA was prepared for RT-PCR analysis of iNOS and COX-2 gene expression in LPS-stimulated BV2 microglial cells. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and actin were used as internal controls for RT-PCR and western blot analysis, respectively. The experiment was repeated 3 times and similar results were obtained.

with an ELISA microplate reader operating at 450 nm. Three ELISA experiments were conducted, with each sample tested in duplicate as previously described (23).

Immunofluorescence analysis. For the detection of NF- κ B p65 translocation, cells were grown on glass coverslips for 24 h and then treated with 500 ng/ml LPS. The cells were either pre-treated or not with PGS for 1 h. The cells were fixed with 3.7% paraformaldehyde, treated with 0.2% Triton X-100, and blocked with 2% BSA. Cells were then sequentially incubated with anti-NF- κ B p65 antibody, FITC-conjugated donkey anti-rabbit IgG and DAPI solution. They were then examined under a fluorescence microscope (Carl Zeiss, Jena, Germany).

Statistical analysis. Data values represent the means \pm SD. Statistical significance was determined using an analysis of variance followed by the Student's t-test. A value of $p < 0.05$ was considered to indicate a statistically significant difference.

Results

Effect of PGS on NO and PGE₂ production in LPS-stimulated BV2 microglial cells. According to the NO detection assay using Griess reagent, treatment with LPS alone markedly induced NO production by the cells when compared to that generated by the control. However, pre-treatment with PGS significantly

repressed the levels of NO production in the LPS-stimulated BV2 microglial cells in a concentration-dependent manner (up to 500 μ g/ml) (Fig. 1A). The amount of PGE₂ present in the culture medium also increased after 24 h of exposure to LPS alone; however, a marked repression was observed after PGS was administered in a concentration-dependent manner (Fig. 1B).

Effect of PGS on iNOS and COX-2 expression in LPS-stimulated BV2 microglial cells. We performed RT-PCR and western blot analysis to determine whether the inhibition of NO and PGE₂ production by PGS in the LPS-stimulated BV2 cells is associated with the decreased levels of iNOS and COX-2, which produce NO and PGE₂ as key mediators of inflammation, respectively. As demonstrated in Fig. 2C, the levels of iNOS and COX-2 proteins were markedly upregulated following 24 h of exposure to LPS; however, PGS significantly inhibited iNOS and COX-2 protein expression in the LPS-stimulated BV2 microglial cells in a concentration-dependent manner. Subsequently, in order to investigate whether PGS suppresses the LPS-mediated induction of iNOS and COX-2 via a pre-translational mechanism, the effects of PGS on iNOS and COX-2 mRNA expression were evaluated. RT-PCR analyses indicated that the reduced iNOS and COX-2 mRNA levels correlated with the corresponding reduction in protein levels (Fig. 2D). These results suggest that PGS-induced reductions

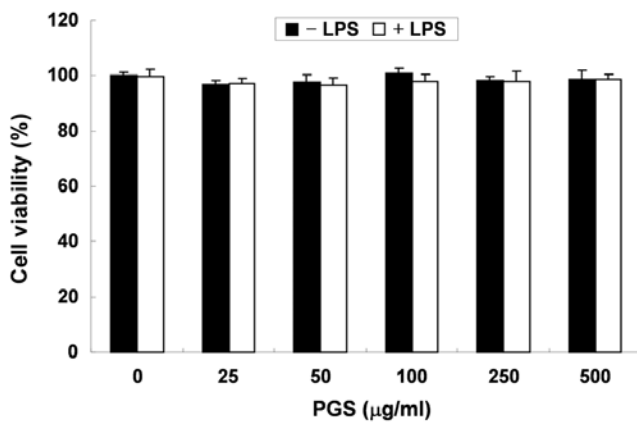


Figure 2. Effects of saponins isolated from *radix platycodi* (PGS) and lipopolysaccharide (LPS) on the cell viability of BV2 microglial cells. Cells were treated with the indicated concentrations of PGS or LPS (500 ng/ml) alone, or pre-treated with PGS for 1 h prior to treatment with LPS; after 24 h, cell viability was assessed using MTT reduction assay. The results are expressed as the percentage of surviving cells over control cells (without the addition of PGS). Each value indicates the mean \pm SD of 3 independent experiments.

in the expression of iNOS and COX-2 inhibited NO and PGE₂ production.

In order to exclude the possibility that the inhibition of NO and PGE₂ production was due to cytotoxicity caused by PGS treatment, MTT assays were performed in BV2 microglial cells treated with PGS for 24 h in the presence or absence of LPS (500 ng/ml). As demonstrated in Fig. 2, at the concentrations (100–500 μ g/ml) used to inhibit NO and PGE₂ production, PGS alone did not affect cell viability. Furthermore, co-treatment with PGS and LPS did not demonstrate any cytotoxic effects. These results clearly indicated that the inhibition of NO and PGE₂ production in LPS-stimulated BV2 cells was not due to the cytotoxic effects of PGS.

Effects of PGS on the LPS-induced production of pro-inflammatory cytokines in BV2 microglial cells. To determine the effects of PGS on LPS-induced inflammatory-associated cytokine production, BV2 cells were treated with various concentrations of PGS in the presence or absence of LPS (500 ng/ml) for 24 h. The production of TNF- α and IL-1 β induced by LPS was evaluated by ELISA. As shown in Fig. 3A, the levels of TNF- α were markedly increased in the culture medium of LPS-stimulated BV2 microglial cells; however, pre-treatment with PGS resulted in a significant decrease in the release of TNF- α in a concentration-dependent manner. In addition, a similar tendency was also observed as regards IL-1 β production (Fig. 3B).

Effects of PGS on the LPS-induced expression of pro-inflammatory cytokines in BV2 microglial cells. Since PGS markedly suppressed the production of TNF- α and IL-1 β in the LPS-treated BV2 cells, we investigated whether the inhibitory effects of PGS on the levels of TNF- α and IL-1 β expression are associated with the inhibitory effects on the release of TNF- α and IL-1 β . As shown in Fig. 3D, the increased IL-1 β and TNF- α mRNA levels as a result of exposure to LPS decreased in a concentration-dependent manner following

treatment with PGS. In a parallel experiment, using western blot analysis, the elevated protein levels of IL-1 β and TNF- α resulting from LPS treatment were also decreased following treatment with PGS (Fig. 3C). These results suggest that PGS is effective in suppressing pro-inflammatory cytokine production by altering the transcriptional levels of IL-1 β and TNF- α in activated microglial cells.

Effect of PGS on LPS-induced NF- κ B translocation in BV2 microglial cells. To further characterize the mechanisms through which PGS inhibits pro-inflammatory responses, we investigated whether PGS prevents the translocation of the p65 subunit of NF- κ B to the nucleus. The immunoblot analysis results presented in Fig. 4A and B show that the amount of NF- κ B p65 in the nucleus was markedly increased following exposure to LPS; however, the LPS-induced p65 level in the nuclear fractions was reduced as a result of pre-treatment with PGS. Furthermore, we wished to confirm the inhibition of LPS-induced NF- κ B activation by PGS by immunofluorescence microscopy assay. As shown in Fig. 4C, similar results were observed. These results suggest that PGS inhibits NF- κ B activation in BV2 microglial cells by suppressing I κ B degradation and the nuclear translocation of NF- κ B.

Inhibition of LPS-induced AKT activation by PGS in LPS-stimulated BV2 microglial cells. Previous studies have indicated that the phosphoinositide 3-kinase (PI3K)/AKT signaling molecule triggers NF- κ B activation through I κ B degradation (24,25). Therefore, we determined the activation levels of AKT at various time points following the stimulation of BV2 cells with LPS and the effect of PGS on LPS-induced AKT activation. As shown in Fig. 5A, although the amount of non-phosphorylated AKT was unaffected by either PGS or LPS treatment, the phosphorylation of AKT showed a marked increase within 15 min following stimulation with LPS. However, pre-treatment with PGS resulted in significant blockage of LPS-induced AKT phosphorylation. These results indicate that the inhibition of pro-inflammatory mediator and cytokine expression by PGS in LPS-stimulated BV2 microglial cells is associated with the inactivation of the PI3K/AKT signaling pathway.

Inhibition of LPS-induced MAPK activation by PGS in LPS-stimulated BV2 microglial cells. To further elucidate the molecular targets of PGS in further upstream signaling pathways, we examined the effect of PGS on the activity of MAPKs, such as extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 MAPK, which regulate the induction of several genes encoding inflammatory factors. As indicated in Fig. 5B, the stimulation of BV2 cells with LPS induced the rapid activation of ERK, JNK and p38 MAPK, with the peak levels of each phosphorylated MAPK occurring 15 to 60 min after the addition of LPS without altering their unphosphorylated forms. However, pre-treatment with PGS significantly inhibited MAPK phosphorylation in LPS-stimulated BV2 microglial cells. These findings suggest that PGS is capable of disrupting MAPK signal transduction pathways activated by LPS in BV2 microglial cells, which subsequently prevents the production of pro-inflammatory mediators and cytokines.

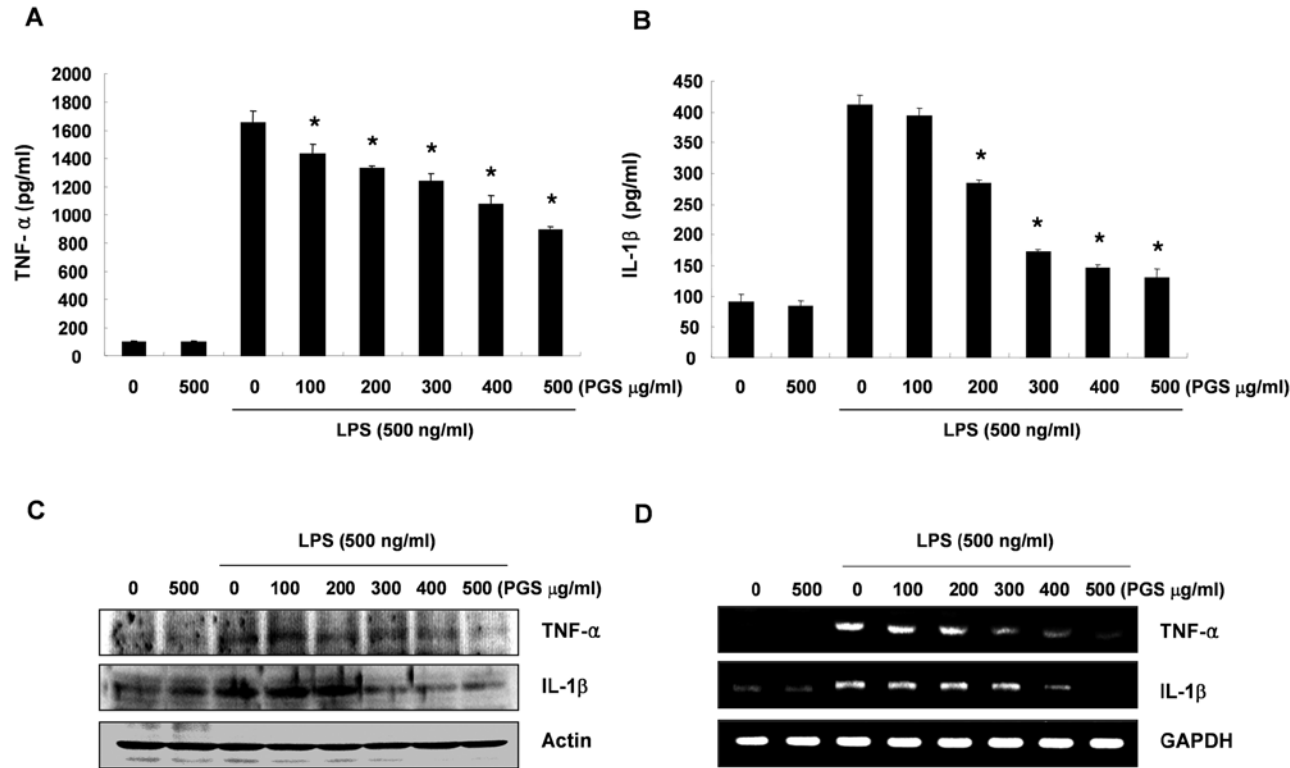


Figure 3. Effect of saponins isolated from *radix platycodi* (PGS) on lipopolysaccharide (LPS)-stimulated tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β) production in BV2 microglial cells. BV2 cells were pre-treated with various concentrations of PGS for 1 h prior to treatment with LPS (500 ng/ml). (A and B) After incubation for 24 h, the levels of TNF- α (A) and IL-1 β (B) present in the supernatants were measured using ELISA kits. Each value indicates the mean \pm SD of 3 independent experiments. * p <0.05, significant difference from the value obtained for cells treated with LPS in the absence of PGS. (C) Cell lysates were then prepared, and western blot analysis was performed using antibodies specific for murine TNF- α and IL-1 β . (D) Following treatment with LPS for 6 h, total RNA was prepared for RT-PCR analysis of TNF- α and IL-1 β gene expression in LPS-stimulated BV2 microglial cells. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and actin were used as internal controls for RT-PCR and western blot analysis, respectively. The experiment was repeated 3 times, and similar results were obtained.

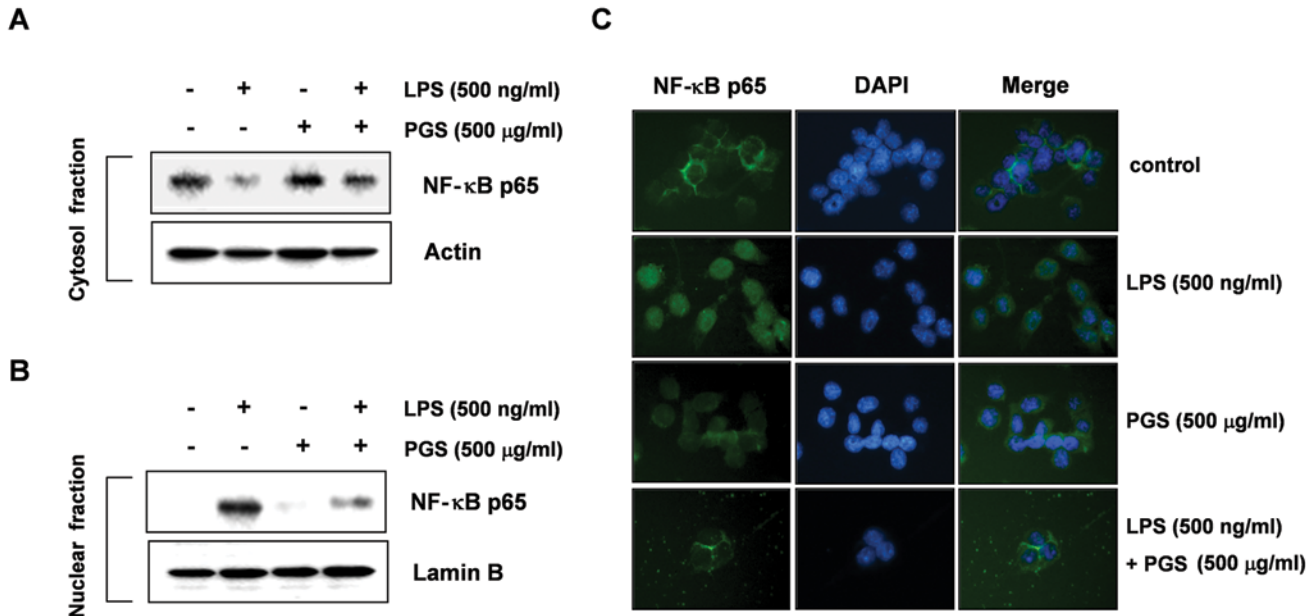


Figure 4. Effects of saponins isolated from *radix platycodi* (PGS) on nuclear factor- κ B (NF- κ B) translocation in lipopolysaccharide (LPS)-stimulated BV2 microglial cells. (A and B) Cells were treated with PGS (500 μ g/ml) for 1 h prior to treatment with LPS (500 ng/ml) for 1 h. (A) Cytosolic and (B) nuclear proteins were subjected to 10% SDS-polyacrylamide gels followed by western blot analysis using anti-NF- κ B p65 antibody. Results are representative of those obtained from 2 independent experiments. Actin and lamin B were used as internal controls for cytosolic and nuclear fractions, respectively. (C) Cells were pre-treated with 500 μ g PGS for 1 h prior to stimulation with LPS (500 ng/ml) for 1 h. Localization of NF- κ B p65 was visualized under a fluorescence microscope after immunofluorescence staining with NF- κ B p65 antibody (green). In addition, the cells were stained with DAPI to visualize nuclei (blue). Results are representative of those obtained from 3 independent experiments.

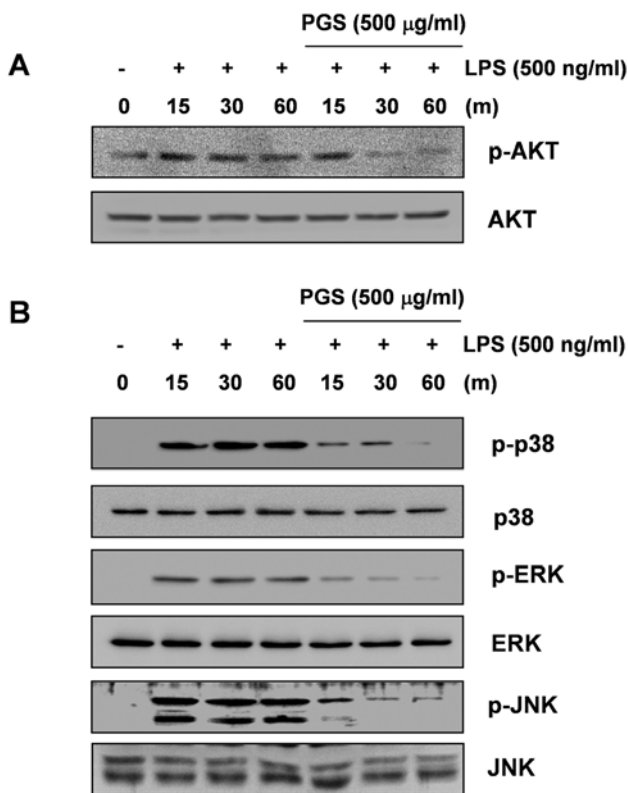


Figure 5. Effects of saponins isolated from *radix platycodi* (PGS) on the phosphorylation of AKT and mitogen-activated protein kinases (MAPKs) induced by lipopolysaccharide (LPS) in BV2 microglial cells. Cells were treated with PGS (500 µg/ml) 1 h prior to treatment with LPS (500 ng/ml) for the indicated periods of time. Total proteins (30-50 µg) were subjected to 10% SDS-polyacrylamide gels, followed by western blot analysis using (A) anti-AKT and (B) anti-p38 MAPK, anti-ERK and anti-JNK anti-bodies. Results are representative of those obtained from 3 independent experiments.

Discussion

In this study, in order to evaluate the cellular and molecular mechanisms by which PGS exerts its anti-inflammatory effects, the inhibitory effects of PGS on the production of LPS-induced pro-inflammatory mediators and cytokines in BV2 microglial cells were investigated. The present results demonstrate that PGS significantly exhibits anti-inflammatory activities via the attenuation of pro-inflammatory factors in LPS-treated BV2 microglial cells. The inhibitory effects were also mediated through the inhibition of the NF-κB, PI3K/AKT and MAPK signaling pathways. Therefore, on the basis of the anti-inflammatory effects of PGS, *P. grandiflorus* may be a possible therapeutic candidate for the treatment of neurodegenerative diseases.

COXs are the enzymes that catalyze the conversion of arachidonic acid to prostaglandin H₂ (PGH₂) and PGH₂ is the precursor of a variety of biologically active mediators, such as PGE₂, prostacyclin and thromboxane A₂. COXs exist as two major isozymes: COX-1, a constitutive COX and COX-2, an isoform that is induced during the response to a number of stimulants and is activated at the site of inflammation (26-28). Previous studies have indicated that LPS, a microbe-derived ligand, significantly activates microglial cells and induces the COX-2 gene, leading to the synthesis of PGE₂ (26,28). A number of studies have also reported that COX-2 is associ-

ated with cytotoxicity in brain diseases since the inhibition of COX-2 induction and/or activity reduces brain injury following ischemia and slows the progression of AD, PD and cerebral ischemia (29,30). In addition, NO has been shown to be an important regulatory molecule for diverse physiological functions, including vasodilation, neural communication and host defense (31,32). In mammalian cells, NO is synthesized from three different isoforms of NOS: endothelial NOS (eNOS), neuronal NOS (nNOS) and iNOS. It has been reported that iNOS is not usually expressed in the brain, but activated microglial cells are a major cellular source of iNOS in the brain. The excessive release of NO by activated microglial cells correlates with the progression of neurodegenerative disorders (33,34). Collectively, inflammatory mediators, including PGE₂ and NO, are responsible for much of the neuronal damage (35,36). In the present study, PGS significantly suppressed LPS-stimulated PGE₂ and NO production in BV2 microglial cells, which appears to be due to the transcriptional suppression of both COX-2 and iNOS (Fig. 1). This inhibition occurred in a concentration-dependent manner and PGS did not exhibit any cytotoxic effects (Fig. 2).

The neuro-inflammatory response in activated microglial cells produces elevated levels of pro-inflammatory cytokines, including TNF-α and IL-1β (37,38). These cytokines have been shown to induce neuronal cell damage; therefore, suppressing their production is important for the prevention of neurodegenerative diseases (39,40). TNF-α is primarily produced by activating monocytes, macrophages and T cells, and it exerts various pro-inflammatory effects. The major producers of TNF-α in the brain are microglial cells, and they may play a role in several pathological conditions in the brain (41,42). IL-1β is also a potent pro-inflammatory cytokine that acts through the IL-1 receptors found on numerous cell types, including neurons and microglial cells. Moreover, IL-1β has been shown to be an important mediator of neuroimmune interactions that participate directly in neurodegeneration (43,44). Thus, the inhibition of cytokine production or function serves as a key mechanism in the control of neurodegeneration. In the current study, treatment with PGS prior to exposure to LPS significantly attenuated the production of IL-1β and TNF-α in BV2 microglial cells and this was associated with the downregulation of their transcriptional activities (Fig. 3). Taken together, our results indicate that PGS at non-toxic concentrations may be a promising candidate for the treatment of neurodegenerative diseases caused by microglial cell activation in the brain.

The excessive production of pro-inflammatory components in over-activated microglial cells may be a risk factor for initiating neurodegenerative disease through a number of cell signaling pathways. Among them, the nuclear transcriptional factor, NF-κB, is a key regulator of inflammation due to its ability to induce the transcription of pro-inflammatory genes, which are modulated by the binding of NF-κB to specific promoter regions (40,45). NF-κB is usually located in the cytoplasm where it is complexed with the inhibitory IκB (IκB) protein. In response to pro-inflammatory stimuli, IκB is phosphorylated and subsequently degraded, and NF-κB is released and translocated to the nucleus where it promotes the expression of inflammation-associated genes that are involved in the production of pro-inflammatory cytokines and enzymes (40,46). In addition, the involvement of the PI3K/AKT signaling pathway in inflam-

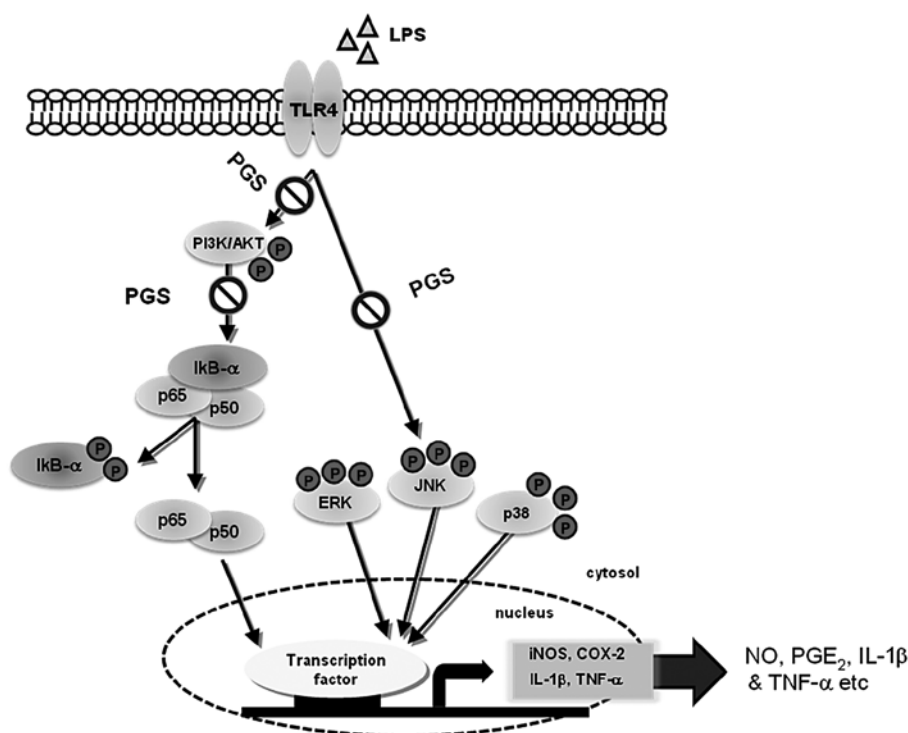


Figure 6. Schematic representation of the mechanism behind the saponins isolated from *radix platycodi* (PGS)-induced anti-inflammatory response. The results revealed that the treatment of BV2 microglial cells with PGS inhibited lipopolysaccharide (LPS)-induced nitric oxide (NO) and prostaglandin E₂ (PGE₂) production by suppressing inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) mRNA and protein expression. PGS also inhibited the production of pro-inflammatory cytokines, such as TNF-α and IL-1β, by suppressing their transcriptional activity. These effects were exerted by the attenuation of NF-κB translocation from the cytoplasm to the nucleus, which was accompanied by the blocking of phosphoinositide 3-kinase (PI3K)/Akt and mitogen-activated protein kinase (MAPK) signaling pathways.

matory mediator expression in microglial cells through NF-κB activation has been demonstrated (25,47,48). Furthermore, it is well known that the blockade of NF-κB transcriptional activity and the PI3K/AKT signaling pathway in microglial cells can also suppress the expression of iNOS, COX-2 and pro-inflammatory cytokines, such as IL-1β, IL-6 and TNF-α (38,46,49). Therefore, modulating the NF-κB and PI3K/AKT signaling pathways is considered a promising strategy for the treatment of several neuropathological disorders. Our results indicated that PGS inhibited the LPS-induced nuclear translocation of the p65 subunit of NF-κB in BV2 microglial cells (Fig. 4), suggesting that PGS inhibits the expression of pro-inflammatory genes by suppressing LPS-induced NF-κB activity. Furthermore, PGS markedly attenuated AKT activation in LPS-stimulated BV2 microglial cells (Fig. 5A), indicating that PGS inhibits LPS-induced NF-κB activation by inactivating the PI3K/AKT signaling pathway. Therefore, the inhibition of the NF-κB and PI3K/AKT signaling pathways in microglial cells as a result of PGS may result in the downregulation of pro-inflammatory mediators and cytokines, resulting in an anti-inflammatory effect.

MAPKs, including ERK, JNK and p38 MAPK, mediate important signaling responses in the immune and inflammatory systems, as well as in the regulation of cellular activities, including mitosis, cell proliferation and survival and gene expression. In general, the JNK and p38 MAPK pathways are activated by pro-inflammatory cytokines, such as IL-1β and TNF-α, microbial endotoxins (e.g., LPS), or cellular stress; however, the ERK pathway is activated by mitogenic

stimuli (50,51). The activation of the p38 MAPK pathway is crucial for a number of immune and inflammatory response-related functions in macrophages (51,52). Furthermore, the expression of TNF-α and IL-1β are strongly regulated by p38 MAPK (53,54). Therefore, further experiments were performed to determine whether PGS regulates MAPK activation to induce anti-inflammatory effects in LPS-stimulated BV2 cells. Our results revealed that LPS induced the phosphorylation of all three classes of MAPKs in BV2 microglial cells, and that PGS markedly inhibited their phosphorylation (Fig. 5B). However, the amounts of non-phosphorylated ERK, JNK and p38 MAPK were unaffected by treatment with PGS and LPS, indicating that PGS diminished the LPS-mediated pro-inflammatory response via the inhibition of MAPK activation in BV2 microglial cells.

In conclusion, we found that PGS significantly attenuated the release of neurotoxic pro-inflammatory mediators, including NO and PGE₂, which are regulated by iNOS and COX-2 and pro-inflammatory cytokines, TNF-α and IL-1β, in LPS-stimulated microglial cells (Fig. 6). The inhibitory effects of PGS were accompanied by the attenuation of NF-κB activity through the prevention of NF-κB translocation from the cytoplasm to the nucleus, which was associated with the inactivation of the PI3K/AKT signaling pathway. In addition, the levels of phosphorylated MAPKs were significantly downregulated by pre-treatment with PGS in LPS-stimulated microglial cells. These results indicate that PGS exerts its anti-inflammatory effects by inhibiting NF-κB activation and the PI3K/AKT and MAPK signaling pathways. The data presented in this study

suggest that PGS may ultimately prove useful in the treatment of inflammatory diseases and several neurodegenerative diseases that are associated with microglial cell activation.

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References

- Knowles RG and Moncada S: Nitric oxide synthases in mammals. *Biochem J* 298: 249-258, 1994.
- O'Banion MK: Cyclooxygenase-2: molecular biology, pharmacology, and neurobiology. *Crit Rev Neurobiol* 13: 45-82, 1999.
- Abrams J: Beneficial actions of nitrites in cardiovascular disease. *Am J Cardiol* 77: 31C-37C, 1996.
- Hou YC, Janczuk A and Wang PG: Current trends in the development of nitric oxide donors. *Curr Pharm Des* 5: 417-441, 1999.
- Rock RB and Peterson PK: Microglia as a pharmacological target in infectious and inflammatory diseases of the brain. *J Neuroimmune Pharmacol* 1: 117-126, 2006.
- Kaur C and Ling EA: Antioxidants and neuroprotection in the adult and developing central nervous system. *Curr Med Chem* 15: 3068-3080, 2008.
- Deng YY, Lu J, Ling EA and Kaur C: Role of microglia in the process of inflammation in the hypoxic developing brain. *Front Biosci (Schol Ed)* 3: 884-900, 2011.
- Perry VH and Gordon S: Macrophages and microglia in the nervous system. *Trends Neurosci* 92: 273-274, 1988.
- Napoli I and Neumann H: Microglial clearance function in health and disease. *Neuroscience* 158: 1030-1038, 2009.
- Eikelenboom P and van Gool WA: Neuroinflammatory perspectives on the two faces of Alzheimer's disease. *J Neural Transm* 111: 281-294, 2004.
- Loane DJ and Byrnes KR: Role of microglia in neurotrauma. *Neurotherapeutics* 7: 366-377, 2010.
- Liu B and Hong JS: Role of microglia in inflammation-mediated neurodegenerative diseases: mechanisms and strategies for therapeutic intervention. *J Pharmacol Exp Ther* 304: 1-7, 2003.
- Germida JJ, Siciliano SD, Freitas JR and Seib AM: Diversity of root-associated bacteria associated with field-grown canola (*Brassica napus* L.) and wheat (*Triticum aestivum* L.). *FEMS Microbiol Ecol* 26: 43-50, 1998.
- Lee JY, Hwang WI and Lim ST: Antioxidant and anticancer activities of organic extracts from *Platycodon grandiflorum* A. De Candolle roots. *J Ethnopharmacol* 93: 409-415, 2004.
- Ishii H, Tori K, Tozjo T and Yoshimura Y: Structures of polygalactin-D and -D2, platycodin-D and -D2, and their monoacetates, saponins isolated from *Platycodon grandiflorum* A. DC., determined by carbon-13 nuclear magnetic resonance spectroscopy. *Chem Pharm Bull* 26: 674-677, 1978.
- Fu WW, Shimizu N, Dou DQ, Takeda T, Fu R, Pei YH and Chen YJ: Five new triterpenoid saponins from the roots of *Platycodon grandiflorum*. *Chem Pharm Bull (Tokyo)* 54: 557-560, 2006.
- Kim YP, Lee EB, Kim SY, Li D, Ban HS, Lim SS, Shin KH and Ohuchi K: Inhibition of prostaglandin E2 production by platycodin D isolated from the root of *Platycodon grandiflorum*. *Planta Med* 67: 362-364, 2001.
- Wang C, Schuller Levis GB, Lee EB, Levis WR, Lee DW, Kim BS, Park SY and Park E: Platycodin D and D3 isolated from the root of *Platycodon grandiflorum* modulate the production of nitric oxide and secretion of TNF-alpha in activated RAW 264.7 cells. *Int Immunopharmacol* 4: 1039-1049, 2004.
- Yoon YD, Kang JS, Han SB, Park SK, Lee HS, Kang JS and Kim HM: Activation of mitogen-activated protein kinases and AP-1 by polysaccharide isolated from the radix of *Platycodon grandiflorum* in RAW 264.7 cells. *Int Immunopharmacol* 4: 1477-1487, 2004.
- Ahn KS, Noh EJ, Zhao HL, Jung SH, Kang SS and Kim YS: Inhibition of inducible nitric oxide synthase and cyclooxygenase II by *Platycodon grandiflorum* saponins via suppression of nuclear factor-kappaB activation in RAW 264.7 cells. *Life Sci* 76: 2315-2328, 2005.
- Chung JW, Noh EJ, Zhao HL, Sim JS, Ha YW, Shin EM, Lee EB, Cheong CS and Kim YS: Anti-inflammatory activity of prosapogenin methyl ester of platycodin D via nuclear factor-kappaB pathway inhibition. *Biol Pharm Bull* 31: 2114-2120, 2008.
- Jang MH, Kim CJ, Kim EH, Kim MG, Leem KH and Kim J: Effects of *Platycodon grandiflorum* on lipopolysaccharide-stimulated production of prostaglandin E2, nitric oxide, and interleukin-8 in mouse microglial BV2 cells. *J Med Food* 9: 169-174, 2006.
- Bae DS, Kim YH, Pan CH, Nho CW, Samdan J, Yansan J and Lee JK: Protopine reduces the inflammatory activity of lipopolysaccharide-stimulated murine macrophages. *BMB Rep* 45: 108-113, 2012.
- Madrid LV, Wang CY, Guttridge DC, Schottelius AJ, Baldwin AS Jr and Mayo MW: Akt suppresses apoptosis by stimulating the transactivation potential of the RelA/p65 subunit of NF-kappaB. *Mol Cell Biol* 20: 1626-1638, 2000.
- Wei J and Feng J: Signaling pathways associated with inflammatory bowel disease. *Recent Pat Inflamm Allergy Drug Discov* 4: 105-117, 2010.
- Fiebig BL, Lieb K, Kammerer N and Hüll M: Synergistic inhibitory effect of ascorbic acid and acetylsalicylic acid on prostaglandin E2 release in primary rat microglia. *J Neurochem* 86: 173-178, 2003.
- Liang X, Wu L, Wang Q, Hand T, Bilak M, McCullough L and Andreasson K: Function of COX-2 and prostaglandins in neurological disease. *J Mol Neurosci* 33: 94-99, 2007.
- Scher JU and Pillinger MH: The anti-inflammatory effects of prostaglandins. *J Investig Med* 57: 703-708, 2009.
- Levi G, Minghetti L and Aloisi F: Regulation of prostanoid synthesis in microglial cells and effects of prostaglandin E2 on microglial functions. *Biochimie* 80: 899-904, 1998.
- Giovannini MG, Scali C, Prosperi C, Bellucci A, Pepeu G and Casamenti G: Experimental brain inflammation and neurodegeneration as model of Alzheimer's disease: protective effects of selective COX-2 inhibitors. *Int J Immunopathol Pharmacol* 16: 31-40, 2003.
- MacMicking J, Xie QW and Nathan C: Nitric oxide and macrophage function. *Ann Rev Immunol* 15: 323-330, 1997.
- Mitchell JA, Larkin S and Williams TJ: Cyclooxygenase-2: regulation and relevance in inflammation. *Biochem Pharmacol* 50: 1535-1542, 1995.
- Mulrennan SA and Redington AE: Nitric oxide synthase inhibition: therapeutic potential in asthma. *Treat Respir Med* 3: 79-88, 2004.
- Cau SB, Carneiro FS and Tostes RC: Differential modulation of nitric oxide synthases in aging: therapeutic opportunities. *Front Physiol* 3: 218, 2012.
- Iadecola C and Ross ME: Molecular pathology of cerebral ischemia: delayed gene expression and strategies for neuroprotection. *Ann NY Acad Sci* 835: 203-217, 1997.
- del Zoppo G, Ginis I, Hallenbeck JM, Iadecola C, Wang X and Feuerstein GZ: Inflammation and stroke: putative role for cytokines, adhesion molecules and iNOS in brain response to ischemia. *Brain Pathol* 10: 95-112, 2000.
- Boka G, Anglade P, Wallach D, Javoy-Agid F, Agid Y and Hirsch EC: Immunocytochemical analysis of tumor necrosis factor and its receptors in Parkinson's disease. *Neurosci Lett* 172: 151-154, 1994.
- Hunot S, Dugas N, Faucheux B, Hartmann A, Tardieu M, Debré P, Agid Y, Dugas B and Hirsch EC: FcepsilonRII/CD23 is expressed in Parkinson's disease and induces, in vitro, production of nitric oxide and tumor necrosis factor-alpha in glial cells. *J Neurosci* 19: 3440-3447, 1999.
- De Nardin E: The role of inflammatory and immunological mediators in periodontitis and cardiovascular disease. *Ann Periodontol* 6: 30-40, 2001.
- Viscido A, Aratari A, Maccioni F, Signore A and Caprilli R: Inflammatory bowel diseases: clinical update of practical guidelines. *Nucl Med Commun* 26: 649-655, 2005.
- Sawada M, Kondo N, Suzumura A and Marunouchi T: Production of tumor necrosis factor-alpha by microglia and astrocytes in culture. *Brain Res* 491: 394-397, 1989.
- Owens T: Identification of new therapeutic targets for prevention of CNS inflammation. *Expert Opin Ther Targets* 6: 203-215, 2002.

43. Rothwell N, Allan S and Toulmond S: The role of interleukin 1 in acute neurodegeneration and stroke: pathophysiological and therapeutic implications. *J Clin Invest* 100: 2648-2652, 1997.
44. Suzumura A, Takeuchi H, Zhang G, Kuno R and Mizuno T: Roles of glia-derived cytokines on neuronal degeneration and regeneration. *Ann NY Acad Sci* 1088: 219-229, 2006.
45. Lee JY, Jhun BS, Oh YT, Lee JH, Choe W, Baik HH, Ha J, Yoon KS, Kim SS and Kang I: Activation of adenosine A3 receptor suppresses lipopolysaccharide-induced TNF- α production through inhibition of PI3-kinase/Akt and NF- κ B activation in murine BV2 microglial cells. *Neurosci Lett* 396: 1-6, 2006.
46. Mankan AK, Lawless MW, Gray SG, Kelleher D and McManus R: NF- κ B regulation: the nuclear response. *J Cell Mol Med* 13: 631-643, 2009.
47. Lee JW, Lee MS, Kim TH, Lee HJ, Hong SS, Noh YH, Hwang BY, Ro JS and Hong JT: Inhibitory effect of infliximab on nitric oxide generation and iNOS expression via inhibition of NF- κ B activation. *Mediators Inflamm* 2007: 93148, 2007.
48. Lee YH, Jeon SH, Kim SH, Kim C, Lee SJ, Koh D, Lim Y, Ha K and Shin SY: A new synthetic chalcone derivative, 2-hydroxy-3',5,5'-trimethoxychalcone (DK-139), suppresses the Toll-like receptor 4-mediated inflammatory response through inhibition of the Akt/NF- κ B pathway in BV2 microglial cells. *Exp Mol Med* 44: 369-377, 2012.
49. Moon DO, Choi YH, Kim ND, Park YM and Kim GY: Anti-inflammatory effects of beta-lapachone in lipopolysaccharide-stimulated BV2 microglia. *Int Immunopharmacol* 7: 506-514, 2007.
50. Garrington TP and Johnson GL: Organization and regulation of mitogen-activated protein kinase signaling pathways. *Curr Opin Cell Biol* 11: 211-218, 1999.
51. Johnson GL and Lapadat R: Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. *Science* 298: 1911-1912, 2002.
52. Ashwell JD: The many paths to p38 mitogen-activated protein kinase activation in the immune system. *Nat Rev Immunol* 6: 532-540, 2006.
53. Cuenda A, Rouse J, Doza YN, Meier R, Cohen P, Gallagher TF, Young PR and Lee JC: SB 203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin-1. *FEBS Lett* 364: 229-233, 1995.
54. Barone FC, Irving EA, Ray AM, Lee JC, Kassiss S, Kumar S, Badger AM, Legos JJ, Erhardt JA, Ohlstein EH, Hunter AJ, Harrison DC, Philpott K, Smith BR, Adams JL and Parsons AA: Inhibition of p38 mitogen-activated protein kinase provides neuroprotection in cerebral focal ischemia. *Med Res Rev* 21: 129-145, 2001.