Purpurogallin exerts anti-inflammatory effects in lipopolysaccharide-stimulated BV2 microglial cells through the inactivation of the NF-κB and MAPK signaling pathways

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Abstract. In this study, we investigated the inhibitory effects of purpurogallin, a naturally occurring phenol, on the production of lipopolysaccharide (LPS)-induced pro-inflammatory mediators and cytokines in BV2 microglia cells. The cells were pre-treated or not with various concentrations of purpurogallin and then stimulated with 0.5 μg/ml LPS. Cell viability was measured by MTT assay. We also measured the production of nictric oxice (NO) and prostaglandin E_2 (PGE_2). Our data indicated that treatment with purpurogallin significantly inhibited the excessive production of NO and PGE_2 in LPS-stimulated BV2 microglial cells. These inhibitory effects were associated with the downregulation of key enzymes for NO and PGE_2, inducible NO synthase (iNOS) and cyclooxygenase-2 (COX2) expression, respectively. Purpurogallin also attenuated the production of pro-inflammatory cytokines, including interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) by suppressing their mRNA and protein expression. The molecular mechanisms underlying the purpurogallin-mediated attenuation of inflammation in BV2 cells closely correlated with the suppression of the translocation of the nuclear factor-κB (NF-κB) p65 subunit into the nucleus and the degradation of the inhibitor of NF-κB (IκB). Moreover, purpurogallin exhibited anti-inflammatory properties by suppressing the phosphatidylinositol 3-kinase/Akt and mitogen-activated protein kinase signaling pathways. These findings suggest that purpurogallin exerts neuroprotective effects through the suppression of pro-inflammatory pathways in activated microglia.

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Introduction

Microglia are the resident immune cells in the central nervous system (CNS) and function as the first and main form of active immune defense in the CNS. Under normal conditions, microglia play a major role in host defense and tissue repair in the CNS (1,2). However, the prolonged activation of microglia can cause chronic neuroinflammation due to the increased production of neurotoxic and pro-inflammatory mediators, including nitric oxide (NO), prostaglandin E_2 (PGE_2), reactive oxygen species (ROS) and pro-inflammatory cytokines, such as interleukin (IL)-1β, IL-6 and tumor necrosis factor-α (TNF-α) (3-5), eventually leading to neuronal death. This is a common characteristic found in the initiation and progression of neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson's disease (PD), cerebral ischemia, multiple sclerosis and trauma (6-8). Thus, regulating microglial activation and the downregulation of pro-inflammatory molecules in microglia may have the therapeutic potential to reduce neuronal injury or death in the treatment of neurodegenerative diseases.

Flavonoids are a diverse group of plant natural products synthesized from phenylpropanoid and acetate-derived precursors. They are becoming an important source of novel agents with pharmaceutical potential and have attracted a great deal of attention over the years for their role in the preven-

tion of chronic diseases (9-12). Among them, purpurogallin (2,3,4,5-tetrahydroxybenzo[7]annulen-6-one) is a benzotropolone-containing natural product, which occurs in the nut gall of *Quercus* spp. (13,14). Purpurogallin is synthesized by the chemical oxidation of pyrogallol and has been shown to have biological properties, such as hydroxyl radical-scavenging properties (15) and to protect erythrocytes against lysis induced by peroxyl radicals (16). Previous studies have indicated that this compound inhibits prolyl endopeptidases (17), human immunodeficiency virus 1 integrase (18) and hydroxyestradiol methylation by catechol-*O*-methyltransferase (19). In addition purpurogallin has also been known to have anticancer activities through the inhibition of the DNA synthesis of tumor cells (20), tyrosine-specific protein kinases (13) and the interaction between Bcl-xL and BH3 peptides (20-22). Moreover, a number of studies have reported the antioxidant and anti-inflammatory effects of purpurogallin (15,23-27); however, the actual molecular mechanisms involving signal transduction cascades underlying the purpurogallin-induced anti-inflammatory effects have not yet been elucidated.

In the present study, we investigated the the anti-inflammatory effects of purpurogallin and the mechanisms by which it inhibits inflammation and the production of inflammatory mediators in lipopolysaccharide (LPS)-stimulated murine BV2 microglial cells. Our findings suggest that purpurogallin may be a candidate for use in the treatment of various neurodegenerative disorders.

Materials and methods

Cell culture and purpurogallin treatment. BV2 murine microglial cells were obtained from Professor I.W. Choi of Inje University College of Medicine (Busan, Korea). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin and were maintained in a humidified incubator with 5% CO_2. Purpurogallin was obtained from Professor T.H. Kim of Daegu Haany University (Gyeongsan, Korea) and dissolved in dimethyl sulfoxide (DMSO) and dilutions were made in DMEM. The final concentration of DMSO in the medium was <0.0005% (v/v) which showed no effects on cell growth. In all the experiments, the cells were pre-treated with the indicated concentrations of purpurogallin for 1 h prior to the addition of LPS (Sigma-Aldrich, St. Louis, MO, USA).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cell viability was measured based on the formation of blue formazan metabolized from colorless MTT (Sigma-Aldrich) by mitochondrial dehydrogenases, which are active only in live cells. In brief, BV2 cells were plated into 24-well plates at a density of $2x10^5$ cells/well for 24 h and then washed. The cells incubated with various concentrations of purpurogallin for 1 h were treated with or without 0.5 μg/ml LPS for 24 h and then incubated in 0.5 mg/ml MTT solution. Three hours later, the supernatant was removed and the formation of formazan was measured at 540 nm using a microplate reader (Dynatech MR-7000; Dynatech Laboratories, El Paso, TX, USA).

Measurement of NO production. Using the Griess reaction, nitrite was measured in the culture supernatants as an indicator of NO production. Aliquots of culture supernatants from each sample were mixed with an equal volume of Griess reagent [1% sulfanilamide/0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride/2.5% H_3PO_4]. NO concentration was determined by measuring the absorbance at 540 nm using a microplate spectrophotometer. Nitrite concentration was calculated with reference to the standard curve of sodium nitrite generated by known concentrations (28).

Measurement of PGE_2 production. BV2 cells were incubated with purpurogallin in the presence or absence of LPS (0.5 μg/ml) for 24 h. Following the manufacturer's instructions, a volume of 100 μl of culture supernatant was collected for the determination of PGE_2 concentration by ELISA (Cayman Chemical Co., Ann Arbor, MI, USA).

Reverse transcriptase-polymerase chain reaction (PCR). Total RNA was prepared using an RNeasy kit (Qiagen, La Jolla, CA, USA) and primed with random hexamers for the synthesis of complementary DNA using AMV Reverse Transcriptase (Amersham Corp., Arlington Heights, IL, USA) according to the manufacturer's instructions. PCR was performed using a Mastercycler (Eppendorf, Hamburg, Germany). The PCR primers were as follows: mouse iNOS (5'-ATG TCC GAA GCA AAC ATC AC-3' and 5'-TAA TGT CCA GGA AGT AGG TG-3'), COX-2 (5'-CAG CAA ATC CTT GCT GTT CC-3' and 5'-TGG GCA AAG AAT GCA AAC ATC-3'), IL-1β (5'-ATG GCA ACT GTT CCT GAA CTC AAC T-3' and 5'-TTT CCT TTC TTA GAT ATG GAC AGG AC-3'), and TNF-α (5'-ATG AGC ACA GAA AGC ATG ATC-3' and 5'-TAC AGG CTT GTC ACT CGA ATT-3'). The following conditions were used for the PCR reactions: 1 x (94˚C for 3 min); 35 x (94˚C for 45 sec; 58˚C for 45 sec; and 72˚C for 1 min); and 1 x (72˚C for 10 min). The resulting amplification products were separated electrophoretically on a 1% agarose gel and visualized by ethidium bromide (EtBr; Sigma-Aldrich) staining. In a parallel experiment, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control.

Protein extraction and western blot analysis. For the preparation of total proteins, the cells were gently lysed for 30 min with lysis buffer (20 mM sucrose, 1 mM EDTA, 20 μM Tris-Cl, pH 7.2, 1 mM DTT, 10 mM KCl, 1.5 mM $MgCl_2$, 5 μg/ml pepstatin A, 10 μg/ml leupeptin and 2 μg/ml aprotinin). Supernatants were collected and protein concentrations were determined using the Bio-Rad Protein Assay kit (Bio-Rad, Hercules, CA, USA). For western blot analysis, an equal amount of protein was subjected to electrophoresis on SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane (Schleicher & Schuell, Keene, NH, USA) by electroblotting. The blots were probed with the desired antibodies for 1 h, incubated with the diluted enzyme-linked secondary antibodies and visualized by enhanced chemiluminescence (ECL) western blotting detection reagents (SuperSignal; Thermo Fisher Scientific, Rockford, IL, USA) according to the recommended procedures. In a parallel experiment, cells were washed with ice-cold PBS and scraped; cytoplasmic and

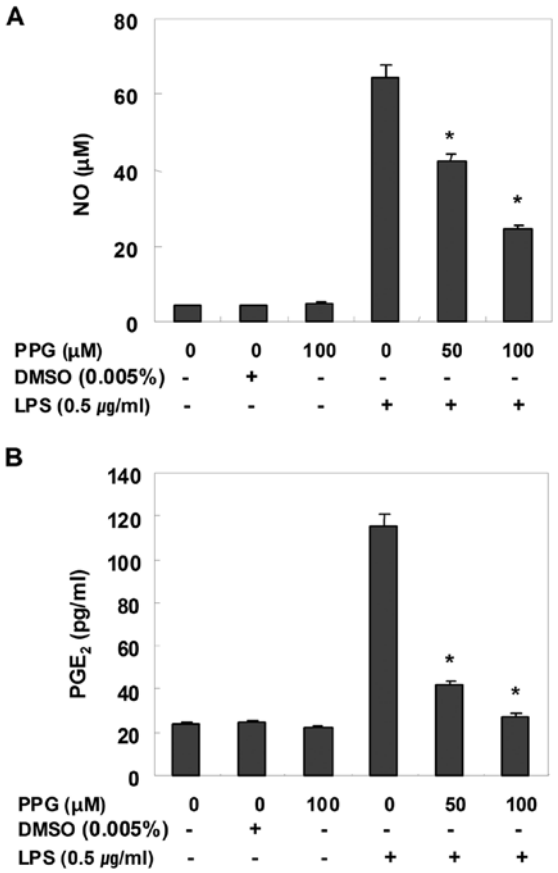

Figure 1. Inhibition of nitric oxide (NO) and prostaglandin E_2 (PGE_2) production by purpurogallin in lipopolysaccharide (LPS)-stimulated BV2 microglial cells. BV2 cells were pre-treated with 50 and 100 μM purpurogallin for 1 h prior to incubation with LPS (0.5 μg/ml) for 24 h. (A) Nitrite content was measured using the Griess reaction. (B) Sample treatment conditions were identical to those described for (A), and a commercially available ELISA kit was used for the measurement of PGE_2 in the resulting supernatants. Each value indicates the mean ± SD and is representative of results obtained from 3 independent experiments. *P<0.05 indicates a significant difference from the value obtained for the cells treated with LPS in the absence of purpurogallin. PPG, purpurogallin.

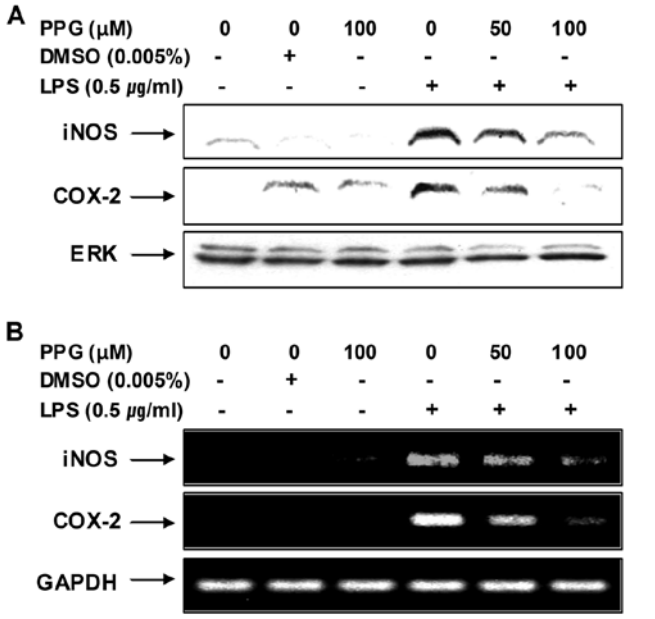

Figure 2. Inhibition of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) expression by purpurogallin in lipopolysaccharide (LPS)-stimulated BV2 microglial cells. (A) BV2 cells were pre-treated with purpurogallin 1 h prior to incubation with LPS (0.5 μg/ml) for 24 h. Cell lysates were then prepared and western blot analysis was performed using anti-iNOS and anti-COX-2 antibodies. (B) 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. ERK and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as internal controls for western blot analysis and RT-PCR, respectively. The experiment was repeated 2 times and similar results were obtained. PPG, purpurogallin.

nuclear proteins were then extracted using NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology, Rockford, IL, USA). Actin and lamin B were used as the internal controls for the cytosolic and nuclear fraction, respectively.

Enzyme immunosolvent assay (ELISA). The levels of IL-1β and TNF-α were measured using ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Briefly, BV2 cells ($5x10^5$ cells/ml) were plated in 24-well plates and pre-treated with the indicated concentrations of purpurogallin for 1 h before treatment with 0.5 μg/ml LPS for 24 h. A total of 100 μl of culture supernatants were collected for the determination of the IL-1β and TNF-α concentration by ELISA as previously described (29).

Statistical analyses. Data represent the means ± SD. Statistical significance was determined using an analysis of variance, followed by a Student's t-test. A value of P<0.05 was considered to indicate a statistically significant difference.

Results

Purpurogallin inhibits NO and PGE_2 production in LPS-stimulated BV2 microglial cells. To determine the inhibitory effects of purpurogallin on LPS-induced NO production in BV2 microglial cells, NO levels in the cell culture medium were measured by the Griess assay. As shown in Fig. 1A, compared with the control, treatment with LPS alone resulted in a marked induction of NO production; however, treatment with purpurogallin resulted in a significant inhibition of LPS-induced NO production in a dose-dependent manner. PGE_2, another important inflammatory mediator, was also evaluated by ELISA. The effects of purpurogallin on the production of PGE_2 in LPS-stimulated BV2 microglial cells were investigated. As shown in Fig. 2B, the treatment of BV2 cells with LPS resulted in a marked increase in the release of PGE_2 when compared with the untreated control; however, treatment with purpurogallin resulted in a dose-dependent decrease in LPS-induced PGE_2 production. These results suggest that pre-treatment with purpurogallin significantly suppresses the expression of LPS-mediated pro-inflammatory mediators, such as NO and PGE_2.

Purpurogallin decreases the expression of LPS-induced inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2) mRNA and protein. In order to determine whether the inhibitory effects of purpurogallin on NO and PGE_2 production were

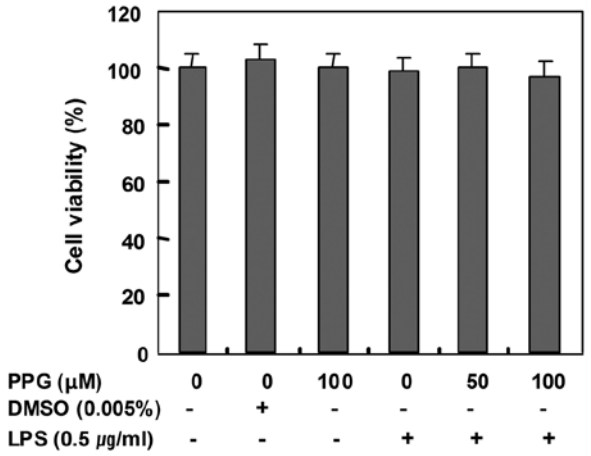

Figure 3. Effects of purpurogallin on the viability of BV2 microglial cells. BV2 cells were incubated in the presence or absence of purpurogallin (50 or 100 μM) and lipopolysaccharide (LPS; 0.5 μg/ml) for 24 h. Cell viability was determined by MTT assay. Values represent the means ± SD of 3 independent experiments. PPG, purpurogallin.

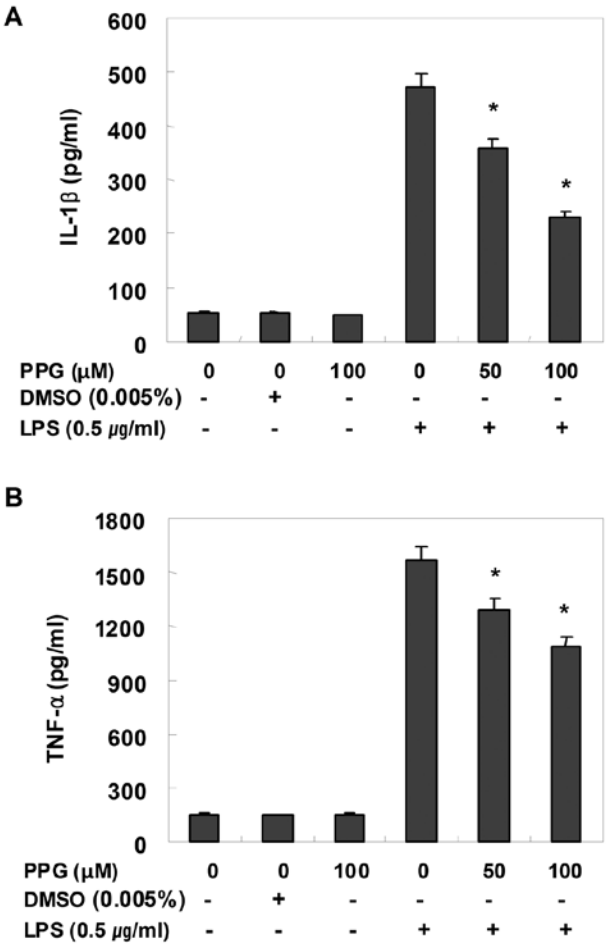

Figure 4. Effects of purpurogallin on lipopolysaccharide (LPS)-induced interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) production in BV2 microglial cells. BV2 cells were pre-treated with the indicated concentrations of purpurogallin for 1 h prior to treatment with LPS (0.5 μg/ml). Following incubation for 24 h, the levels of (A) IL-1β and (B) TNF-α present in the supernatants were measured. Each value indicates the mean ± SD and is representative of results obtained from 3 independent experiments. *P<0.05 indicates a significant difference from the value obtained for the cells treated with LPS in the absence of purpurogallin. PPG, purpurogallin.

associated with the modulation of iNOS and COX-2 expression, we examined the mRNA and protein levels of iNOS and COX-2 by RT-PCR and western blot analysis, respectively. As shown in Fig. 2, iNOS and COX-2 mRNA levels were observed 6 h following treatment with LPS, whereas the protein levels of these enzymes were detected in the whole cell lysates 24 h after treatment with LPS. However, treatment with purpurogallin resulted in a significant decrease in the mRNA and protein induction of iNOS and COX-2 in the LPS-stimulated BV2 microglial cells. These results indicate that the purpurogallin-induced reduction in the expression of iNOS and COX-2 is responsible for the inhibition of NO and PGE_2 production.

Effects of purpurogallin on cell viability. In order to exclude any cytotoxic effects of purpurogallin in BV2 microglial cells, we evaluated the viability of BV2 cells incubated with or without 0.5 μg/ml LPS in the absence or presence of purpurogallin using an MTT assay. The concentrations (50 to 100 μM) used for the inhibition of NO and PGE_2 production in the present study did not affect cell viability (Fig. 3), confirming that the inhibition of NO and PGE_2 production in the LPS-stimulated BV2 cells was not due to the cytotoxic effects of purpurogallin.

Purpurogallin suppresses the induction of inflammatory cytokines in LPS-stimulated BV2 microglial cells. Using EIA and RT-PCR, we also analyzed the effects of purpurogallin on pro-inflammatory cytokines, such as TNF-α and IL-1β. For this study, BV2 cells were incubated with 50 and 100 μM purpurogallin in the presence or absence of LPS for 24 h and then cytokine levels in the culture medium were measured. As shown in Fig. 4, TNF-α and IL-1β levels were significantly increased in the culture medium of LPS-stimulated BV2 microglial cells. However, pre-treatment with purpurogallin resulted in a significant decrease in the levels of these cytokines in a concentration-dependent manner. In a parallel experiment, using RT-PCR, we studied the effects of purpurogallin on LPS-induced IL-1β and TNF-α mRNA expression. As shown in Fig. 5, IL-1β and TNF-α mRNA transcription also decreased following treatment with purpurogallin. These results suggest that purpurogallin is effective in the suppression of pro-inflammatory cytokine production through the alteration of the transcription levels of IL-1β and TNF-α in activated microglia.

Purpurogallin blocks nuclear factor-κB (NF-κB) activation in LPS-stimulated BV2 microglial cells. NF-κB is the most important transcription factor involved in the regulation of pro-inflammatory mediators and cytokines, including iNOS, COX-2, TNF-α and IL-1β in activated microglial cells (30-32); therefore, we investigated whether purpurogallin blocks the NF-κB signaling pathway. For this purpose, cytosolic and nuclear levels of inhibitor of NF-κB (IκB) and NF-κB p65 subunits were analyzed. As indicated in Fig. 6, LPS stimulation induced IκB degradation in the cytosol and the translocation of the NF-κB p65 subunit into the nucleus of BV2 cells;

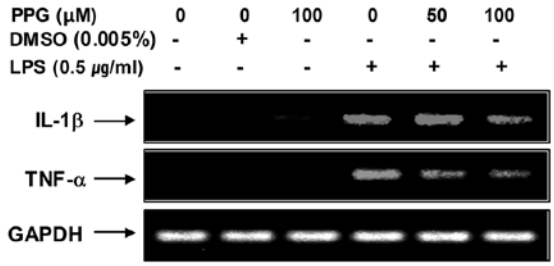

Figure 5. Effects of purpurogallin on lipopolysaccharide (LPS)-induced interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) mRNA expression in BV2 microglial cells. BV2 cells were pre-treated with the indicated concentrations of purpurogallin for 1 h prior to treatment with LPS (0.5 μg/ml), and total RNA was isolated 6 h after treatment with LPS. RT-PCR was performed to determine the levels of IL-1β and TNF-α mRNA. The experiment was repeated 2 times and similar results were obtained. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. PPG, purpurogallin.

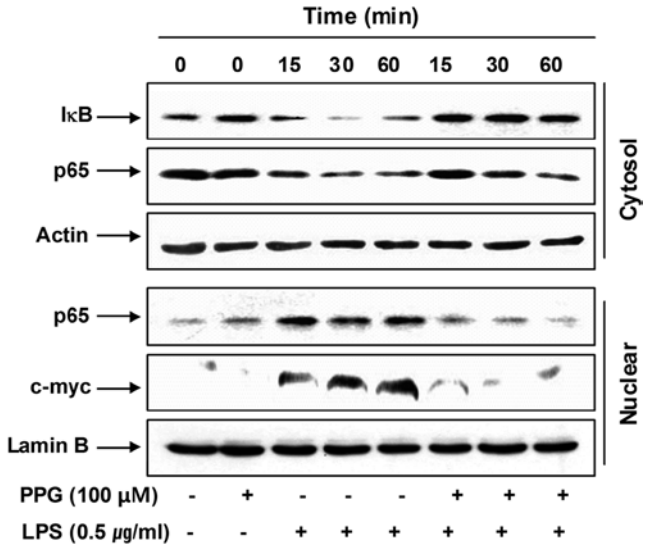

Figure 6. Effects of purpurogallin on lipopolysaccharide (LPS)-induced nuclear factor-κB (NF-κB) translocation and inhibitor of NF-κB (IκB) degradation in BV2 microglial cells. Cells were treated with 100 μM purpurogallin for 1 h before treatment with LPS (0.5 μg/ml) for the indicated periods of time. Nuclear and cytosolic proteins were subjected to 10% SDS-polyacrylamide gels followed by western blot analysis using anti-NF-κB p65, anti-IκB-α and c-myc antibodies. Results are representative of those obtained from 2 independent experiments. Actin and lamin B were used as internal controls for nuclear and cytosolic fractions, respectively. PPG, purpurogallin.

however, treatment with purpurogallin attenuated the degradation of IκB and the nuclear levels of the NF-κB p65 subunit in LPS-stimulated BV2 cells.

As c-myc is one of the transcription factors involved in inflammation and its transcription is regulated by NF-κB (33), we then investigated whether purpurogallin has an effect on LPS-induced c-myc expression. As shown in Fig. 6, although c-myc was expressed at very low levels, exposure to LPS markedly induced the accumulation of c-myc proteins in the nucleus of BV2 cells. However, the results demonstrated that, in the presence of purpurogallin, the levels of c-myc expression were significantly reduced to levels similar to those of the controls. These results indicate that purpurogallin inhibits LPS-induced c-myc expression mediated by NF-κB. Taken

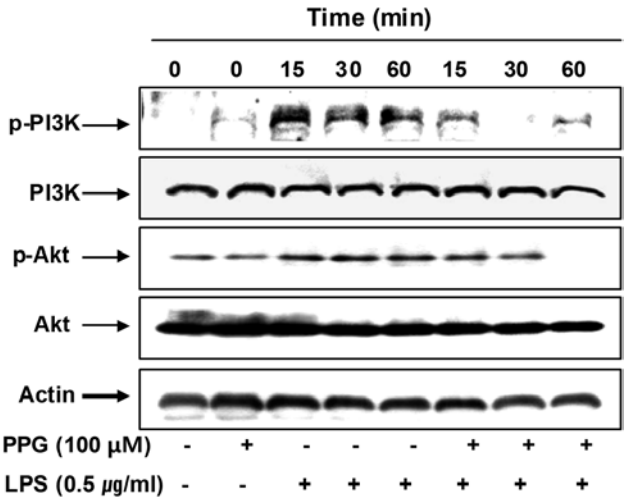

Figure 7. Effects of purpurogallin on phosphatidylinositol 3-kinase (PI3K)/Akt activation by lipopolysaccharide (LPS) in BV2 microglial cells. BV2 cells were treated with 100 μM purpurogallin for 1 h prior to treatment with LPS (0.5 μg/ml) for the indicated periods of time. Total proteins were subjected to 10% SDS-polyacrylamide gels, followed by western blot analsysi using the indicated antibodies. Proteins were visualized using an ECL detection system. Actin was used as an internal control. PPG, purpurogallin.

together, these findings indicate that purpurogallin suppresses iNOS, COX-2, TNF-α and IL-1β expression, at least in part, through an NF-κB-dependent mechanism.

Purpurogallin reduces LPS-induced phosphorylation of phosphatidylinositol 3-kinase (PI3K)/Akt and mitogen-activated protein kinases (MAPKs) in LPS-stimulated BV2 microglial cells. To investigate other intracellular mechanisms responsible for the inhibitory effects of purpurogallin on inflammatory mediators, we examined the effects of purpurogallin on the PI3K/Akt and MAPK signaling pathways. As shown in Fig. 7, the phosphorylation of PI3K and Akt was markedly increased within 15 min after LPS stimulation; however, pre-treatment with purpurogallin resulted in significant blockage of the LPS-induced PI3K and Akt phosphorylation, indicating that the anti-inflammatory effects of purpurogallin are associated with the inactivation of the PI3K/Akt signaling pathway.

Furthermore, the stimulation of BV2 cells with LPS induced the rapid activation of MAPKs, such as p38MAPK, ERK and JNK, with the peak levels of each phospho-MAPK occurring 15 to 60 min after the addition of LPS. However, pre-treatment with purpurogallin significantly inhibited the phosphorylation of MAPKs in the LPS-stimulated BV2 microglial cells (Fig. 8). Moreover, the elevated nuclear levels of c-Jun protein, a common target phosphorylated through the stress-activated protein kinases, p38MAPK and JNK (34), were also markedly blocked by purpurogallin.

To assess the mechanisms by which purpurogallin and LPS co-treatment suppress ERK activation, we evaluated the effects of LPS on the small molecular weight G protein, Ras, in BV2 cells, given that Ras is a known upstream activator of ERK (35). As shown in Fig. 8, LPS potently stimulated Ras expression as early as 15 min after treatment, which was an effect that persistd for at least 60 min. Of note, upon co-stimulation with LPS and purpurogallin, we observed a marked

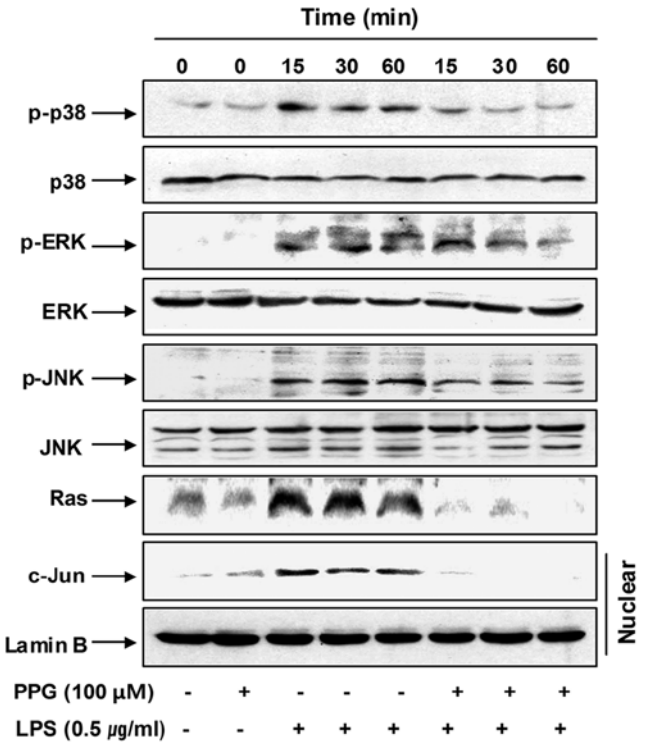

Figure 8. Effects of purpurogallin on mitogen-activated protein kinase activation by lipopolysaccharide (LPS) in BV2 microglial cells. BV2 cells were treated with the indicated concentrations of purpurogallin for 1 h prior to treatment with LPS (0.5 μg/ml) for the indicated periods of time. Total proteins were subjected to 10% SDS-polyacrylamide gels, followed by western blot analysis using the indicated antibodies. Proteins were visualized using an ECL detection system. PPG, purpurogallin.

reduction in LPS-mediated Ras expression. These data suggest that purpurogallin blocks the phosphorylation of ERK through the downregulation of Ras.

Discussion

In the present study, we demonstrate that purpurogallin inhibits inflammation in BV2 microglial cells. Purpurogallin significantly reduced the LPS-induced production of pro-inflammatory mediators, such as NO and PGE_2, as well as the production of cytokines, including TNF-α and IL-1β. Furthermore, the anti-inflammatory properties of purpurogallin were mediated by the downregulation of NF-κB activity, and the inactivation of the Akt and MAPK signaling pathways.

Among pro-inflammatory mediators released by activated microglia, NO and PGE_2 are the main cytotoxic mediators participating in the innate response in mammals (36-38). Several lines of evidence have shown that the activation of microglia induced by injury to the CNS or infection is associated with neurodegenerative disorders and the release of NO and PGE_2 and with the subsequent release of pro-inflammatory cytokines and chemokines (3-5). A number of studies have shown that the expression of COX-2 and iNOS, key enzymes for NO and PGE_2, are upregulated in activated glial cells. In addition, pro-inflammatory cytokines activate the transcription of COX-2 and iNOS genes, and anti-inflammatory drugs may also effectively reduce NO and PGE_2 production (3-5,39). Thus, downregulators of these inflammatory molecules have been considered as potential candidates of anti-inflammatory agents to alleviate the progression of neurodegenerative diseases caused by the activation of microglia. The results of this study demonstrated that purpurogallin significantly inhibited NO and PGE_2 production by downregulating iNOS and COX-2 mRNA and protein expression, indicating that the effects of purpurogallin occur at the transcriptional level. Notably, the inhibitory effects of purpurogallin on the LPS-induced production of NO and PGE_2 in BV2 microglial cells were not due to the cytotoxicity of this compound, as assessed by an MTT assay.

Several studies have demonstrated that pro-inflammatory cytokines, such as IL-1β and TNF-α, are initiators of the inflammatory response and mediators of the development of chronic inflammatory diseases (40,41). Therefore, the consequent overproduction of pro-inflammatory cytokines can be considered to be a histopathological hallmark of various neurological diseases and controlling microglial activation and neuroinflammatory processes may prove to be a therapeutic benefit in the treatment of depression. In the present study, our results revealed that purpurogallin significantly inhibited the production of IL-1β and TNF-α by suppressing their mRNA expression in LPS-treated BV2 microglial cells. Our data indicate that the inhibitory effects of purpurogallin on the production of these pro-inflammatory cytokines occur at the transcriptional level.

Various intracellular signaling molecules are involved in the modulation of inflammatory responses. Among them, the transcription factor, NF-κB, is a primary regulator of genes that are involved in the production of pro-inflammatory cytokines and enzymes involved in the inflammatory process (30-32). In addition, the involvement of the PI3K/Akt pathway in the expression of inflammatory mediators in microglia through the activation of NF-κB has been shown (42,43). As a result of its key role in several pathological conditions, NF-κB is a major drug target in a variety of diseases, and the blockade of NF-κB transcriptional activity in microglia is known to suppress the expression of iNOS, COX-2 and pro-inflammatory cytokines (44-46). Therefore, many putative anti-inflammatory therapies seek to block NF-κB activity. Our results demonstrated that purpurogallin markedly inhibited the LPS-induced IκB-α degradation and NF-κB translocation, which was associated with the inhibition of c-myc accumulation in the nucleus. Furthermore, purpurogallin significantly inhibited PI3K/Akt activation in LPS-stimulated BV2 microglial cells. These data indicate that purpurogallin inhibits LPS-induced NF-κB activation through the inactivation of the PI3K/Akt signaling pathway, and that the inhibitory effects of purpurogallin on LPS-induced NF-κB and PI3K/Akt activation may be associated with its anti-inflammatory mechanism(s).

MAPKs are also important signaling molecules involved in the production of pro-inflammatory mediators and cytokines and the modulation of NF-κB in microglia (47-49). MAPKs have previously been implicated in the signaling pathways relevant to LPS-induced inflammation and LPS is also known to activate a series of MAPKs, such as ERK, p38MAPK and JNK in microglial cells (50). Therefore, in this study, we conducted experiments to determine whether purpurogallin tightly regulates the activation of 3 MAPKs to induce anti-inflammatory effects in LPS-stimulated BV2 microglial

cells. LPS enhanced the activation of MAPKs, whereas purpurogallin decreased the LPS-induced activation of MAPKs, which was accompanied by alterations in the nuclear levels of c-Jun, a common target of p38MAPK and JNK (34) and Ras, an upstream activator of ERK (35). These results suggest that the purpurogallin-mediated inhibition of pro-inflammatory mediators and cytokines is associated with the downregulation of the MAPK signaling pathways.

In conclusion, the results presented in this study demonstrate that purpurogallin inhibits LPS-induced NO and PGE_2 production by suppressing iNOS and COX-2 mRNA and protein expression in BV2 microglial cells. Purpurogallin also inhibits the production of pro-inflammatory cytokines (TNF-α and IL-1β) by suppressing their transcriptional activity. The inhibitory effects of purpurogallin were mediated by the prevention of NF-κB activation and by the inhibition of IκB-degradation, which was accompanied by the blocking of the PI3K/Akt and MAPKs pathways. Our findings suggest that purpurogallin may provide an effective treatment strategy for a number of inflammatory and neurodegenerative disorders.

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References

1. Mosley RL, Benner EJ, Kadiu I, Thomas M, Boska MD, Hasan K, Laurie C and Gendelman HE: Neuroinflammation, oxidative stress and the pathogenesis of Parkinson's disease. Clin Neurosci Res 6: 261-281, 2006.
2. Reynolds A, Laurie C, Mosley RL and Gendelman HE: Oxidative stress and the pathogenesis of neurodegenerative disorders. Int Rev Neurobiol 82: 297-325, 2007.
3. Perry VH, Nicoll JA and Holmes C: Microglia in neurodegenerative disease. Nat Rev Neurol 6: 193-201, 2010.
4. Rivest S: Molecular insights on the cerebral innate immune system. Brain Behav Immun 17: 13-19, 2003.
5. Das S and Basu A: Inflammation: a new candidate in modulating adult neurogenesis. J Neurosci Res 86: 1199-1208, 2008.
6. Stadelmann C: Multiple sclerosis as a neurodegenerative disease: pathology, mechanisms and therapeutic implications. Curr Opin Neurol 24: 224-229, 2011.
7. Lull ME and Block ML: Microglial activation and chronic neurodegeneration. Neurotherapeutics 7: 354-365, 2010.
8. Long-Smith CM, Sullivan AM and Nolan YM: The influence of microglia on the pathogenesis of Parkinson's disease. Prog Neurobiol 89: 277-287, 2009.
9. Dixon RA and Steele CL: Flavonoids and isoflavonoids - a gold mine for metabolic engineering. Trends Plant Sci 4: 394-400, 1999.
10. Le Marchand L, Murphy SP, Hankin JH, Wilkens LR and Kolonel LN: Intake of flavonoids and lung cancer. J Natl Cancer Inst 92: 154-160, 2000.
11. Szejtli J and Szente L: Elimination of bitter, disgusting tastes of drugs and foods by cyclodextrins. Eur J Pharm Biopharm 61: 115-125, 2005.
12. Chen D, Chen MS, Cui QC, Yang H and Dou QP: Structure-proteasome-inhibitory activity relationships of dietary flavonoids in human cancer cells. Front Biosci 12: 1935-1945, 2007.
13. Abou-Karam M and Shier WT: Inhibition of oncogene product enzyme activity as an approach to cancer chemoprevention. Tyrosine-specific protein kinase inhibition by purpurogallin from *Quercus* sp. nutgall. Phytother Res 13: 337-340, 1999.
14. Wu TW, Zeng LH, Wu J, Fung KP, Weisel RD, Hempel A and Camerman N: Molecular structure and antioxidant specificity of purpurogallin in three types of human cardiovascular cells. Biochem Pharmacol 52: 1073-1080, 1996.
15. Prasad K and Laxdal VA: Evaluation of hydroxyl radical-scavenging property of purpurogallin using high pressure liquid chromatography. Mol Cell Biochem 135: 153-158, 1994.
16. Sugiyama H, Fung KP and Wu TW: Purpurogallin as an antioxidant protector of human erythrocytes against lysis by peroxyl radicals. Life Sci 53: PL39-PL43, 1993.
17. Inamori Y, Muro C, Sajima E, Katagiri M, Okamoto Y, Tanaka H, Sakagami Y and Tsujibo H: Biological activity of purpurogallin. Biosci Biotechnol Biochem 61: 890-892, 1997.
18. Farnet CM, Wang B, Hansen M, Lipford JR, Zalkow L, Robinson WE Jr, Siegel J and Bushman F: Human immunodeficiency virus type 1 cDNA integration: new aromatic hydroxylated inhibitors and studies of the inhibition mechanism. Antimicrob Agents Chemother 42: 2245-2253, 1998.
19. Lambert JD, Chen D, Wang CY, Ai N, Sang S, Ho CT, Welsh WJ and Yang CS: Benzotropolone inhibitors of estradiol methylation: kinetics and in silico modeling studies. Bioorg Med Chem 13: 2501-2507, 2005.
20. Fung KP, Wu TW and Lui CP: Purpurogallin inhibits DNA synthesis of murine fibrosarcoma L-929 and human U-87 MG glioblastoma cells in vitro. Chemotherapy 42: 199-205, 1996.
21. Kitada S, Leone M, Sareth S, Zhai D, Reed JC and Pellecchia M: Discovery, characterization, and structure-activity relationships studies of proapoptotic polyphenols targeting B-cell lymphocyte/leukemia-2 proteins. J Med Chem 46: 4259-4264, 2003.
22. Tang G, Yang CY, Nikolovska-Coleska Z, Guo J, Qiu S, Wang R, Gao W, Wang G, Stuckey J, Krajewski K, Jiang S, Roller PP and Wang S: Pyrogallol-based molecules as potent inhibitors of the antiapoptotic Bcl-2 proteins. J Med Chem 50: 1723-1726, 2007.
23. Doulias PT, Nousis L, Zhu BZ, Frei B and Galaris D: Protection by tropolones against H_2O_2-induced DNA damage and apoptosis in cultured Jurkat cells. Free Radic Res 39: 125-135, 2005.
24. Méchin V, Baumberger S, Pollet B and Lapierre C: Peroxidase activity can dictate the in vitro lignin dehydrogenative polymer structure. Phytochemistry 68: 571-579, 2007.
25. Moniruzzaman M, Kamiya N and Goto M: Biocatalysis in water-in-ionic liquid microemulsions: a case study with horseradish peroxidase. Langmuir 25: 977-982, 2009.
26. Cheong MH, Lee SR, Yoo HS, Jeong JW, Kim GY, Kim WJ, Jung IC and Choi YH: Anti-inflammatory effects of *Polygala tenuifolia* root through inhibition of NF-κB activation in lipopolysaccharide-induced BV2 microglial cells. J Ethnopharmacol 137: 1402-1408, 2011.
27. Kim TH, Ku SK, Lee IC and Bae JS: Anti-inflammatory functions of purpurogallin in LPS-activated human endothelial cells. BMB Rep 45: 200-205, 2012.
28. Lee YA, Choi HM, Lee SH, Yang HI, Yoo MC, Hong SJ and Kim KS: Synergy between adiponectin and interleukin-1β on the expression of interleukin-6, interleukin-8, and cyclooxygenase-2 in fibroblast-like synoviocytes. Exp Mol Med 44: 440-447, 2012.
29. Lee SH, Kim DW, Eom SA, Jun SY, Park M, Kim DS, Kwon HJ, Kwon HY, Han KH, Park J, Hwang HS, Eum WS and Choi SY: Suppression of 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced skin inflammation in mice by transduced Tat-Annexin protein. BMB Rep 45: 354-359, 2012.
30. Atreya I, Atreya R and Neurath MF: NF-kappaB in inflammatory bowel disease. J Intern Med 263: 591-596, 2008.
31. Wright JG and Christman JW: The role of nuclear factor kappa B in the pathogenesis of pulmonary diseases: implications for therapy. Am J Respir Med 2: 211-219, 2003.
32. Sebban H and Courtois G: NF-kappaB and inflammation in genetic disease. Biochem Pharmacol 72: 1153-1160, 2006.
33. Duyao MP, Buckler AJ and Sonenshein GE: Interaction of an NF-kappa B-like factor with a site upstream of the c-myc promoter. Proc Natl Acad Sci USA 87: 4727-4731, 1990.
34. Turjanski AG, Vaqué JP and Gutkind JS: MAP kinases and the control of nuclear events. Oncogene 26: 3240-3253, 2007.
35. Montagut C and Settleman J: Targeting the RAF-MEK-ERK pathway in cancer therapy. Cancer Lett 283: 125-134, 2009.
36. Garden GA and Möller T: Microglia biology in health and disease. J Neuroimmune Pharmacol 1: 127-137, 2006.
37. Sugama S, Takenouchi T, Fujita M, Conti B and Hashimoto M: Differential microglial activation between acute stress and lipopolysaccharide treatment. J Neuroimmunol 207: 24-31, 2009.
38. Schwartz M and Shechter R: Systemic inflammatory cells fight off neurodegenerative disease. Nat Rev Neurol 6: 405-410, 2010.

39. Gebicke-Haerter PJ: Microglia in neurodegeneration: molecular aspects. Micros Res Tech 54: 47-58, 2001.
40. Inoue K: The function of microglia through purinergic receptors: neuropathic pain and cytokine release. Pharmacol Ther 109: 210-226, 2006.
41. Merson TD, Binder MD and Kilpatrick TJ: Role of cytokines as mediators and regulators of microglial activity in inflammatory demyelination of the CNS. Neuromolecular Med 12: 99-132, 2010.
42. 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.
43. 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-alpha production through inhibition of PI 3-kinase/Akt and NF-kappaB activation in murine BV2 microglial cells. Neurosci Lett 396: 1-6, 2006.
44. Shin WS, Szuba A and Rockson SG: The role of chemokines in human cardiovascular pathology: enhanced biological insights. Atherosclerosis 160: 91-102, 2002.
45. Nam NH: Naturally occurring NF-kappaB inhibitors. Mini Rev Med Chem 6: 945-951, 2006.
46. Luqman S and Pezzuto JM: NFkappaB: a promising target for natural products in cancer chemoprevention. Phytother Res 24: 949-963, 2010.
47. Kaminska B: MAPK signalling pathways as molecular targets for anti-inflammatory therapy - from molecular mechanisms to therapeutic benefits. Biochim Biophys Acta 1754: 253-262, 2005.
48. Kaminska B, Gozdz A, Zawadzka M, Ellert-Miklaszewska A and Lipko M: MAPK signal transduction underlying brain inflammation and gliosis as therapeutic target. Anat Rec (Hoboken) 292: 1902-1913, 2009.
49. Wei J and Feng J: Signaling pathways associated with inflammatory bowel disease. Recent Pat Inflamm Allergy Drug Discov 4: 105-117, 2010.
50. Pyo H, Jou I, Jung S, Hong S and Joe EH: Mitogen-activated protein kinases activated by lipopolysaccharide and beta-amyloid in cultured rat microglia. Neuroreport 9: 871-874, 1998.