



Galangin Suppresses Pro-Inflammatory Gene Expression in Polyinosinic-Polycytidylic Acid-Stimulated Microglial Cells

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Abstract

Galangin (3,5,7-trihydroxyflavone) is a polyphenolic compound abundant in honey and medicinal herbs, such as *Alpinia officinarum*. In this study, we investigated the anti-inflammatory effects of galangin under *in vitro* and *in vivo* neuroinflammatory conditions caused by polyinosinic-polycytidylic acid (poly(I:C)), a viral mimic dsRNA analog. Galangin suppressed the production of nitric oxide, reactive oxygen species, and pro-inflammatory cytokines in poly(I:C)-stimulated BV2 microglia. On the other hand, galangin enhanced anti-inflammatory interleukin (IL)-10 production. Galangin also suppressed the expression of pro-inflammatory markers in poly(I:C)-injected mouse brains. Further mechanistic studies showed that galangin inhibited poly(I:C)-induced nuclear factor (NF)- κ B activity and phosphorylation of Akt without affecting MAP kinases. Interestingly, galangin increased the expression and transcriptional activity of peroxisome proliferator-activated receptor (PPAR)- γ , known to play an anti-inflammatory role. To investigate whether PPAR- γ is involved in the anti-inflammatory function of galangin, BV2 cells were pre-treated with PPAR- γ antagonist before treatment of galangin. We found that PPAR- γ antagonist significantly blocked galangin-mediated upregulation of IL-10 and attenuated the inhibition of tumor necrosis factor (TNF)- α and IL-6 in poly(I:C)-stimulated microglia. In conclusion, our data suggest that PI3K/Akt, NF- κ B, and PPAR- γ play a pivotal role in mediating the anti-inflammatory effects of galangin in poly(I:C)-stimulated microglia.

Key Words: Galangin, Poly(I:C), Microglia, PI3K/Akt, NF- κ B, PPAR- γ signaling

INTRODUCTION

Microglia are resident immune cells in the brain and play a role in neuronal survival during brain development via growth factors and phagocytosis of apoptotic cells and debris throughout the entire brain (Graeber and Streit, 2010; Ulland *et al.*, 2015). By challenge with various immunogens (e.g., lipopolysaccharides) and pathogens (e.g., bacteria, viruses), resting microglia transform into an active form and secrete reactive oxygen species, prostaglandins, matrix metalloproteinases (MMPs), and pro-inflammatory cytokines (Hoogland *et al.*, 2015). If the acute inflammation shifts to an anti-inflammatory/resolution phase, it leads to tissue repair and homeostasis. However, a persistent and unresolved inflammation leads to neuronal cell death and contributes to the generation of neurodegenerative disorders (Glass *et al.*, 2010; Cherry *et al.*, 2014).

Recent studies reported that systemic inflammation plays an important role in the occurrence and progression of neu-

roinflammatory disorders. Systemic challenges with infectious stimuli such as lipopolysaccharide (LPS), polyinosinic-polycytidylic acid (poly(I:C)), lipoteichoic acid, and live bacteria result in microglial activation and upregulate inflammatory responses in the brain (Cunningham *et al.*, 2007; Qin *et al.*, 2007; Hoogland *et al.*, 2015). These neuroinflammatory conditions may eventually lead to neurodegenerative diseases. Thus, the development of compounds that can control systemic inflammation and/or microglial activation has been suggested as a potential therapeutic target for neurodegenerative diseases.

Galangin (3,5,7-trihydroxyflavone) is a polyphenolic compound enriched in honey and medicinal herbs, such as *Alpinia officinarum* (Jaganathan and Mandal, 2009; Honmore *et al.*, 2016). Galangin is known to have anti-tumor, anti-oxidant, anti-inflammatory, and anti-microbial activities (Meyer *et al.*, 1997; Cushnie *et al.*, 2007; Ruweler *et al.*, 2008; Chien *et al.*, 2015; Honmore *et al.*, 2016). The anti-tumor effects of galangin have been reported in human leukemia, hepatoma, and

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breast cancer cells (Heo *et al.*, 2001; Jaganathan and Mandal, 2009; Chien *et al.*, 2015). In addition, the anti-inflammatory effects of galangin have been reported in several animal models such as paw edema, asthma, and rheumatoid arthritis (Huh *et al.*, 2013; Liu *et al.*, 2015; Honmore *et al.*, 2016). Galangin also showed neuroprotective effects in a model of acute ischemic stroke by restoring cortical blood flow and ameliorating apoptosis and mitochondrial dysfunction (Li *et al.*, 2012). Moreover, galangin inhibited β -amyloid production and acetylcholinesterase activity, suggesting therapeutic potential for Alzheimer disease as well as cerebral ischemia (Guo *et al.*, 2010; Zeng *et al.*, 2015).

Although the pharmacological activities of galangin have been reported in many papers, the anti-inflammatory effects of galangin in the brain have not been fully demonstrated. In this study, we examined the effects of galangin in poly(I:C)-induced neuroinflammatory conditions and analyzed its underlying molecular mechanisms. Our data showed that galangin exerts anti-inflammatory effects by modulating PI3K/Akt, NF- κ B, and PPAR- γ signaling pathways.

MATERIALS AND METHODS

Reagent

Galangin (3,5,7-trihydroxyflavone), poly(I:C), and PPAR- γ antagonist (T0070907) were obtained from Sigma-Aldrich (St. Louis, MO, USA). All reagents used for cell culture were purchased from Welgene (Gyeongsan, Korea). Trizol reagent was obtained from Thermo Fisher Scientific (Waltham, MA, USA). Antibodies against phospho-/total forms of p38, JNK/SARK, ERK1/2, and Akt were supplied by Cell Signaling Technology (Beverly, CA, USA). The structure of galangin is shown in Fig. 1.

Microglial cell culture

The immortalized mouse BV2 microglial cell line (obtained from Dr. Hong JS, NIEHS, USA) was grown and maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, streptomycin (10 μ g/mL), and penicillin (10 U/mL) at 37°C under 5% CO₂.

Measurement of nitric oxide, cytokine, and intracellular ROS levels

BV2 cells (1 \times 10⁵ cells per well in a 24-well plate) were pretreated with galangin for 1 h and stimulated with poly(I:C) (10 μ g/ml). After 16 h, the supernatants were collected and the accumulated nitrite was measured using the Griess reagent (Promega, Madison, WI, USA). The concentrations of TNF- α , IL-6, and IL-10 in the supernatants were measured by ELISA according to the procedure recommended by the supplier (BD Biosciences, San Jose, CA, USA). The intracellular accumulation of ROS was measured with 2',7'-dichlorodihydrofluorescein diacetate (H₂DCF-DA) (Sigma-Aldrich). In brief, microglia were stimulated with poly(I:C) for 16 h and stained with 20 mM H₂DCF-DA in PBS for 1 h at 37°C. DCF fluorescence intensity was measured at 485-nm excitation and 535-nm emission, using a fluorescence plate reader (Molecular Devices, Sunnyvale, CA, USA).

RT-PCR

BV2 cells (7.5 \times 10⁵ cells per well in a 6-well plate) were

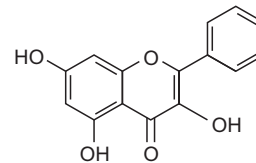


Fig. 1. The chemical structure of galangin (3,5,7-trihydroxyflavone).

treated with poly(I:C) in the presence or absence of galangin, and total RNA was extracted using TRI reagent (Thermo Fisher Scientific). Cortical brain tissue was homogenized using a homogenizer (Fisher Scientific, Pittsburgh, PA, USA), and total RNA was extracted using TRI reagent. For RT-PCR, total RNA (1 μ g) was reverse-transcribed in a reaction mixture containing 0.1 μ g of random primers, 3 mM MgCl₂, 0.5 mM dNTP, 1 \times RT buffer, and 10 U reverse transcriptase (Promega). The synthesized cDNA was used as a template for the PCR reaction using Go Taq polymerase (Promega) and primers for the target gene. The primers used in the PCR reaction are shown in Table 1.

Mice

Adult male ICR mice (*Mus musculus*, 28-32 g, 7 weeks old) were purchased from Samtako Inc (Osan, Korea). All animal experiments were approved by the Institutional Animal Care and Research Ethics Committee at the School of Medicine, Ewha Womans University (IACUC #2014-0274), and were carried out in accordance with the guidelines of the National Institute of Health's Guide for the Care and Use of Laboratory Animals.

Poly(I:C)-induced inflammation and administration of galangin

Poly(I:C) (12 mg/kg) was administered intraperitoneally (i.p.) to induce neuroinflammation in male ICR mice, as previously described (Jeong *et al.*, 2017). Galangin (50 mg/kg) dissolved in vehicle solution (normal saline containing 1% dimethylsulfoxide), was administered daily (i.p.) for 4 days before the poly(I:C) treatment. The brains were obtained 3 h after LPS administration and analyzed for the expression of inflammatory markers.

Electrophoretic mobility shift assay (EMSA)

BV2 cells were pretreated with galangin for 1 h and stimulated with poly(I:C) for 3 h. The nuclear extracts from the cells were prepared as previously described (Lee *et al.*, 2015). Double-stranded DNA oligonucleotides containing consensus sequences of NF- κ B were end-labeled using T4 polynucleotide kinase (New England Biolabs, Beverly, MA, USA) in the presence of [γ -³²P] ATP. Nuclear proteins (5 μ g) were incubated with a ³²P-labeled probe on ice for 30 min, resolved on a 5% polyacrylamide gel, and visualized with autoradiography.

Transient transfection and luciferase assay

BV2 cells (2 \times 10⁵ cells per well on a 12-well plate) were transfected with 1 μ g of plasmid DNA ([κ B]₃-luc or PPRE-luc) using the Metafectene transfection reagent (Biontex, Martinsried/Planegg, Germany). The effect of galangin on reporter gene activity was determined by pre-treating the cells with galangin prior to stimulation with poly(I:C) (10 μ g/mL) for 6 h. After preparing the cell lysates, the luciferase assay was

Table 1. Primers used in RT-PCR reactions

Gene	Forward Primer (5'→3')	Reverse Primer (5'→3')	Size
iNOS	GCTTGGGTCTTGTCTACTCC	GGCCTTGTGGTGAAGAGTGT	385 bp
TNF- α	CCTATGTCTCAGCCTCTTCT	CCTGGTATGAGATAGCAAAT	354 bp
IL-6	CCACTTCACAAGTCGGAGGCTT	CCAGCTTATCTGTTAGGAGA	214 bp
IL-1 β	GGCAACTGTTCCCTGAACTCAACTG	CCATTGAGGTGGAGAGCTTTTCAGC	447 bp
COX-2	TTCAAAGAAGTGCTGGAAAAGGT	GATCATCTCTACCTGAGTGTCTTT	304 bp
IL-10	GCCAGTACAGCCGGGAAGACAATA	GCCTTGTAGACACCTTGGTCTT	185 bp
MMP8	ATTCAGTCCCTCTATGGA	CTCCAGTATTTGTCTCTAC	245 bp
PPAR- γ	CCGAAGAACCATCCGATT	CGGAAGGACTTTATGTA	271 bp
GAPDH	TTCACCACCATGGAGAAGGC	GGCATGGACTGTGGTCATGA	236 bp

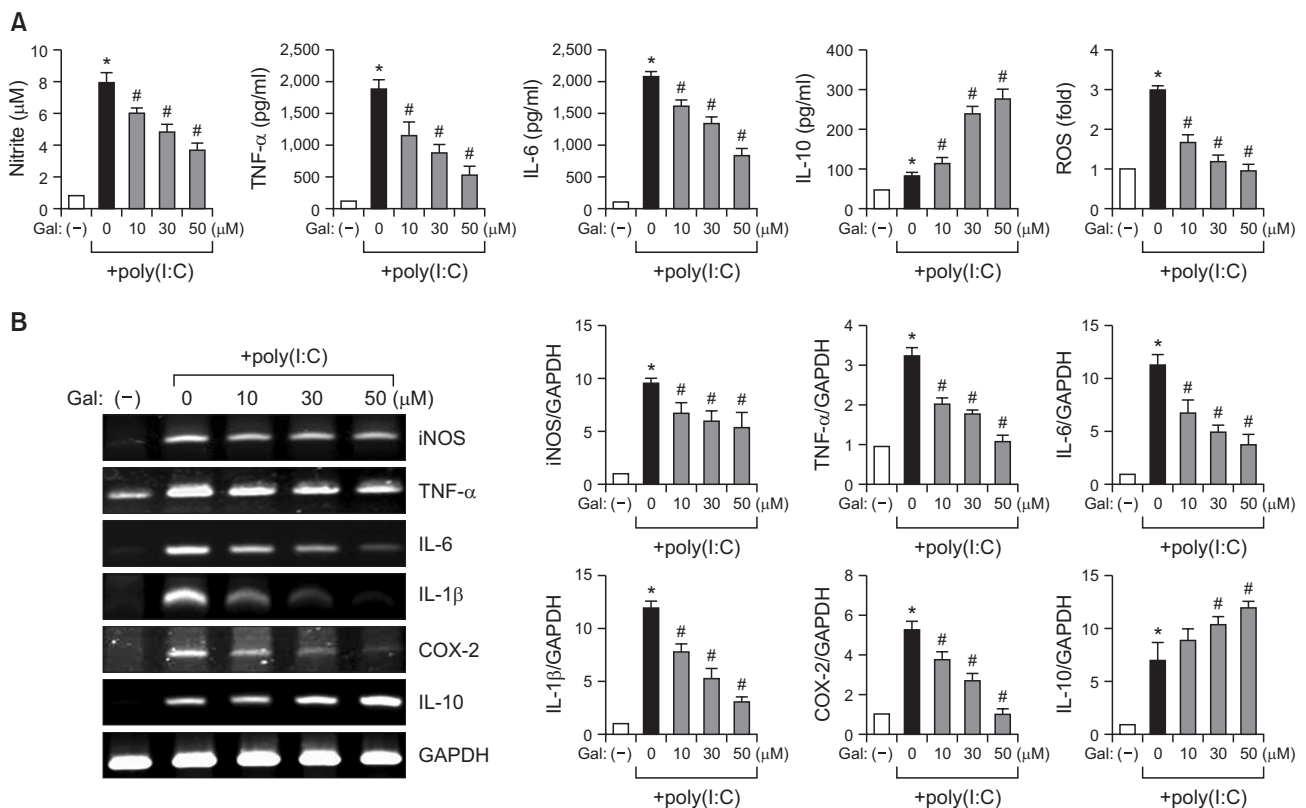


Fig. 2. Effects of galangin on the expression of pro-/anti-inflammatory molecules in poly(I:C)-stimulated BV2 microglia. (A) Cells were pre-treated with galangin (Gal) at the indicated concentrations (10, 30, 50 μ M) for 1 h, and then subjected to treatment with poly(I:C) (10 μ g/ml) for 16 h. The amounts of NO, TNF- α , IL-6, and IL-10 released into the medium were measured using Griess reagent or ELISA. Intracellular ROS levels were determined by the DCF-DA method. (B) Cells were pre-treated with galangin for 1 h, and then treated with poly(I:C) (10 μ g/ml) for 6 h. An RT-PCR was performed to measure the mRNA expression of pro-/anti-inflammatory molecules. The quantification data are shown in the right panel. The data are expressed as the mean \pm SEM of three independent experiments. * $p < 0.05$, vs. control samples. # $p < 0.05$, vs. poly(I:C)-treated samples.

performed as previously described (Lee *et al.*, 2015).

Western blot analysis

Whole cell protein lysates were prepared in a lysis buffer (10 mM Tris, pH 7.4, 30 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.1% sodium deoxycholate, and 1 mM EDTA) containing a protease inhibitor cocktail. Protein samples were separated by SDS-PAGE, transferred to nitrocellulose membranes, and incubated with antibodies against the phospho- or the total

form of MAP kinase (1:1000), Akt (1:1000) (Cell Signaling), and β -actin (1:1000) (Sigma). After thoroughly washing with TBST, HRP-conjugated secondary antibodies (1:2000 dilution in TBST; Bio-Rad, Hercules, CA, USA) were applied, and the blots were developed using an enhanced chemiluminescence detection kit (Thermo Fisher Scientific).

Statistical analysis

Data are expressed as mean \pm standard error of the mean

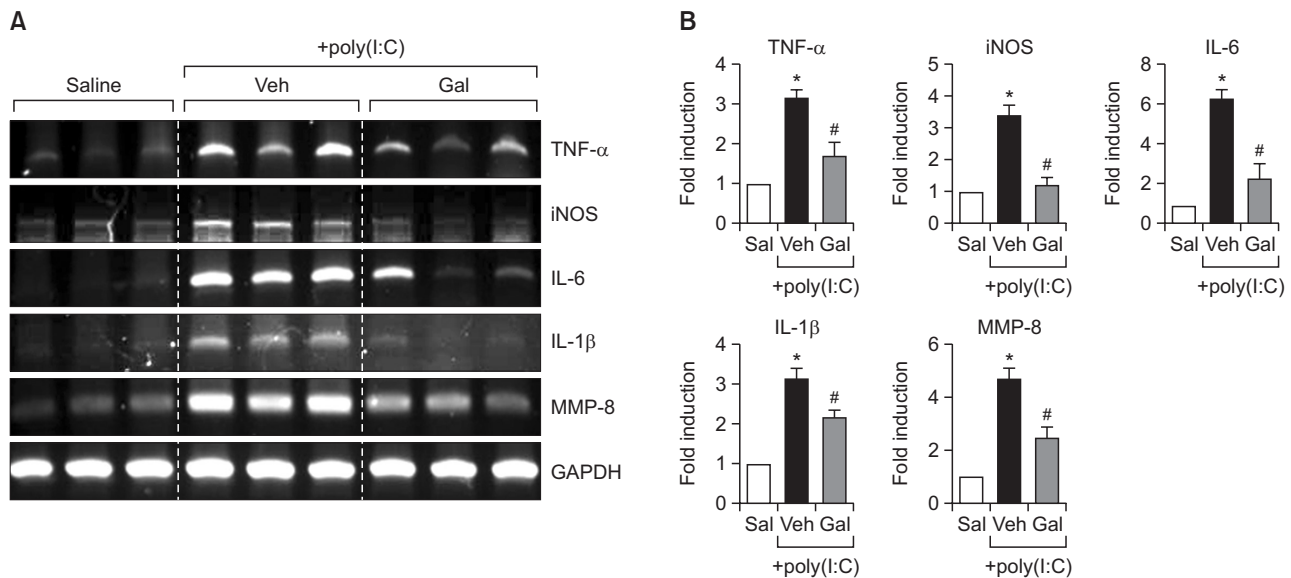


Fig. 3. Effect of galangin on mRNA expression of inflammatory markers in poly(I:C)-injected mouse brains. (A, B) Effects of galangin (50 mg/kg) on mRNA levels of iNOS, pro-inflammatory cytokines (IL-6, IL-1 β , TNF- α), and MMP-8 in the cortex of poly(I:C)-injected mice. Representative gels (A), and quantification data (B) are shown (n=5). * p <0.05, vs. control samples. # p <0.05, vs. poly(I:C)-treated samples.

(SEM), and statistical analyses were performed using a one-way ANOVA followed by Newman-Keuls post-hoc tests or t -tests. The calculations were performed using the GraphPad Prism software (version 4.0; GraphPad Software, Inc., La Jolla, CA, USA). A p -value of less than 0.05 indicated statistical significance.

RESULTS

Galangin inhibits poly(I:C)-induced production of NO, pro-inflammatory cytokines, and ROS with enhancement of anti-inflammatory IL-10 in BV2 microglial cells

To examine the anti-inflammatory effects of galangin, BV2 cells were stimulated with poly(I:C) (10 μ g/m) in the presence or absence of galangin. Poly(I:C) is a viral mimic dsRNA analog that activates the toll-like receptor 3 (TLR3) pathway (Cunningham *et al.*, 2007). Galangin suppressed poly(I:C)-induced NO, TNF- α , and IL-6 production in a concentration-dependent manner (Fig. 2A). Moreover, galangin inhibited intracellular ROS production. On the other hand, galangin remarkably increased anti-inflammatory IL-10 production (Fig. 2A). Next, RT-PCR analysis was performed to determine the effect of galangin on mRNA expression of pro-/anti-inflammatory molecules in poly(I:C)-stimulated microglia. As shown in Fig. 2B, galangin significantly reduced the mRNA levels of iNOS, TNF- α , IL-6, IL-1 β , and COX-2, induced by poly(I:C), while galangin increased IL-10 mRNA levels. The results suggest that galangin modulates the expression of iNOS, COX-2, and several pro-/anti-inflammatory cytokines at the transcriptional level.

Galangin suppresses the expression of pro-inflammatory markers in poly(I:C)-injected mouse brains

To confirm the effects of galangin under neuroinflammatory conditions *in vivo*, galangin was intraperitoneally injected

into mice prior to the administration of poly(I:C). The expression levels of proinflammatory markers were determined in poly(I:C)-injected mouse brains 3 hours after injection. RT-PCR analysis showed that galangin inhibited the mRNA expression of iNOS, TNF- α , IL-6, and IL-1 β in poly(I:C)-injected mouse brains (Fig. 3). Galangin also suppressed the expression of MMP-8, which plays a role as a pro-inflammatory mediator in activated microglia (Fig. 3) (Lee *et al.*, 2015).

Galangin inhibits poly(I:C)-induced NF- κ B activity and phosphorylation of Akt without affecting MAPK signaling

To determine the molecular mechanism underlying the anti-inflammatory effects of galangin, we examined the effect of galangin on the TLR3 signaling pathway. After dsRNA binds to TLR3, it activates intracellular signaling molecules such as PI3K, MAPKs, and downstream transcription factors such as NF- κ B, AP-1, and IRFs, which lead to the expression of various immune genes (Moynagh, 2005; Sen and Sarkar., 2005). Therefore, in the present study, we examined the effects of galangin on a key transcription factor NF- κ B as well as signaling kinases such as PI3K/Akt and three types of MAP kinases. The EMSA data showed that galangin significantly inhibited poly(I:C)-induced NF- κ B DNA binding activity (Fig. 4A). Moreover, galangin inhibited NF- κ B reporter gene activity (Fig. 4B). We also found that galangin significantly inhibited poly(I:C)-induced phosphorylation of Akt, without affecting p38 MAPK, ERK, or JNK phosphorylation (Fig. 4C). The data suggest that PI3K/Akt and NF- κ B signaling pathways may be involved in the anti-inflammatory mechanism of galangin.

PPAR- γ signaling appears to mediate anti-inflammatory activity of galangin in poly(I:C)-stimulated microglia

PPAR- γ is a nuclear receptor that acts as an anti-inflammatory regulator in macrophage and microglial activation (Bouhlel *et al.*, 2007; Saijo *et al.*, 2013). In addition, many papers reported that activation of PPAR- γ can suppress the inflamma-

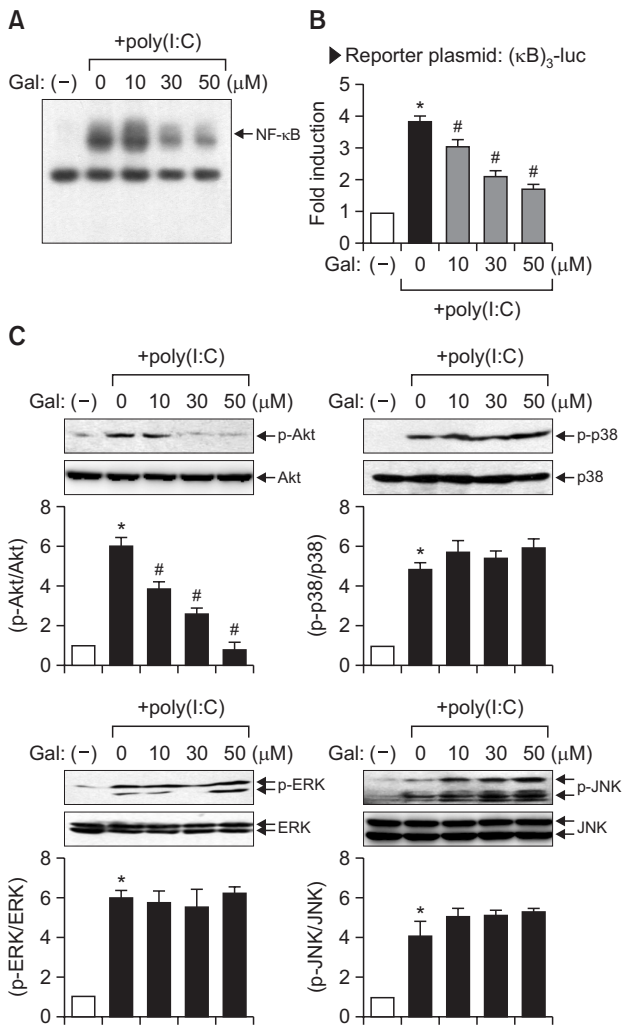


Fig. 4. Effect of galangin on NF- κ B activity and the phosphorylation of Akt and MAP kinases in poly(I:C)-stimulated BV2 cells. (A) Nuclear extracts were prepared from BV2 cells after treatment with poly(I:C) (10 μ g/ml) for 3 h, in the absence or presence of galangin, and EMSA was performed to determine the DNA binding activity of NF- κ B. The arrow indicates the DNA-protein complex of NF- κ B. (B) BV2 cells transfected with the reporter plasmid ($[\kappa B]_3$ -luc) were pretreated with galangin for 1 h prior to poly(I:C) treatment. After 6 h, cells were harvested and a luciferase assay was performed. (C) Western blot analysis for MAPKs and PI3K/Akt activities. Cell extracts were prepared from BV2 cells treated with poly(I:C) for 30 min, in the absence or presence of galangin, and a western blot analysis was performed using antibodies against phospho- or total forms of three types of MAPKs or Akt. The blots are representative of three independent experiments. Quantification of data from western blot analysis is shown in the bottom panel. The levels of the phosphorylated forms of MAPKs or Akt were normalized to the levels of each total form and expressed as relative fold changes versus the untreated control group. The results were obtained from three independent experiments and expressed as the mean \pm SEM. * p <0.05, vs. control samples. # p <0.05, vs. poly(I:C)-treated samples.

tory response through inhibition of NF- κ B (Saijo *et al.*, 2013). In the present study, we found that galangin increased PPAR- γ -mediated transcriptional activity in poly(I:C)-stimulated BV2 cells (Fig. 5A), and pre-treatment with PPAR- γ antagonist

(T0070907) abolished the increased PPRE-luc activity (Fig. 5A). These data indicate that the upregulation of PPRE-luc activity by galangin was mediated by PPAR- γ . Furthermore, we found that the treatment of BV2 cells with poly(I:C) significantly suppressed PPAR- γ expression, and treatment with galangin restored PPAR- γ expression (Fig. 5B).

To investigate whether PPAR- γ mediates the anti-inflammatory effects of galangin, BV2 cells were treated with T0070907 before the addition of poly(I:C) and/or galangin. As shown in Fig. 5C, treatment with T0070907 significantly blocked galangin-mediated upregulation of IL-10, and the inhibition of IL-6 and TNF- α in poly(I:C)-stimulated microglia. However, it did not affect NO production. These data collectively suggest that PPAR- γ is at least partly involved in anti-inflammatory mechanism of galangin in activated microglia.

DISCUSSION

The present study showed the anti-inflammatory effects of galangin in poly(I:C)-induced neuroinflammatory conditions *in vitro* and *in vivo*. Galangin suppressed the expression of pro-inflammatory molecules such as iNOS, COX-2, and pro-inflammatory cytokines, and enhanced anti-inflammatory IL-10 in poly(I:C)-stimulated microglia (Fig. 2). Moreover, galangin inhibited the expression of proinflammatory markers in poly(I:C)-injected mouse brains (Fig. 3). Detailed mechanistic studies showed that PI3K/Akt, NF- κ B, and PPAR- γ are involved in mediating the anti-inflammatory effects of galangin in poly(I:C)-stimulated BV2 microglia (Fig. 4, 5). The data collectively suggest the therapeutic potential of galangin in neuroinflammatory disorders.

Previous studies demonstrated the role of PPAR- γ in neuroinflammation (Zolezzi *et al.*, 2017). PPAR- γ is expressed in microglia and mediates anti-inflammatory activity by inducing microglial polarization into the M2-like phenotype (Saijo *et al.*, 2013). Moreover, PPAR- γ is known to suppress the expression of pro-inflammatory cytokines, chemokines, and MMPs via inhibition of transcription factors, such as NF- κ B, STAT, and AP-1 (Bernado and Minghetti, 2006). In particular, several mechanisms have been proposed underlying the NF- κ B regulation by PPAR- γ . First, PPAR- γ competes with NF- κ B for binding to coactivators, such as p300/CBP, and leads to inhibition of a NF- κ B-mediated inflammatory response (Zhao *et al.*, 2015). Second, PPAR- γ directly binds to NF- κ B subunits p50/p65 and inhibits the DNA binding activity of NF- κ B (Chung *et al.*, 2008). Lastly, PPAR- γ inhibits NF- κ B-mediated transcription via transrepression through preventing clearance of co-repressor complexes from NF- κ B (Saijo *et al.*, 2013). Therefore, at least one of these mechanisms might be related to PPAR- γ -mediated inhibition of NF- κ B in galangin+poly(I:C)-treated microglial cells. Further studies are necessary to unravel the detailed mechanism.

In the present study, we found that galangin inhibited the phosphorylation of Akt in LPS-stimulated microglia (Fig. 4). Several papers have reported that PI3K/Akt signaling pathways play an important role in pro-inflammatory gene expression in activated microglia (Kim *et al.*, 2004; Saponaro *et al.*, 2012). Kim *et al.* (2004) reported that the PI3K inhibitor LY294002 suppressed the expression of iNOS, pro-inflammatory cytokines, and MMP-9 in LPS-stimulated BV2 cells. In addition, Saponaro *et al.* (2012) reported that the PI3K/Akt path-

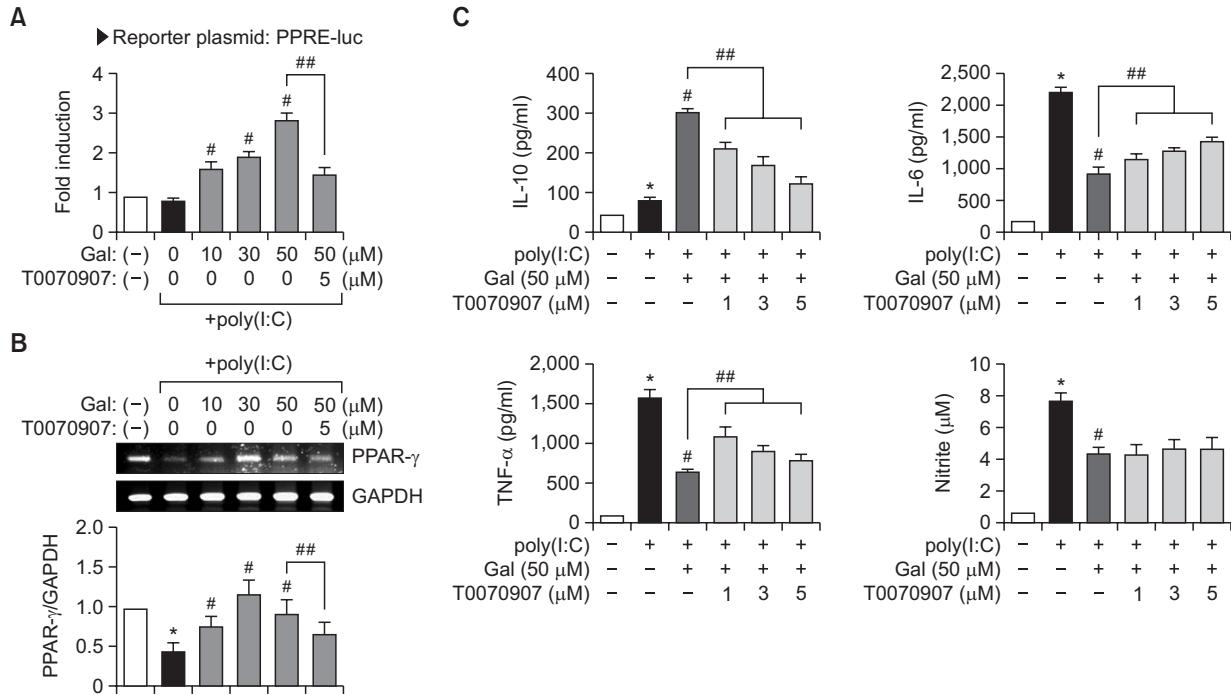


Fig. 5. Galangin increases PPAR- γ transcriptional activity and expression, and PPAR- γ antagonist reverses the anti-inflammatory effect of galangin in poly(I:C)-stimulated BV2 cells. (A) BV2 cells were transfected with PPRE-luc and treated with poly(I:C) with or without galangin. After 6 h, the cells were harvested and the luciferase assay was performed. The PPAR- γ antagonist, T0070907 (5 μ M), was added to BV2 cells 1 h before treatment with galangin. (B) BV2 cells were pretreated with galangin for 1 h prior to incubation with poly(I:C) for 6 h. The mRNA level of PPAR- γ was determined by RT-PCR. Quantification data are shown in the bottom panel. (C) The effect of T0070907 on the production of IL-10, IL-6, TNF- α , and NO in poly(I:C) +galangin-treated cells. BV2 cells were pre-treated with the indicated concentrations (1, 3, 5 μ M) of T0070907 for 1 h, and then galangin (50 μ M) for 1 h, followed by treatment with poly(I:C) (10 μ g/ml) for 16 h. The amounts of IL-10, IL-6, TNF- α , and NO released into the medium were determined. Data are expressed as mean \pm SEM from four independent experiments. * p <0.05, vs. control samples. # p <0.05, vs. poly(I:C)-treated samples. ## p <0.05, vs. poly(I:C) +galangin-treated samples.

way plays a role in NF- κ B-dependent inflammatory responses of activated microglia. Therefore, in addition to PPAR- γ , the PI3K/Akt signaling pathway appears to be involved in the inhibition of NF- κ B and subsequent downregulation of proinflammatory gene expression by galangin in poly(I:C)-stimulated BV2 microglial cells.

Recent studies have reported on the therapeutic benefits of galangin in several neurological disorders. Galangin showed neuroprotective effects in a model of focal cerebral ischemia by improving regional cortical blood flow and attenuating mitochondrial dysfunction and apoptosis (Li *et al.*, 2012). Moreover, galangin showed therapeutic potential for Alzheimer disease. It inhibited acetylcholine esterase activity (Guo *et al.*, 2010) and decreased β -amyloid production by suppressing BACE activity, which may be related to PPAR- activation (Sastre *et al.*, 2003; Descamps *et al.*, 2013). Recently, galangin has been suggested as one of the candidate compounds for the treatment of Alzheimer disease (Ahmad *et al.*, 2016). Therefore, the anti-inflammatory effects of galangin may provide therapeutic potential for various neurodegenerative diseases that are associated with neuroinflammation.

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