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Galangin regulates LPS-induced immune responses through the inhibition of dendritic cell and microglial cell activation

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이 논문을 이학석사학위 신청 논문으로 제출함

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CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

APCs	Antigen presenting cells
CNS	Central nervous system
COX-2	Cyclooxygenase-2
DCs	Dendritic cells
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide
ECL	Enhanced chemiluminescence
ELISA	Enzyme-linked immune sorbent assay
ERK	Extracellular signal-regulated kinases
FACS	Fluorescence activated cell sorter
FITC	Fluorescein isothiocyanate
GM-CSF	Granulocyte-macrophage colony stimulating factor
iDCs	Immature DCs
IFN-γ	Interferon-γ
Ig	Immunoglobulin
IL	Interleukin
iNOS	Inducible nitric oxide synthase
JNK	c-Jun N-terminal kinases
LPS	Lipopolysaccharide
mDCs	Mature DCs
MAPK	Mitogen-activated protein kinase
MFI	Mean fluorescence intensity
MHC	Histocompatibility complex

MTT	3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide
NF-κB	Nuclear factor-κappa-B
NO	Nitric oxide
PBS	Phosphate-buffered saline
PI	Propidium iodide
PE	Phycoerythrin
PVDF	Polyvinylidene difluoride
rm	Recombinant murine
RT	Reverse transcription
SDS	Sodium dodecyl sulphate
TCR	T cell receptor
Th1	T helper cell type 1
Th2	T helper cell type 2
TLR	Toll-like receptor
TNF	Tumor necrosis factor

ABSTRACT

Galangin regulates LPS-induced immune responses through the inhibition of dendritic cell and microglial cell activation

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Natural compound has been widely used for the treatment of various diseases, such as pneumonia, diarrhea, and hepatitis. Recent studies have demonstrated that natural compounds possess a wide range of pharmacological and biological activities, including anti-inflammatory, anti-microbial, anti-oxidant, and anti-tumor properties. Galangin is a natural compound derived from *Alpinia officinarum*, and propolis, and has a flanovol backbone. Microglia and denritic cells are originated from myeloid progenitor cells and act as antigen presenting cells in immune system. In previous studies, it has been reported that galangin has an anti-inflammatory effect on RAW 264.7 murine macrophages. However, the effect of galangin on microglial cells and dendritic cells, a kind of phagocytes, is still unknown. In the present study, I demonstrated that inhibitory activity of galangin on BV-2 murine microglial cells and bone marrow-derived dendiritc cells (DCs).

I found that galangin decreases nitric oxide (NO) production at non-cytotoxic concentration and the mRNA expression of pro-inflammatory factors such as inducible-NO synthesis (iNOS),

NO, interleukin (IL)-6, tumor necrosis factor (TNF)-α, and IL-1β on lipopolysaccharide (LPS)-stimulated BV-2 cells in a dose-dependent manner. In addition, galangin suppressed the protein expression of iNOS, and the secretion of IL-1β in LPS-activated BV-2 cells. I determined that galangin inhibited the phosphorylation of extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinases (JNK), p38, and nuclear factor-κappa-B (NF-κB) and degradation of NF-κB inhibitor (IκB-α). These results indicated that galangin has anti-neuroinflammatory effect on BV-2 murine microglial cells *via* inhibition of MAPKs and NF-κB activation.

I also found that galangin was not cytotixic to DCs and decreases the expression of co-stimulatory molecules such as cluster of differentiation (CD) 80, CD86, major histocompatibility (MHC) I, and MHC II on LPS-stimulated DCs maturation. On DCs, LPS down-regulated the antigen-uptake activity and galangin induced the LPS-decreased phagocytic activity. I ascertain the galangin suppresses the phophorylation of ERK, and JNK in LPS-stimulated DCs. And to determine the inhibitory effect of galangin on LPS-induced DCs maturation *in vivo*, oral administration of galangin reduced co-stimulatory molecules on splenic DCs of C57BL/6 mice. These results suggested that galangin has an inhibitory effect on LPS induced-DCs maturation through the suppression of ERK and JNK activation.

In summary, these findings provide the elucidation of immunopharmacological functions of galangin, and galangin should be considered potential therapeutic adjuvants for excessive immunological reactions and immune-related disease.

국문초록

Galangin 의 수지상 세포와 미세아교세포 활성화의 억제를 통해 LPS 로 유도된 면역 반응 조절

박푸름

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천연물은 폐렴, 설사 및 간염과 같은 다양한 질병의 치료에 널리 사용되어왔다. 최근 연구에 따르면 천연물들은 항염증, 항균, 항산화 및 항암 활성을 비롯한 광범위한 약리학적 및 생물학적 활성을 보유하고 있음이 입증되었다. Galangin은 *Alpinia officinarum* 및 propolis에서 추출한 천연물로 flanovol 구조를 가지고 있다. 미세아교세포와 수지상세포는 골수성 전구세포에서 유래되며, 항원제시세포로써의 기능을 가진다. 이전 연구들에서, galangin은 마우스의 대식세포인 RAW 264.7에 대해 항염증 효과가 있다고 보고되었다. 그러나 또 다른 식세포인 미세아교세포와 수지상세포에 미치는 영향은 아직 알려지지 않았다. 본 연구에서는, 마우스의 미세아교세포인 BV-2 세포와 골수 유래 수지상세포에서 galangin의 저해 활성을 관찰하였다.

지질다당체 (LPS)로 자극된 BV-2 세포에서 독성을 나타내지 않는 농도에서 galangin은 일산화 질소 (NO) 생성을 감소시키고, 유도형 NO생성효소 (iNOS), 인터루킨-6, 종양 괴사 인자-α (TNF-α) 및 인터루킨-1β와 같은 염증성 인자의 mRNA 발현을 농도 의존적으로 감소시켰다. 또한, galangin은 LPS로 활성화된 BV-2 세포에서 iNOS의 단백질 발현과 인터루킨-1β의 분비를 억제하였다. Galangin은 ERK, JNK, p38 및 NF-κB의 인산화와 IκB-α의 분해를 저해하였다.

더욱이 세포독성을 가지지 않는 농도의 galangin은 LPS로 자극된 수지상세포의 공동자극분자인 CD80, CD86, MHC I 및 MHC II의 발현을 감소시켰다. 또한 galangin은 LPS에 의해 감소된 수지상세포의 항원포식능을 개선하였으며, 이러한 효능은 ERK와 JNK 신호전달경로의 인산화를 억제함으로써 나타난다는 것을 확인하였다. 나아가 LPS로 유도된 수지상세포의 성숙에 대한 galangin의 억제 효과를 마우스의 생체 내에서 확인한 결과, galangin을 경구 투여했을 때 C57BL/6 마우스 비장의 수지상세포에서 공동자극분자 발현이 저해됨을 관찰하였다.

따라서, 본 연구는 미세아교세포와 수지상세포에 대한 galangin의 면역약리학적인 기능들을 규명하고, 과민성 면역반응과 그에 관련된 질병에 대한 치료보조제로서 가능성을 제시하였다.

Ⅰ. INTRODUCTION

1. Immunity

Immunity is a defense mechanism against to both exogenous antigens such as bacteria, virus, and parasites and endogenous antigens including tumor-antigen and auto-antigen. The immune response consists of an innate immune response that is nonspecific to the antigen and an adaptive immune response which is specific for the antigen (Thomas J. Kindt 2007).

1) Innate immunity

The innate immune system, also known as the nonspecific immune response, is an important primary immune reaction and consists of various immune cells which protect the host from infection by other microbes (Grasso 2002). The immune cells involved in innate immune system generally recognize and respond to pathogens. As contrast with the adaptive immune system, innate immunity does not provide long-lasting or specific immunity (Bruce Alberts 2002). In addition, the innate immune system which is found in all animals and plants, provides immediate defense mechanism against infection (Charles A Janeway 2001).

The primary function of the innate immune system of vertebrates is to recruit immune cells into the area of infection through the production of cytokines and chemokines. Then it activates the complement cascade and promotes clearance of immune complex (antigen-antibody complex) and dead cells. It also eliminates foreign bodies by specialized phagocytes in blood, tissues, organs, and lymphatic system. Some innate immune cells such as macrophages and dendritic cells can present the antigens and initiate adaptive immune response.

(1) Inflammation

Inflammation is major symptoms of the innate immune system to non-specific

infections. The inflammatory reaction is characterized by the following symptoms, locally increased blood circulation, erythema, mucus swollen, and fever (Parker and Picut 2005; Khansari, Shakiba et al. 2009; Pohl and Benseler 2013). Inflammation is induced by chemical factors released of damaged cells. On the other hands, it establishes physical barriers to infection spreading, and promotes healing of damaged tissues following pathogen removal (Reid, Jass et al. 2003; Martin and Leibovich 2005; Kawai and Akira 2006; Miller 2006; Ferrero-Miliani, Nielsen et al. 2007).

The process of acute inflammation is initiated by resident cells such as macrophages, dendritic cells, and mast cells in injured tissues (Yutin, Wolf et al. 2009). These cells express receptors called pattern recognition receptors (PRRs) on cell surface or intracellular space. PRRs recognize molecules as known as pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). And then, it can provide and widely shares the pathogen information to other immune cells. When a burn, infection, or other wound occurs, the one of the PRR expressed on activated cells recognizes PAMP and releases inflammatory cytokines such as interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α, subsequently the cytokines cause symptoms of inflammation (Vinay Kumar 1998). The inflammatory mediators cause local vasodilatation, and attraction of phagocytes, especially neutrophils. Neutrophils cause the additional immunological response by chemokines which recruit white blood cells and lymphocytes. The cytokines and chemokines are produced from macrophages and other immune cells under the inflammatory condition.

(2) Neuro-inflammation

Neuro-inflammation is inflammatory response of the nervous system. It can be initiated by a variety of stimuli including toxic metabolites, traumatic brain injury, infection, and autoimmunity (Gendelman 2002). Under the resting-state, the central nervous system (CNS) is immunologically privileged because the peripheral immune

cells are blocked by the intact blood brain barrier (BBB), a special structure composed of astrocytes and endothelial cells (Das Sarma 2014). However, circulating peripheral immune cells may infiltrate through a compromised BBB and encounter neurons and glial cells expressing major histocompatibility complex molecules (MHC), perpetuating the immune response (t Hart and den Dunnen 2013). These reaction usually has protective effect in the CNS, however the reaction can lead to excessive or chronic inflammation as well as additional migration of immune cells through the BBB (Gendelman 2002). In the neuro-inflammation, microglia plays a critical role in defense mechanism, and acts as phagocyte and antigen presenting cells (APCs) (Reid, Jass et al. 2003).

2) Antigen presenting cells

(1) Macrophage

Macrophages are a type of leukocyte that digests cellular debris, microbes, foreign organisms, and tumor cells in a process called phagocytosis. These cells are found in all kinds of tissues and protect them against pathogens through amoeboid movements. Macrophages play an effective role in nonspecific defense (innate immunity) and help to initiate specific defense mechanisms (adaptive immunity) by recruiting immune cells such as various lymphocytes (Gordon 1998; Ovchinnikov 2008; Locati, Mantovani et al. 2013).

Macrophages divided into M1 and M2 phenotype, and they have an opposite characters each other. M1 macrophages are involved in inflammatory response and the dysfunctions of M1 macrophages cause several immune-related disorders. On the other hands, M2 macrophages have a tissue repair activities and produce the anti-inflammatory cytokines (Akira, Uematsu et al. 2006; Moreira and Hogaboam 2011; Mills 2012).

(2) Microglia

Microglia, as known as phagocytes in the brain, accounts for 10 to 15% of total brain cells (Lawson, Perry et al. 1992; Ginhoux, Lim et al. 2013). It has important role in neuro-inflammatory response which is major immune defense mechanism against exogenous pathogen and injury in the CNS (Gehrmann, Matsumoto et al. 1995; Filiano, Gadani et al. 2015) This process is very sensitive to small pathological changes in the CNS, as it must be efficient in preventing potential and catastrophic damage (Dissing-Olesen, Ladeby et al. 2007).

If a person directly infected by pathogens which cross the BBB, the microglia rapidly reacts to eliminate the infectious agent and reduce inflammation before tissue injury. Microglia can act as APCs which recognize and present the antigen, and prime the T cells

activation in CNS (Gehrmann, Matsumoto et al. 1995; Aloisi 2001).

(3) Dendritic cells

DCs are a kind of immune cells that make up the immune system of mammals. It is originated from both lymphoid progenitor and myeloid progenitor derived hematopoietic stem cells in bone marrow. And myeloid-derived DCs are originated from same lineage as macrophages and microglia. It exists in tissues that are exposed to the external environment, such as the skin, nasal cavity, lungs, stomach, and intestines. DCs are strongest APCs under the stimulated condition, while they can generally be found immature state in the blood on resting condition. They can uptake the pathogens *via* endocytosis, and break down their proteins into small pieces which are presented on the cell surface as specific antigen using MHC classes (Fig. 1) (Austyn 1998; Banchereau and Steinman 1998; Lo and Clare-Salzler 2006). They can provide the information of pathogen and regulate T cells activation by MHC-T cell receptor (TCR) complex and co-stimulatory molecules such as CD80 (B7.1) and CD86 (B7.2) (Maverakis, Kim et al. 2015).

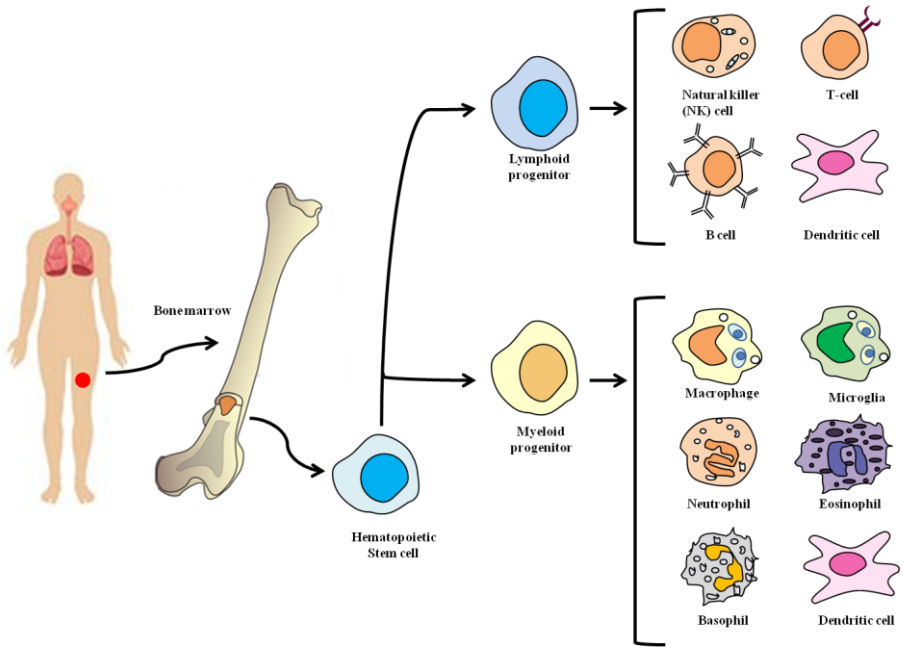

Fig. 1. Immune cells

3) Adaptive immunity

The adaptive immune system is pathogen-specific immune strategy found in vertebrate animals. In contrast to innate immunity, adaptive immune response provides immediate and long-lasting reaction. It generates immunological memory by an initial challenge of particular pathogen, therefore they can induce an immediate response to same pathogen infections. Adaptive immune system includes both humoral immunity and cell-mediated immunity.

DCs, major APCs of adaptive immunity, uptake infected pathogens and present pathogen-specific antigen on the MHC classes which bind to TCR expressed on Th cells. The Th cells are differentiated into type 1 helper T cell (Th1) and type 2 helper T cell (Th2) depend on co-stimulatory molecules and cytokines of mature DCs. Th1 cells trigger the cytotoxic T cells (Tc cells) activation, it can directly attack on virus, bacteria, and tumor cells using perforin and granzyme. Th2 cells induce switching the immature B cells to plasma cells or memory B cells. The plasma cells produce and secrete pathogen-specific antibodies, which can be tagged with complement, and the memory B cells can lead the stronger and more rapid antibody response to re-challenge of the same pathogens (Fig. 2). (Janeway 2001; Alberts 2002; Thomas J. Kindt 2007; Comeau, Hale et al. 2010; Spencer and Weller 2010).

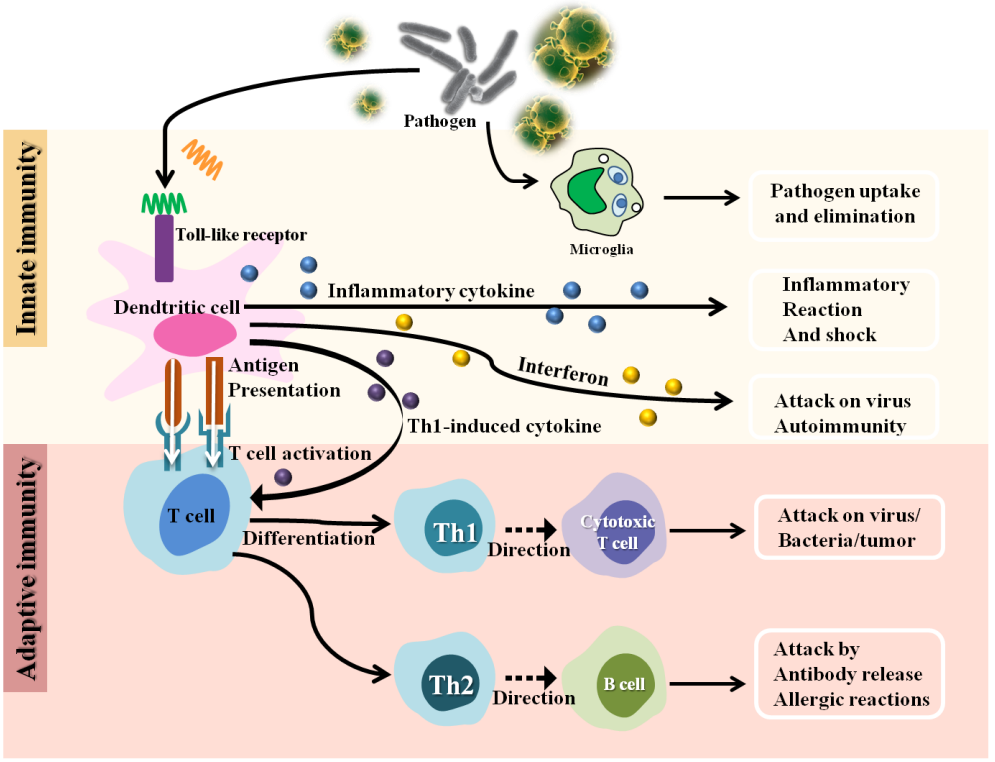

Fig. 2. Innate immunity and adaptive immunity.

2. Galangin

Flavonoids consist of over 4000 polyphenolic compounds that naturally exist in a variety of foods, including vegetables, herbs, grains and fruits. The flavonoid has a carbon skeleton structure of C_6-C_3-C_6 type in which two phenyl groups are bonded through a C_3 chain. It is divided into isoflavones, flavonols, and flavones, depending on where the substituent is attached (Leonard, Yan et al. 2006; Wang, Lee et al. 2009).

Flavonoids have been previously reported to be effective against anti-oxidants, anti-cancer and adult diseases, and people tend to be interested in foods containing flavonoids nowadays (Seelinger, Merfort et al. 2008; Xie, Kang et al. 2012).

Galangin,3, 5, 7-trihydroxyflavone, has three hydroxyl groups and carbon rings. It belongs to the flavonols which is included in flavonoids (Fig. 3). It is abundant in *Zingiber officinale* (ginger), *Alpinia officinarum* and *Helichrysum aureonitens* (Afolayan and Meyer 1997; Ciolino and Yeh 1999). In previous studies, it has been reported that galangin has useful biological activities such as anti-fibrosis, anti-cancer, and anti-oxidants (So, Guthrie et al. 1996; Meyer, Afolayan et al. 1997; So, Guthrie et al. 1997; Cushnie and Lamb 2005; Cushnie and Lamb 2006; Zhang, Luo et al. 2010; Wen, Wu et al. 2012; Wang, Gong et al. 2013; Zhang, Tang et al. 2013).

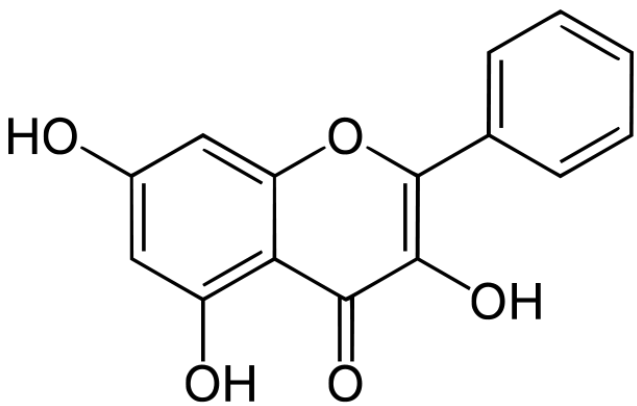

Fig. 3. Structure of galangin

3. Signaling pathway

1) Mitogen-activated protein kinase pathway

Mitogen-activated protein kinase (MAPK) family is a serine/threonine-specific protein kinase consists of extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK. MAPKs are involved in diverse cellular responses triggered by a variety of stimuli such as stresses, mitogenic stimulants, and pro-inflammatory cytokines. It also modulates cellular activity including differentiation, proliferation, transcription, cell survival, division, homeostasis, and cell death. (Meriin, Mabuchi et al. 2001).

The ERK signaling pathway is involved in cell proliferation, division, differentiation, and maturation. They are associated with Ras or Raf proteins (Rao and Reddy 1994; Orton, Sturm et al. 2005; Matsuda and Fukumoto 2011; Kim and Sim 2012). JNK is derived from three genes (*JNK1*, *JNK2*, and *JNK3)* and is involved in the stress condition, cell death, and development of insulin resistance (Hirosumi, Tuncman et al. 2002; Oltmanns, Issa et al. 2003). p38 MAPK is involved in cell differentiation and, apoptosis respond to various stimuli (Segales, Perdiguero et al. 2016).

MAPKs cascade are regulated by upstream signal transduction such as MAP2Ks and MAP3Ks. MAP2Ks phosphorylated by MAP3Ks can induce the activation of MAPKs cascade, subsequently MAPKs can regulate several downstream signals including nuclear transcription factors. In the immune responses, LPS can bind to toll-like receptor (TLR) 4 and then trigger the downstream signal transduction mediated by myeloid differentiation primary response gene 88 (MyD88) under the LPS-stimulated inflammatory condition. Activated MyD88 can lead the phosphorylation of MAPKs and IKK mediated by IL-1 receptor-associated kinase (IRAK) and TNF receptor associated factor (TRAF) activation (Derijard, Raingeaud et al. 1995; Roberts and Der 2007).

2) Nuclear factor κ-light chain-enhancer of activated B cells pathway

Nuclear factor κ-light chain-enhancer of activated B cells (NF-κB) is transcription factor and a heterodimeric protein complex that regulates cytokine production, DNA transcription and cell survival. NF-κB is found in almost animal cells and is involved in cellular responses to stimuli such as cytokines, oxidative stress, growth factors, and exogenous antigen (Gilmore 1999; Tian and Brasier 2003; Brasier 2006; Gilmore 2006; Perkins 2007). NF-κB has a critical role in the several cellular functions including tumor growth, infections, autoimmunity, and immune responses (Freudenthal, Locatelli et al. 1998; Albensi and Mattson 2000; Merlo, Freudenthal et al. 2002; Meffert, Chang et al. 2003; Levenson, Choi et al. 2004; Park and Youn 2013).

NF-κB family consists of two classes (class I and II) which include five subunits. Class I is comprised of NF-κB1 (p50) and NF-κB2 (p52), and class II is constituted by RelA (p65), RelB, and c-Rel. They generally exist as heterodimer form in various cells. In NF-κB signaling pathway, NF-κB inhibitor (IκB) kinase (IKK) is activated by MAPKs phosphorylation or directly phosphorylated by MyD88 mediated signaling pathway. Activated IKK induces IκB-α phosphorylation, continuously p-IκB-α is ubiquitinated and degraded by proteosome (Karin 1999). NF-κB, freed from IκB-α, can expose the nuclear localization sequence (NLS) of RelA (p65). NF-κB-p65 subunit is phosphorylated by kinases such as MAPKs and translocated to the nucleus from cytosol. P-p65 binds to specific DNA-binding site, and regulates the transcription of various immune-associated genes including pro-inflammatory cytokines, chemokines, and CD molecules. (Mercurio, Zhu et al. 1997; Regnier, Song et al. 1997; Jacobs and Harrison 1998). Thus, inflammatory cytokines and inducible enzymes are expressed (Fig. 4) (Monaco, Andreakos et al. 2004).

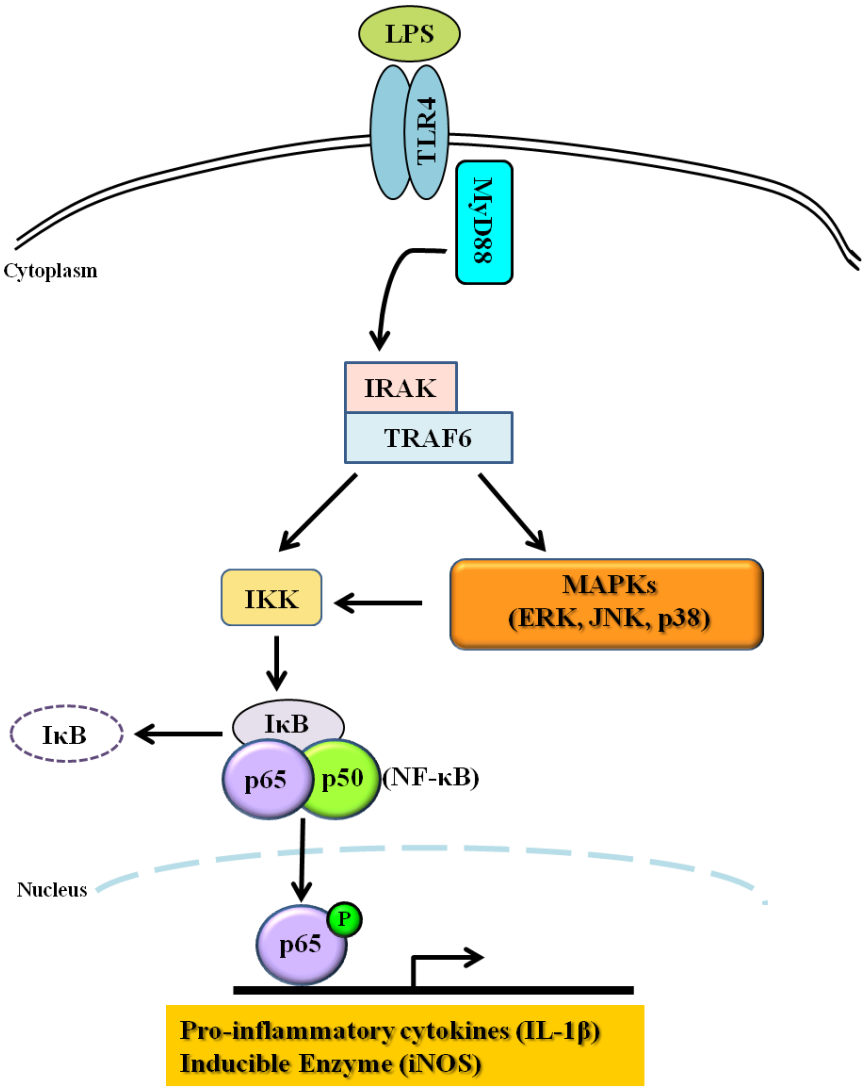

Fig. 4. MAPKs pathway and NF-κB pathway

Ⅱ. MATERIALS AND METHODS

1. Reagents

Galangin, 3-(4, 5-Dimethyl-2-thiazolyl)-2, TRI reagent, Griess reagent and 5-diphenyl-2H-tetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). e-Taq DNA Polymerase kit and and RedSafe Nucleaic Acid Staining Solution were purchased from Solenget (Daejeon, Korea) and Intron Biotechnology (Seongnam-Si, Korea), respectively. Recombinant mouse (rm) GM-CSF and rmIL-4 were purchased for Invitrogen Corporation (Carlsbad, CA, USA).

2. Cell culture

Murine microglial cell line BV-2 was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 200 IU/ml penicillin, 200 μg/ml streptomycin, 4 mM L-glutamine and 1 mM sodium pyruvate at 37℃ in the presence of 5% CO^2.

3. Galangin treatment

Galangin was dissolved in dimethyl sulfoxide (DMSO) and diluted to the desired concentration in DMEM. Equal amount of DMSO to the final concentration of galangin was used as a control (untreated) samples.

4. Cell viability assay using MTT assay

Cell viability was assessed by colorimetric MTT assay. The cells (1×10^4 cells/well) were seeded in 96-well culture plates with DMEM. Cells were treated with various galangin concentrations (0, 5, 10, 20, and 30 μM) and incubated at 37℃ for 24 hr. After incubation, the medium containing galangin was sucked and MTT solution (0.5 mg/ml) was added to each well. After incubation at 37℃ for 4 hr, MTT solution was removed and formazan product was

dissolved in solvent (1:1 = DMSO:ethanol). Absorbance of dissolved formazan solution was quantified by an Enzyme-linked immunosorbent assay (ELISA) micro reader at 570 nm.

5. NO assay

BV-2 cells (2×10^4 cells/well) were seeded 96-well culture plate in DMEM. The cultured cells were pretreated with various concentrations of galangin (0 - 30 μM) for 2 hr, and then the cells were incubated for 22 hr with or without LPS (200 ng/ml). The cultured medium was then mixed with an equal volume of 1× Griess reagent (40 mg/ml) and incubated at room temperature for 15 min. After incubation, the absorbance was quantified by ELISA microplate reader at 540 nm.

6. Reverse transcription (RT)-PCR

BV-2 cells (3×10^5 cells/well) were seeded in 12-well culture plate. Cultured cells were pretreated with various concentration of galangin (0 - 30 μM) for 2 hr. After treatment, cells were incubated for 6 hr with or without LPS (200 ng/ml). Incubated cells were collected by centrifugation and total RNA was isolated from the cells using TRI reagent according to manufacturer's protocol. To synthesize cDNA, 1 μg of total RNA was primed with oligodT and reacted with mixture of dNTP, M-MLV RTase, and reaction buffer (Promega, WI, USA). To quantify the mRNA level of inflammatory genes including iNOS, IL-1β, and COX-2, I designed the primers for target genes (Table. 1) (Bioneer, Daejeon, Korea). And cDNA was amplified using e-Taq DNA polymerase kit (solenget, Daejeon, Korea) and Gene Atlas G02 gradient thermal cycler system (Astec, Japen). And then, PCR products were visualized using RedSafe Nucleaic Acid Staining Solution (Intron Biotechnology, Seongnam-Si, Korea), NEO image for NaBI and Nucleic acid Bioimaging Instrument (NeoScience Co., Ltd., Suwon, Korea).

Table. 1. Primers used in RT-PCR

Target		Sequence
i-NOS	F	5'-CTTGCCCCTGGAAGTTTCTC-3'
	R	5'-GCAAGTGAAATCCGATGTGG-3'
COX-2	F	5'-TGGGTGTGAAGGGAAATAAGG-3'
	R	5'-CATCATATTTGAGCCTTGGGG-3'
IL-6	F	5'-CCTTCCTACCCCAATTTCCA-3'
	R	5'-CGCACTAGGTTTGCCCACTA-3'
IL-1β	F	5'-GTGTCTTTCCCGTGGACCTT-3'
	R	5'-TCGTTGCTTGGTTCTCCTTG-3'
TNF-α	F	5'-GGCCTCTCTACCTTGTGCC-3'
	R	5'-TAGGCGATTACAGTCACGGC-3'
GPADH	F	5'-TGCACCACCAACTGCTTAG-3'
	R	5'-GGATGCAGGGATGATGTTC-3'

7. Western blot analysis

To confirm the protein expression level of iNOS (NOS2) and COX-2, BV-2 cells were pretreated with various concentration of galangin (0 - 30 μM) for 2 hr and then treated with or without LPS (200 ng/ml). For verification of signal transduction, BV-2 cells were serum starved for 4 hr in serum-free medium and then pretreated with 30 μM galangin for 2 hr. After incubation, LPS (200 ng/ml) was added to the cells in the absence or presence of galangin for 13, 30, or 45 min. Following chemical treatment, the cells were washed with cold 10 mM PBS and lysed with modified RIPA buffer containing 150 mM sodium chloride, 0.5% sodium deoxycholate, 1% Triton X-100, 50 mM Tris (pH 8.0), 0.1% sodium dodecyl sulphate (SDS), 1 mM phenylmethylsufonyl fluoride (PMSF), 1 μg/mL pepstatin, 2 μg/mL leupeptin, 1 mM sodium orthovanadate, and 100 mM sodium fluoride using VCX130 Ultrasonic Processor (Sonics & Materials, Inc., CT, USA) at 4℃. The protein content of cell lysates was assessed using the Micro BCA assay kit (Pierce, Rockford, IL, USA). Equal amounts of proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to Polyvinylidene Difluoride (PVDF) membrane. The membrane was blocked with blocking solution (2% BSA) at room temperature for 1 hr. And then, the membrane was incubated with primary antibodies (listed in Table. 2) overnight (Santa Cruz Biotechnology, CA, USA). Horseradish peroxidase-conjugated anti-rabbit IgG and anti-mouse IgG antibodies (Santa cruze Biotechnology) were used as the secondary antibodies. Band detection was performed using the ECL (Enhanced chemiluminescence) detection system and exposed to radiation film. Pre-stained blue markers were used for molecular weight determination.

Table. 2. Primary antibodies used in western blotting

1st antibody	2nd antibody	Dilution ratio	Company (Cat No.)
ERK1/2	Rabbit	1:2000	Cell signaling #9102
phospho-ERK1/2	Mouse	1:2000	Santa Cruz Sc-7378
JNK1/2	Rabbit	1:2000	Santa Cruz Sc-571
phospho-JNK1/2	Mouse	1:2000	Santa Cruz Sc-6254
p38	Rabbit	1:2000	Cell signaling #9212
phospho-p38	Mouse	1:2000	Santa Cruz Sc-166182
NF-κB-p65	Mouse	1:2000	Santa Cruz Sc-8008
phospho-NF-κB-p65	Rabbit	1:1000	Santa Cruz Sc-33020
IκB-α	Rabbit	1:2000	Santa Cruz Sc-847
NOS2	Mouse	1:2000	Santa Cruz Sc-8310
Cox-2	Rabbit	1:2000	Santa Cruz Sc-166475
β-actin	Mouse	1:5000	Santa Cruz Sc-47778

8. Enzyme-linked immune sorbent assay (ELISA)

BV-2 cells (2×10^4 cells/well) were seeded in 96-well culture plate. The cells were pretreated with various concentration of galangin (0 – 30 μM) for 2 hr, and then incubated in absence or presence of LPS for 22 hr. Supernatant was used as samples and the quantification of IL-1β release was measured by Mouse IL-1β ELISA MAX^{TM} Deluxe Sets (BioLegend, CA, USA), according to manufacturer's protocol. Briefly, standard and samples were incubated on capture antibody coated plate at 4℃, overnight. Detection antibody was incubated for 3 hr and Avidin-HRP bind to detection antibody. To visualization, substrate solution was added to each well, and stopped by stop solution (2N H_2SO_4). Absorbance was measured by ELISA micro plate reader at 405 nm wavelength.

9. Animals

7-week-old male C57BL/6 (H-2K^b and I-A^b) mice were purchased from the Orient Bio (Sungnam, Korea). The mice were housed in a specific pathogen-free environment and acclimatized for at least 1 week before use. Mice were fed with galangin dissolved in corn oil by oral administration for 3 days (p.o.). On the 4th day, LPS (1 mg/kg) was administered by intraperitoneal injection (i.p.). After 24 hr, they were euthanatized by CO_2 gas. I measured the body weight everyday and isolated the whole splenocyte. All mouse work was approved by the IACUC.

10. Generation and culture of DCs

Immature DCs were generated from C57BL/6 mice bone marrow. DCs were cultured in RPMI-1640 medium (welgene, Gyeongsangbuk-do, South Korea), containing 10% heat-inactivated FBS, 1% penicillin, 4 mM L-glutamine and 1 mM sodium bicarbonate and 20 ng/ml GM-CSF at 37℃ in the presence of 5% CO_2. DCs were seeded in 6-well culture plates (2×10^6 cells/ml; 2ml/well). GM-CSF was newly supplied with new media every other day for

6 days. On day 6, immature DCs were treated with galangin or stimulated with 100 ng/ml LPS.

12. Antigen uptake assay

DCs were seeded in 12-well culture plate (1×10^6 cells/ml; 1 ml/well). The cultured cells were pretreated with galangin (60 μM) for 2 hr, and then the cells were incubated for 22 hr in the absence and presence of LPS (100 ng/ml) at 37℃ in the presence of 5% CO_2. After 22 hr, it was washed with 1X PBS and were stained with FITC-dextran and cultured at 4℃ or 37℃, respectively. Exposure to dextran-FITC at 4℃ was used as a negative control (non-specific binding) for endocytic activity. After 45 min, both 4℃ and 37℃ were washed with 1X cold PBS. Subsequently, DCs were stained with a PE-conjugated anti-CD11c antibody. Antigen uptake was assessed by flow cytometry.

13. Cell viability assay using Annexin/PI staining

Galangin was dissolved in DMSO and added to isolate DCs in 6-well plates (2×10^6 cells/ml; 2 ml/well). DMSO alone (0.06% v/v) was used as a control. For the analysis of cytotoxicity, DCs were treated with various concentration of galangin (0, 20, 40, and 60 μM). Additionally, cytotoxicity was analyzed by FITC Annexin V Apoptosis Detection Kit I (BD biosciences, CA, USA) and FC500 (Beckman coulter, CA, USA) according to the manufacturer's instruction.

14. Flow cytometric analysis

On day 6, harvested BM-DCs were washed with PBS and resuspended in flow cytometry washing buffer (1% fetal bovine serum in PBS). The cells were stained with phycoerythrin (PE)-conjugated anti-H-$2K^b$ [major histocompatibility complex (MHC) class I], anti-I-A^b (MHC class II), anti-CD80, and anti-CD86 with FITC-conjugated anti-CD11c (eBioscience, CA, USA) for 30 min at 4℃. The stained cells were measured by FC500 and analyzed by

Kaluza Flow Cytometry Analysis Software (Beckman coulter, CA, USA)

15. Statistical analysis

All results were represented as the means ± SD of the indicated number of experiments and significance was estimated using a Student' s t-test for unpaired observations, and the differences were compared with regard to statistical significance by one-way ANOVA, followed by LSD *post hoc* test. A P value of < 0.05 was considered significant.

Ⅲ. RESULTS

PART Ⅰ. Anti-inflammatory effects of galangin on LPS-stimulated microglial activation *via* MAPKs and NF-κB pathway regulation

1) Galangin inhibits the production of NO in LPS-stimulated microglia

First, I examined the cytotoxicity of galangin in BV-2 cells (such as microglial cells) using MTT assay. Microglia was cultured for 24 hours in the presence of various concentrations of galangin (0, 5, 10, 20 and 30 μM). Galangin have no cytotoxicity to BV-2 cells up to 30 μM (Fig. 5). The Griess assay was used to investigate whether galangin could effectively regulate NO production at these concentrations. Microglia was pretreated with galangin for 2 hr before stimulation with LPS (200 ng/ml). The LPS-induced NO production was significantly decreased by galangin treatment in a dose-dependent manner (Fig. 6). These results indicate that galangin effectively inhibits NO production in LPS-stimulated microglia.

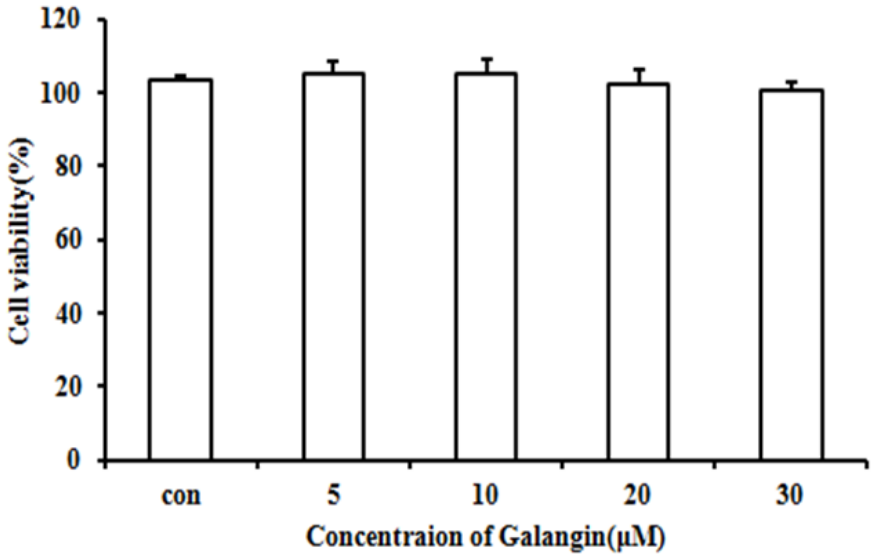

Fig. 5. Galangin has no effect on cell viability in microglia. Microglia was seeded in 96-well cell culture plates. Various concentrations of galangin (0 – 30 μM) and control (0.03% DMSO) were added and the number of viable cells was assessed by MTT assay after 24 hr as described in *materials and methods*. Data are reported as the number of viable cells present as a percentage of control cells exposed to 0.03% DMSO. The data represent the average (± SD) of four replicate wells and are representative of three separate experiments.

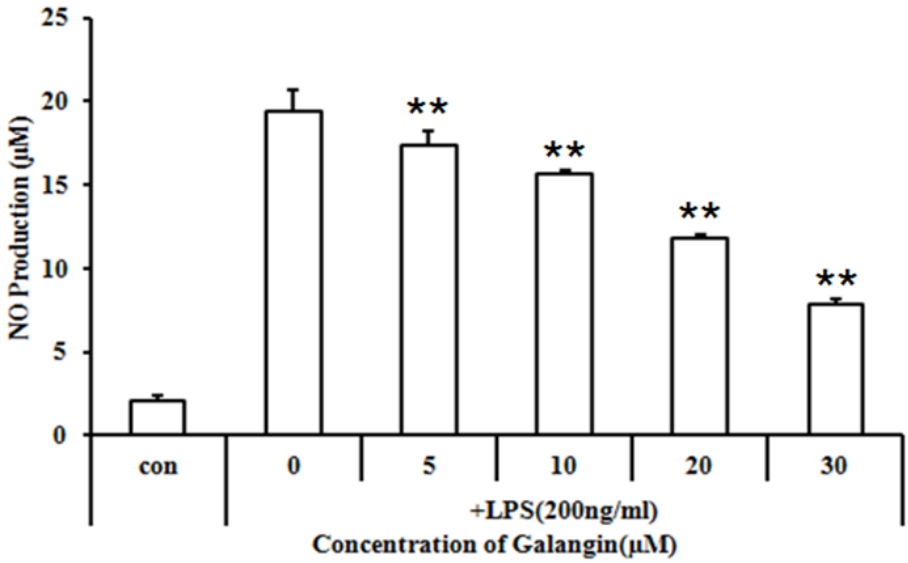

Fig. 6. Galangin reduces LPS-induced NO in microglia. Microglia was seeded in 96-well cell culture plates. Microglia was pretreated with various concentrations of galangin for 2 hr and stimulated with LPS (200 ng/ml). After 22 hr, NO production levels were determined using the NO assay described in *materials and methods*. Supernatants were mixed with Griess reagent, and absorbance was measured by ELISA microplate reader. The data represent the average (± SD) of four replicate wells and are representative of three separate experiments (**$P < 0.01$ *vs.* LPS only groups).

2) Galangin inhibits LPS-stimulated pro-inflammatory factors.

I investigated whether galangin could inhibit the levels of the pro-inflammatory genes including IL-1β, IL-6, and TNF-α as well as pro-inflammatory enzymes such as iNOS and COX-2. RT-PCR analysis was used to determine the expression level of pro-inflammatory genes. Galangin was pretreated for 2 hours and then stimulated with LPS (200 ng/ml) for 6 hours. As shown in Fig. 7A, mRNA expression levels of iNOS, COX-2, IL-6, TNF-α, and IL-1β were increased in LPS-stimulated microglia, among them iNOS, COX-2, and IL-1β were significantly reduced by galangin treatment in a dose-dependent manner. Because galangin effectively reduced the mRNA level of iNOS, I confirmed the protein expression level of iNOS and COX-2. As a result, the protein level of iNOS was decreased by galangin treatment in LPS-stimulated microglia (Fig. 7B). However, COX-2 mRNA and protein levels were not regulated by galangin (Fig. 7A and B). These results indicate that galangin inhibits mRNA and protein expression level of iNOS in LPS-stimulated microglia.

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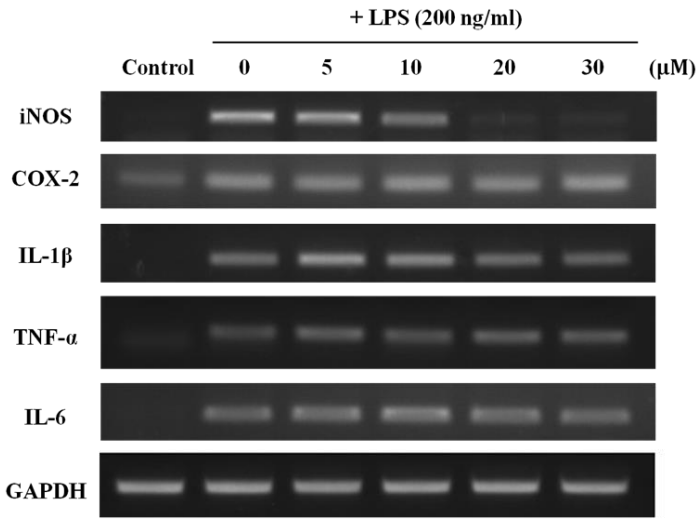

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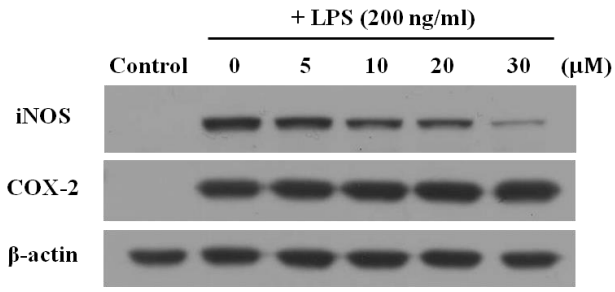

Fig. 7. Galangin suppresses the mRNA and protein expression levels of pro-inflammatory factors. (A) Microglia was pretreated with galangin (0 - 30 μM) for 2 hr and stimulated with LPS for 6 hr; mRNA expression levels of inflammatory genes were determined by RT-PCR. GAPDH was used as an internal control. (B) Microglia was stimulated with LPS for 24 hr following pretreatment of galangin. Western blots were used to determine protein levels. β-actin was used as an internal control.

3) Galangin decreases the cytokine release of IL-1β

IL-1β is a proinflammatory cytokine and released from microglia by LPS or other inflammatory stimuli. Previous result shows reduced IL-1β mRNA expression levels by treatment of galangin. Therefore, ELISA assay was performed to determine whether galangin regulates cytokine release of IL-1β. As shown Fig. 8, stimulation of microglia with LPS increased the secretion level of IL-1β, which was significantly decreased by galangin treatment in a dose-dependent manner. However, TNF-α and IL-6 cytokine secretions were not regulated by galangin. These results suggest that galangin reduced the cytokine secretion of IL-1β from LPS-stimulated microglia.

4) Galangin treatment reduces LPS-stimulated activation of MAPKs

MAPKs pathway includes ERK1/2, JNK1/2, and p38 and are involved in the regulation of inflammatory mediators through the activation of transcription factors, particularly NF-κB. To demonstrate the molecular mechanism of inhibitory effect of galangin, the LPS-induced MAPKs phosphorylation was determined by western blot analysis. AS shown in Fig. 9, phosphorylation of ERK 1/2, JNK 1/2, p38 was increased by LPS stimulation at 15, 30, and 45 min. And treatment of galangin significantly reduced the LPS-stimulated phosphorylation of ERK 1/2, JNK 1/2 and p-p38. This result indicate that galangin altered LPS-stimulated microglia activation by inhibition of MAPKs phosphorylation.

Fig. 8. Galangin attenuates secretion of IL-1β from microglia. Microglia was pretreated with various concentrations (0 - 30 μM) of galangin, and stimulated with LPS for 24 hr. Supernatants were examined for cytokine release, including IL-1β, TNF-α and IL-6 were measured by ELISA. Data are expressed as the average (±SD) of triplicate cultures (**$P < 0.01$ *vs*. LPS only groups).

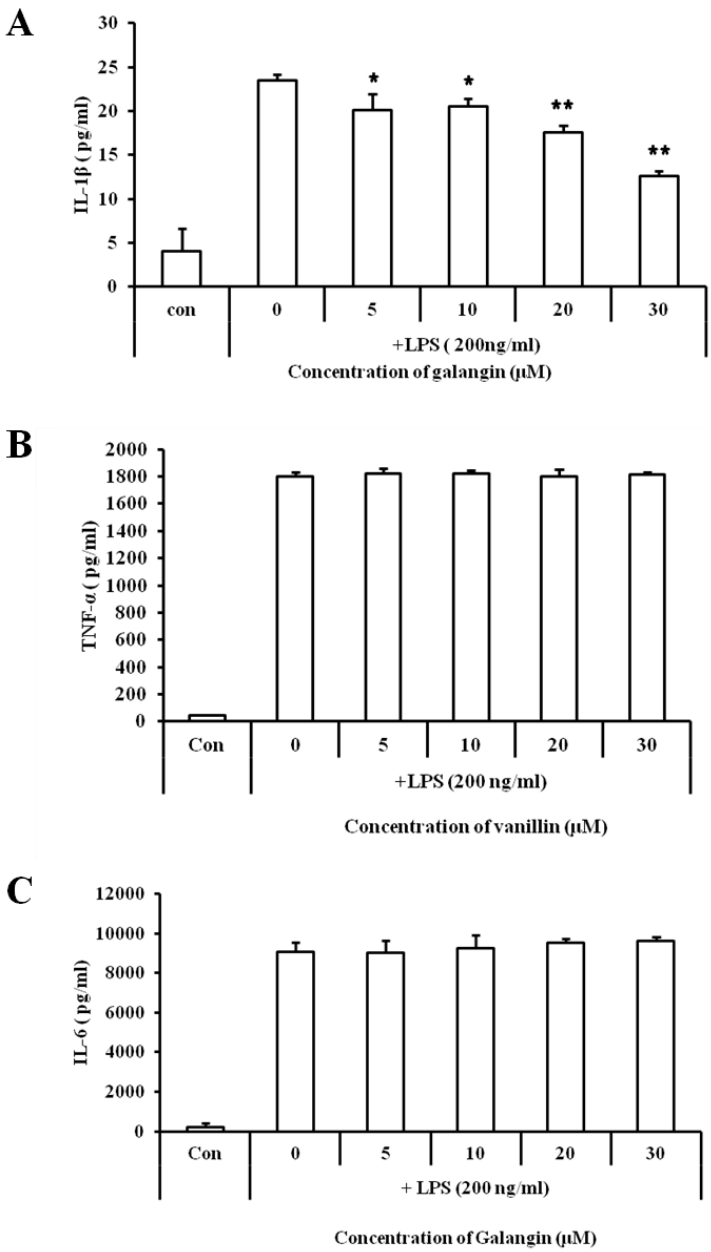

Fig. 8. (continued)

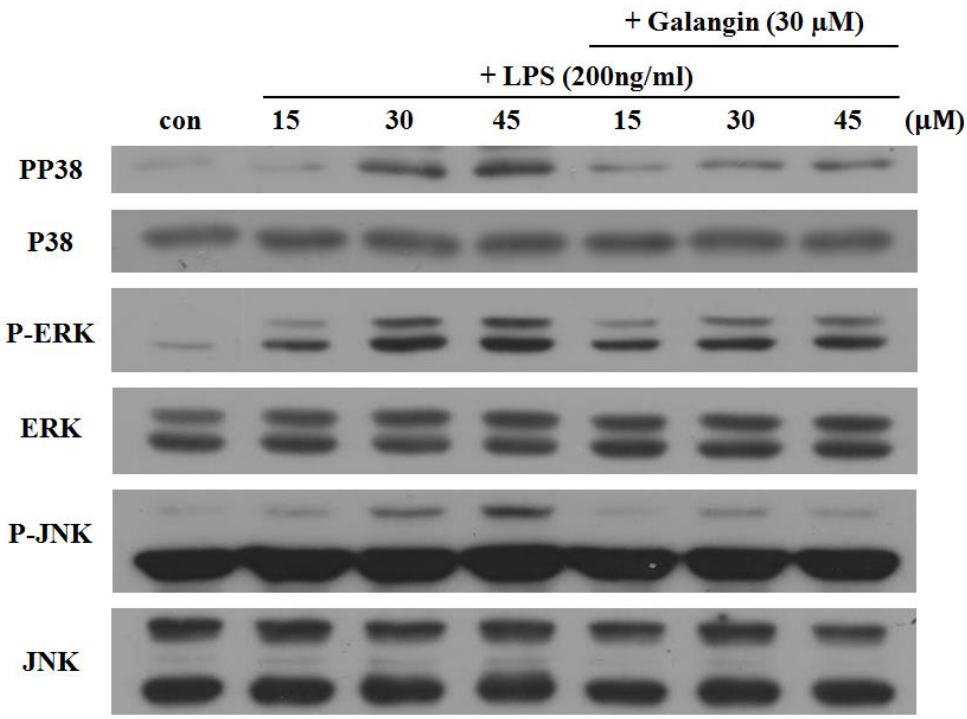

Fig. 9. Galangin inhibits LPS-stimulated ERK, JNK and p38 phosphorylation. Microglia was starved in serum-free DMEM for 4 hr and pretreated with 30 μM galangin for 2 hr. The cells were then stimulated with 200 ng/ml LPS for 15, 30 and 45 min. The phosphorylation of MAPKs was analyzed by western blot analysis using anti-ERK, anti-p-ERK, anti-JNK, anti-p-JNK, anti-p-38, anti-p-p-38 antibodies. This result is from one representative experiment, and is one of three that represent a similar pattern.

5) Galangin attenuates LPS-induced NF-κB activation

Phosphorylated MAPKs can activate IKK, activated IKK phosphorylates Iκ-B. Phosphorylated Iκ-B was subsequently ubiquitinated and degraded by proteosome. Degradation of IκB-α, which is the inhibitor protein of NF-κB, allowed NF-κB translocation from cytosol to nuclear. As shown Fig. 10, IκB-α were decreased after 15, 30 and 45 min of LPS-stimulated microglia activation, and galangin slightly recovered IκB-degradation. NF-κB activation plays an important role in the induction of inflammatory genes transcription such as iNOS, COX-2, and IL-1β in LPS-activated microglia. Thus, the effect of galangin on the activation of NF-κB subunit NF-κB-p65 was determined in LPS-activated microglia. Galangin reduced the LPS-induced phosphorylation of the NF-κB subunit NF-κB-p65 in microglia. These results suggest that the MAPKs pathway, along with the NF-κB pathway, may contribute to the inhibitory effect of galangin on the down-regulation of pro-inflammatory mediators such as iNOS and IL-1β.

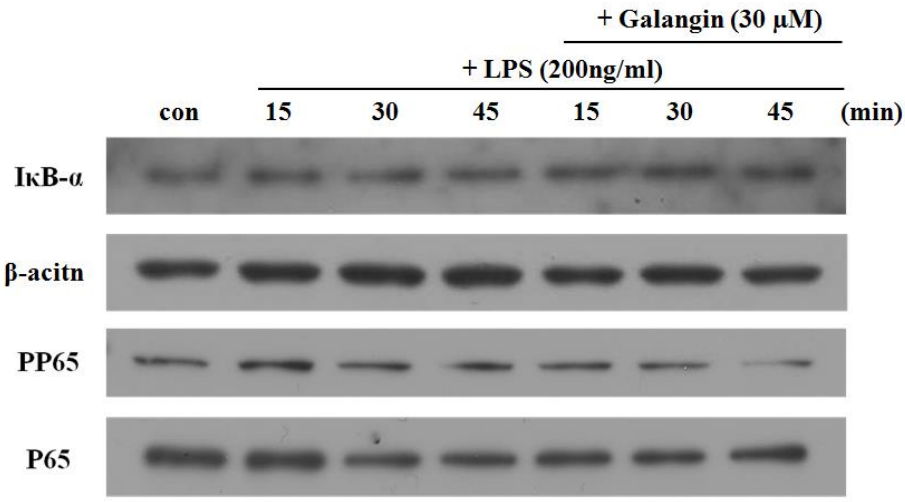

Fig. 10. Galangin regulates the NF-κB pathway. Microglia was starved in serum-free DMEM for 4 hr and pretreated with 30 μM galangin for 2 hr. The cells were then stimulated with 200 ng/ml LPS for 15, 30 and 45 min. Cells were lysed and equal amount of whole cell proteins were separated by western blot analysis using anti-IκB-α, anti-p-65 and anti-p-p-65 antibodies. β-actin was used as an internal control. This result is from one representative experiment, and is one of three that represent a similar pattern.

PART Ⅱ. Galangin regulates LPS-induced immune response through the inhibition of dendritic cell maturation.

1) Galangin inhibits LPS-induced DCs maturation

First, it was investigated whether galangin could affect DCs maturation. Bone marrow-derived monocytes were differentiated into DCs in media supplemented with GM-CSF and IL-4 for 6 days. After 6 days, DCs were cultured for 24 hours in the presence of various concentrations of galangin (0, 20, 40 and 60 μM) and the cytotoxicity was analyzed by annexin/PI staining. As shown Fig. 11A, galangin did not indicate cytotoxicity to DCs up to 60 μM. Next, the protein expression levels of the co-stimulatory molecules DC80 (B7-1), CD86 (B7-2), MHC class I and II were measured in LPS-stimulated DCs to determine if galangin had a modulating effect on DCs maturation. Galangin treatment significantly decreased the up-regulation of CD80, CD86, and MHC class I on LPS-induced DCs maturation, MHC II was also slightly decreased on LPS-activated DCs (Fig. 11B). These results indicated that the galangin suppresses LPS-induced DCs maturation.

Fig. 11. Galangin suppresses the expression of co-stimulatory molecules in during DCs maturation. DCs were generated as written in the *Materials and Methods*. (A) On day 6, galangin was treated for 24 hr at concentrations of 20, 40 and 60 μM. DCs were stained for annexin-V and PI. (B) The expression of surface molecules was analyzed by two-color flow cytometry. Galangin was pretreated for 2 hr and stimulated with 100 ng/ml LPS for 24 hr. These results represent three separate experiments.

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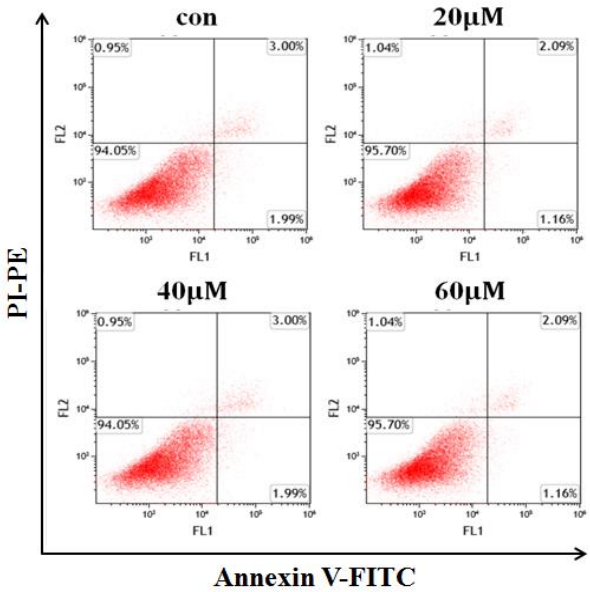

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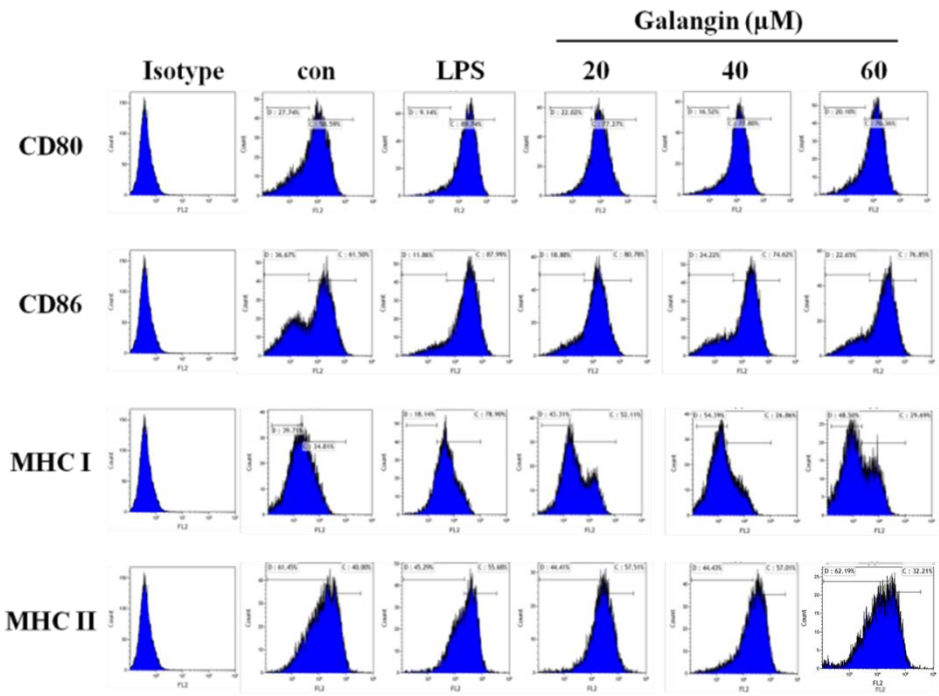


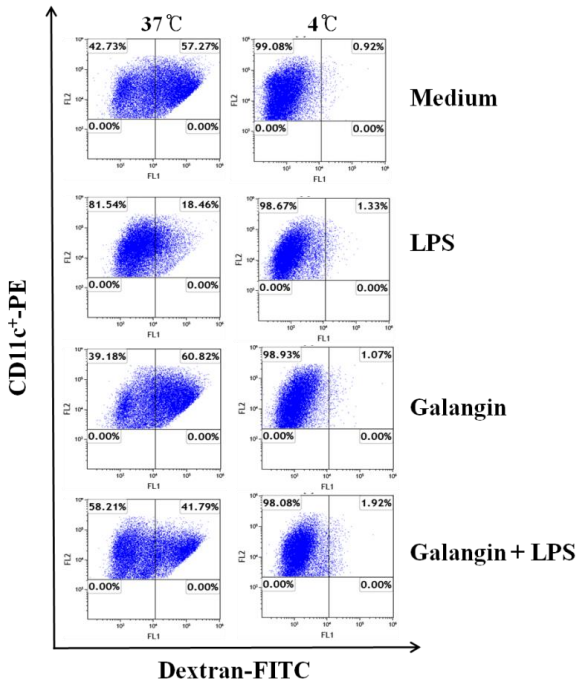
Fig. 11. (continued)

2) Galangin enhances the endocytic capacity

As previous results, the alteration of co-stimulatory molecules indicated that galangin induce the inhibitory effect on DCs maturation. Therefore, I investigated whether the antigen uptake ability of DCs was altered when galangin was treated. DCs which are exposed to galangin in the presence or absence of LPS, incubated with the media containing FITC-conjugated dextran to measure endocytic activity. And then the DCs were double-stained with PE-conjugated anti-CD11c. In results, there was no difference in the percentage of double-positive cells ($CD11c^{+}$-PE with dextran-FITC) between galangin treated and untreated DCs (control). In addition, LPS reduced the antigen uptake activity of DCs and the galangin-treated DCs showed enhanced endocytic capacity for dextran-FITC compared to LPS-stimulated DCs. A same set of the experiment was performed at 4°C, in results, and the result showed that the uptake of dextran-FITC was inhibited by low temperature (Fig. 12A and B). These results demonstrated that galangin rehabilitate the reduced antigen uptake activity of LPS-stimulated DCs, suggesting that galangin enhanced DCs immaturity.

Fig. 12. Galangin rehabilitates the antigen uptake capacity of LPS-stimulated DCs. DCs were incubated with or without LPS (100 ng/ml) for 24 hr following pre-treatment of 60 μM galangin. (A) The ability of uptake of FITC conjugated-dextran by DCs was measured by flow cytometry. DCs were co-stained with a PE-conjugated anti-CD11c antibody. Exposure to FITC-dextran at 4℃ was used as a negative control (non-specific binding). Antigen uptake capacity was represented as the number of dextran-FITC and $CD11c^{+}$-PE double-positive cells. (B) The result indicated the number of dextran-FITC and $CD11c^{+}$-PE double-positive cells as a bar graph.

A



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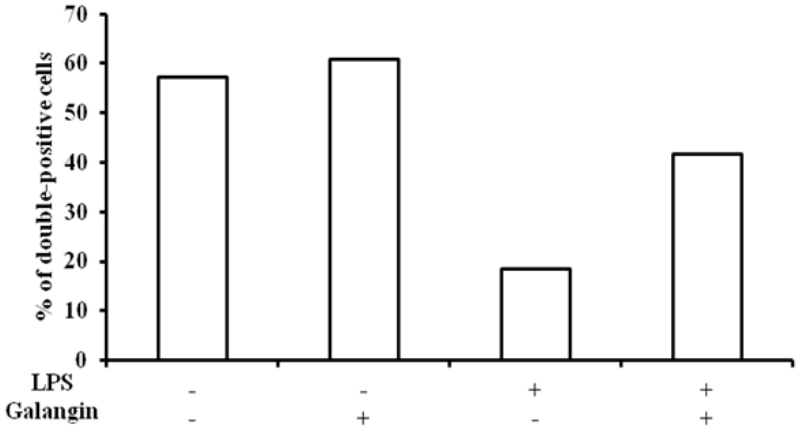

Fig. 12. (continued)

3) Galangin inhibits LPS-induced ERK and JNK phosphorylation on DCs

MAPKs pathway such as ERK1/2 and JNK1/2 can be phosphorylated during DCs maturation and subsequently induce the activation of transcription factors. To demonstrate the molecular mechanism of inhibitory effect of galangin, the MAPKs phosphorylation was assessed in LPS-activated DCs. As shown in Fig. 13, LPS treatment induced ERK and JNK phosphorylation on DCs, treatment of galangin significantly reduced the LPS-induced phosphorylation of ERK and JNK on DCs. This results show that galangin suppresses the phosphorylation of ERK and JNK during DCs maturation.

4) Galangin modulates the expression of co-stimulatory molecules *in vivo*

Previous results indicated that galangin effectively suppresses the co-stimulatory molecules including CD80, CD86, MHC I and II and immunological functions on DCs. Therefore, I investigated whether galangin regulates the expression of co-stimulatory molecule *in vivo*. First, Seven weeks old mice were fed 5 mg/kg galangin by oral administration for 3 days and then sensitized with LPS on the 4th day. After 24hr, the expression of co-stimulatory molecules such as CD80, CD86, MHC I, and MHC II were observed in the splenic DCs (Fig. 14A). As shown in Fig. 14B, I confirmed that galangin was not affect the body weight of the mice. To determine whether galangin alters the expression of co-stimulatory molecules of splenic DCs, whole splenocyte were harvested from mice and double-stained with anti-CD11c (DCs marker) and anti-CD80, anti-CD86, anti-MHC I, or anti-MHC II antibodies. While the expression of the co-stimulatory molecule was significantly increased in the LPS-sensitized group, the upregulated co-stimulatory molecules were markedly suppressed by galangin administration (Fig. 15). Taken together, galangin inhibited the expression of CD80, CD86, MHC class I and MHC class II *in vitro* as well as *in vivo*.

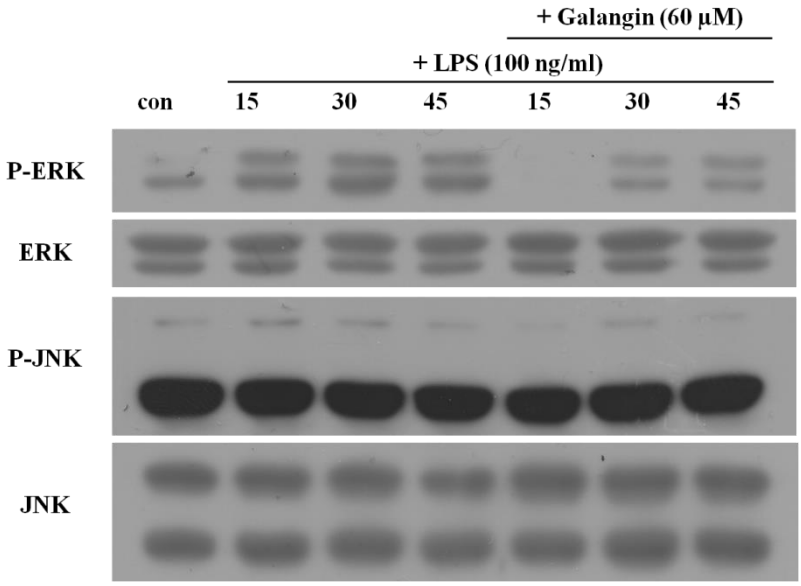

Fig. 13. Galangin inhibits LPS-induced ERK and JNK phosphorylation. DCs were starved in serum-free RPMI for 4 hr and pretreated with 60 μM galangin for 2 hr. The cells were then stimulated with 100 ng/ml LPS for 15, 30 and 45 min. Cells were lysed and equal amount of whole cell proteins were separated by SDS-PAGE and transferred to PVDF membranes. The membranes were probed with anti-ERK, anti-p-ERK, anti-JNK, anti-p-JNK antibodies. This result is from one representative experiment, and is one of three that represent a similar pattern.

Fig. 14. Galangin has no cytotoxicity. (A) *In vivo* experimental design and galangin administration scheme. Galangin dissolved in corn oil was orally administered by a syringe fitted feeding needle catheter for 3 days. On the 4th day, the mice were administered with LPS (1 mg/kg) by intraperitoneal injection (i.p.). After 24 hr, the splenocyte was isolated from the mice. (B) Body weight of the mice was measured for 3 days before LPS administration. (n = 3 per group)

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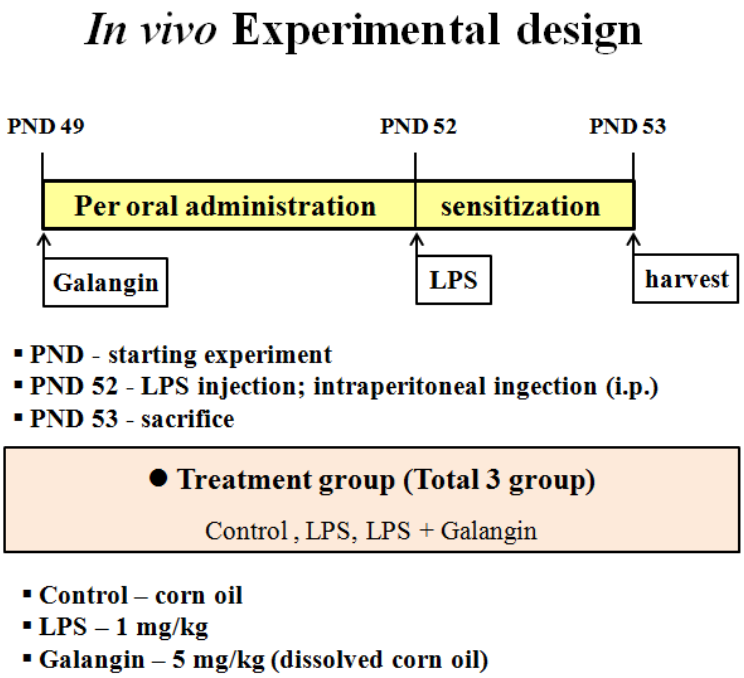

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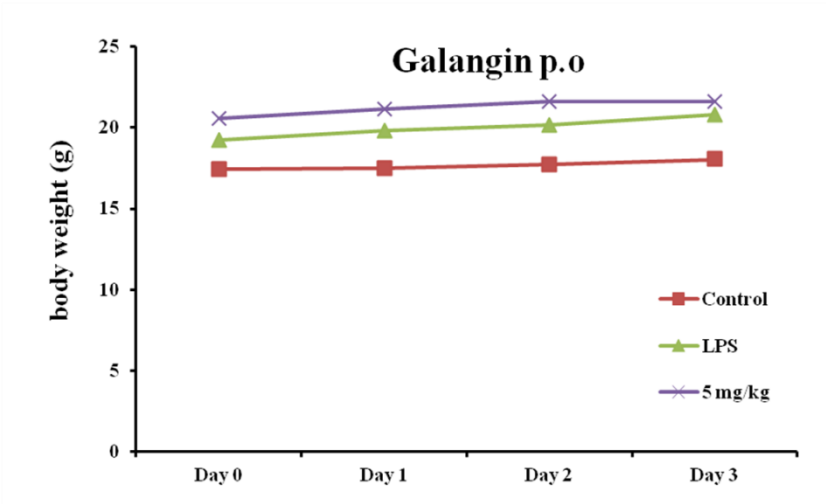

Fig. 14. (continued)

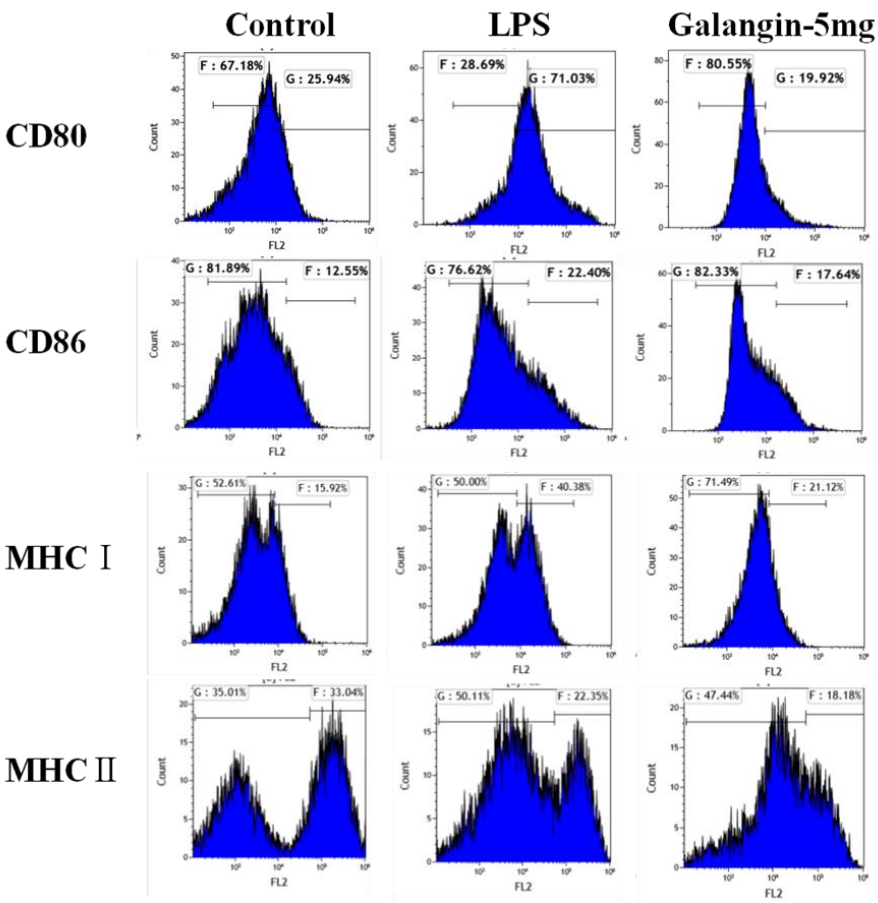

Fig. 15. Galangin suppresses the expression of co-stimulatory molecules on splenic DCs *in vivo*. Galangin (5 mg/kg) was administrated for 3 days and then sensitized with 1 mg/kg LPS for 24 hr. After that, whole splenocyte was isolated from the mice and stained with anti-CD11c, anti-CD80, anti-CD86, anti-MHC I, and anti-MHC II antibodies. The expression of co-stimulatory molecules was analyzed by two-color flow cytometry. Histograms indicate a number of cells.

Ⅳ. DISCUSSION

Microglia is an important immune cell in the brain and acts as the first major safeguard of CNS. Microglia has a phagocytic activity and antigen presenting functions (Lawson, Perry et al. 1992; Gehrmann, Matsumoto et al. 1995; Ginhoux, Lim et al. 2013; Filiano, Gadani et al. 2015). In the present study, galangin inhibited the production of pro-inflammatory mediators such as NO, iNOS, and IL-1β in LPS-induced microglia. Moreover, galangin regulated LPS-induced MAPKs and NF-κB phosphorylation.

NO is a biological marker of inflammatory responses and can be regulated by iNOS expression, and other NO metabolites. NO is induced by activated microglia and inflammatory stimuli, and plays an important role not only in acute but also in chronic inflammation (Evans 1995; Park, Kim et al. 2010; Park, Jeon et al. 2012; Jung, Kim et al. 2014). Therefore, suppression of NO production can be a principal therapeutic approach in the development of anti-neuroinflammatory agents. In current study, I found that galangin reduced NO production and iNOS expression in LPS-stimulated microglia. However, the expression of COX-2 was not altered by galangin treatment (Fig. 6 and Fig. 7).

MAPKs family is a serine/threonine kinases that have crucial roles in iNOS modulation in diverse biological systems (Tsatsanis, Androulidaki et al. 2006; Zhou, Shin et al. 2008). MAPKs are known to modulate the expression of cell survival genes as well as pro-inflammatory enzymes and cytokines by various stimuli (Kim, Ko et al. 2013; Li, Su et al. 2013). Expression of iNOS was dependent on MAPKs activation in LPS-stimulated microglia. Thus, I determined the effect of galangin on MAPKs phosphorylation in LPS-stimulated microglia. As shown in Fig. 9 and Fig. 10, phosphorylation of ERK 1/2, JNK 1 2, p38 and p65 was significantly suppressed by galangin pretreatment in LPS-activated microglia.

In summary of PART I, the results showed that galangin inhibits LPS-induced iNOS and IL-1β gene expression in microglia and the inhibitory effect of galangin is associated with MAPKs

and NK-κB pathways.

DCs are strongest APCs which could be mediator between innate and adaptive immune responses (Steinman 1991; Hart 1997; Banchereau and Steinman 1998; Manel, Hogstad et al. 2010). First, I investigated whether galangin has an effect on DCs phenotype and functional maturation. I found that galangin reduces LPS-stimulated DCs maturation. I also examined the effect of galangin on antigen uptake capacity in DCs using dextran-FITC. As a result, the endocytic capacity of galangin-pretreated DCs was significantly enhanced compared with LPS only treated DCs, suggesting that galangin regulates DCs maturation (Fig. 11 and Fig. 12).

Previous reports have shown that MAPKs is an important signal transduction in DCs maturation (Soukup, Halfmann et al. 2015). I observed activation of MAPK members ERK, p38 and JNK in LPS-stimulated DCs, however galangin treatment markedly suppressed the LPS-induced MAPKs activation (Fig. 13). In addition, I confirmed the inhibitory effect of galangin on DCs maturation *in vivo*. In the result, I found that the expression of co-stimulatory molecules including CD80, CD86, MHC class I, and II is inhibited by galangin administration *in vivo* (Fig. 15).

In conclusion, I verified the various biological functions of galangin in myeloid derived-phagocytes including microglia and DCs. Galangin showed the anti-neuroinflammatory activity in microglial and regulatory activity of DCs maturation. Taken together, these results suggest that galangin may contribute to immunotherapy strategy and could be an immune-adjuvant for neuroinflammation and DCs-related diseases.

Ⅴ. REFERENCES

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감사의 글

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시간을 함께 나누었던 우리 실험실 식구들에게도 감사의 말 전합니다. 가장 가까이에서 기쁠 때나 힘들 때나 묵묵히 옆을 지켜주며 항상 위로해주고 다독여준 주용이 오빠, 내 일도 항상 자기 일처럼 함께 고민해주고 동생이지만 때로는 언니 같았던 인애한테도 너무너무 고마워. 함께 실험실 생활을 시작해 어느덧 3년이라는 시간이 훌쩍 흘러 비록 먼저 실험실을 떠나게 되었지만 우리가 함께했던 추억들을 잊지 못할 거야. 또 비록 지금은 다른 분야에서 일 하고 있는 우리 은별이, 열심히 공부중인 유리도 항상 밝은 기운 더해줘서 고마웠어. 누구보다도 더한 열정으로 연구하고 있는 순효, 막내지만 듬직하고 묵묵하게 자기의 일을 해나가는 준휘에게도 너무 고마워. 지치고 힘들 때마다 너희들이 있어 웃음이 멈추질 않았고 잘 될 거야, 할 수 있어 라는 따뜻한 말로 응원해 줘서 너무 고마워.

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드리고 자주 뵙진 못하지만 언제나 저를 생각해주시고 위로해주시는 할머니, 할아버지께도 감사 말씀 드립니다.

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