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The Regulation Effects of Hesperetin on Hypersensitivity in Human Mast Cells and Microglia Cells

조선대학교 대학원

생명과학과

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Hesperetin에 의한 인간비만세포 및 신경아교세포의 과민성 면역반응 조절 연구

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ABBREVIATIONS

AD	Alzheimer’s disease
ADCC	Antibody-dependent cell mediated cytotoxicity
Ag	Antigen
APC	Antigen presenting cell
BBB	Blood-brain barrier
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 immunosorbent assay
ERKs	Extracellular signal-regulated kinase
FBS	Fetal bovine serum
GM-CSF	Granulocyte-macrophage colony stimulating factor
IFN-γ	Interferon- γ
Ig	immunoglobulin
IL	Interleukin
IMEM	Iscove’s modified Dulbecco’s Medium
iNOS	Inducible nitric oxide synthase
IKK	IκB kinase
JNK	c-Jun N-terminal kinase
LPS	Lipopolysaccharides
MAPK	Mitogen-activated protein kinase

MTT	3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium Bromide
MS	Multiple sclerosis
NK	Natural killer
NO	Nitric oxide
NOS	NO synthase
NF-κB	Nuclear factor-κappa-B
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate-buffered saline
PD	Parkinson's disease
PGD	prostaglandin D
PMA	Phorbol-12-myristate-13-acetate
PPRs	Pattern recognition receptors
PVDF	Polyvinylidene difluoride
SCF	Stem cell factor
SDS	Sodium dodecyl sulfate
Th1	T helper cell type 1
Th2	T helper cell type 2
TSLP	Thymic stromal lymphopoietin
TLR	Toll-like receptor
TNF	Tumor necrosis factor

ABSTRACT

The Regulation Effects of Hesperetin on Hypersensitivity in Human Mast Cells and Microglia Cells

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Immune response is an essential defense mechanism to protect our bodies. However, inadequate immune response causes hypersensitivity leading to various diseases. Hypersensitivity is divided into four types. The present study focuses on type I and type IV hypersensitivity. Type I hypersensitivity is an allergic response and induced by histamine and cytokines released from mast cells. Atopic dermatitis is typical chronic inflammatory disease skin caused by a persistent allergic reaction. Type IV hypersensitivity is a inflammatory reactions caused by phagocytes such as macrophage and microglia. Neuroinflammation is a specific or nonspecific immunological reaction in the central nervous system that is distinct from peripheral inflammatory responses. Neuroinflammation is also induced by activation of microglial cells that acts as resident phagocytes in the brain. Activated microglial cells release a variety of pro-inflammatory mediator that lead to neuronal cell death and neurodegenerative diseases, such as Alzheimer's and Parkinson's disease.

Hesperetin is one of the flavanones and is present in the peel of citrus. Hesperetin is reported to have anti-inflammatory, anti-cancer, and antioxidant effects. However, the anti-allergic and anti-neuroinflammatory effects of hesperetin on human mast cells and microglia cells are still unknown.

Therefore, this study focused on the regulatory effects of hespretin on hypersensitivity through modulation of immune cells.

In the study of Part I, I examined whether hesperetin regulates the allergic inflammatory response in activated human mast cells. I found that hesperetin significantly inhibited gene expression of pro-allergic inflammatory cytokine such as interleukin (IL)-6, TNF-α and IL-1β and secretion of IL-6 in PMA/A23187-stimulated human mast cells. In addition, I found that hesperetin inhibited the phosphorylation of the mitogen-activated protein kinase (MAPK) pathway including extracellular signal-regulated kinase (ERK) 1/2 and p38. Moreover, hesperetin suppressed atopic dermatitis and infiltration of mast cells in epidermis of DNCB-induced atopic dermatitis mouse model.

In Part II, I investigated the anti-neuroinflammatory effects of hesperetin on lipopolysaccharide (LPS)-stimulated BV-2 microglial cells. I found that hesperetin strongly inhibited nitric oxide (NO) production, as well as expression of inducible nitric oxide synthase (iNOS) in LPS-stimulated BV-2 microglial cells. Hesperetin also significantly reduced secretion of inflammatory cytokines, including IL-1β and IL-6. Furthermore, I found that hesperetin down-regulated the phosphorylation of ERK1/2 and p38 in MAPK pathway, resulting in anti-inflammatory effects. Moreover, Hesperetin suppressed astrocytes and microglial cells activation in LPS-challenged mice brain.

Therefore, my findings indicate that hesperetin inhibits allergic inflammation and neuroinflammation and has potential prophylactic application in treating allergic disease and neurodegenerative diseases by modulation of hypersensitivity mediated mast cells and microglia cells.

국문초록

Hesperetin에 의한 인간비만세포 및 신경아교세포의 과민성 면역반응 조절 연구

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면역반응은 우리 몸에서 일어나는 중요한 방어 기작이다. 하지만 비정상적인 면역반응은 다양한 질병을 일으키는 과민성 면역반응을 초래한다. 과민성 면역반응은 4가지 유형으로 나뉘어진다. 본 연구는 I형과 IV형 과민성 면역반응에 초점을 두고 진행했다. 알러지 반응은 전형적인 I형 과민성 면역반응이며 비만 세포에 의해 분비된 히스타민과 사이토카인에 의해 유도된다. 아토피 피부염은 대표적인 만성 염증 피부질환으로 지속적인 알러지 반응에 의해 발병된다. IV형 과민성 면역반응은 대식세포 또는 미세아교세포와 같은 식세포에 의해서 매개되는 염증성 면역반응이다. 신경염증은 말초염증반응과는 다르게 중추신경계에서 특이적 또는 비특이적 면역반응이며, 뇌에 상주하는 식세포 작용을 하는 미세아교세포의 활성화에 의해 유도된다. 활성화된 미세아교세포는 알츠하이머병과 파킨슨병과 같은 신경 세포의 사멸과 신경 퇴행성 질환을 유발하는 다양한 염증 매개 물질을 방출한다.

Hespretin은 플라보논 중 하나이며 감귤류의 껍질에 풍부하게 존재한다. Hesperetin은 항염증, 항암 및 항산화 효과가 있다고 보고되어 있으나, microglia에 대한 hesperetin의

항신경염증 효과는 아직 알려지지 않았다. 따라서, 본 연구에서는 면역세포 활성조절을 통한 hesperetin의 과민성 면역반응 조절 효과에 대하여 확인하였다.

첫번째 연구에서 hesperetin이 PMA/A23187에 의해 자극된 인간비만세포에 의해 발생되는 알러지성 염증반응을 조절하는지 알기 위해 실험하였다. 실험결과에서 hesperetin이 PMA/A23187에 의해 자극된 인간비만세포에서 IL-6와 TNF-알파, IL-1베타와 같은 알러지염증성 사이토카인의 유전자 발현수준과 IL-6의 분비를 감소시켰으며, 이러한 효과는 ERK1/2와 p38을 포함하는 MAPK 신호전달 경로의 인산화 억제를 통하여 나타나는 것을 확인하였다. 또한, hesperetin은 DNCB에 의해 유도된 아토피 피부염 마우스 모델에서 아토피 피부염 병변의 표피로의 비만세포 침투를 억제하였다.

두번째 연구에서는 hesperetin의 LPS에 의해 자극된 BV-2 microglia 세포에 대한 항신경염증 효과를 조사하였다. 실험결과는 hesperetin이 LPS에 의해 자극된 BV-2 미세아교세포에서 iNOS와 NO의 생성의 강하게 억제한다는 것을 나타낸다. Hesperetin은 또한 IL-1베타와 IL-6를 포함한 염증성 사이토카인의 분비를 유의하게 감소시킨다. 또한, 이 연구는 hesperetin이 MAPK경로의 ERK1/2 와 p38의 인산화를 낮추어 항신경염증 효과를 유발한다는 것을 발견했다. Hesperetin은 LPS에 의해 자극된 쥐의 뇌에서 성상교세포와 미세아교세포의 활성화를 억제했다.

본 연구 결과를 통하여 hesperetin이 알러지염증과 신경염증을 억제하는 것을 확인하였으며, 이는 hesperetin이 인간비만세포와 미세아교세포의 활성화를 억제함으로써 알러지 질환과 신경퇴행성 질환치료에 잠재적인 예방치료제로 이용될 수 있음을 시사한다.

I. Introduction

1. Hypersensitivity in immune response

Hypersensitivity refers to an inappropriate response, including hypersensitive allergies and autoimmunity. In general, hypersensitivity is overreaction of immune system accompanied by cough, pain, and itching, and requires the host's pre-sensitization. There are four types of hypersensitivity: Type I has immediate hypersensitivity, type II has antibody-mediated hypersensitivity, type III has immune complex-mediated hypersensitivity, and type IV has cell-mediated hypersensitivity (Fig. 1).

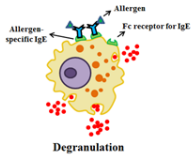
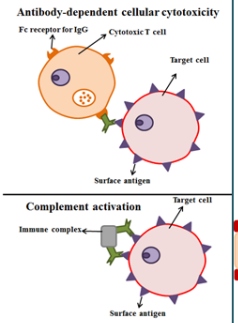
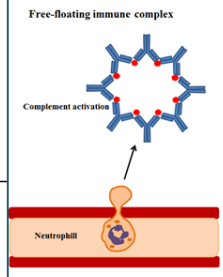
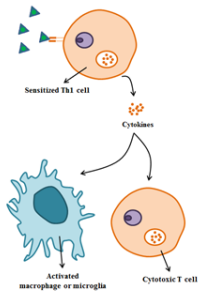
Hypersensitivity	< Type Ⅰ >	< Type Ⅱ >	< Type Ⅲ >	< Type Ⅳ >
Reaction type	 IgE-mediated hypersensitivity	 IgG- or IgM-mediated cytotoxic hypersensitivity	 Immune complex-mediated hypersensitivity	 Cell-mediated hypersensitivity
Mechanism of activation	Allergen-specific IgE antibodies bind to mast cells via their Fc receptor	IgG or IgM antibody binds to cellular antigne, leading to complement activation and cell lysis	Antigen-antibody complexes are deposited in tissune	Th1 cell secrete cytokines, which activate macrophages and cytotoxic T cell
Exampler of hypersensitivity reaction	Local and systemic anaphylaxis, seasonal hay fever, food allergies, drug allergies	Red blood cell destruction after transfusion with mismatched blood types	Post-streptococcal glomerulonephritis, rheumatoid arthritis	Contact dermatitis, type I diabetes mellitus, multiple sclerosis

Fig. 1. Types of hypersensitivity and major characteristics.

A. Type I of hypersensitivity

In type I, immediate hypersensitivity is a typical allergy response which is an immune system overreaction to harmful substances in environment. These allergic reactions include various diseases such as pollen allergies, food allergies, atopic dermatitis, allergic asthma and anaphylaxis. In the mechanism of allergy, allergens cause production of immunoglobulin E (IgE) which is bound to FcεRI of mast cells and basophils. And then mast cells and basophils secretes chemicals such as histamine and leukotriene, respectively. These chemicals cause excessive inflammation in the local area (Johansson et al., 2004).

In early stage of allergic reaction, antigen presenting cell (APC)s provides the specific antigen (Ag) of allergen to Th2 lymphocytes. IL-4 secreted by Th2 lymphocytes stimulate B cells and lead to begin mass production of specific antibodies known as immunoglobulin E in B cells. Secreted IgE binds to IgE-specific receptors on the surface of immune cell such as mast cells in the blood. At this stage, IgE-coated cells become sensitive to allergens. Later, when exposed to the same allergen, allergens can bind IgE molecules attached to the surface of mast cells or basophils. Activated mast cells and basophils cause allergic inflammatory reaction through degranulation allergic inflammatory chemicals (Eder et al., 2006; Galli, 2000).

(1) Mast Cells

Mast cell is a kind of granulocyte which has a lot of granules and typical allergy related cells. Bone marrow (BM)-derived mast cell progenitors are differentiated to immature mast cells by stem cell factor (SCF) and various cytokines such as IL-3, IL-4, and IL-10. SCF and cytokines bind to receptor c-kit that induced differentiation, proliferation, and hematopoiesis. Furthermore, SCF is also important for the regulation of adhesion, migration, survival, and histamine release in mast cell (Amin, 2012). When allergen infiltrates in body, APC uptake and presented the allergen to T cells. Helper T cells are divided to two types including Th1

and Th2 T cells. Th1 cells have high-affinity interaction and produced interferon gamma (IFN-γ). These cytokines inhibit activation of Th2 cells. Whereas, Th2 cells act as a low-affinity interaction with APC and IL-4 which is produced by Th2 cells. The Th2 cells downregulate Th1 cells and switches B cells for production of IgE. Furthermore, IL-3, IL-5, and granulocyte-macrophage colony-stimulating factor (GM-CSF) activate basophils and eosinophils. IgE release from B cells binds to IgE-specific receptors on mast cells. This IgE-specific receptor called FcεR I, also known as the high-affinity IgE receptor. When IgE bind to FcεR I, mast cells sensitize to specific allergen but do not trigger allergic inflammation. After second infiltration of allergen, allergens bind to FcεR I of mast cells and mast cells produce histamine, cytokine, proteases and prostaglandin D (PGD) (Fig. 2). Degranulation is critical process in mast cells mediated allergic inflammatory response. After production of allergic inflammation mediator, cytoplasmic granules fuse with plasma membrane and allergic inflammation mediator releases on external environment. In addition, mast cells secrete various factors such as serotonin, heparin, chondroitin sulphate and tryptases, chymases, carboxypeptidases through degranulation (Fig.2) (Galli et al., 2008).

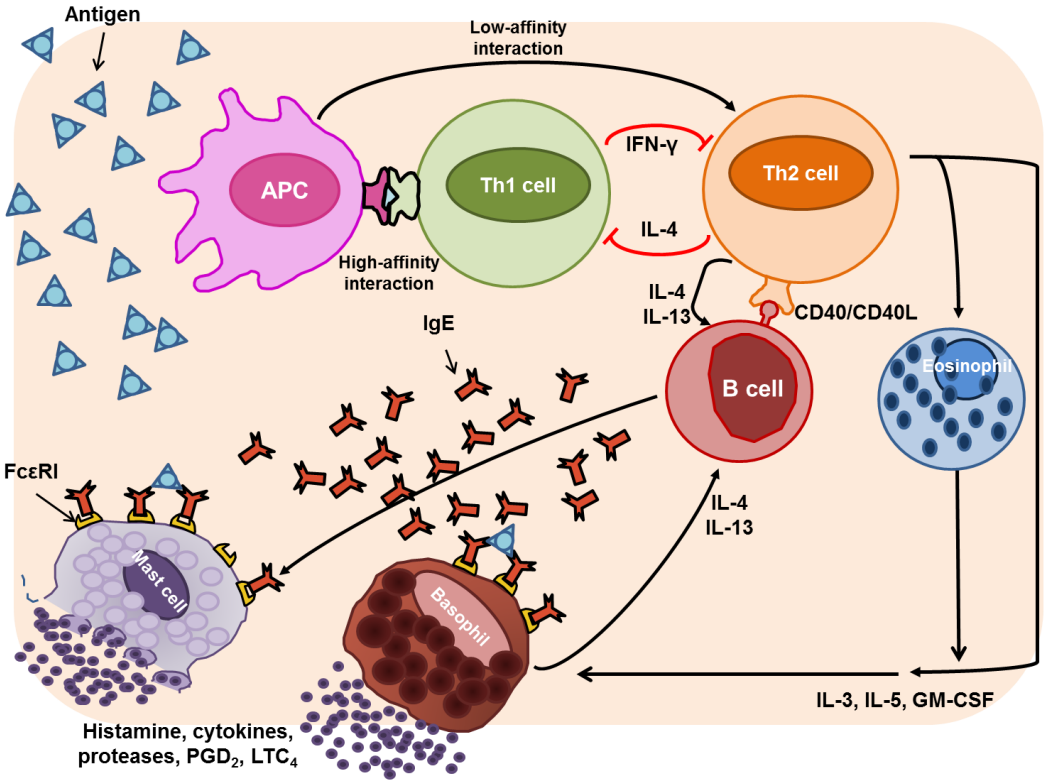


Fig. 2. Mechanism of allergic inflammation through mast cells. APCs phagocytose specific Ag and then presented to Th2 cells. Activated Th2 cells produces IL-4 and interacts with B cells. IL-4 induces the switching of B cells to plasma B cells and then plasma B cells produces Ag specific-IgE. Secreted IgE bind to FcεR I on mast cells. When primed mast cells bind to specific Ag, histamine and cytokines are secreted by degranulation in mast cells.

(2) Atopic dermatitis

Atopic dermatitis is a chronic or chronic relapsing inflammatory skin disease that is a worldwide problem. The atopic dermatitis occurs in various age groups, from children to adults (Akdis et al., 2006). It also causes eczema lesions on the scalp, nape, cheeks, and limbs, and then the skin becomes more prominent and thicker in adults. Symptoms of the atopic dermatitis such as inflammatory skin disease, skin barrier function collapse are cuased by IgE-mediated sensitization, and perivascular infiltration of lymphocytes and mast cells (Chan et al., 2001).

Atopic dermatitis develops an acute response and a chronic response. When Dendritic cells (DCs) recognize allergen, it provides information about the allergen to T cells. At times, the keratinocytes, DCs, and the mast cells promote differentiation of Th2 cells by secretion of thymic stromal lymphopoietin (TSLP), IL-4, and IL-10. IL-4, IL-5, and IL-13 secreted by Th2 cells cause acute skin lesions by inducing allergic inflammation. Acute skin lesions are accompanied intensely pruritic, erythematous papules. However, chronic response is caused by various factors. When macrophages and eosinophils infiltrate into the epidermis in the blood vessels, they secrete IL-12 and promote differentiation of Th1 cells. Chronic inflammatory reaction is caused by IFN-gamma produced by Th1 cells. In addition, IL-5 and GM-CSF increase the survival of macrophages and eosinophils, resulting in chronic reactions. Finally, collagen deposition occurs by IL-11 and tissue remodeling and thickened plaques occur (Hamid et al., 1994; Leung et al., 2004).

B. Type II of hypersensitivity

Type II hypersensitivity is antibody-dependent cell mediated cytotoxicity (ADCC), and induces degradation of target cells. Target cells are coated with specific antibodies on the membrane. These ADCCs are involved in immunoglobulin G (IgG) and are included in the adaptive immune response. In addition, ADCC differs from the immune response through complement reaction and is closely related to natural killer (NK) cells that interact with IgG (Shang et al., 2002).

A typical ADCC response is achieved by antibody-induced NK cells activation. These NK cells recognize the IgG through binding to the Fc receptor. The Fc receptor of NK cells is called FcεRIII, as known as CD16. If the virus are infected, the viral protein is replicated in the cells and expressed on the cell surface. IgG bound to the NK cells recognize the viral protein of the infected cell. Therefore, NK cells secrete granzyme, perforin, and protease. The granzyme breaks down the target cell to prevent the virus from spreading throughout the body (Fig. 3) (Vivier et al., 2008).

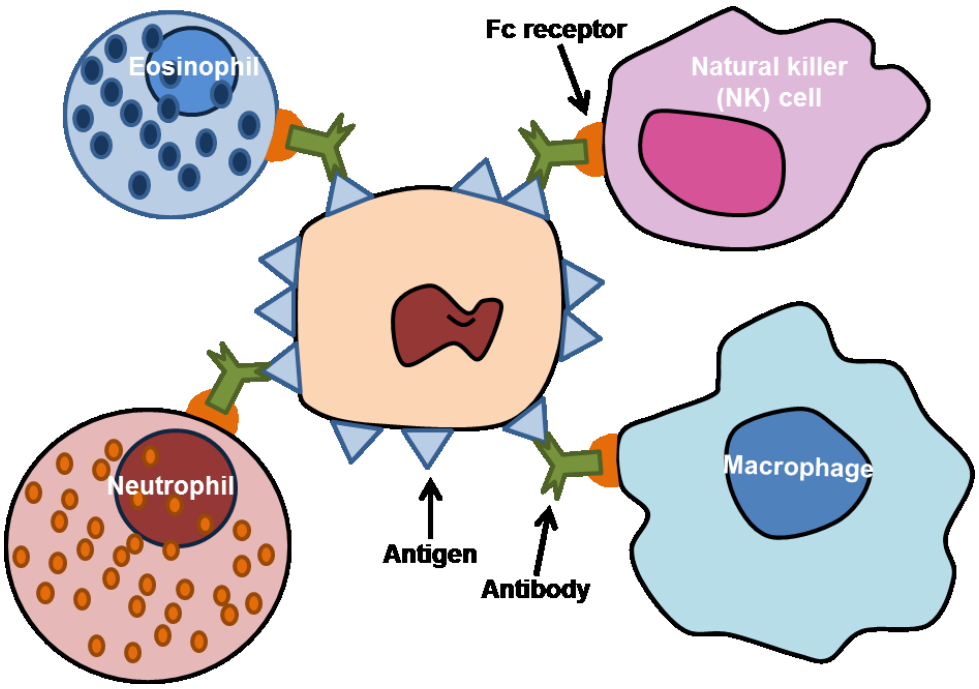


Fig. 3. Type II of hypersensitivity. When primed immune cells recognize specific Ags, perforin induces cell death in pathogen infected-target cells. Neutrophils and macrophages can also mediate ADCC by secreted granzymes. Eosinophil recognizes parasites and secretes protease to cause ADCC reaction. Especially, NK cells are primed by IgG and secrete granzymes, perforin and protease.

C. Type III of hypersensitivity

Type III hypersensitivity is immune complex response and also called antigen-antibody complex which is formed by the binding of circulating soluble Ag and antibody in the body. Ag and antibody act like a single body and act as specific epitopes, and become effective Ags themselves. The resulting immune complexes react with complement deposition, opsonization, and phagocytosis. In addition, the immune complexes themselves become illness members and are classified as type III hypersensitivity. The autoimmune disease is typical type III hypersensitivity disease and occurs when the immune cells in our body recognize the immune complex as an Ag (Fig. 4) (Cohen et al., 1971; Polack et al., 1999).

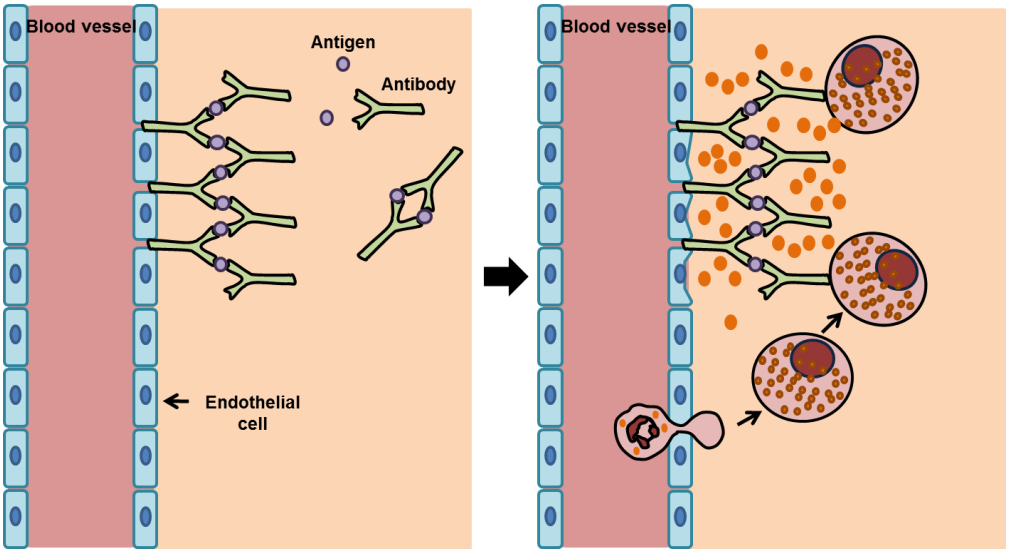


Fig. 4. Type III of hypersensitivity. The small immune complex consists of Ags and antibodies. Small immune complexes interact to form large immune complexes. Large immune complexes are deposited on the blood vessel wall and are recognized by neutrophils. Activated neutrophils secrete enzymes and damage endothelial cells.

D. Type IV of hypersensitivity

Type IV hypersensitivity is called cell-mediated hypersensitivity and consists of sensitization and effect steps. It takes a long time to become a sensitization, but the effect step caused by the secondary infection occurs very quickly. In addition, the immune response by antibodies do not occur, but inflammation reaction and tissue damage are caused by the inflammatory cells such as Th1 cells, macrophages and microglia cells. When Ag penetrates into our bodies, APCs recognize to Ag and present it to CD4 T cells. Once same Ag is infected again, sensitized Th1 cells secrets various cytokines and chemokines such as IL-2, IL-3, IFN-γ, MCP-1, CCL2, and CXCL8. Subsequently, the various factors induce the activation of resting phagocytic cells such as macrophage and microglia that produce Oxygen radicals, Nitric oxide (NO), lytic enzyme (Huber et al., 1976).

(1) Neuroinflammatory response

The innate immune response is a defense response in which the body responds to the pathogen. It is also the earliest and most rapidly developing immune response in our body. The white blood cells, macrophages and microglia cells, are involved in the innate immune response. The biggest function of Macrophages and microglia cells is phagocytosis. When the pathogen is recognized by the receptor, the macrophages and microglia cells phagocyte to take up the Ag, forming a phagosome, killing the pathogen, or releasing an inflammatory factor or cytokine, thereby induce an inflammatory response. Another innate immune response is characterized by a non-specific immune response. Rather than reacting only to specific Ag, the innate immune response occurs by recognizing the pathogen-associated molecular patterns (PAMPs) produced by Ag into pattern-recognition receptors (Medzhitov et al., 2002).

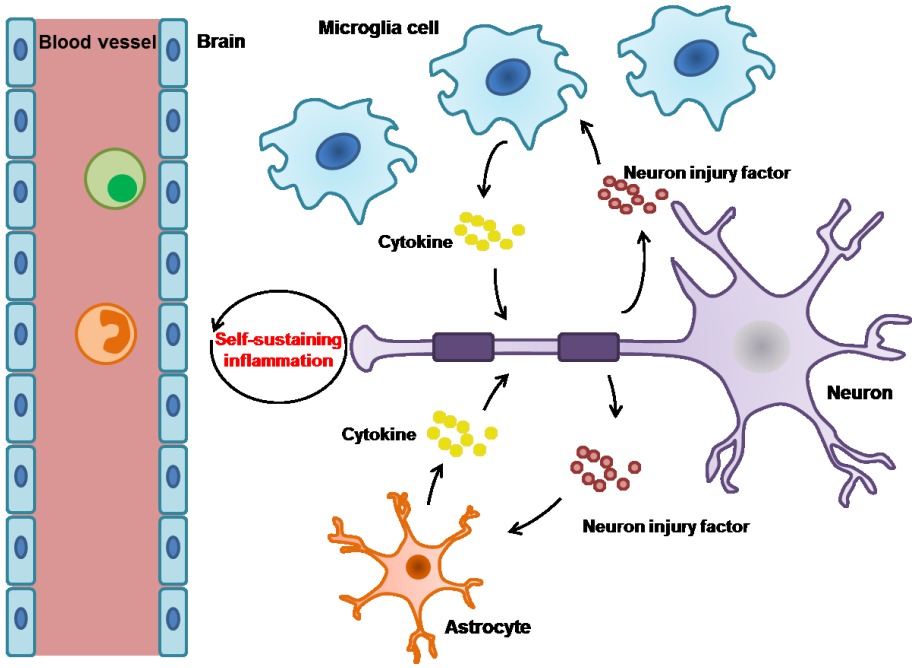
Conversely, the adaptive immune response is a specific-immune response. The role of

DCs in APCs is important for specific immune response. After the phagocytosis of DCs, DCs transfer information about Ag to T cells. T cells that receive information about a specific Ag will undergo polarization to CD4 and CD8 T cells. CD8 T cells are cytotoxicity T cells, which directly attack the infected cells in the Ag, leading to a cell-mediated immune response. CD4 T cells are helper T cells, which transfer B cells information about Ag. In this case, B cells forms antibody and memory for Ag, which causes humoral immune response. Thus, T cells and B cells that received antigen-specific information by APC are called specific-immune responses because they act only on specific antigens (Iwasaki et al., 2010; Medzhitov et al., 1997, 1998).

The increasing prevalence of neurological and brain-related diseases is an important issue in an aging society(Mecocci et al., 2018). These brain-related diseases are associated with neuroinflammation. Neurodegenerative disease is defined by progressive dysfunction of the nervous system and includes Alzheimer's disease (AD) and Parkinson's disease (PD) (Pennisi et al., 2017; Reale et al., 2010; Tiwari et al., 2017). The neuroinflammatory response defends against exogenous antigens, but chronic neuroinflammation contributes to various neurodegenerative disorders. In the central nervous system (CNS), innate immune surveillance is mainly coordinated by the microglia cells of the brain. Microglia cells counteract disturbances in immunological homeostasis in order to protect neurons that have a limited regeneration capacity. The activation and proliferation of microglia induce neuroinflammation and can cause various neurodegenerative diseases, including AD, PD, and multiple sclerosis (MS) (Schetters et al., 2018). In their normal state, microglia cells protect the nervous system by killing pathogens, removing debris, and promoting the immune response. However, in cases of brain injury or neurodegenerative disease, activated microglial cells release pro-inflammatory cytokines and neurotoxic materials, including NO, IL-1β, IL-6, and tumor necrosis factor (TNF)-α (Mairuae et al., 2018; Xu et al., 2018). Overproduction of deleterious inflammatory mediators may be involved in the progression

of neuronal degeneration. Therefore, down-regulation of these inflammatory processes in microglia might slow the progress of neurodegenerative disease (Fig. 5).

A. Resident-mediated neuroinflammation (Acute response)



B. Periphery-mediated neuroinflammation (Chronic reposnse)

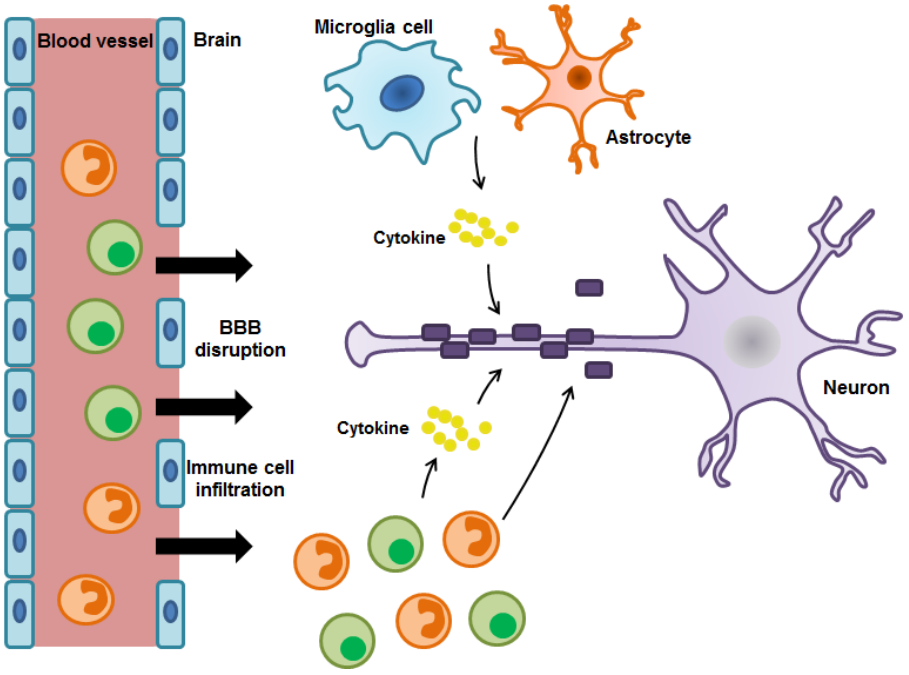


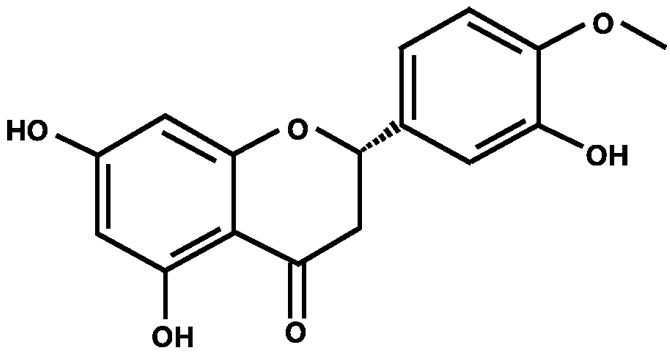
Fig. 5. Mechanism of neuroinflammation. Resident immune cell-mediated neuroinflammation is an acute response. Microglial cells and astrocytes are activated by pathogens and secrete neurotoxic factors. Neurons are damaged by neurotoxic factors and neuron injury factors reactivate microglia cells and astrocytes. This reaction is called self-sustaining inflammation (A). If Blood-brain barrier (BBB) disruption occurs due to persistent inflammation, lymphocytes infiltrate into the CNS. Infiltrated lymphocytes directly attack neurons or activate microglia cells and astrocytes, leading to chronic reactions (B).

2. Hesperetin

Natural compounds are substances or chemical compound derived from nature resource such as plants, seaweeds, and microorganisms. The several natural compounds have been considered as pharmacological and physiological therapeutic agents, these biological activities help searching for new drugs (Berdy, 2005; Harvey, 2008).

Flavonoid is consists of five subclass such as anthoxanthins, flavanones, flavanonols, and anthocyanidins. When 15 carbon atoms are used to form two phenyl rings and a heterocyclic ring, it is called a flavonoid. These flavonoids are mainly secondary metabolites of plants and fungi and exhibit various effects depending on various environments.

Hesperetin (3',5,7-trihydroxy-4-methoxyflavanone) is a member of the flavanone subclass of flavonoids and large quantities are found in citrus fruits, including oranges and grapefruit (Shirzad et al., 2017). Previous studies have demonstrated that hesperetin have significant antioxidant, anti-inflammatory, anti-apoptotic, and anti-tumor effects (Parhiz et al., 2015; Smina et al., 2015; Ye et al., 2012). Furthermore, hesperetin has also been shown to inhibit inflammation in various cell types by regulating ERK1/2, p38 MAPK signaling pathways (Kim, 2014). Recently, hesperetin has been reported to have protective effects against impairment of recognition memory during elevated oxygen stress in a rat model of AD (Fig. 6) (Moghaddam et al., 2018).



Hesperetin

Fig. 6. Structure of hesperetin.

3. Inflammatory signaling pathway

A.Mitogen-activated protein kinase (MAPK) pathway

Mitogen-activated protein kinase (MAPK) is serine/threonine specific-kinase. The members of MAPK are extracellular signal-regulated kinase (ERK), c-Jun N terminal kinase (JNK) and p38. This family is essential in regulating many cellular processes including inflammation, cell stress response, cell differentiation, cell division, cell proliferation, metabolism, motility and apoptosis. Activation of the MAPK signaling pathway begins in the cell membrane where small GTPases and various protein kinases phosphorylate and activate MAPKKK. Subsequently, MAPKKK phosphorylates MAPKK and phosphorylated MAPKK phosphorylates MAPK. Activated MAPK interact with numerous cytoplasmic substrates, phosphorylate and induce transcription by activating specific transcription factors. Depending on which transcription factor is activated, a corresponding response occurs (Johnson et al., 2002; Zhang et al., 2002).

The ERK signal pathway is activated by growth factors and cytokines. Raf in MAPKKK regulates the ERK signal. When Raf is activated, phosphorylation of MAPKK, MEK1/2, occurs. Phosphorylated MEK1/2 activates ERK 1/2. ERK1/2 activates various substrates such as MNK1/2, Ets, Myc, and STAT1/3. By controlling these substrates, the ERK signal pathway is involved in proliferation, differentiation, and development (Kolch, 2000).

The p38 signal pathway is related to proliferation, apoptosis, and stress response. It also plays a major role in inflammation of the MAPK signal pathway. p38 signal pathway are activated by cellular stress including UV irradiation, heat shock, lipopolysaccharide (LPS), protein synthesis inhibitors, proinflammatory cytokines and certain mitogens. Activation of MEKK1-4 and MLK phosphorylates MKK3/6 and MKK4, and phosphorylation of p38 finally results in various cellular responses (Zarubin et al., 2005).

The JNK signal pathway is also activated by stress, growth factors, and ceramides and regulates substrates such as c-jun and NFAT4. Thus, the JNK signal pathway regulates proliferation, differentiation, and apoptosis (Fig. 7) (Weston et al., 2007).

B. Nuclear factor kappa-B (NF-κB) pathway

NF-κB regulate various cellular responses such as immune response, cell death, and inflammation. NF-κB is heterodimeric transcription factors and is consist of RelA (p65), RelB, c-Rel, p50, and p52. This NF-κB members contain the Rel-homology domain which mediates DNA binding and dimerization. While in and resting state, NF-κB complex what consist of RelA, p50, and IκB located in the cytosol. When external signal bind to receptors, IκB kinase (IKK) is activated. IκB are phosphorylated by activated IKK and lead to ubiquitination. Degradated IκB induce activation of NF-κB that releases NF-κB and allows it to translocate to the nucleus where it bind to specific gene. RelA, p50, and IκB complex is major transcription factor that regulate innate immune response and inflammation. When LPS bind to TLR4 on immune cell membrane, NF-κB complex is phosphorylated by IKK on ser536 of RelA and IκB. Phosphorylated RelA localize in nuclear and bind to target gene such as TNF-α, IL-β, Inducible nitric oxide synthase (iNOS), Cyclooxygenase-2 (COX-2), and MCP-1 for its transactivation functions. Pro-inflammatory factor induce inflammation on wound region (Fig. 8) (Lawrence, 2009; Tak et al., 2001).

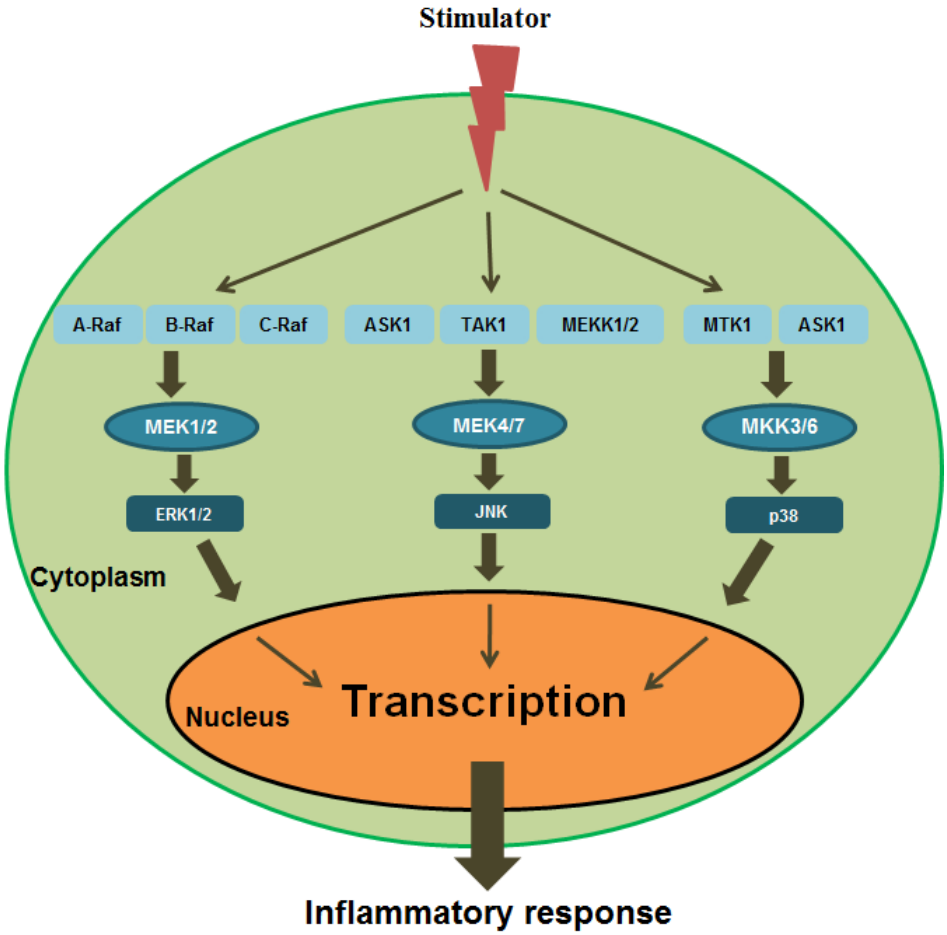


Fig. 7. MAPK signal pathway.

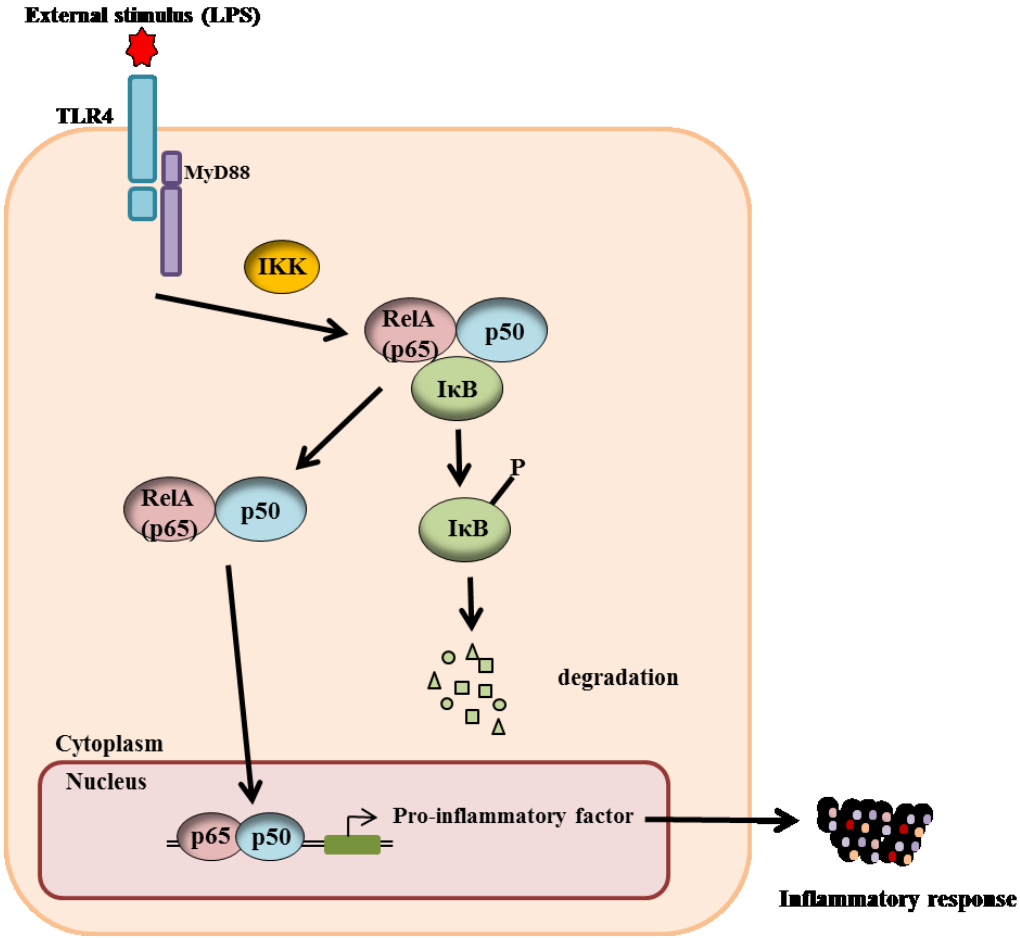


Fig. 8. NF-κB signal pathway.

4. Purpose of study

Hypersensitivity is induced over-reactive immune response. Atopic dermatitis and neuroinflammatory disorder are typical hypersensitivity-induced diseases and a problem that needs to be solved in various age groups and worldwide. Hesperetin is a member of the flavanone glycoside. Previous studies have demonstrated that hesperetin has significant antioxidant, anti-inflammatory, anti-apoptotic, and anti-tumor effects. However, hesperetin is incompletely understood regulation effect on hypersensitivity-induced disease. To understand how hesperetin regulates allergic- and neuro-inflammation, this study investigate the effect of hesperetin on immune cell regulation in hypersensitivity.

In part I, to investigate whether hesperetin regulate allergic inflammation in Phorbol-12-myristate-13-acetate (PMA) /A23187-stimulated human mast cells, Treatment with hesperetin reduced PMA/A23187-induced expression of pro-inflammatory cytokines and MAPK phosphorylation. To address the relevance of atopic dermatitis, I analyzed effect of hesperetin on DNCB-induced atopic dermatitis and found that hesperetin also reduced DNCB-induced atopic dermatitis.

In part II, to determine whether the anti-neuroinflammatory effect of hesperetin in LPS-stimulated BV-2 microglial cells and the underlying regulation of this mechanism, Treatment with hesperetin reduced LPS-induced expression of pro-inflammatory cytokines, NO production, and MAPK phosphorylation. Hesperetin also reduced LPS-induced activation of microglia in the hippocampus of LPS-injected mice.

To summarize the above results, hesperetin effectively regulated allergic inflammation and neuroinflammtion in hypersensitivity-induced diseases.

II. Materials and Methods

1. Mice

Male C57BL/6 mice (9 weeks) and male BALB/c mice (8 weeks) were purchased from Orientbio, Inc. (Seongnam, Gyeonggi-do, Korea). Mice were cared for pathogen-free environment for one week. The mice were maintained at reasonable temperature and humidity in a 12 hr light/dark cycle.

2. Chemicals and reagents

3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), Griess reagent, LPS (L2880, L2630), and TRI reagent were purchased from Sigma Chemical Co. (St. Louis, MO, USA). TNF-α, IL-1β, and IL-6 ELISA detection kit were purchased from BioLegend (San Diego, CA, USA). Antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA) and Santa Cruz Biotechnology (Dallas, TX, USA). Iscove's modified Dulbecco's Medium (IMDM), Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), and penicillin/streptomycin were purchased from WELGENE, Inc. (Gyeongsan-si, Gyeongsangbuk-do, Korea)

3. Cell culture and Chemical treatment

The human mast cell-1 (HMC-1) line were provided by Dr. D.K Kim (Chonbuk national university) and Murine microglial cell line BV-2 were purchased from American Type Culture Collection (Manassas, VA, USA). These cells were cultured at 37°C with 5% CO_2 in IMDM, DMEM supplemented with 10% FBS, 200 IU/ml penicillin, 200 μg/ml streptomycin, 4 mM L-glutamine, and 1 mM sodium pyruvate (complete medium). Hesperetin was reconstituted in

dimethyl sulfoxide (DMSO) at a concentration of 100 mM and then diluted to the desired concentration in IMDM and DMEM (final DMSO concentration 0.1% v/v). The culture media containing of the equal concentration of DMSO was used as the control.

4. Annexin V & PI staining

Cell viability was measured by Annexin & PI staining. Cells (2×10^6 cells/flask) were seeded in T25 flask in IMDM. Cultured cells were pretreated with various concentrations of hesperetin (0, 25, 50, 100, 200 μM) and incubated in 37°C for 24 hr. After the treatment, Cells were harvested and washed twice with cold 1 X phosphate-buffered saline (PBS). Then cells double stained with Annexin V and PI according to the manufacture's instructions. The stained cells were analyzed using a FC500 flow cytometer (Beckman Coulter, Brea, CA) and FlowJo (FlowJo LCC, Ashland, OR, USA).

5. MTT assay

Cell viability was measured by MTT assay. BV-2 microglia cells were seeded in 96-well culture plates in DMEM. Cultured cells were pretreated with various concentrations of hesperetin (0, 5, 25, 50, 100, 200 μg/ml) and incubated in 37°C for 24 hr. After the treatment, medium containing hesperetin were removed and MTT (0.5 mg/ml) in media was added to each wells. After incubation in 37°C for 4 hr, MTT solution was removed and formazan product was dissolved in solubilization solution (1:1 = dimethyl sulfoxide: ethanol) into a colored solution. Absorbance of the formazan solution was measured at 570 nm using an Epoch microplate reader (BioTek instrument, Inc., Winooski, VT, USA).

6. 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 hesperetin (0, 25, 50, 100, 200 μM). Cultured cells were pretreated with various concentrations of hesperetin (0, 25, 50, 100, 200 μM) for 2 hr, and then incubated in the presence or absence of LPS for 22 hr. After incubation, the culture medium were mixed with an equivalent volume of 1× Griess reagent and incubated for 15 min at room temperature. Absorbance was measured at 450 nm using an Epoch microplate reader (BioTek Instrument, Inc.; Winooski, VT, USA)

7. Reverse transcription (RT)-PCR

BV-2 microglia cells (3×10^5 cells/well) were seeded in 12-well culture plate. Cultured cells were pretreated with various concentrations of hesperetin (0, 25, 50, 100, 200 μM). HMC-1 cells (5×10^4 cells/flask) in complete medium were seeded into a T25 culture flask. Cultured cells were pretreated with various concentrations of hesperetin (0, 25, 50, 100, 200 μM) for 2 hr, and then cells were incubated for 6 hr in the absence or presence of LPS or PMA/A23187. After incubation, the cells were collected by centrifugation and total RNA was isolated from hesperetin-treated cells using TRI reagent according to manufacturer's protocol. To synthesize cDNA, 0.5 μg of total RNA was primed with oligo dT and reacted with mixture of M-MLV RTase, dNTP, and reaction buffer (promega, WI). To measure the mRNA level of inflammatory cytokines, cDNA was amplified using Gene atlas G02 gradient thermal cycler system (Astec, Japan) e-Taq DNA polymerase kit (solgent, Daejeon, Korea) and indicated primers. And then, PCR products were visualized by fluorescent dye and NaBI Gel-doc system (NEO science, Suwon, Gyeonggi-do, Korea).

Table. 1. Primers used in RT-PCR

Species	Target gene	primer	Sequence
Mus musculus	*IL-1β*	Forward	5'-GTGTCTTTCCCGTGGACCTT-3'
		Reverse	5'-TCGTTGCTTGGTTCTCCTTG-3'
	IL-6	Forward	5'-CCTTCCTACCCCAATTTCCA-3'
		Reverse	5'-CGCACTAGGTTTGCCCACTA-3"
	TNF-α	Forward	5'-GGCCTCTCTACCTTGTGCC-3'
		Reverse	5'-TAGGCGATTACAGTCACGGC-3'
	COX-2	Forward	5'-TGGGTGTGAAGGGAAATAAGG-3
		Reverse	5'-CATCATATTTGAGCCTTGGGG-3'
	i-NOS	Forward	5'-CTTGCCCCTGGAAGTTTCTC-3'
		Reverse	5'-GCAAGTGAAATCCGATGTGG-3
	GAPDH	Forward	5'-TGCACCACCAACTGCTTAG-3'
		Reverse	5'-GGATGCAGGGATGATGTTC-3'
Homo sapiens	*IL-1β*	Forward	5'-GTACCTGAGCTCGCCAGTGA-3'
		Reverse	5'-TGAAGCCCTTGCTGTAGTGG-3'
	IL-6	Forward	5'-GCTGTGCAGATGAGTACAAA-3'
		Reverse	5'-CTGCATAGCCACTTTCCATT-3'
	TNF-α	Forward	5'-CCATCAGAGGCCCTGTACCT-3'
		Reverse	5'-CAGACTCGGCAAAGTCGAGA-3'
	GAPDH	Forward	5'-AAGGGTCATCATCTCTGCCC-3'
		Reverse	5'-GTGATGGCATGGACTGTGGT-3'

8. Western blot analysis

HMC-1 cells (5×10^6 cells) were seeded in T25 cell culture flask and starved with serum-free IMDM for 4 hr. After starvation, cells were pretreated with hesperetin for 2 hr, and then stimulated with PMA/A23187, for 15, 30 and 45 min.

To confirm the protein expression level of NO synthase (NOS) 2 and COX-2, BV-2 microglia cells were pretreated with various concentrations of hesperetin (0, 25, 50, 100, 200 μM) for 2 hr and then treated with or without LPS (200 ng/ml). For verification of signal transduction, BV-2 microglia cells were serum starved for 4 hr in serum-free medium and then pretreated with 200 μM hesperetin for 2 hr. After incubation, LPS (200 ng/ml) was added to the cells in the absence or presence of hesperetin for 15, 30, or 45 min.

Cells were treated with stimulator such as PMA/A23187 and LPS in the absence or presence of 200 μM of hesperetin. Following 5, 10, 20 min and 24 hr of incubation at 37°C, cells were washed twice with cold PBS and lysed by ultra-sonication in RIPA buffer and using for 30 min at 4°C. Lysates were collected by centrifuging at 12,000×g for 15 min at 4°C. Cells were washed twice with cold PBS and lysed with modified RIPA buffer (150 mM sodium chloride, 1% Triton X-100, 0.5% sodium deoxy cholate, 0.1% sodium dodecyl sulfate (SDS), 50 mM Tris (pH 8.0), 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 μg/mL leupeptin, 1 μg/mL pepstatin, 1 mM sodium orthovanadate, and 100 mM sodium fluoride) for 30 min at 4°C. Lysates were cleared by centrifuging at 14,000 ×g for 15 min at 4°C. The protein content of cell lysates was determined using the Micro BCA assay kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. Equivalent amounts of protein were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was placed into a blocking solution (5% non-fat milk) at room temperature for 1 hr. After blocking, blots were incubated with anti-ERK1/2, anti-phospho-ERK1/2 (p-ERK), anti-p38, and phospho-p38 (p-p38)

antibodies overnight (Santa Cruz Biotechnology, CA, USA). Horseradish peroxidase-conjugated anti-rabbit and anti-mouse antibodies were used as the secondary antibodies (Table 3). Band detection was performed using the enhanced chemiluminescence (ECL) kit (ELPIS-Biotech Inc., Daejeon, Korea) detection system and exposed to radiographic film. Pre-stained blue markers were used for molecular weight determination.

9. Enzyme-linked immunosorbent assay (ELISA)

HMC-1 cells (1×10^6 cells/well) were seeded in 96-well u-bottom culture plates. BV-2 microglia cells (2×10^4 cells/well) were seeded in 96-well culture plate. The cells were pretreated with various concentrations of hesperetin for 2 hr, and then incubated in absence or presence of stimulator (PMA/A23187 and LPS) for 22 hr. HMC-1 cells and BV-2 cells were incubated in 96-well cell u-bottom culture plate or 96-well culture plates overnight and incubated with or without hesperetin for 2 hr. And then the cells were incubated with or without PMA/A23187or LPS for 22 hr. Supernantant was used for samples and the cytokine production was measured by ELISA MAX™ Deluxe Sets (BioLegend, CA, USA), according to manufacturer's protocol. Briefly, standards and samples were incubated on capture antibody coated plate at 4°C, overnight. Detection antibody was incubated for 1 hr and Avidin-HRP bind to detection antibody at room temperature. Next, substrate solution was added to each well and the reaction was stopped by stop solution (2N H_2SO_4). Absorbance was measured by ELISA microplate reader at 450 nm wavelength.

10. Animal model

All experiments were approved and performed in accordance with the regulations of the Chosun University Care and Use Committee (IACUC). For neuroinflammation experiment in vivo, C57/BL6 male mice were randomly divided in to 3 groups (Control, LPS,

LPS+hesperetin). Hesperetin was dissolved in sterile saline and pre-administered by intraperitoneal injection (i.p.) (5 mg/kg) for 3 days prior to LPS injection. Also, LPS group was injected intraperitoneally (1 mg/kg) for 24 hr. For atopic dermatitis mouse model,BALB/c male mice were randomly divided in to 4 groups (Control, DNCB, 200 μM hesperetin, 2 mM hesperetin). All BALB/c mice were shaved on dorsal skin and sensitized treatment of 1% DNCB solution in acetone/olive oil (3:1) for 4 days. After 3 days, hesperetin (200 μM, 2 mM) solution and 0.5% DNCB solution challenged on the dorsal skin for 11 days. On day 19, all BALB/c mice were sacrificed.

11. Histological analysis

The dorsal skin was extracted and post-fixed in 4% paraformaldehyde for 24 hr at 4°C. These skin samples were embedded in OCT for frozen sections, and then coronally sectioned at 10 μm using a freezing microtome (MICROM, Walldorf, Germany). For examine the epidermal hypertrophy and thickness, skin sections were stained with hematoxylin (St. Louis, MO, USA) and eosin (St. Louis, MO, USA). In addition, skin section were stained with toluidine blue O (St. Louis, MO, USA) for evaluate infiltration of mast cell. All sections were captured using light microscope (Nikon, Tokyo, Japan).

12. Immunhistochemistry

The mice were intra-cardially perfused with 10 mM PBS and subsequently 4% paraformaldehyde. The brain was extracted and post-fixed in 4% paraformaldehyde for 24 hr at 4°C. Fixed brain was transferred to 30% sucrose solution. These brain samples were embedded in OCT for frozen sections, and then coronally sectioned at 40 μm using a freezing microtome (MICROM, Walldorf, Germany). Brain sections were washed and blocked with blocking solution for 30 min at room temperature. The sections were incubated with anti-

ionized calcium-binding adapter molecule 1 (Iba-1, microglia maker) antibody and glial fibrillary acidic protein (GFAP, astrocytes maker) (Wako Chemical USA. Inc., Richmond, VA, USA) in TBS-TS at 4% overnight. Brain sections were washed with TBS and incubated with anti-mouse IgG labeled with Alexa Fluor 488 and 568 for 3 hr at room temperature, respectively. Confocal fluorescence images were acquired FV10i fluoview confocal microscope (Olympus; Tokyo).

13. Statistical analysis

All results were expressed as the mean ± SE of the indicated number of experiments. Statistical significance was estimated using Student's t-test for unpaired observations, and the differences were compared with regard to statistical significance by one-way ANOVA, followed by Scheffe's post-hoc test using SPSS (SPSS, Armonk, NY). The differences were considered statistically significant at $p < 0.01$.

Table. 2. Primary antibodies used in western blotting

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

III. Results

Part I. Anti-allergic effect of hesperetin on human mast cell activation via MAPK pathway regulation.

1. Hesperetin had no effect on cytotoxicity in human mast cells.

First, I investigated the cytotoxicity of hesperetin on human mast cells. Human mast cells were treated with various concentrations of hesperetin (0, 25, 50, 100, 200 μM) for 24 hr and analyzed to an Annexin V/PI staining. Annexin V/PI staining was an analysis to measure cytotoxicity. Annexin V is apoptotic marker and associated with phosphatidic serines in apoptotic cells. PI is necrosis marker and bind to the nucleus under the necrotic cell death. Annexin V^+/PI^- cells are early apoptotic cells and Annexin V^-/PI^+ cells are necrotic cells. In addition, annexin V^+/PI^+ cells indicated lately apoptotic cells. As shown in Fig. 9, percentage of early apoptotic cells, necrotic ells, and late apoptotic cells are 0%, 1.34%, and 0.3% in control, respectively. When hesperetin was treated up to 200 μM, there was no significant difference in percentage of apoptotic/necrotic cells compared to control. This result indicated that hesperetin had no cytotoxic effects on human mast cells. Therefore, this range of hesperetin concentrations was used to determine the anti-allergic effect of hesperetin on human mast cell activation.

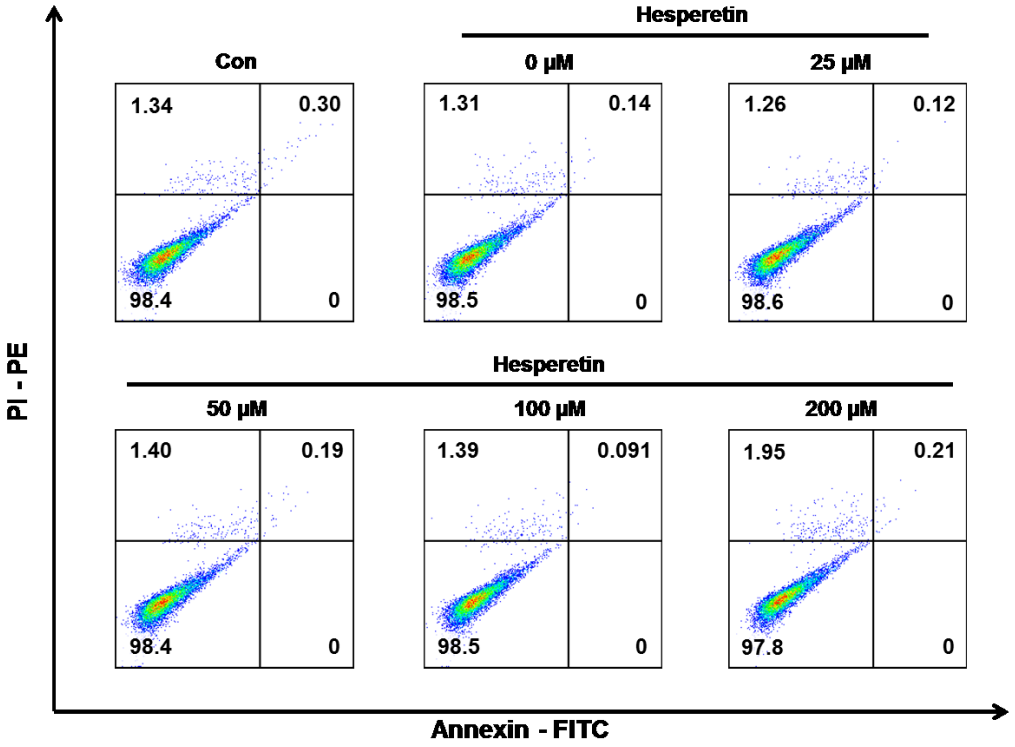


Fig. 9. Hesperetin was not cytotoxicity in human mast cells. HMC-1 were treated with hesperetin for 24 hr and stained with Annexin-V-FITC and PI-phycobiliproteins (PE). Fluorescence intensity was measured by flow cytometry and presented as a percentage of each quadrant.

2. Hesperetin suppresses the gene expression levels of pro-inflammatory cytokines in human mast cell activation.

Pro-allergic cytokines were important mediators of allergic inflammation, cell recruitment and allergenic response decided to further investigate the anti-inflammatory effects of hesperetin by measuring the expression of inflammatory mediators in PMA/A23187-stimulated human mast cells (Bradding et al., 1993). Moreover, IL-1β is a potent inflammatory cytokine and induced by various factors such as inflammation, prostaglandin synthesis, and pyrogen (Turner et al., 2014). To evaluate the effect of hesperetin on the mRNA expression of pro-inflammatory cytokines, human mast cells were pretreated with hesperetin and then stimulated with PMA (20 nM) and A23187 (1 μM) for RT-PCR analysis. As shown in Fig. 10, a hesperetin significantly suppressed the gene expression level of *IL-1β*, *TNF-α*, and *IL-6* in PMA/A23187-stimulated human mast cells in dose dependent manners.

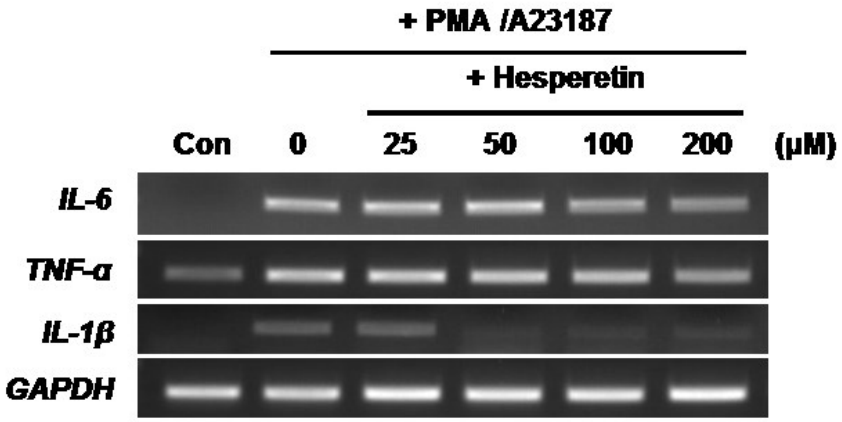


Fig. 10. Hesperetin suppressed PMA/A23187-induced pro-inflammatory factors in human mast cells. HMC-1 cells were pretreated with various concentrations of hesperetin for 2 hr and then stimulated by PMA (20 nM) and A23187 (1 μM) for 4 hr. The mRNA expression level of *IL-6*, *TNF-α* and *IL-1β* genes was determined by RT‑PCR.

3. Hesperetin significantly inhibits allergic inflammatory cytokine production in human mast cell activation.

IL-6 is an important cytokine for activation and maturation of mast cells. Protein kinase C (PKC) induces IL-6 production leading histamine production and secretion in mast cell (Kalesnikoff et al., 2002). As shown in Fig. 10, IL-6 gene expression dramatically increased by PMA/A23187 treatment; however, PMA/A23187-induced IL-6 gene expression was significantly inhibited by hesperetin treatment. Therefore, to further examine the effects of hesperetin on allergic inflammatory cytokine production in activated human mast cells, I measured the secretion of IL-6 using an ELISA. As shown Fig. 11, PMA/A23187-induced IL-6 secretion was significantly reduced by hesperetin treatment, in a dose-dependent manner. These results suggest that hesperetin effectively suppressed the production and secretion of IL-6 in PMA/A23187-stimulated human mast cells.

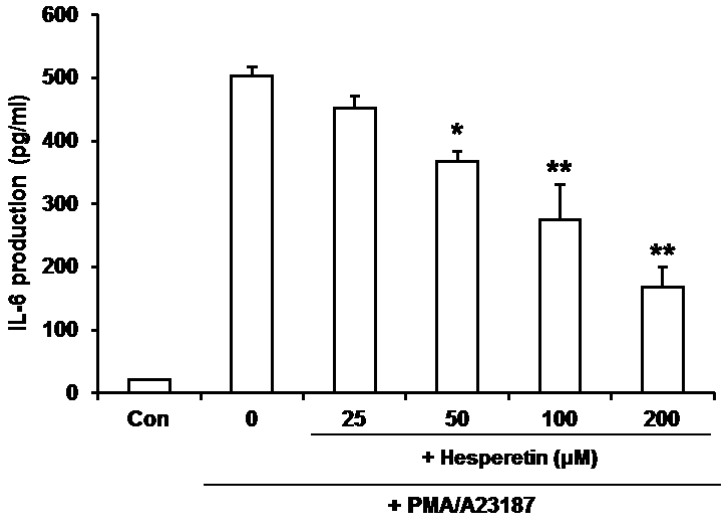


Fig. 11. Hesperetin inhibited PMA/A23187-induced inflammatory cytokines in human mast cells. HMC-1 cells were pre-treated with hesperetin (25, 50, 100, 200 μM) for 2 hr, followed by treatment of PMA (20 nM) and A23187 (1 μM) for 24 hr. The IL-6 production level in the supernatant was measured by ELISA. Each bar indicates the mean ± SE of four independent experiments. *p < 0.05, **p < 0.01, significantly different from PMA/A23187-treated group.

4. Hesperetin inhibits phosphorylation of ERK and p38 MAPK pathway in activated human mast cells.

To investigate the mechanism of inhibitory effect of hesperetin on human mast cell, I examined the MAPK signaling pathways. MAPK and NF-κB pathway are important signal pathway that regulate inflammatory cytokines including IL-1β and IL-6 (Min et al., 2007). To investigate the regulation effect of hesperetin on the MAPK pathways, human mast cells were pretreated with 200 μM hesperetin and activated by 20 nM PMA and 1 μM A23178. As shown in Fig. 12, I found that phosphorylation of ERK1/2, p38 MAPK increased by PMA/A23187 stimulation at 15, 30, and 45 min. However, pre-treatment of hesperetin significantly decreased PMA/A23187-induced phosphorylation of ERK1/2, p-38 MAPK. These results indicate that hesperetin regulates PMA/A23187-stimulated human mast cells activation through inhibiting the phosphorylation of ERK1/2 and p38 MAPK.

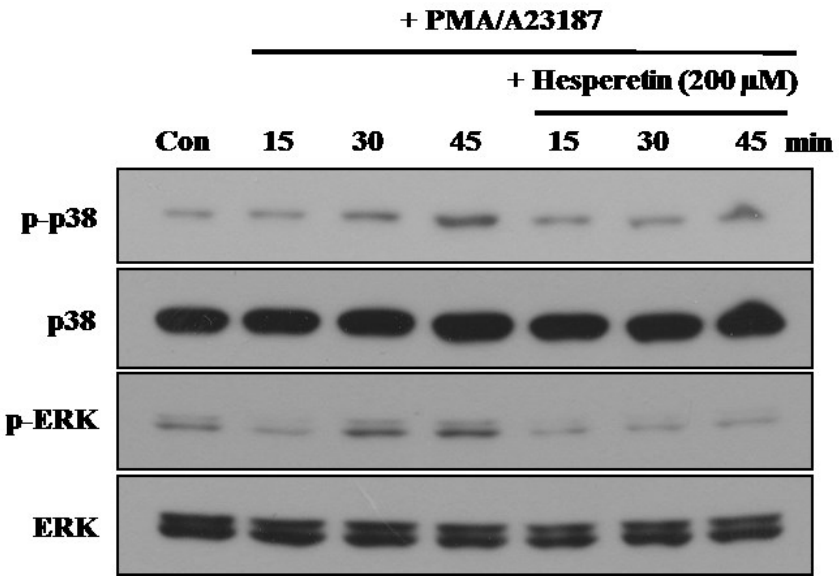


Fig. 12. Hesperetin inhibited PMA/A23187-induced ERK and p38 MAPK phosphorylation. HMC-1 cells were pre-treated with hesperetin (200 μM) for 2 hr, followed by treatment of PMA (20 nM) and A23187 (1 μM) for 15, 30, 45 min. The expression of phosphorylated proteins was measured by Western blot. The total extracts were assayed with antibodies specific for p-ERK1/2 and p-38 MAPK.

5. Hesperetin attenuates epidermal hypertrophy and mast cell infiltration in DNCB-induced atopic dermatitis.

To investigate the effect of hesperetin on atopic dermatitis, DNCB-induced atopic dermatitis was examined in mouse experiment. On day 19, the dorsal skins of DNCB group highly increased atopic dermatitis compared with control groups. DNCB-induced atopic dermatitis were significantly decreased by administration of hesperetin (Fig. 13). Furthermore, I examined change of skin lesion using histological experiment. Epidermal hypertrophy and thickness of DNCB treated group increased with compared control group. However, treatment of hesperetin effectively decreased DNCB-induced epidermal hypertrophy and thickness. In the same manner, infiltration of mast cell and immune cell increased in DNCB group with compared control group. In addition, these DNCB-induced infiltration were significantly decreased by hesperetin treatment. These data indicate that hesperetin suppresses the DNCB-induced atopic dermatitis by inhibiting epidermal hypertrophy and mast cell infiltration.

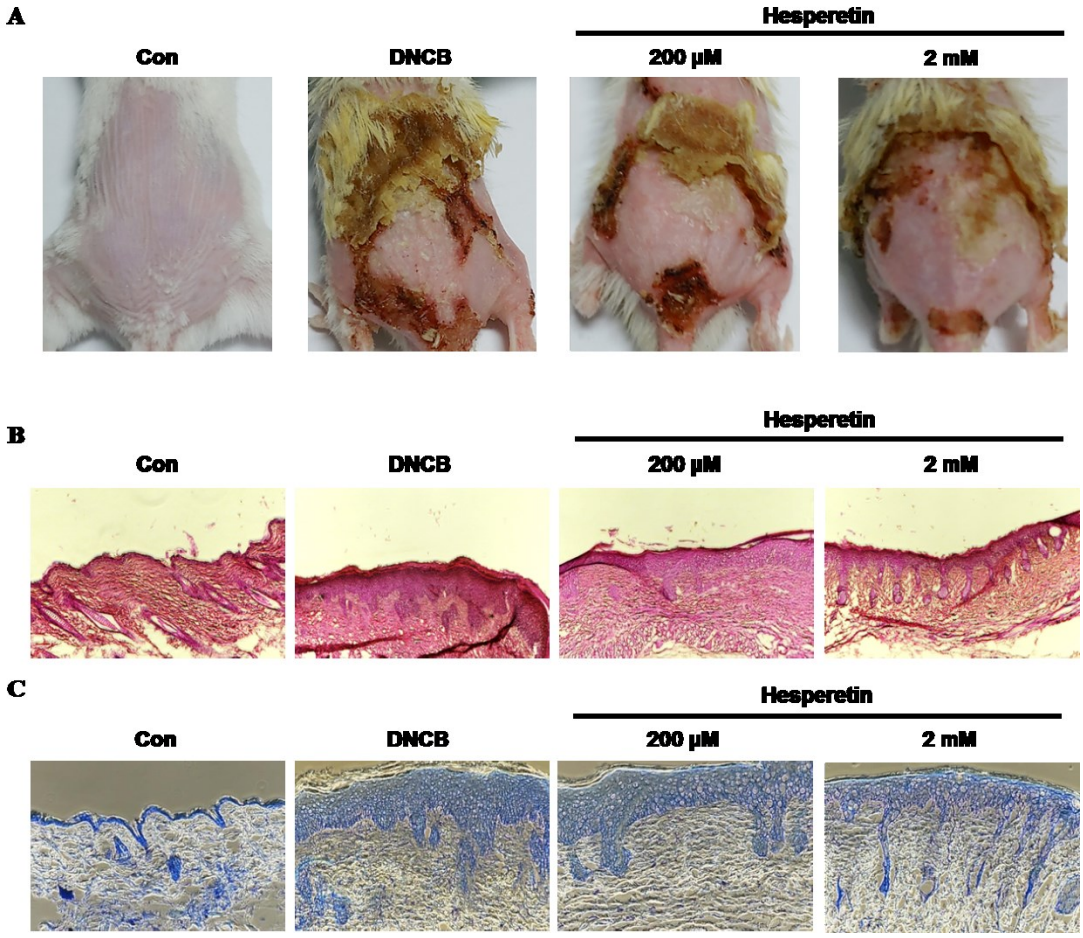


Fig. 13. Hesperetin decreased epidermal hypertrophy and mast cell infiltration in DNCB-induced atopic dermatitis. All BALB/c mice were shaved on dorsal skin and sensitized treatment of 1% DNCB solution in acetone/olive oil (3:1) for 4 days. After 3 days, hesperetin (200 μM, 2 mM) solution and 0.5% DNCB solution challenged on the dorsal skin for 11 days. (A) Effect of hesperetin on skin lesion of a DNCB-induced atopic dermatitis. (B) H&E staining of atopic dermatitis dorsal skin (magnification ×100). (C) Toluidine blue stained of atopic dermatitis dorsal skin (magnification ×200).

Part II. Hesperetin regulates LPS-induced neuroinflammation on microglia by suppressing pro-inflammatory cytokines and MAPK phosphorylation

1. Hesperetin inhibits NO production in LPS-stimulated BV-2 microglial cells

To investigate the effect of hesperetin on cell viability and NO regulation, I measured cell viability using the MTT assay. BV-2 microglial cells were cultured for 24 hr in the presence of various concentrations of hesperetin (0, 5, 25, 50, 100, or 200 μM). I found that hesperetin had no detectable cytotoxic effects on BV-2 microglial cells at doses up to 200 μM. Subsequently, I chose five non-cytotoxic doses of hesperetin to test for anti-neuroinflammatory effects. To this end, I measured the levels of NO released in the culture medium, using an NO detection assay. BV-2 microglial cells were pretreated with hesperetin for 2 hr and then stimulated with LPS (200 ng/ml). I used the NO assay to investigate whether hesperetin could effectively regulate NO production at the given concentrations. I found that LPS-induced NO production was significantly decreased by hesperetin treatment, in a dose-dependent manner (Fig. 14). These results indicate that hesperetin effectively inhibited NO production in LPS-stimulated BV-2 microglial cells.

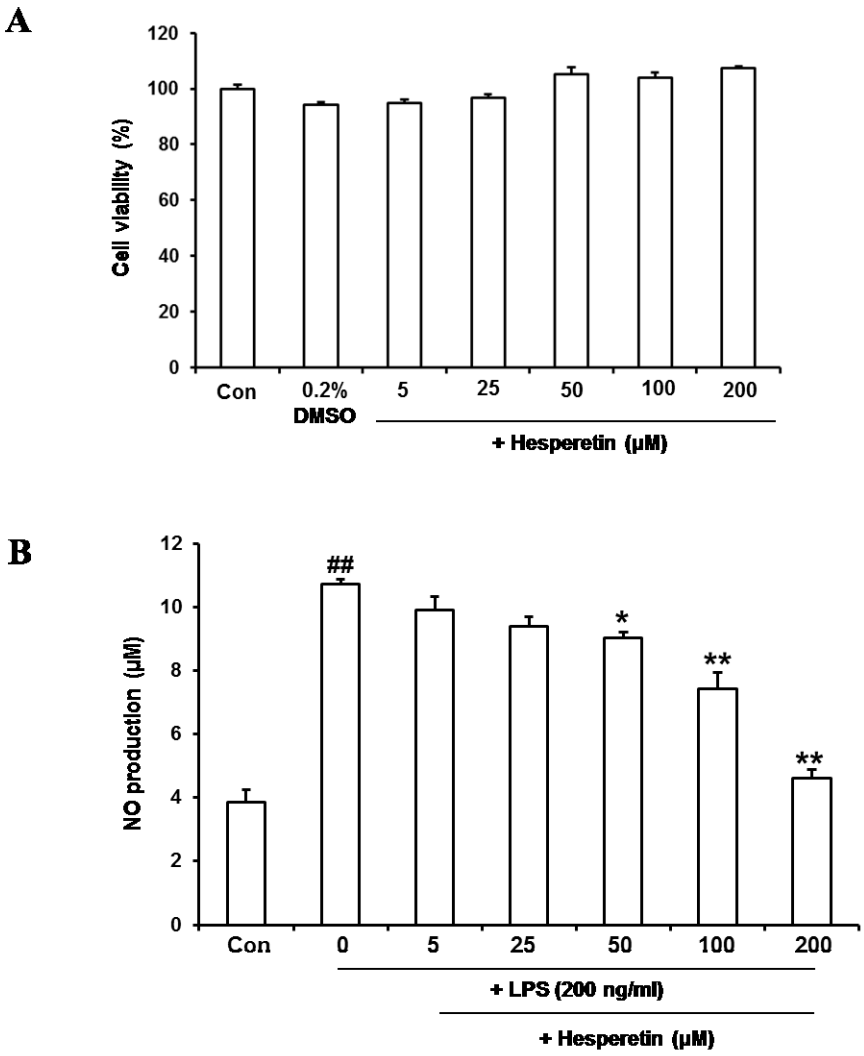
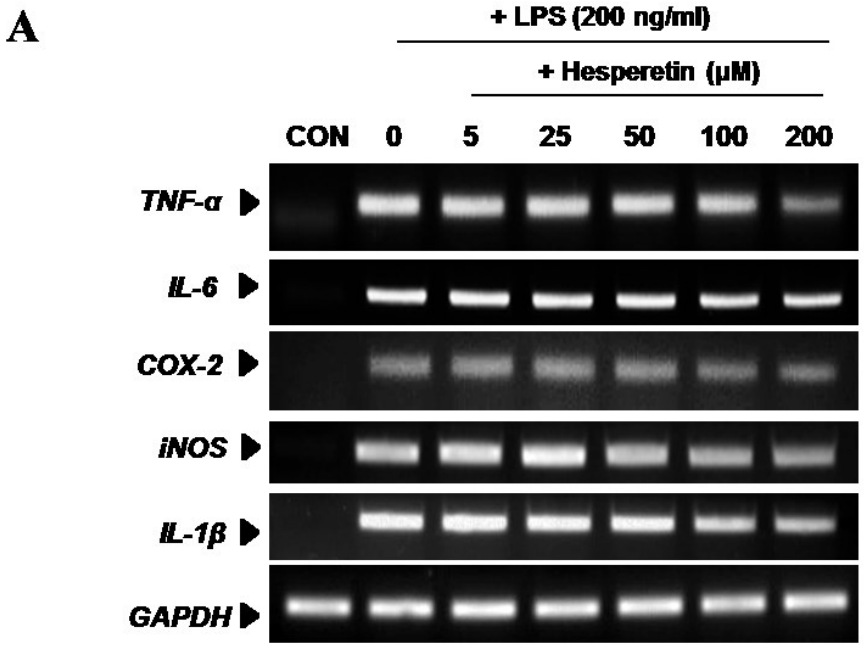


Fig. 14. Regulatory effect of hesperetin on NO production in LPS-stimulated BV-2 microglial cells. Cell viability was determined by MTT assay after hesperetin treatment with BV-2 microglial cells (A). After hesperetin treatment of BV-2 microglial cells stimulated with LPS (200 ng/ml), NO production ability was determined by Griess assay (B). The result is representative of repeated four independent experiments. Experimental results were indicated as mean (± SE). *$p < 0.05$, **$p < 0.01$, significantly different from LPS-treated group; ## $p < 0.01$, significantly different from LPS-untreated group

2. Hesperetin inhibits the expression of pro-inflammatory cytokines and iNOS in LPS-stimulated BV-2 microglial cells.

Given the strong inhibitory effect of hesperetin on NO production in LPS-stimulated microglial cells, I decided to further investigate the anti-inflammatory effects of hesperetin by measuring the expression of inflammatory mediators in LPS-stimulated BV-2 microglial cells. Specifically, I investigated the expression of pro-inflammatory genes, *IL-1β*, *IL-6*, and *TNF-α*, as well as the levels of the pro-inflammatory enzymes, inducible *iNOS* and *COX*-2. I used RT-PCR to determine whether hesperetin inhibited the expression of pro-inflammatory genes and inducible enzymes. As shown in Figure. 15A, mRNA expression levels of *iNOS*, *COX-2*, *IL-6*, *TNF-α*, and *IL-1β* increased in LPS-stimulated BV-2 microglial cells. Interestingly, the LPS-induced expression of *iNOS* and *IL-1β* were significantly decreased by hesperetin treatment, in a dose-dependent manner. I next investigated whether hesperetin treatment also reduced the LPS-induced expression of iNOS and COX-2 protein. As shown in Figure. 15B, the levels of iNOS protein decreased with hesperetin treatment in LPS-stimulated BV-2 microglial cells. However, COX-2 protein levels were affected by hesperetin treatment. These results indicate that hesperetin treatment inhibited the expression of *IL-1β*, *TNF-α*, and *iNOS* mRNA, as well as iNOS protein, in LPS-stimulated microglia.



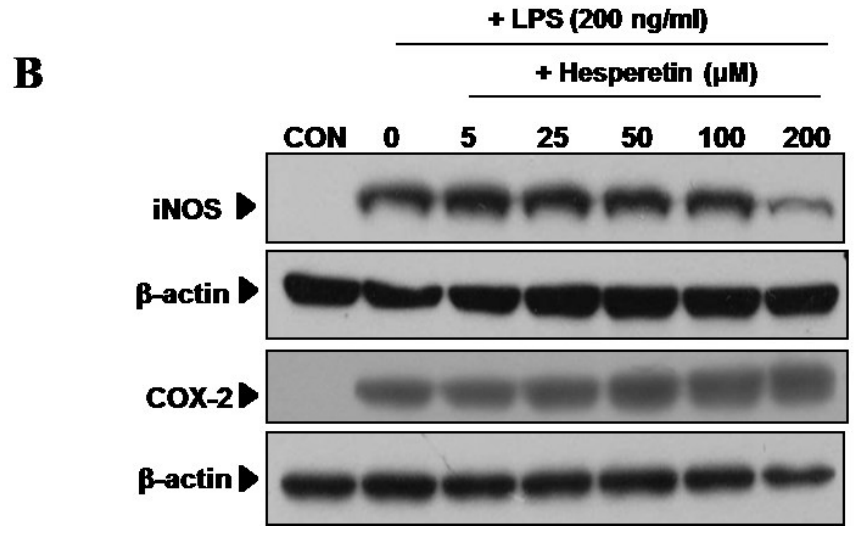


Fig. 15. Effect of hesperetin on mRNA and protein expression of iNOS and COX-2. Hesperetin was pretreated with BV-2 microglial cells 2 hr, and then incubated with 200 ng/ml LPS for 6 hr. RNA was isolated and analyzed by RT-PCR. *iNOS*, *COX-2*, *TNF-α*, *IL-1β*, and *IL-6* gene expression was confirmed, respectively (A). Hesperetin was pretreated with BV-2 microglial cells 2 hr, and then incubated with 200 ng/ml LPS for 22 hr. iNOS and COX-2 protein expression was determined by Western blot (B). The result is representative of repeated three independent experiments.

3. Hesperetin reduces the production of pro-inflammatory cytokine such as TNF-α, IL-1β, and IL-6

IL-1β was a pro-inflammatory cytokine that is released by microglia after exposure to LPS or other inflammatory stimuli. As shown in Fig. 15A, IL-1β expression dramatically increased in response to LPS treatment; however, its expression was significantly inhibited by hesperetin treatment. Therefore, to further examine the effects of hesperetin treatment on inflammatory cytokine production in LPS-stimulated BV-2 microglial cells, I measured the production levels of IL-1β, TNF-α and IL-6 protein by an ELISA. As shown Fig. 16A, B, and C, LPS stimulation of microglia increased the secretion of IL-1β, IL-6, and TNF-α, which was significantly reduced by hesperetin treatment, in a dose-dependent manner. These results suggest that hesperetin selectively decreased the production of IL-1β, IL-6, and TNF-α proteins in LPS-stimulated BV-2 microglial cells.

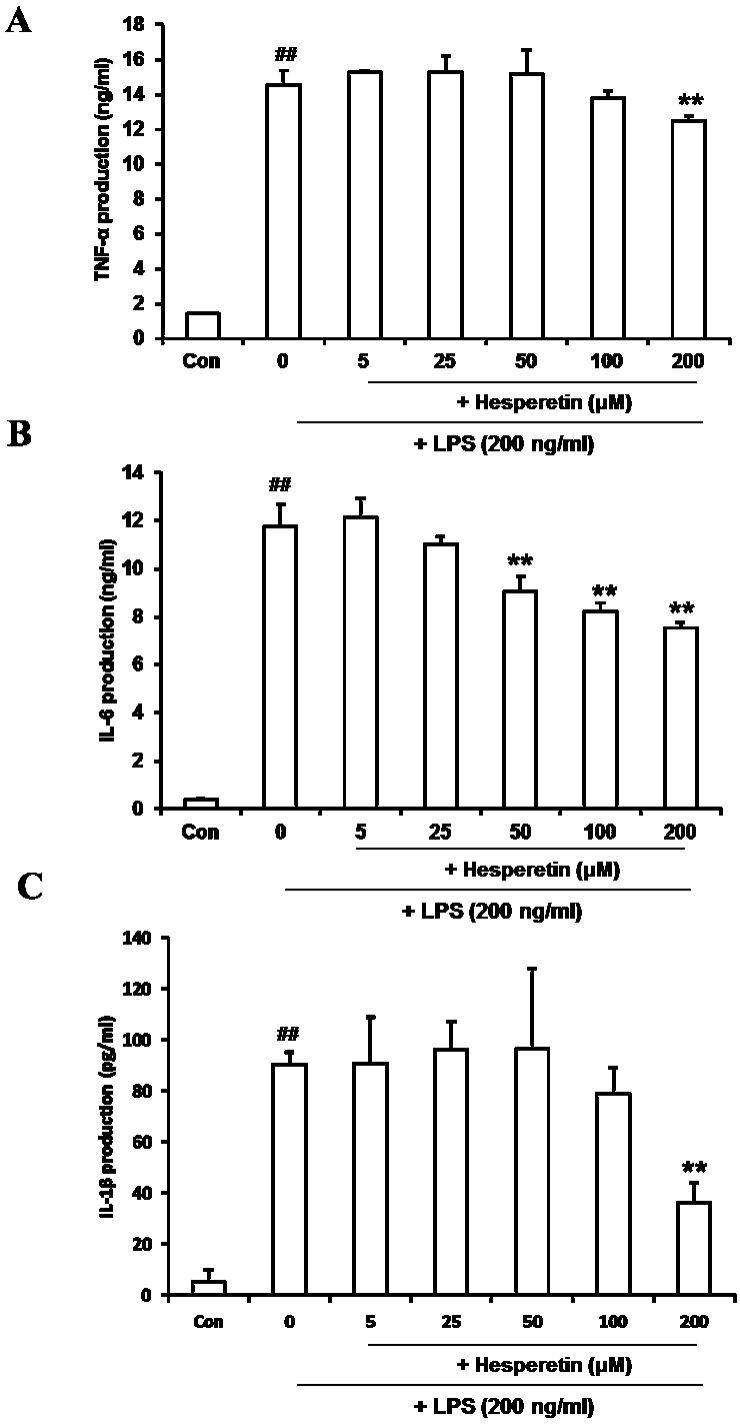


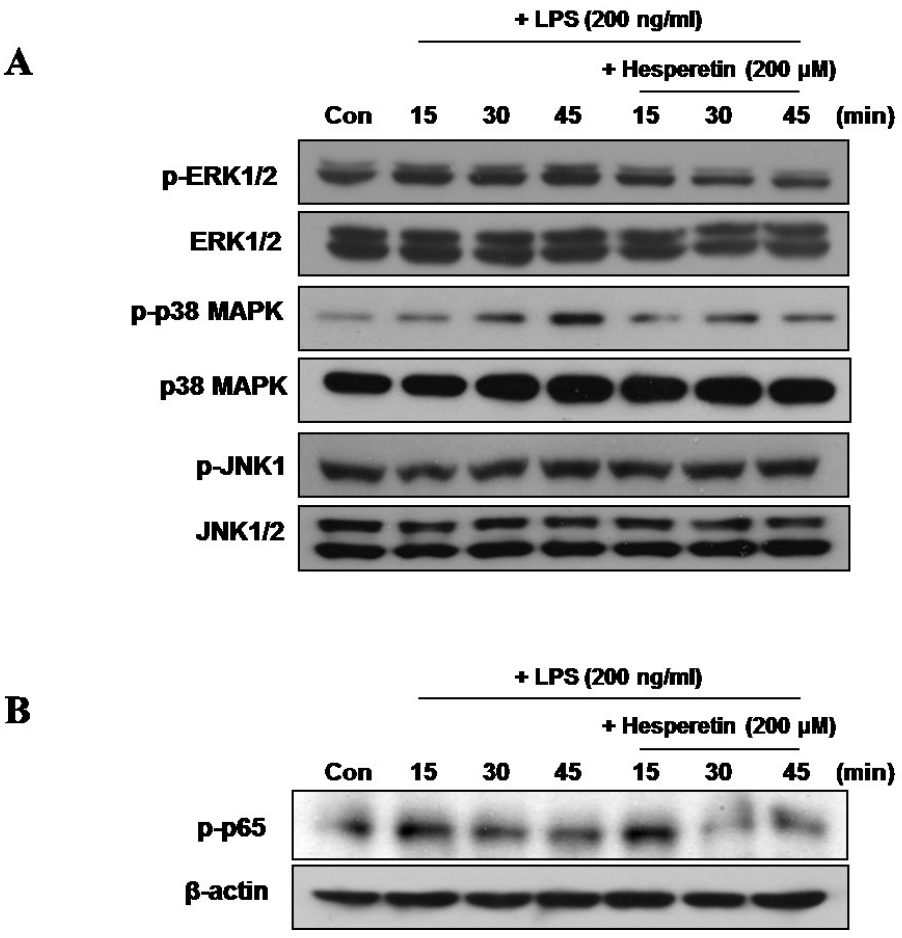
Fig. 16. Effect of hesperetin on the production levels of IL-1β, IL-6 and TNF-α in LPS-stimulated BV-2 microglial cells. The cells were pre-treated with hesperetin (5, 25, 50, 100, 200 μM) for 2 hr, followed by treatment of LPS (200 ng/ml) for 24 hr; the levels in the supernatants were measured by ELISA. Each bar indicates the mean ± SE of four independent experiments. **p < 0.01, significantly different from LPS-treated group; ## p < 0.01, significantly different from LPS-untreated group.

4. Hesperetin inhibits phosphorylation of p38 MAPK and ERK in LPS-stimulated BV-2 microglial cells.

To investigate the mechanism by which hesperetin treatment exerts anti-inflammatory effects, I examined the MAPK signaling pathways. These pathways play important roles in pro-inflammatory gene expression by modulating transcription factors, such as nuclear factor kappa B (NF-κB). The MAPK pathways also include ERK 1/2, JNK 1/2, and p38 MAPK. I measured LPS-induced phosphorylation of these members of the MAPK pathway using western blot analysis. I found that phosphorylation of ERK1/2, JNK1, p38 MAPK, and NF-κB increased with LPS stimulation at 15, 30, and 45 min. However, treatment with hesperetin significantly decreased LPS-induced phosphorylation of ERK1/2, p-38 MAPK and NF-κB. These results indicate that hesperetin regulates LPS-stimulated microglial activation by inhibiting the phosphorylation of p38 MAPK, ERK1/2, and NF-κB (Fig. 17A and B).

5. Hesperetin attenuate astrocyte and microglia activation in the LPS-administrated mouse brain

To confirm anti-neuroinflammatory activity of hesperetin in brain-resident glial cells, LPS-induced astrocyte and microglia activation were examined in mouse brain. GFAP, astrocyte marker was slightly increased and Iba-1, microglia marker was highly expressed in hippocampus in LPS-injected groups compared with control groups. LPS-induced astrocyte and microglia activation in hippocampus was significantly decreased by hesperetin administration (Fig. 17C). These data indicated that hesperetin suppresses the LPS-induced neuroinflammatory response by inhibiting astrocyte and microglia activation.



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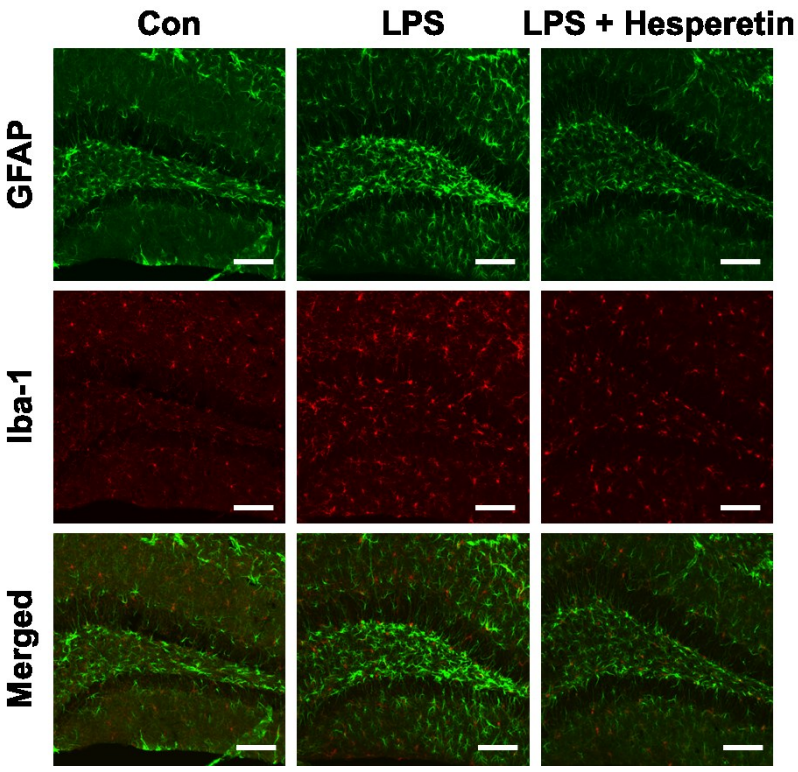

Fig. 17. Hesperetin attenuated LPS-induced MAPK phosphorylation and microglia activation. BV-2 microglial cells were pre-treated with various concentrations of hesperetin for 2 h, followed by treatment of LPS (200 ng/ml) for 15, 30, 45 min. The expression of phosphorylated proteins was measured by Western blot. The total extracts were assayed with antibodies specific for p-ERK1/2, p-38 MAPK, p-JNK1, and p-p65 (NF-κB). β-actin was used as internal control for total fraction. The blots are representative of three independent experiments (A and B). Brain sections were immunostained with anti-GFAP or anti-Iba-1 antibody as described in the Materials and Methods section (C) (Scale bar = 100 μm). The result is representative of repeated three independent experiments

IV. Discussion

Part I

The allergic response is representative immediate type hypersensitivity. These reactions cause various diseases such as atopic dermatitis, anaphylaxis, and asthma, which is a problem around the world (Rhinitis et al., 2001). Allergy is a kind of hypersensitivity and mediated by activated mast cells. In this study, I showed that the anti-allergic effect of hesperetin is mediated by inhibiting the production of pro-allergic inflammatory cytokines, IL-6, TNF-α and IL-1β in PMA/A23187-activated human mast cells. In addition, this result indicates that hesperetin treatment suppressed the mast cell activation and recruitment in DNCB-induced atopic dermatitis mice.

In the study, I conform that hesperetin had no cytotoxicity effect on human mast cells using Annexin V/PI staining. When 200 μM hesperetin is treated in human mast cells, hesperetin do not induce cell death consistent with the control. PMA/A23178 are used to activation of human mast cells. These activators are increased the permeability of cell membrane to calcium and induced phosphorylation of PKC. Activated PKC caused expression of histamine, various cytokines such as IL-1β, IL-6, and TNF-α through MAPK signal pathway. These inflammatory factors induce allergic response (Barrett et al., 2009). Treatment of PMA/A23187 induces increased expression levels of cytokines in human mast cells. As shown in Fig 10 and 11, I demonstrate that hesperetin treatment suppressed the expression of IL-1β, IL-6, and TNF-α in PMA/A23187-stimulated human mast cells. The cytokines expression is typically regulated by the MAPK signaling pathway. In agreement with previous reports on the MAPK signal pathway human mast cells activation, I observed that PMA/A23187-stimulated human mast cells activation were related to MAPK pathway including ERK1/2 and p38. MAPK has been found to be upstream signal pathway of inflammatory cytokines and degranulation in allergic

inflammation (Gilfillan et al., 2006; Rivera et al., 2006). In addition, I found that hesperetin notably inhibited the ERK1/2 phosphorylation in PMA/A23187-stimulated human mast cells. In order to confirm the inhibitory effect of hesperetin on atopic dermatitis model, BALB/c male mice induced atopic dermatitis-like skin using DNCB (Fujii et al., 2009). DNCB-induced atopic dermatitis was suppressed by treatment of hesperetin. Epidermal hypertrophy, thickness, and infiltration of mast cells were also inhibited by application of hesperetin.

In conclusion, I investigated whether hesperetin regulate allergic inflammation on human mast cells. Hespertin inhibits the gene expression of pro-inflammatory cytokines IL-1β, IL-6, and TNF-α levels and suppress secretion of IL-6. Additionally, hesperetin downregulates activation of the ERK signaling pathway. DNCB-induced atopic dermatitis suppress by treatment of hesperetin. This result indicates that hesperetin effectively inhibited allergic inflammation.

Part II

BV-2 cells are involved in the immune response and regulate the neuroinflammatory response in the brain. The development of chronic inflammatory diseases can lead to neurodegenerative diseases, such as PD, AD, and Huntington's disease (McGeer et al., 1988; Sapp et al., 2001; Zhang et al., 2005). In present study, I showed that the anti-inflammatory activity of hesperetin is mediated by inhibiting the production of pro-inflammatory molecules, iNOS, NO, IL-6, and IL-1β, in LPS-activated BV-2 cells. In addition, this result demonstrates that hesperetin treatment inhibited astrocytes and microglia activation in LPS-injected mince.

NO is a key factor in promoting the inflammatory response. NO is regulated by iNOS expression and other NO metabolites. Moreover, NO production from microglia is a mediator of the inflammatory response and can promote neurotoxicity (Meda et al., 1995). When NO is continuously activated, the inflammatory response develops into a chronic inflammatory reaction, which can cause neurodegenerative disease (Teismann et al., 2003). As shown in Fig. 15, I found that iNOS expression and NO production were increased in LPS-stimulated BV-2 cells. However, COX-2 mRNA and protein expression was not regulated by hesperetin treatment. Interestingly, in a previous study, inhibition of iNOS in LPS-stimulated microglia resulted in neuro-protective effects (Gan et al., 2015).

Increased expression of pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α can induce neuro-inflammatory responses. Activated microglia also increase the production of neuro-inflammatory cytokines and CNS inflammation, as shown in previous studies (Qin et al., 2007; Vargas et al., 2005). As shown in Fig. 16, I demonstrate that hesperetin treatment inhibits the production of IL-1β, IL-6, and TNF-α in LPS-stimulated BV-2 microglial cells. The expressions of these cytokines are regulated by the MAPK signaling pathway. Consistent with previous reports on the signaling pathways that underlie microglial activation, I observed that MAPK, including ERK1/2, JNK, and p38 MAPK, were involved in LPS-stimulated BV-2 microglial cells

activation. MAPK have been shown to be important upstream regulators of inflammatory cytokines and NO production in a variety of biological systems. Interestingly, I found that hesperetin markedly inhibited the phosphorylation of ERK1/2, p38 MAPK, and NF-κB in LPS-stimulated BV-2 microglia cells.

Astrocyte and microglia activation is important in neuroinflammatory responses and also generally induced in various CNS injuries including Parkinson's disease and Alzheimer's disease (Jahn, 2013; Teismann et al., 2004). Astrocyte and microglia produce pro-inflammatory substances such as NO and pro-inflammatory cytokines, which can act as neurotoxic chemicals in brain. In addition, astrocyte and microglia interact with each other under neuroinflammatory condition (Sofroniew, 2009). In order to confirm the inhibitory effect of hesperetin on neuroinflammation, As a result of LPS injection into mice pretreated with hesperetin, astrocyte and microglia were activated in the group treated with LPS only. However, astrocyte and microglia activation were suppressed in the group pretreated with hesperetin. This result suggests that hesperetin has a preventive effect on neuroinflammation.

I found that hesperetin decreased expression of NO, the inflammatory cytokines IL-1β and IL-6, and iNOS in LPS-stimulated BV-2 microglial cells. In addition, hesperetin significantly suppressed astrocytes and microglial activation in LPS-challenged mouse brain. Taken together, I conclude that hesperetin may be a good candidate for preventive or therapeutic treatments for inflammation-mediated neurodegenerative disease.

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감사의 글

2014년도 12월에 처음으로 실험실에 들어와 학부 3학년, 4학년, 석사 2년이라는 시간이 정말로 눈 깜짝할 사이에 지나버렸습니다. 4년이라는 시간들이 제 인생에 있어서 정말 소중하고 매우 중요한 시기라고 생각합니다. 그렇기에 학위과정 동안 많은 위로와 도움을 주신 고마운 분들께 짧게나마 감사한 마음으로 글을 남기고자 합니다. 먼저 항상 저에게 큰 힘이 되고 보금자리가 되어주신 부모님이 생각납니다.. 공부하고 들어온 저에게 항상 따듯한 말과 사랑으로 저에게 큰 힘과 용기를 주시고 그 누구보다 저를 걱정하고 배려해주셔서 감사합니다. 또한 제가 하고 싶은 일을 하는데 있어서 그 누구보다 지지하고 응원해 주셨기에 제가 지금 이렇게 성장하고 이 자리에 있을 수 있었습니다. 우리 사랑하는 동생들 지형이와 진언이 다들 표현이 조금은 서툴지만 같이 너희와 커갈수록 정말 너희 소중함을 느끼고 너희만한 동생들도 없다는 것을 느끼고 있어. 너희와 대화를 하다 보면 너희에게 힘이 되어주고 싶다가도 되려 내가 너희에게 힘을 받아가고 나에게 없던 자세나 행동을 배워가는 생각이 매번 들더구나. 항상 우리 셋이 앞으로 살아가면서 더욱 끈끈하고 우애 깊은 삼형제가 되었으면 좋겠어.

실험실에 들어오기 전부터 석사 학위 생활까지 부족한 저를 항상 조언과 격려로 이끌어 주신 지도교수님이신 이준식 교수님께 감사 드립니다. 교수님께 처음 면역학이라는 학문을 배워 나가면서 많은 것을 느낄 수 있었습니다. 교수님의 제자로서 부끄럽지 않고 떳떳한 사람이 되고자 항상 노력하겠습니다. 많이 부족하지만 저의 논문을 심사해 주시며 많은 조언들을 해주신 전택중 교수님과 조광원 교수님께 감사 립니다. 생태학이라는 과목을 통해 생물학에 대한 거시적인 시점을 알려주신

윤성명 교수님, 전반적인 식물들의 생리적인 특징을 가르쳐 주신 박현용 교수님, 해양생물들의 특징과 분류를 알려주신 조태오 교수님 감사합니다. 또한 유전학과 분자생물학을 가르쳐 주신 송상기 교수님 감사합니다. 항상 저의 장단점을 통해 저를 칭찬해 주시는 정현숙 교수님, 언제나 인자한 미소로 인사해 주시는 이현화 교수님 감사합니다.

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