



Galangin attenuates mast cell-mediated allergic inflammation



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ABSTRACT

A great number of people are suffering from allergic inflammatory disease such as asthma, atopic dermatitis, and sinusitis. Therefore discovery of drugs for the treatment of these diseases is an important subject in human health. In this study, we investigated anti-allergic inflammatory effect of galangin and underlying mechanisms of action using *in vitro* and *in vivo* models. Galangin inhibited histamine release by the reduction of intracellular calcium in phorbol 12-myristate 13-acetate plus calcium ionophore A23187-stimulated human mast cells (HMC-1). Galangin decreased expression of pro-inflammatory cytokines, such as tumor necrosis factor (TNF)- α , interleukin (IL)-6, IL-1 β , and IL-8. The inhibitory effect of galangin on these pro-inflammatory cytokines was related with c-Jun N-terminal kinases, and p38 mitogen-activated protein kinase, nuclear factor- κ B, and caspase-1. Furthermore, galangin attenuated IgE-mediated passive cutaneous anaphylaxis and the expression of histamine receptor 1 at the inflamed tissue. The inhibitory effects of galangin were more potent than cromolyn, a known anti-allergic drug. Our results showed that galangin down-regulates mast cell-derived allergic inflammatory reactions by blocking histamine release and expression of pro-inflammatory cytokines. In light of *in vitro* and *in vivo* anti-allergic inflammatory effects, galangin could be a beneficial anti-allergic inflammatory agent.

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1. Introduction

Mast cells are effector cells in the initiation of inflammatory reactions associated with allergic disorders such as asthma, atopic dermatitis, and sinusitis. In vertebrates, mast cells are widely distributed throughout vascularized tissues, particularly near surfaces exposed to the external environment, including the skin, airways, conjunctiva and gastrointestinal tract (Grimbaldeston et al., 2006).

Upon exposure of an allergen, which is recognized by immunoglobulin (Ig)E antibodies bound to the high affinity IgE receptor (Fc ϵ RI) expressed on mast cell surface, aggregation of Fc ϵ RI triggers a intracellular signaling process (Rivera et al., 2008). Phosphorylation of Src family kinases (Lyn, Syk and Fyn) and phospholipase C- γ are followed by calcium mobilization and activation of protein kinase C, mitogen-activated protein kinases (MAPKs) and nuclear factor (NF)- κ B (Galli et al., 2008). As a result, mast cells secrete both preformed and newly synthesized mediators including histamine, eicosanoids, proteases, and several pro-inflammatory cytokines/chemokines. Using these products, mast cells contribute not only

immediate-type hypersensitivity but also late reaction, like inflammatory responses.

Histamine released from activated mast cells is major mediator leading immediate-type hypersensitivity. Histamine causes vasodilation and increases permeability of vessels near the allergic site. By the release of chemotactic and pro-inflammatory mediators such as tumor necrosis factor (TNF)- α , interleukin (IL)-6, IL-1 β and IL-8, mast cells affect on the late phase responses of an allergic inflammation (Galli et al., 2008). These cytokines released from mast cells change terminal microenvironment and attract neutrophils and basophils. Therefore reduction of these pro-inflammatory cytokines is one of key indicators of relieved allergic inflammatory symptoms.

MAPKs are mediators that respond to extracellular stimuli and regulate diverse cellular activities. Extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinases (JNK) and p38 are major subfamily of MAPKs. In mast cells, MAPKs play a crucial role in the regulation of pro-inflammatory cytokine (Scheller et al., 2011; Turner et al., 2012). NF- κ B is a transcriptional factor which regulates expression of pro-inflammatory cytokines (Azzolina et al., 2003). For this reason, NF- κ B is an obvious target of anti-inflammatory treatment (Kim et al., 2006). Caspase-1 is also involved in regulation of inflammatory cytokines. Caspase-1, a member of cysteine protease also known as IL-1 β converting enzyme, is found predominantly in the cytoplasm of cells, where it proteolytically convert pro-IL-1 β into mature form, that is involved in inflammation (Lamkanfi et al., 2011).

Abbreviations: HMC, human mast cell; PCA, passive cutaneous anaphylaxis; DNP, dinitrophenyl; PMA, phorbol 12-myristate 13-acetate; TNF, tumor necrosis factor; JNK, c-Jun N-terminal kinase.

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Galangin (3,5,7-trihydroxyflavone) is a natural flavonol, which is constituent of propolis, a resinous mixture that made by honey bee from the juice of plants, vegetable and fruits (Schnitzler et al., 2010). Galangin has been demonstrated to possess anti-cancer effect by evaluating apoptotic activity or decreasing Bcl-2 and cyclin D3 (Bestwick and Milne, 2006; Murray et al., 2006; Tolomeo et al., 2008). Galangin has anti-oxidative activity by reducing NADH oxidation and as a scavenger of free radical (Russo et al., 2002). Some studies reported that galangin has anti-inflammatory effect caused by phospholipase A2 inhibition (Lattig et al., 2007) and decrease of adhesion molecule expression. Galangin also has anti-viral (Schnitzler et al.), anti-microbial (Cushnie et al., 2007), and vasorelaxant activity (Morello et al., 2006). However, in spite of the beneficial biological effects, effect of galangin on the allergic inflammation was not elucidated. The aim of this study is to evaluate the effect of galangin on the mast cell-mediated allergic inflammation and determine the involving mechanism of these effects.

2. Materials and methods

2.1. Reagents and cell culture

Anti-DNP IgE, DNP-human serum albumin (HSA), phorbol 12-myristate 13-acetate (PMA), calcium ionophore A23187, *o*-phthalaldehyde, galangin, and cromolyn were purchased from Sigma (St. Louis, MO, USA). Human mast cells (HMC)-1 were grown in IMDM (GIBCO, Grand Island, NY, USA) supplemented with 100 units/mL penicillin/streptomycin, and heat-inactivated 10% fetal bovine serum (GIBCO) in 5% CO₂ at 37 °C.

2.2. Animals

The male ICR mice (6 weeks) were purchased from Dae-Han Experimental Animal Center (Daejeon, Korea). The animals were housed in a laminar air flow room maintained under a temperature of 22 ± 2 °C and relative humidity of 55 ± 5% throughout the study. The care and treatment of the animal were in accordance with the guidelines established by the Public Health Service Policy on the Humane Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee.

2.3. Cell viability

Cell viability was assayed using XTT assay kit (WELGENE, Seoul, Korea). HMC-1 cells (2 × 10⁴ cells/well in 96-well plates) were treated with galangin for 24 h followed by incubation with XTT plus phenazine methosulfate reagent for 4 h. Absorbance was read at 450 nm using an Anthos 2010 spectrophotometer (Salzburg, Austria). Cell viability was calculated as relative absorbance compared with the control and expressed as percentage of the control.

2.4. Histamine contents

Histamine contents in cultured cell supernatants were measured by the *o*-phthalaldehyde spectrofluorometric procedure as previously described (Singh et al., 2012). HMC-1 cells were preincubated with galangin at 37 °C for 30 min, and then stimulated with PMA plus A23187 for 8 h. The fluorescent intensity was measured at emission 438 nm and excitation 353 nm using spectrofluorometer.

2.5. Intracellular calcium

To determine intracellular calcium, Fluo-3/AM (Molecular Probes, Eugene, OR, USA) was used according to the manufacturer's protocol, a brief description of which follows. HMC-1 cells were preincubated with Fluo-3/AM for 30 min at 37 °C. After washing the dye from the cell surface, galangin (10 µg/mL) was pretreated 30 min prior to the PMA plus A23187 stimulation. The fluorescent intensity was measured using fluorometer (Molecular Devices, Sunnyvale, CA, USA) and visualized in a fluorescence microscope (Olympus BX51, Center Valley, PA, USA).

2.6. RNA extraction and PCR

The total cellular RNA was isolated from the cells (1 × 10⁶/well in 24-well plate) after stimulation of PMA (40 nM) plus A23187 (1 µM) with or without galangin using a RNeasy Plus (TakaraBio, Shiga, Japan) according to the manufacturer's protocol. The first strand complementary DNA (cDNA) was synthesized using Maxime RT Premix (iNtRON Biotech, Sungnam, Korea). A reverse-transcriptase polymerase chain reaction (RT-PCR) was used to analyze the expression of mRNA for TNF-α,

IL-6, IL-1β, IL-8, and β-actin (internal control). H1 receptor mRNA level was determined by real-time PCR. The primer sets for cytokines and H1 receptor are summarized in Supplementary Table 1. Total RNA was isolated from PCA-reacted spots of skin in mice. PCA-reacted spots were detached and frozen in liquid nitrogen. Frozen skins smashed using a homogenizer with RNeasy Plus. Total RNA was extracted and dissolved in DEPC-water. The conditions for the reverse transcription and PCR steps were similar to those previously described (Kim et al., 2006). The primer sets were chosen by the Primer3 program (Whitehead Institute, Cambridge, MA, USA). The cycle number was optimized in order to ensure product accumulation in the exponential range. The amplified products were separated by electrophoresis on 2% agarose gel containing ethidium bromide, documented using a molecular imager gel doc XR system (Bio-Rad, Hercules, CA, USA).

After the PCA reaction, PCA-reacted ears were detached and total cellular RNA was isolated from the tissue. The expression level of H1R and β-actin were determined by quantitative real-time PCR. Quantitative real-time PCR was carried out using the Thermal Cycler Dice TP850 (TakaraBio Inc.) according to the manufacturer's protocol. Briefly, 2 µL of cDNA (100 ng), 1 µL of sense and antisense primer solution (0.4 µM), 12.5 µL of SYBR Premix Ex Taq (TakaraBio Inc.), and 9.5 µL of dH₂O were mixed together to obtain a final 25 µL reaction mixture in each reaction tube. The amplification conditions were 10 s at 95 °C, 40 cycles of 5 s at 95 °C and 30 s at 60 °C, 15 s at 95 °C, 30 s at 60 °C, and 15 s at 95 °C. Relative quantification of mRNA expression was performed using the TP850 software.

2.7. Nuclear protein extraction

After HMC-1 cell (3 × 10⁶ in a 6-well plate) were activated for 2 h, cells were washed in 1 mL of ice-cold PBS, centrifuged at 1200g for 5 min, resuspended in 400 µL of ice-cold hypotonic buffer (10 mM HEPES/KOH, 2 mM MgCl₂, 0.1 mM EDTA, 10 mM KCl, 1 mM DTT, 0.5 mM PMSF, pH 7.9), left on ice for 10 min, vortexed, and centrifuged at 15,000g for 30 s. After several times of washing, pelleted nuclei were resuspended in 50 µL of ice-cold saline buffer (50 mM HEPES/KOH, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 10% glycerol, 1 mM DTT, 0.5 mM PMSF, pH 7.9), left on ice for 20 min, vortexed, centrifuged at 15,000g for 5 min at 4 °C, and supernatant gathered.

2.8. Western blotting

HMC-1 (3 × 10⁶ in a 6-well plate) extracts were prepared by detergent lysis procedure (Lee et al., 2011). Samples of protein (50 µg) were electrophoresed using 8–12% SDS-PAGE, and then transferred to nitrocellulose membrane. The nucleus and cytosolic p65 NF-κB, IκBα, caspase-1, actin were assayed using anti-NF-κB (p65), anti-IκBα, anti-caspase-1 and actin antibodies (Santa Cruz Biotech, Santa Cruz, CA, USA), respectively. The phosphorylation of JNK, p38 and ERK was determined using anti-phospho-JNK, anti-phospho-p38 and anti-phospho-ERK antibody (Cell Signaling, Beverly, MA, USA). Immunodetection was done using supersignal west pico chemiluminescent substrate (Thermo scientific, Waltham, MA, USA).

2.9. Caspase-1 activity assay

The enzymatic activity of caspase-1 was measured according to the manufacturer's specification using a caspase assay kit (R&D Systems Inc, Minneapolis, MN, USA). The cell lysate was centrifuged at 10,000g for 1 min. The protein supernatant was incubated with 50 µL reaction buffer and 5 µL substrates (WEHD-pNA) at 37 °C for 2 h. The absorbance was measured using a plate reader at a wavelength of 405 nm. Equal amounts of the total protein from each lysate were quantified using a bicinchoninic acid protein quantification kit (Sigma).

2.10. Transient transfection and luciferase activity

HMC-1 cells were seeded at 1 × 10⁶ cells in 12-well plate 4 h before the transient transfection. NF-κB luciferase reporter gene constructs (pNF-κB-LUC, plasmid containing NF-κB binding site; STANTAGEN, Grand Island, NY, USA) were transiently transfected into HMC-1 with FBS and antibiotics free IMDM containing 8 µL of lipofectamin 2000 reagent (Invitrogen, Carlsbad, CA, USA). After 6 h of incubation, medium was replaced with IMDM containing 10% FBS and antibiotics. After transfected cells were incubated for 18 h, stimulated with PMA plus A23187 at 37 °C for 2 h. Galangin was pretreated to cells for 30 min prior to stimulation. Cells were harvested and washed in ice-cold PBS before lysis in 100 µL of cell lysis buffer (Luciferase assay kit; Promega), according to the manufacturer's protocol. Luciferase activity was determined using the luminometer.

2.11. Passive cutaneous anaphylaxis (PCA)

An IgE-dependent cutaneous reaction was carried out as described previously (Bae et al., 2011). The mice were injected intradermally into the ear with 0.5 µg of anti-DNP IgE. After 48 h, each mice were received an injection of 1 µg of DNP-HSA containing 4% Evans blue (1:4) via the tail vein. Galangin and cromolyn were intraperitoneally administered at doses 1 and 10 mg/kg BW 1 h before the challenge. Thirty minutes after the challenge, the mice were killed and the ears were

removed for measurement of the pigment area. The amount of dye was determined colorimetrically after extraction with 1 mL of 1 M KOH and 9 mL of a mixture of acetone and phosphoric acid (5:13). The intensity of absorbance was measured at 620 nm in a spectrophotometer (UV-1201; Shimadzu, Kyoto, Japan).

2.12. Statistical analysis

Statistical analyses were performed using SAS statistical software (SAS Institute, Cary, NC, USA). Treatment effects were analyzed using analysis of variance, followed by Duncan's multiple range tests. $P < 0.05$ was to indicate significance.

3. Results

3.1. Effect of galangin on histamine release and intracellular calcium

First, to check the effect of galangin on the cell viability, XTT assay was performed. HMC-1 cells were treated with various concentrations (0.1–100 $\mu\text{g/mL}$) of galangin and incubated for 24 h. As shown in Fig. 1A, up to 100 $\mu\text{g/mL}$ of galangin did not alter the cell viability of HMC-1.

We assessed the ability of galangin on histamine release from HMC-1 cells. PMA (40 nM) plus A23187 (1 μM) stimulated HMC-1 released a high level of histamine (Fig. 1B). The release of histamine was dose-dependently decreased in galangin treated cells. To investigate the mechanisms of galangin on the reduction of histamine release, we assayed the intracellular calcium. Calcium movements across membranes of mast cells are critical to histamine

release (Eisenhut and Wallace, 2011). We used Fluo-3/AM for intracellular calcium assay. The stimulation with PMA (40 nM) plus A23187 (1 μM) increased the intracellular calcium (Fig. 2). When HMC-1 cells were preincubated with galangin (10 $\mu\text{g/mL}$), intracellular calcium level is decreased.

3.2. Effect of galangin on the expression of pro-inflammatory cytokines

We tested the effect of galangin on expression of pro-inflammatory cytokines such as TNF- α , IL-6, IL-1 β and IL-8 in HMC-1 cells. HMC-1 cells stimulated with PMA (40 nM) plus calcium ionophore A23187 (1 μM) for 4 h expressed all cytokines at a high level. Galangin (0.1–10 $\mu\text{g/mL}$) dose-dependently inhibited gene expression of cytokines (Fig. 3A). To confirm the inhibition, quantitative real-time PCR was also performed. The results of quantitative real-time PCR showed reduction of all the cytokines (Fig. 3B).

3.3. Effect of galangin on the activation of NF- κB and MAPKs

To find out the mechanism responsible for the inhibitory effect of galangin on the cytokine expression, we next investigated the effect of galangin on NF- κB translocation and MAPK phosphorylation using Western blot. Stimulation of HMC-1 cells with PMA (40 nM) plus A23187 (1 μM) induced translocation of p65 NF- κB to the nucleus following degradation of I $\kappa\text{B}\alpha$. Galangin hindered the PMA plus A23187-induced degradation of I $\kappa\text{B}\alpha$ and translocation of p65 NF- κB (Fig. 4A). To confirm the inhibitory effect of galangin on NF- κB translocation, we examined the effect of galangin on the NF- κB -dependent gene reporter assay. After transient transfection with NF- κB -luciferase reporter construct, stimulation of cells with PMA plus A23187 enhanced the luciferase activity (Fig. 4B). Galangin significantly reduced PMA plus A23187-induced luciferase activity. MAPKs also play a crucial role in the regulation

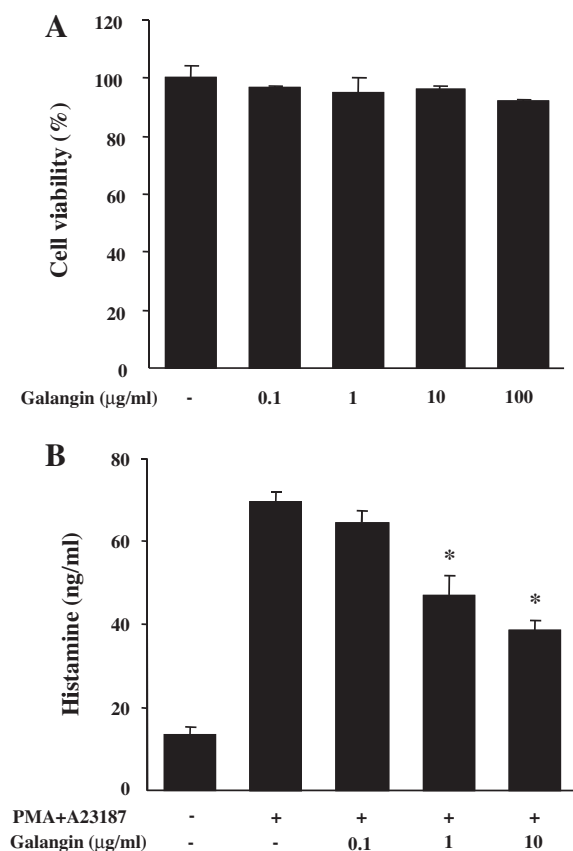


Fig. 1. Effect of galangin on cell viability and histamine release. (A) HMC-1 cells (2×10^4 cells/well in 96-well plates) were treated with various concentration of galangin for 24 h. The cell viability was determined using XTT assay. Cell viability was represented by relative absorbance compared to control. (B) For histamine assay, cells (1×10^5 cells/well in 24-well plates) were pretreated with galangin for 30 min and stimulated with PMA (40 nM) plus A23187 (1 μM) for 8 h. Supernatant was used to analyze using the *o*-phthalaldehyde spectrometric procedure. Each bar represents the mean $\pm$ SE of three independent experiments. *Significant difference from PMA plus A23187 value at $P < 0.05$.

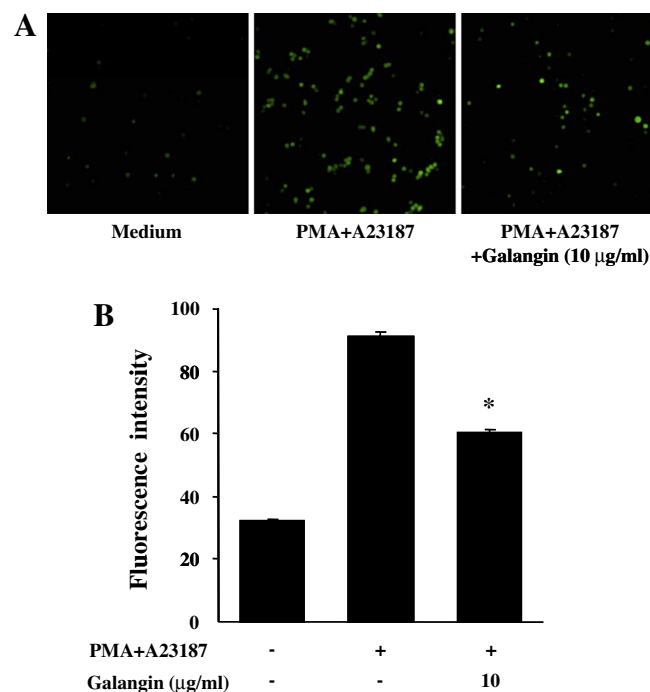


Fig. 2. Effect of galangin on the intracellular calcium. HMC-1 cells were preincubated with galangin (10 $\mu\text{g/mL}$) for 30 min and then another 10 min with PMA (40 nM) plus A23187 (1 μM). Intracellular calcium level was detected by fluorescence microscope (A) and fluorescent plate reader (B). Each bar represents the mean $\pm$ SE of three independent experiments. *Significant difference from the PMA plus A23187 value at $P < 0.05$.

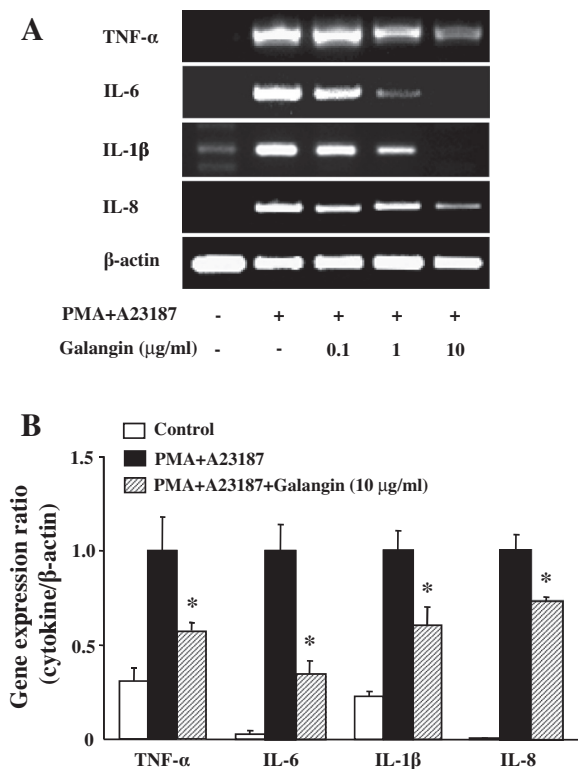


Fig. 3. Effect of galangin on expressions of pro-inflammatory cytokines. HMC-1 (1×10^6 cells/well in 24-well plates) were pretreated with galangin for 30 min prior to being stimulated with PMA (40 nM) plus A23187 (1 μM) for 4 h. The expression of TNF-α, IL-6, IL-1β and IL-8 were determined by RT-PCR (A) and confirmed by quantitative real-time PCR (B). Each bar represents the mean $\pm$ SE of three independent experiments. *Significant difference from the PMA plus A23187 value at $P < 0.05$.

of pro-inflammatory molecules on cellular responses (Newton and Dixit, 2012). PMA plus A23187 induced phosphorylation of ERK, JNK and p38. Galangin reduced phosphorylation of JNK and p38, but not ERK (Fig. 4C). To confirm the role of JNK, p38 and NF-κB on the gene expression of pro-inflammatory cytokines, specific inhibitors for JNK (SP600125), p38 (SB203580) and NF-κB (PDTC) were used. According to the XTT assay, we adopted the optimal concentrations of SB203580 (10 μM), SP600125 (2 μM) and PDTC (50 μM) without any cell toxicity (Supplementary Fig. 1). As shown in Fig. 4D, pro-inflammatory cytokines from PMA plus A23187 stimulated HMC-1 cells were inhibited by SP600125, SB203580 and PDTC, even though the inhibitory degree is different depends on cytokines and inhibitors.

3.4. Effect of galangin on the caspase-1 activation

To find out additional underlying mechanisms, we assayed caspase-1 activation. Caspase-1 cleave precursor of IL-1β to biologically active form. Active caspase-1 is derived from the cleavage of the 45 kDa pro-caspase-1. We examined pro-caspase-1 in HMC-1 cells. PMA plus A23187 induced decrease of pro-caspase-1, that is, caspase-1 activation is increased and galangin reduced caspase-1 activation (Fig. 5A). To confirm the attenuation of caspase-1, we measured the caspase-1 activity. The enhanced caspase-1 activity by PMA plus A23187 was markedly decreased by galangin (Fig. 5B).

3.5. Effect of galangin on the IgE-mediated local anaphylaxis

To examine the effects of galangin on IgE-mediated allergic reaction *in vivo*, passive cutaneous anaphylaxis (PCA) was used.

PCA is one of the most appropriate *in vivo* models of local allergic reaction (Kim et al., 2006). After injection of 4% Evans blue mixed with antigen, PCA reaction site showed markedly increased vascular permeability as indicated by the amount of Evans blue dye extravasation. Administration of galangin (1 and 10 mg/kg BW) visibly reduced PCA reaction. In comparison, cromolyn showed relatively low reduction (Fig. 6A and B). In allergic reactions, histamine is one of the important mediators. Action of histamine depends on expression level of H1R. To evaluate effect of galangin on the level of H1R mRNA, we analyzed mRNA expression of H1R in PCA-reacted areas. As shown in Fig. 6C, 10 mg/kg of galangin and cromolyn similarly attenuated H1R mRNA expression.

4. Discussion

In the present study, we showed that galangin relieved mast cell-mediated allergic inflammation using *in vitro* and *in vivo* models. Mast cells function as a key mediator of allergic inflammation. After allergen exposure, mast cells are activated by crosslinking of adjacent IgE, aggregation of FcεRI triggers intracellular signaling process that results in the degranulation and secretion of histamine, protease, cytokines, and chemokines. Histamine was originally considered as a mediator of acute inflammatory and immediate hypersensitivity responses. Histamine also induces actin polymerization and chemotaxis (Jutel et al., 2002). Numerous reports established that stimulation of mast cells with compound 48/80 or IgE initiates the activation of the signal transduction pathway, which leads to histamine release (Amin, 2012; Kim et al., 2006). Recently it has been reported that histamine affects chronic inflammation and regulates several essential events of immune response such as immune cell maturation, polarization and lymphocyte responsiveness (O'Mahony et al., 2011).

Regulation of intracellular calcium is critical to the histamine release of mast cells. Calcium movements across membranes of mast cells represent a major target for effective anti-allergic drugs, as these are essential events linking stimulation to secretion (Ma and Beaven, 2011; Manikandan et al., 2012). In the present study, we evaluated the effects of galangin on PMA plus A23187-induced histamine release and intracellular calcium. The mode of action of galangin is likely related the prevention of histamine release from mast cells due to inhibition of the intracellular calcium. Our results showing an attenuation of intracellular calcium in mast cells following galangin treatment are consistent with other reports (Dambros et al., 2005; Kim et al., 2011). According from these observations, we strongly speculate that decreased intracellular calcium might be involved in the inhibitory effect of galangin on histamine release.

The HMC-1 is one of the useful cells for studying cytokine activation pathways (Kim et al., 2006). The variously types of cytokines produced by HMC-1 with PMA plus A23187 stimulation support the well recognized role of mast cells in immediate-type hypersensitivity. Mast cell-derived pro-inflammatory cytokines, especially TNF-α, IL-6, IL-1β and IL-8 have a critical biological role in the allergic inflammation. Mast cells are one of the major sources of TNF-α in human dermis (Sarchio et al., 2012). TNF-α promotes inflammation, granuloma formation and tissue fibrosis and is thought to be an initiator of cytokine related inflammatory states by stimulating cytokine production (Vandenabeele et al., 2010). TNF-α is involved in eosinophils survival, thereby contributes to chronic inflammation (Walczak, 2011). IL-6 is also produced from mast cells and its local accumulation is associated with PCA reaction (Mican et al., 1992). These reports indicate that reduction of TNF-α and IL-6 from mast cells is one of the key indicators of reduced allergic inflammatory symptoms. IL-1β induces expression of adhesion molecules on endothelium to enhance neutrophil transmigration

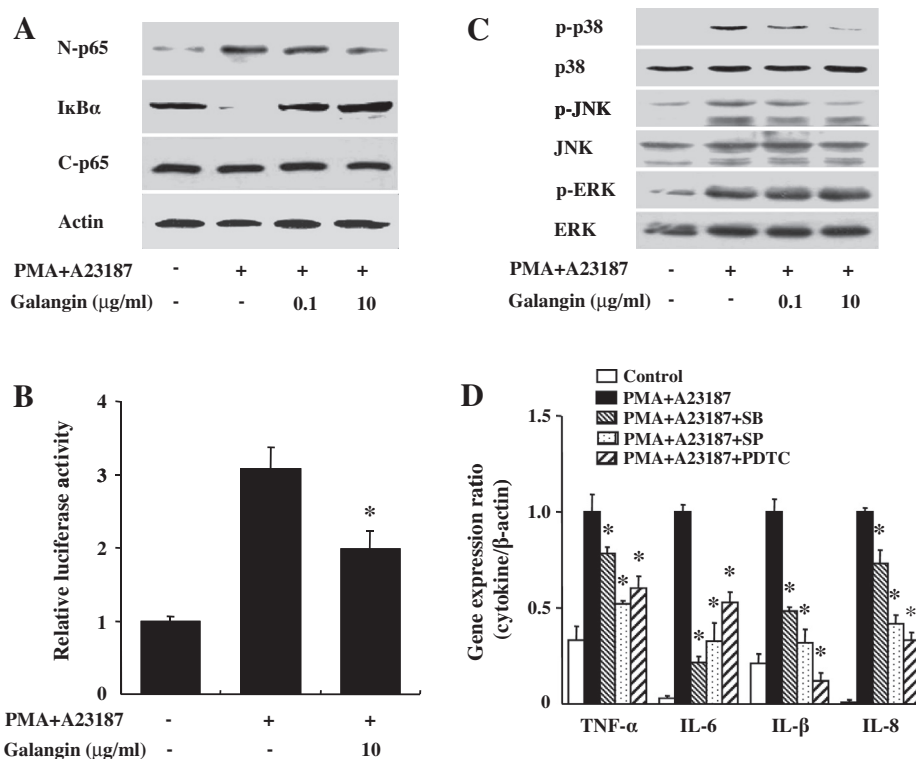


Fig. 4. Effect of galangin on the activation of NF- κ B and MAPKs. HMC-1 (3×10^6 cells/well in 6-well plates) were pretreated with galangin for 30 min prior to PMA (40 nM) plus A23187 (1 μ M) stimulation. Then cells were further incubated 2 h for NF- κ B and 30 min for MAPKs. (A) Nuclear translocation of p65 NF- κ B and degradation of I κ B α were assayed by Western blot (N-p65, nucleus NF- κ B; C-p65, cytoplasmic NF- κ B). (B) Cells were transiently transfected with the NF- κ B-luciferase reporter construct gene. Then, cells were incubated with or without galangin and stimulated with PMA plus A23187. NF- κ B dependent transcriptional activity was determined by luciferase activity assay. (C) Phosphorylation of MAPKs was assayed by Western blot. (D) HMC-1 (1×10^6 cells/well in 24-well plates) were pretreated with specific inhibitors (JNK for SP600125, p38 for SB203580 and NF- κ B for PDTTC) for 30 min prior to being stimulated with PMA (40 nM) plus A23187 (1 μ M) for 4 h. The expression of TNF- α , IL-6, IL-1 β and IL-8 were determined by quantitative real-time PCR. These data are representative of three independent experiments. *Significant difference from the PMA plus A23187 value at $P < 0.05$.

(Lin et al., 2002). IL-8 released from mast cells acts on surrounding cells such as neutrophils and eosinophils, and induces migration and activation on inflammatory effector cells (Castellani et al., 2010). In our study, galangin decreased the elevated gene expression of TNF- α , IL-6, IL-1 β and IL-8 in mast cells. These data suggest that galangin shows anti-inflammatory effects by the inhibition of pro-inflammatory cytokines. Galangin is one of the major constituents and active components of propolis. Recently it was reported that the extract of propolis attenuates IL-4 and MCP-1 production from RBL-2H3 cells (Nakamura et al., 2010) and galangin inhibits TNF- α and IL-1 β gene expression in collagen-induced arthritis mice (Huh et al., 2012). These accumulated reports support our results showing the inhibition of pro-inflammatory cytokines in mast cells.

Intracellular calcium is crucially involved in gene transcription of inflammatory cytokines as well. Several reports have demonstrated associations between intracellular calcium and inflammatory cytokine production from mast cells (Law et al., 2011; Tanaka et al., 2005). Depletion of intracellular calcium with calcium blockers inhibits IgE-induced cytokine production in mast cells. Because of the reducing effect of galangin on the intracellular calcium, we suggest that one possible mechanism of the inhibitory effect of galangin on the inflammatory cytokines is mediated by the reduction of intracellular calcium in mast cells.

Among pro-inflammatory cytokines, IL-1 β is synthesized in cytoplasm and is processed and released from cells by a mechanism involving an enzyme called IL-1 β converting enzyme, known as caspase-1. Pro-IL-1 β is biologically inactive and must be converted to the mature IL-1 β to acquire the ability to bind to recep-

tors and activate cells. Caspase-1 presents in a catalytically inactive 45 kDa proenzyme and is activated by cleavage into two non-identical subunits, p20 and p10. Active caspase-1 can also cleave other members of the IL-1 family, IL-18 and IL-33 (Strowig et al., 2012). The administration of caspase-1 inhibitors attenuates the course of collagen-induced arthritis and inhibits the pro-inflammatory responses of monocytes isolated from patients with familial cold auto-inflammatory syndrome (Ku et al., 1996; Stack et al., 2005). In our study, we observed that PMA plus A23187-stimulated caspase-1 activation was inhibited by galangin in mast cells. Through that result, we assume that galangin inhibits IL-1 β via the regulation of IL-1 β converting enzyme activation.

NF- κ B is a transcription factor that induces the transcription of a variety of genes. Many of these genes encode molecules important in inflammatory processes such as cytokines and adhesion molecules. The role of NF- κ B and its regulation of cytokine production in allergic inflammation have been characterized (DiDonato et al., 2012). NF- κ B regulates the expression of multiple inflammatory and immune genes and plays a critical role in chronic inflammatory diseases (Pasparakis, 2012). Activation of NF- κ B required phosphorylation and proteolytic degradation of the inhibitory protein I κ B α , an endogenous inhibitor that binds to NF- κ B in the cytoplasm (Nakagomi et al., 2012). To evaluate the mechanisms of inhibition of galangin on expression of pro-inflammatory cytokines, we examined the effect of galangin on NF- κ B pathway. In our study, galangin decreased the degradation of I κ B α and nuclear translocation of p65 NF- κ B. These results indicate that inhibitory effects of galangin on inflammatory cytokines were due to the regulation of NF- κ B pathway. This finding is in agreement with recent

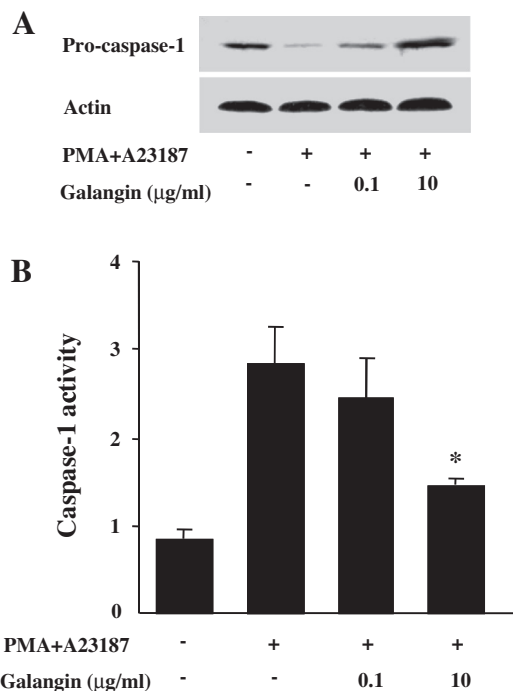


Fig. 5. Effect of galangin on the caspase-1 activation. HMC-1 cells were pretreated with galangin for 30 min prior to PMA (40 nM) plus A23187 (1 µM) stimulation for 2 h. (A) Pro-caspase-1 was assayed by Western blot. (B) The enzymatic activity of caspase-1 was assayed by caspase-1 colorimetric assay. Each bar represents the mean $\pm$ SE of three independent experiments. *Significant difference from the PMA plus A23187 value at $P < 0.05$.

reports showing that galangin restrains collagen-induced arthritis by regulating NF- κ B translocation (Huh et al., 2012) and oxidative damage by inhibiting NF- κ B activation in fructose-fed rats (Sivaku-

mar and Anuradha, 2011). The MAPK cascade is also an important signaling pathway in immune responses and cross talks with NF- κ B to regulate inflammation signaling (Wang and Liu, 2007). The expression of pro-inflammatory cytokines is known to be regulated by MAPKs in mast cells (Kim et al., 2006). The precise signaling pathways among the three types of MAPKs, i.e., ERK, JNK and p38 are still unclear. However, the induction of inflammatory cytokine genes requires phosphorylation of all three types of MAPKs. In this study, galangin reduced PMA plus A23187 induced phosphorylation of JNK and p38, but not ERK. Studies with Fyn deficient mast cells showed an important role for JNK and p38 but not ERK in cytokine secretion (Gomez et al., 2005). This report supports our results showing the regulatory role of JNK and p38 in activated mast cells. Our data suggest that the inhibitory effect of galangin on the pro-inflammatory cytokines is mediated by NF- κ B, and JNK and p38 pathway.

Cromolyn is a clinically used medication for treating asthma and allergy and it widely characterized works by stabilizing mast cells. In PCA reaction, the inhibitory effect of galangin was superior to cromolyn. Local injection of anti-DNP IgE followed by an intravenous antigenic challenge induced local extravasation. Various agents via mast cell degranulation are commonly associated with IgE-mediated reaction in humans and other mammals. Histamine, one of the various degranulated mediator, is known to be extremely important on allergic reactions. The regulatory character of histamine in cellular events is attributed to its binding to four distinct subtypes of G-protein-coupled receptors, specified H1, H2, H3, and H4 that are differentially expressed in various cell types (Zucchi et al., 2006). Above all, H1 receptors are widely distributed throughout the periphery and central nervous system, and play a critical role in regulating inflammatory responses (Jutel et al., 2009). H1 antagonists, also known as H1 blockers or antihistamines, are the most commonly used first-line medication for allergic symptom (Simons and Simons, 2011). Therefore, we checked expression of H1 receptors in PCA-reacted area. Inhibitory effect

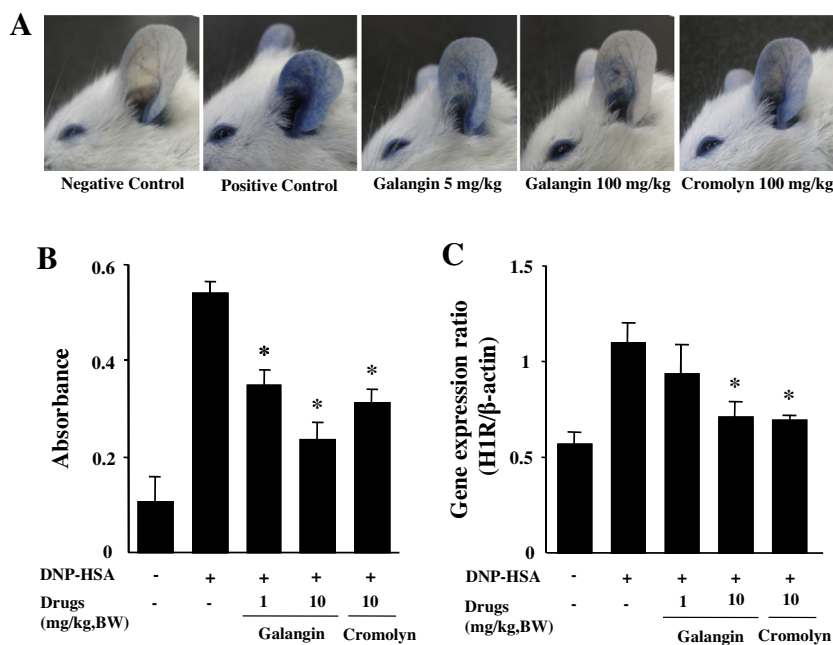


Fig. 6. Effect of galangin on PCA reaction. The mice were injected intradermally into the ear with 0.5 µg of anti-DNP IgE. After 48 h, each mice were received an injection of 1 µg of DNP-HSA containing 4% Evans blue (1:4) via the tail vein. Galangin or cromolyn were administered 1 h prior to the challenge with antigen. (A) Blue color indicates inducing level of PCA reaction. (B) Each amount of dye was determined colorimetrically. The intensity of absorbance was measured at 620 nm using a spectrophotometer. (C) PCA-reacted ears were detached and expression level of H1R and β -actin were determined by quantitative real-time PCR. Each bar represents the mean $\pm$ SE of three independent experiments. *Significant difference at $P < 0.05$. BW, body weight. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

of galangin in PCA reaction was higher than cromolyn, however the gene expression level of H1 receptors were same manners. Based on these results, we speculated that the inhibitory effect of galangin in PCA reaction was regulated through correlation of histamine receptors and other routes.

In conclusion, our results suggest that galangin reduces histamine release via regulating intracellular calcium and inhibits inflammatory cytokines through regulating of caspase-1, NF- κ B and MAPKs. We provide evidence that galangin could contribute to the prevention or treatment of mast cell-mediated allergic inflammatory disorders.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.fct.2013.03.015>.

References

- Amin, K., 2012. The role of mast cells in allergic inflammation. *Respir. Med.* 106, 9–14.
- Azzolina, A., Bongiovanni, A., Lampiasi, N., 2003. Substance P induces TNF- α and IL-6 production through NF- κ B in peritoneal mast cells. *Biochim. Biophys. Acta* 1643, 75–83.
- Bae, Y., Lee, S., Kim, S.H., 2011. Chrysin suppresses mast cell-mediated allergic inflammation: involvement of calcium, caspase-1 and nuclear factor- κ B. *Toxicol. Appl. Pharmacol.* 254, 56–64.
- Bestwick, C.S., Milne, L., 2006. Influence of galangin on HL-60 cell proliferation and survival. *Cancer Lett.* 243, 80–89.
- Castellani, M.L., De Lutiis, M.A., Toniato, E., Conti, F., Felaco, P., Fulcheri, M., Theoharides, T.C., Caraffa, A., Antinolfi, P., Conti, P., Cuccurullo, C., Ciampoli, C., Felaco, M., Orso, C., Salini, V., Cerulli, G., Kempuraj, D., Tete, S., Shaik, B., 2010. Impact of RANTES, MCP-1 and IL-8 in mast cells. *J. Biol. Reg. Homeos. Ag.* 24, 1–6.
- Cushnie, T.P., Hamilton, V.E., Chapman, D.G., Taylor, P.W., Lamb, A.J., 2007. Aggregation of *Staphylococcus aureus* following treatment with the antibacterial flavonoid galangin. *J. Appl. Microbiol.* 103, 1562–1567.
- Dambros, M., van Deutekom, M., de Jongh, R., van Koeveeringe, G.A., De Mey, J.G., van Kerrebroeck, P., 2005. The inhibitory effect of the flavonoid galangin on urinary bladder smooth muscle contractility is mediated in part by modulation of Ca²⁺ release from intracellular stores. *Planta Med.* 71, 962–964.
- DiDonato, J.A., Mercurio, F., Karin, M., 2012. NF- κ B and the link between inflammation and cancer. *Immunol. Rev.* 246, 379–400.
- Eisenhut, M., Wallace, H., 2011. Ion channels in inflammation. *Pflugers Arch.* 461, 401–421.
- Galli, S.J., Tsai, M., Piliponsky, A.M., 2008. The development of allergic inflammation. *Nature* 454, 445–454.
- Gomez, G., Gonzalez-Espinosa, C., Odom, S., Baez, G., Cid, M.E., Ryan, J.J., Rivera, J., 2005. Impaired Fc ϵ sR1-dependent gene expression and defective eicosanoid and cytokine production as a consequence of Fyn deficiency in mast cells. *J. Immunol.* 175, 7602–7610.
- Grimbaldeston, M.A., Metz, M., Yu, M., Tsai, M., Galli, S.J., 2006. Effector and potential immunoregulatory roles of mast cells in IgE-associated acquired immune responses. *Curr. Opin. Immunol.* 18, 751–760.
- Huh, J.E., Jung, I.T., Choi, J., Baek, Y.H., Lee, J.D., Park, D.S., Choi, D.Y., 2012. The natural flavonoid galangin inhibits osteoclastic bone destruction and osteoclastogenesis by suppressing NF- κ B in collagen-induced arthritis and bone marrow-derived macrophages. *Eur. J. Pharmacol.*
- Jutel, M., Watanabe, T., Akdis, M., Blaser, K., Akdis, C.A., 2002. Immune regulation by histamine. *Curr. Opin. Immunol.* 14, 735–740.
- Jutel, M., Akdis, M., Akdis, C.A., 2009. Histamine, histamine receptors and their role in immune pathology. *Clin. Exp. Allergy* 39, 1786–1800.
- Kim, S.H., Jun, C.D., Suk, K., Choi, B.J., Lim, H., Park, S., Lee, S.H., Shin, H.Y., Kim, D.K., Shin, T.Y., 2006. Gallic acid inhibits histamine release and pro-inflammatory cytokine production in mast cells. *Toxicol. Sci.* 91, 123–131.
- Kim, H.H., Yoo, J.S., Lee, H.S., Kwon, T.K., Shin, T.Y., Kim, S.H., 2011. *Elsholtzia ciliata* inhibits mast cell-mediated allergic inflammation: role of calcium, p38 mitogen-activated protein kinase and nuclear factor- κ B. *Exp. Biol. Med.* (Maywood) 236, 1070–1077.
- Ku, G., Faust, T., Lauffer, L.L., Livingston, D.J., Harding, M.W., 1996. Interleukin-1 beta converting enzyme inhibition blocks progression of type II collagen-induced arthritis in mice. *Cytokine* 8, 377–386.
- Lamkanfi, M., Walle, L.V., Kanneganti, T.D., 2011. Deregulated inflammasome signaling in disease. *Immunol. Rev.* 243, 163–173.
- Lattig, J., Bohl, M., Fischer, P., Tietbohl, C., Menschikowski, M., Gutzeit, H.O., Metz, P., Pisabarro, M.T., 2007. Mechanism of inhibition of human secretory phospholipase A2 by flavonoids: rationale for lead design. *J. Comput. Aid. Mol. Des.* 21, 473–483.
- Law, M., Morales, J.L., Mottram, L.F., Iyer, A., Peterson, B.R., August, A., 2011. Structural requirements for the inhibition of calcium mobilization and mast cell activation by the pyrazole derivative BTP2. *Int. J. Biochem. Cell Biol.* 43, 1228–1239.
- Lee, S., Yun, H.S., Kim, S.H., 2011. The comparative effects of mesoporous silica nanoparticles and colloidal silica on inflammation and apoptosis. *Biomaterials* 32, 9434–9443.
- Lin, T.J., Garduno, R., Boudreau, R.T., Issekutz, A.C., 2002. *Pseudomonas aeruginosa* activates human mast cells to induce neutrophil transendothelial migration via mast cell-derived IL-1 α and β . *J. Immunol.* 169, 4522–4530.
- Ma, H.T., Beaven, M.A., 2011. Regulators of Ca(2+) signaling in mast cells: potential targets for treatment of mast cell-related diseases? *Adv. Exp. Med. Biol.* 716, 62–90.
- Manikandan, J., Kothandaraman, N., Hande, M.P., Pushparaj, P.N., 2012. Deciphering the structure and function of Fc ϵ sR1/mast cell axis in the regulation of allergy and anaphylaxis: a functional genomics paradigm. *Cell Mol. Life Sci.* 69, 1917–1929.
- Mican, J.A., Arora, N., Burd, P.R., Metcalfe, D.D., 1992. Passive cutaneous anaphylaxis in mouse skin is associated with local accumulation of interleukin-6 mRNA and immunoreactive interleukin-6 protein. *J. Allergy Clin. Immunol.* 90, 815–824.
- Morello, S., Vellecco, V., Alfieri, A., Mascolo, N., Cicala, C., 2006. Vasorelaxant effect of the flavonoid galangin on isolated rat thoracic aorta. *Life Sci.* 78, 825–830.
- Murray, T.J., Yang, X., Sherr, D.H., 2006. Growth of a human mammary tumor cell line is blocked by galangin, a naturally occurring bioflavonoid, and is accompanied by down-regulation of cyclins D3, E, and A. *Breast Cancer Res.* 8, R17.
- Nakagomi, D., Suzuki, K., Nakajima, H., 2012. Critical roles of I κ B kinase subunits in mast cell degranulation. *Int. Arch. Allergy Immunol.* 158 (Suppl. 1), 92–95.
- Nakamura, R., Watanabe, K., Oka, K., Ohta, S., Mishima, S., Teshima, R., 2010. Effects of propolis from different areas on mast cell degranulation and identification of the effective components in propolis. *Int. Immunopharmacol.* 10, 1107–1112.
- Newton, K., Dixit, V.M., 2012. Signaling in innate immunity and inflammation. *Cold Spring Harb Perspect. Biol.* 4.
- O'Mahony, L., Akdis, M., Akdis, C.A., 2011. Regulation of the immune response and inflammation by histamine and histamine receptors. *J. Allergy Clin. Immunol.* 128, 1153–1162.
- Pasparakis, M., 2012. Role of NF- κ B in epithelial biology. *Immunol. Rev.* 246, 346–358.
- Rivera, J., Fierro, N.A., Olivera, A., Suzuki, R., 2008. New insights on mast cell activation via the high affinity receptor for IgE. *Adv. Immunol.* 98, 85–120.
- Russo, A., Longo, R., Vanella, A., 2002. Antioxidant activity of propolis: role of caffeic acid phenethyl ester and galangin. *Fitoterapia* 73 (Suppl. 1), S21–S29.
- Sarchio, S.N., Kok, L.F., O'Sullivan, C., Halliday, G.M., Byrne, S.N., 2012. Dermal mast cells affect the development of sunlight-induced skin tumours. *Exp. Dermatol.* 21, 241–248.
- Scheller, J., Chalaris, A., Schmidt-Arras, D., Rose-John, S., 2011. The pro- and anti-inflammatory properties of the cytokine interleukin-6. *Biochim. Biophys. Acta* 1813, 878–888.
- Schnitzler, P., Neuner, A., Nolkemper, S., Zundel, C., Nowack, H., Sensch, K.H., Reichling, J., 2010. Antiviral activity and mode of action of propolis extracts and selected compounds. *Phytother. Res.* 24 (Suppl. 1), S20–S28.
- Simons, F.E., Simons, K.J., 2011. Histamine and H1-antihistamines: celebrating a century of progress. *J. Allergy Clin. Immunol.* 128 (1139–1150), e1134.
- Singh, T.S., Lee, S., Kim, H.H., Choi, J.K., Kim, S.H., 2012. Perfluorooctanoic acid induces mast cell-mediated allergic inflammation by the release of histamine and inflammatory mediators. *Toxicol. Lett.* 210, 64–70.
- Sivakumar, A.S., Anuradha, C.V., 2011. Effect of galangin supplementation on oxidative damage and inflammatory changes in fructose-fed rat liver. *Chem. Biol. Interact.* 193, 141–148.
- Stack, J.H., Beaumont, K., Larsen, P.D., Straley, K.S., Henkel, G.W., Randle, J.C., Hoffman, H.M., 2005. IL-converting enzyme/caspase-1 inhibitor VX-765 blocks the hypersensitive response to an inflammatory stimulus in monocytes from familial cold autoinflammatory syndrome patients. *J. Immunol.* 175, 2630–2634.
- Strowig, T., Henao-Mejia, J., Elinav, E., Flavell, R., 2012. Inflammasomes in health and disease. *Nature* 481, 278–286.
- Tanaka, S., Mikura, S., Hashimoto, E., Sugimoto, Y., Ichikawa, A., 2005. Ca²⁺ influx-mediated histamine synthesis and IL-6 release in mast cells activated by monomeric IgE. *Eur. J. Immunol.* 35, 460–468.

- Tolomeo, M., Grimaudo, S., Di Cristina, A., Pipitone, R.M., Dusancho, L., Meli, M., Crosta, L., Gebbia, N., Invidiata, F.P., Titone, L., Simoni, D., 2008. Galangin increases the cytotoxic activity of imatinib mesylate in imatinib-sensitive and imatinib-resistant Bcr-Abl expressing leukemia cells. *Cancer Lett.* 265, 289–297.
- Turner, M.D., Chaudhry, A., Nedjai, B., 2012. Tumour necrosis factor receptor trafficking dysfunction opens the TRAPS door to pro-inflammatory cytokine secretion. *Biosci. Rep.* 32, 105–112.
- Vandenabeele, P., Declercq, W., Van Herreweghe, F., Vanden Berghe, T., 2010. The role of the kinases RIP1 and RIP3 in TNF-induced necrosis. *Sci. Signal* 3, re4.
- Walczak, H., 2011. TNF and ubiquitin at the crossroads of gene activation, cell death, inflammation, and cancer. *Immunol. Rev.* 244, 9–28.
- Wang, X., Liu, Y., 2007. Regulation of innate immune response by MAP kinase phosphatase-1. *Cell. Signal.* 19, 1372–1382.
- Zucchi, R., Chiellini, G., Scanlan, T.S., Grandy, D.K., 2006. Trace amine-associated receptors and their ligands. *Brit. J. Pharmacol.* 149, 967–978.