

Original Article

Eupatilin suppresses the allergic inflammatory response *in vitro* and *in vivo*Eun-Hye Song^{a,1}, Kyung-Sook Chung^b, Yun-Mi Kang^a, Jong-Hyun Lee^c, Minho Lee^{b,*},
Hyo-Jin An^{a,*}^a Department of Pharmacology, College of Korean Medicine, Sangji University, 83 Sangjidae-gil, Wonju-si, Gangwon-do 220-702, Republic of Korea^b Catholic Precision Medicine Research Center, College of Medicine, The Catholic University of Korea, 222, Banpo-daero, Seocho-gu, Seoul, 06591, Republic of Korea^c Department of Pharmacy, College of Pharmacy, Dongduk Women's University, Seoul 136-714, Republic of Korea

ARTICLE INFO

Keyword:

Allergic inflammation
Eupatilin
Human mast cell
NF-κB
Cytokine

ABSTRACT

Introduction: Eupatilin, a pharmacologically active ingredient found in *Artemisia asiatica*, has been reported to have anti-oxidative, anti-inflammatory, and anti-apoptotic activities. However, molecular mechanisms underlying its anti-allergic properties are not yet clear. In this study, we investigated the effects of eupatilin on allergic inflammation in phorbol 12-myristate 13-acetate plus calcium ionophore A23187 (PMACI)-stimulated human mast cells and a compound 48/80-induced anaphylactic shock model.

Methods: Cytokine assays, histamine assays, quantitative real-time polymerase chain reaction analysis, western blot analysis and compound 48/80-induced anaphylactic shock model were used in this study.

Results: Eupatilin significantly suppresses the expression and production of pro-inflammatory cytokines, such as interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and IL-6 *in vitro* and *in vivo*. In addition, eupatilin inhibits nuclear factor kappa B (NF-κB) activation by regulating the phosphorylation and degradation of IκBα via the Akt/IKK(α/β) pathway. Eupatilin treatment also attenuates the phosphorylation of p38, ERK, and JNK MAPKs. Furthermore, eupatilin blocked anaphylactic shock and decreased the release of histamine.

Conclusions: Anti-allergic inflammation may involve the expression and production of regulating pro-inflammatory cytokines via Akt/IKK(α/β) and MAPK activation of NF-κB. On the basis of these data, eupatilin is a potential candidate for the treatment of allergic diseases.

Introduction

Allergic diseases are inflammatory disorders that include allergic asthma, hay fever, food allergies, and anaphylaxis and are induced by the interaction of antigen-specific immunoglobulin E (IgE) and T-helper-2 (Th2) cells (Hawrylowicz and O'Garra, 2005). The prevalence of allergic disease is increasing dramatically as societies become developed and industrialized. Thus, the economic burden of asthma is also growing (Pawankar, 2014). Allergic reactions are characterized by an abnormal immune response to generally harmless environmental antigens that trigger mast cell activation (Holgate and Polosa, 2008). Mast cells are tissue-based inflammatory cells that play important roles in immediate and delayed allergic reactions through degranulation (Stone et al., 2010). Mast cells activated by IgE release histamine, pro-inflammatory cytokines such as interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and IL-6 (Amin, 2012). These cytokines play an

important role in regulating the allergic inflammatory response. IL-1β was originally identified as an endogenous pyrogen (Turner et al., 2014); it is also a pro-inflammatory cytokine and potent mediator of inflammatory processes that play a crucial role in allergic responses. IL-1β is released into nasal secretions during early-phase and late-phase allergic reactions (Da Silva et al., 2002). TNF-α is secreted from activated mast cells and plays an important role in initiating allergic asthmatic inflammation and the occurrence of airway hypersensitivity. It is also a powerful inducer of other inflammatory cytokines including IL-1β and IL-6 (Rasheed et al., 2010). IL-6 is produced by mast cells in response to various stimuli, including allergens that have a biological role in allergic inflammation. IL-6 is regarded as an important regulator of Th2 differentiation and inhibition, as well as CD4 T cell differentiation (Rincon and Irvin, 2012). Therefore, reductions in these mediators are considered indicators of symptomatic relief from allergic inflammation.

Abbreviations: HMC-1, human mast cell line; PMACI, phorbol 12-myristate 13-acetate plus calcium ionophore A23187; TNF-α, tumor necrosis factor-α; NF-κB, nuclear factor kappa B; MAPKs, mitogen-activated protein kinases; IMDM, Iscove's modified Dulbecco's medium; FBS, fetal bovine serum; DMSO, dimethyl sulfoxide; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide

* Corresponding authors.

E-mail addresses: MinhoLee@catholic.ac.kr (M. Lee), hjan@sj.ac.kr (H.-J. An).

¹ Present address: Jeonnam Development Institute for Korean Traditional Medicine, 288, Udeuraendeu-gil, Jangheung-gun, Jeonnam 529-851, Republic of Korea.

<https://doi.org/10.1016/j.phymed.2017.08.027>

Received 13 December 2016; Received in revised form 21 April 2017; Accepted 31 August 2017
0944-7113/ © 2017 Elsevier GmbH. All rights reserved.

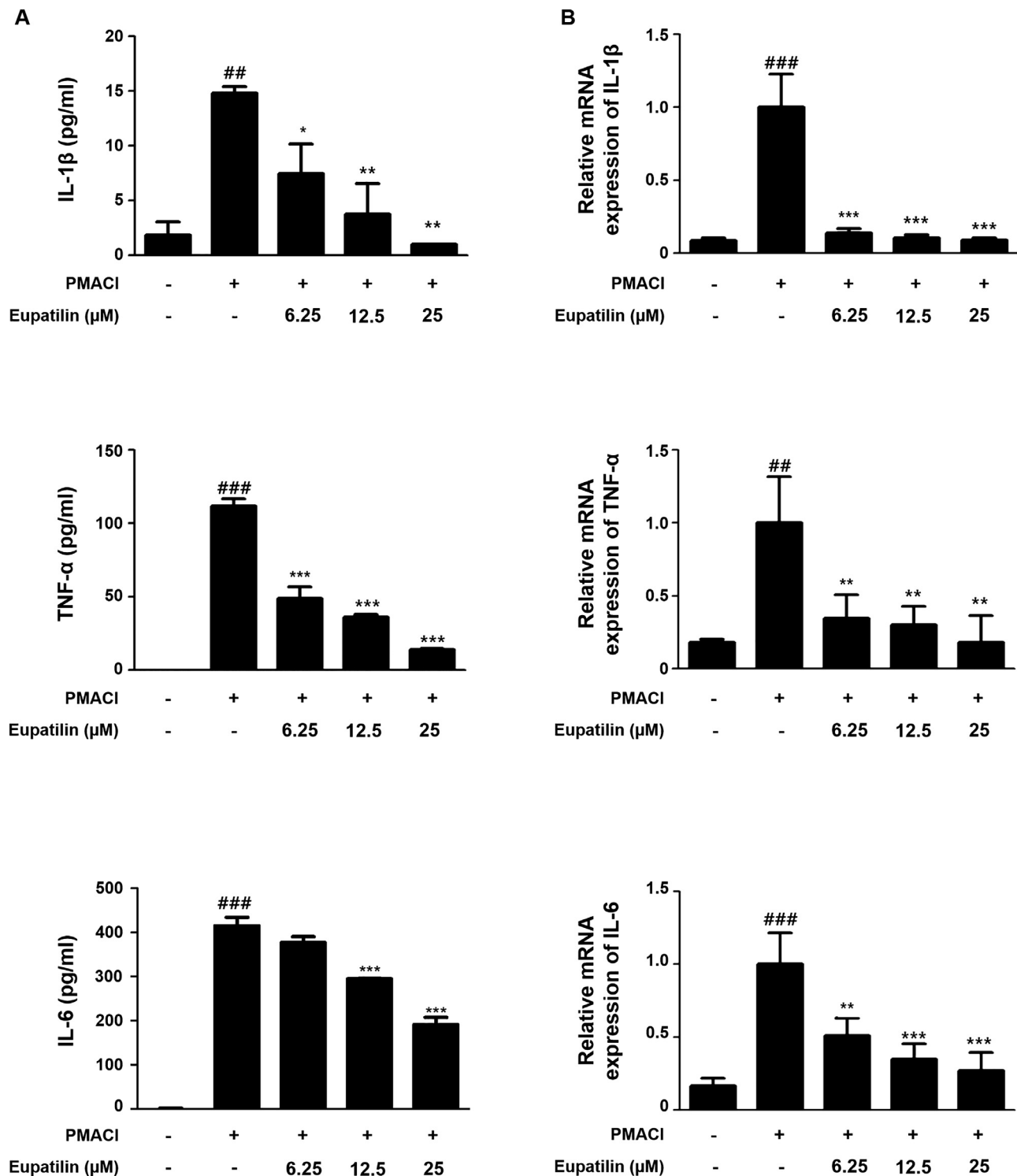


Fig. 1. Effects of eupatilin on pro-inflammatory cytokines in PMACI-stimulated HMC-1 cells. HMC-1 cells were pretreated with 6.25, 12.5 and 25 μ M of eupatilin for 30 min and stimulated by PMA (40 nM) plus A23187 (1 μ M), and the cells were further incubating 24 h (A) or 6 h (B). The cytokines productions were measured using EIA and cytokine mRNA expression levels were measured using real-time PCR. The data shown represent mean $\pm$ SD of three independent experiments. ## $p < 0.01$ and ### $p < 0.001$ vs the control group, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs the PMACI-treated group.

Nuclear factor kappa B (NF- κ B) is bound to inhibitor of κ B (I κ B) proteins that are separated in the cytoplasm. NF- κ B activation is controlled by the I κ B kinase (IKK) complex, which mediates the phosphorylation of I κ B. When stimulated, I κ B is phosphorylated and then degraded; NF- κ B is then released and translocated to the nucleus, where it can activate promoters (Perkins, 2007). NF- κ B is a critical regulator of allergic inflammation, playing a role in the expression of cytokines such

as IL-1 β , TNF- α , and IL-6, which mediate allergic responses to inflammatory stimuli (Barnes, 2011). It has been reported that Akt regulates the transcriptional activity of NF- κ B by inducing phosphorylation of IKK (Romashkova and Makarov, 1999). Akt is a serine/threonine protein kinase that has been reported to regulate cell apoptosis, proliferation, and survival signals in response to cytokines and growth factors (Madrid et al., 2000). Akt is also involved in regulating the

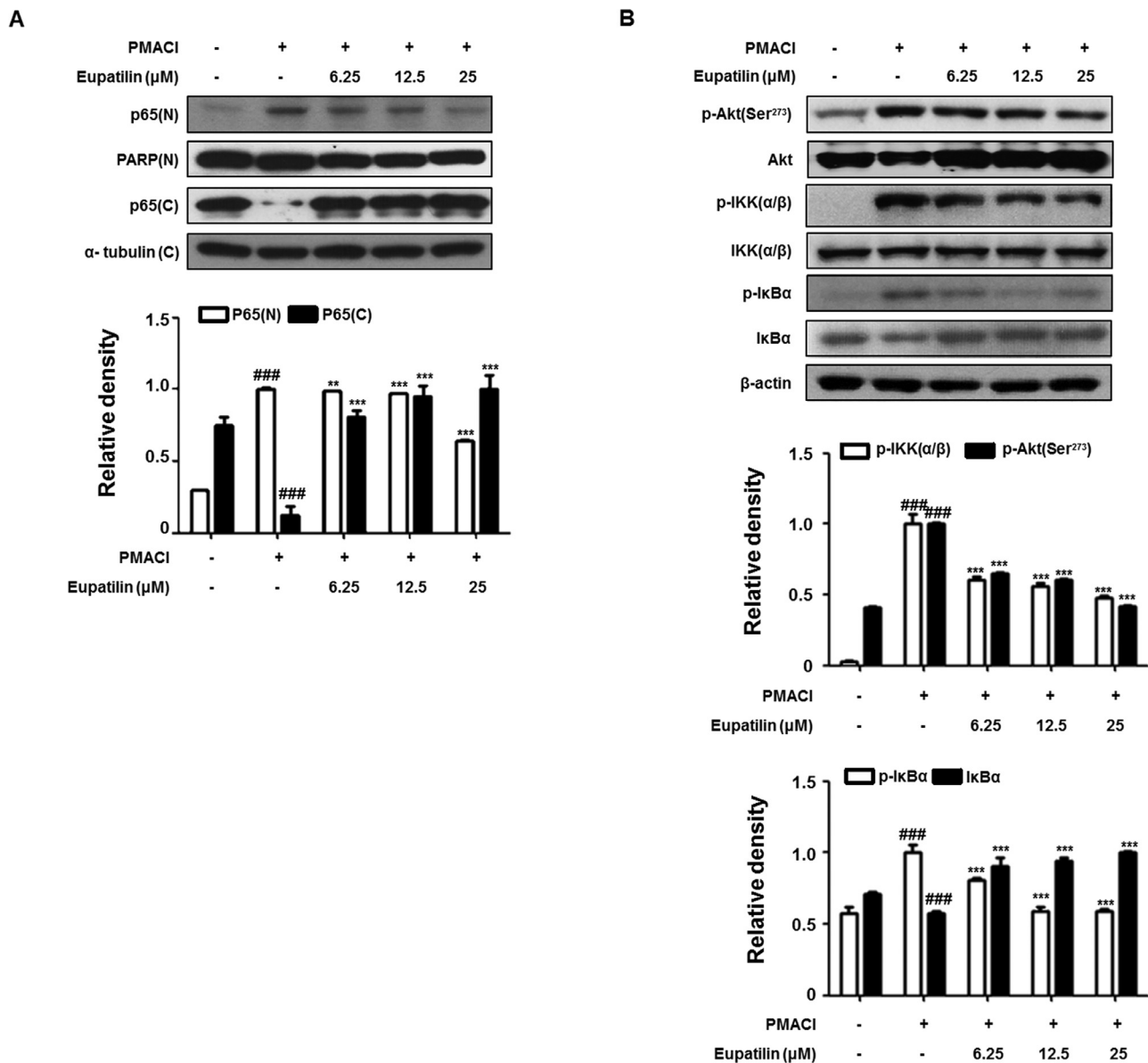


Fig. 2. Effects of eupatilin on the NF- κ B activation in PMACI-stimulated HMC-1 cells. HMC-1 cells were pretreated with eupatilin for 30 min and stimulated by PMA (40 nM) plus A23187 (1 μ M) for (A) 30 min (NF- κ B p65) or (B) 15 min (p-I κ B) or 10 min (Akt and IKK). Nuclear (N) and cytosolic (C) extracts were isolated, and the levels of p65 in each fraction were determined by western blot analysis. PARP and α -tubulin were used as internal controls. Densitometry analysis of the blots was done using ImageJ software. The data shown represent mean $\pm$ SD of three independent experiments. ### $p < 0.001$ vs the control group, ** $p < 0.01$ and *** $p < 0.001$ vs the PMACI-treated group.

inflammatory response. Several studies have reported the significance of Akt in inflammation-mediated diseases such as asthma, psoriasis, multiple sclerosis, and rheumatoid arthritis (Di Lorenzo et al., 2009).

Mitogen-activated protein kinases (MAPKs) are components of intracellular signal transduction pathways that play an important role in the regulation of pro-inflammatory molecules. MAPKs are activated in response to stimuli in mast cells and play a key role in signal pathways (Kim et al., 2006). MAPK signals are composed of three pathways, including the extracellular signal-regulated p38 MAPKs, extracellular signal-regulated kinase 1 and 2 (ERK), and c-Jun N-terminal kinases (JNK). MAPKs are involved in the activation of NF- κ B in the cytoplasm and play a significant role in signaling cytokine expression (Dhawan and Richmond, 2002). NF- κ B activation and MAPK phosphorylation are important events in the allergic inflammatory response; therefore, regulation of their activity can help in the treatment of allergic disease.

5, 7-Dihydroxy-3, 4, 6-trimethoxyflavone (eupatilin) is a pharmacologically active ingredient found in *Artemisia asiatica*. Eupatilin has

antioxidant, anti-inflammatory, anti-apoptotic, and neuroprotective properties (Cai et al., 2012). Eupatilin has also been reported to be therapeutically helpful in cases of gastric mucosal injury (Seol et al., 2004). Despite the known biological effects of eupatilin, there are no reports of its anti-allergic molecular mechanisms, including anaphylactic activity. In this study, we investigated the effects of eupatilin on allergic inflammation in phorbol 12-myristate 13-acetate plus calcium ionophore A23187 (PMACI)-stimulated human mast cells and a compound 48/80-induced anaphylactic shock model.

Materials and methods

Reagents

Eupatilin was purchased from Adipogen Corp ($\geq 98\%$ purity). Phorbol 12-myristate 13-acetate (PMA), calcium ionophore A23187 (Calcimycin; C29H37N3O6), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) liquefied in dimethyl sulfoxide

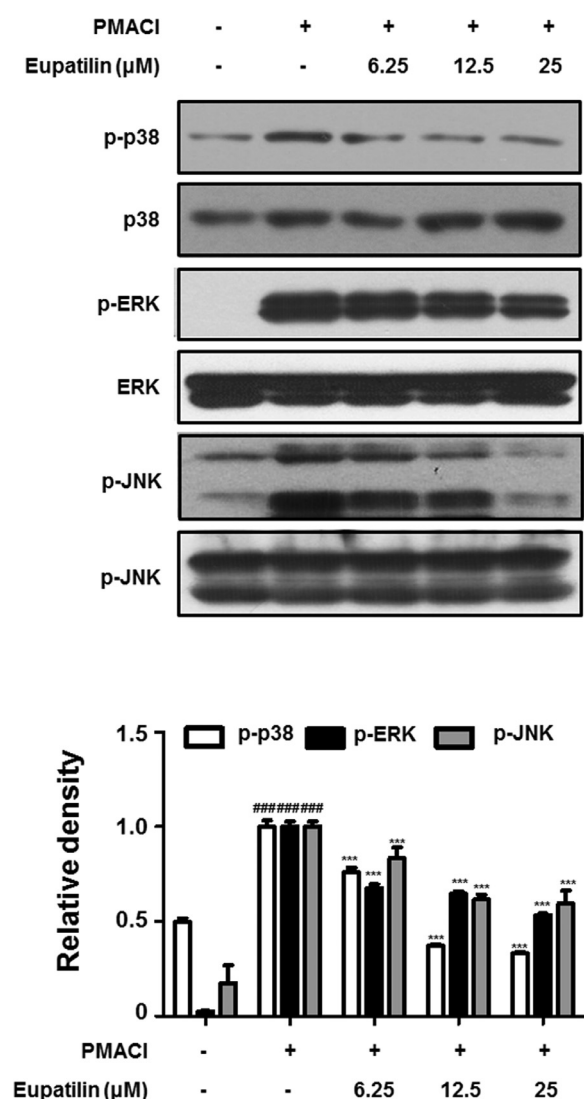


Fig. 3. Effects of eupatilin on the MAPKs phosphorylation in PMACI-stimulated HMC-1 cells. HMC-1 cells were pretreated with eupatilin for 30 min and stimulated by PMA (40 nM) plus A23187 (1 μM) for 15 min. Densitometry analysis of the blots was done using ImageJ software. The data shown represent mean $\pm$ SD of three independent experiments. ### $p < 0.001$ vs the control group, *** $p < 0.001$ vs the PMACI-treated group.

(DMSO), and all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Iscove's modified Dulbecco's medium (IMDM), fetal bovine serum (FBS), and Gibco® antibiotic-antimycotic were purchased from Life Technologies (Grand Island, NY, USA). EIA kits for IL-1 β , TNF- α , and IL-6 were purchased from Becton Dickinson and Company (BD) Biosciences (San Jose, CA, USA). Histamine EIA kits were purchased from Enzo Life Sciences (East Farmingdale, NY, USA). Power SYBR® Green Master Mix was purchased from Applied Biosystems (Foster City, CA, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), IL-1 β , TNF- α , and IL-6 oligonucleotide primers were purchased from Bioneer (Daejeon, Republic of Korea). NF- κ B p65, p-I κ B- α , I κ B- α , IKK(α / β), Akt1/2/3, caspase-1, β -actin, and α -tubulin antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). ERK, p-p38, p-ERK, p-JNK, p-Akt, p-IK α / β , p38, JNK, and PARP antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA).

Cell culture and sample treatment

The human mast cell line (HMC-1) was kindly provided by Prof. Jae-

Young Um (Kyung Hee University, Republic of Korea). Cells were cultured in IMDM supplemented with 10% FBS and 1% Gibco® antibiotic-antimycotic (containing 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B) in an incubator at 37 °C and 5% CO₂. HMC-1 cells were treated with various doses of eupatilin (6.25, 12.5, and 25 μM) for 30 min before stimulation with 40 nM PMA and 1 μM A23187 for the indicated time. Compounds at various concentrations were dissolved in DMSO and added to the PMACI. DMSO (0.05%) was used as a vehicle control.

Compound 48/80-induced anaphylactic shock model

ICR mice (male, 4 weeks old) were purchased from Daehan Biolink (Daejeon, Republic of Korea). Mice were maintained at 22–25 °C, with a 12 h light/dark cycle and relative humidity of 40–60%. Anaphylactic shock was induced with compound 48/80 as previously described (Shin, 2005); specifically, ICR mice were injected intraperitoneally with phosphate-buffered saline (PBS) or compound 48/80 (8 mg/kg dissolved in PBS). Eupatilin (20 mg/kg dissolved in saline) and disodium cromoglycate (DSCG, positive control, 25 mg/kg, dissolved in saline) were injected orally for 1 h before injecting compound 48/80. Mortality was observed for 1 h after injecting compound 48/80. At the end of the experiment, blood was drawn from the heart of each mouse and allowed to clot for 1 h at room temperature and then centrifuged for 20 min at 3000 $\times$ g and 4 °C to obtain serum for analysis of histamine and cytokine production. The study was approved by Sang-ji University Institutional Animal Care and Use Committees (Reg. No. 2015-08).

Cytokine assays

IL-1 β , TNF- α , and IL-6 levels were measured in the culture media and serum of mice with compound 48/80-induced anaphylaxis using EIA kits (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions.

Histamine assays

Mouse serum histamine levels were measured using commercially available EIA kits (Enzo Life Sciences, Farmingdale, NY, USA) according to the manufacturer's recommendations.

Quantitative real-time polymerase chain reaction analysis

Total RNA was isolated from cells or liver tissue using Trizol reagent, and cDNA was obtained using isolated total RNA (2 μg), d(T)16 primers, and AMV reverse transcriptase (Intron Biotechnology, Seongnam, Republic of Korea). Relative gene expression was quantified using real-time PCR (Real Time PCR System 7500, Applied Biosystems, Foster City, CA, USA) with SYBR Premix Ex Taq. The oligonucleotide primers for IL-1 β were TGGACCTCTGCGCTCTGGAT (forward) and GGCAGGGAACCAGCATCTTC (reverse). The oligonucleotide primers for TNF- α were GCTGGAGAAGGGTGACCGAC (forward) and GTTCGTCTCTCACAGGGC (reverse). The oligonucleotide primers for IL-6 were ATTCGCGGAACGAAAGAGAA (forward) and TCTTCTCTGGGGTACTGG (reverse). The oligonucleotide primers for GAPDH were CTCCTCCACCTTTGACGCTG (forward) and CTCTGTGCTCTTGCTGGG (reverse). The synthesized cDNA was 200 bp. Results are expressed as the ratio of the optical density of the target locus to GAPDH.

Western blot analysis

Cells or liver tissue were re-suspended in protein extraction solution (PRO-PREP™, Intron Biotechnology, Seongnam, Republic of Korea) and incubated for 20 min at 4 °C. Cell debris was eliminated by micro-centrifugation followed by immediately freezing the supernatant. Protein concentrations in the supernatant were measured using the Bio-Rad

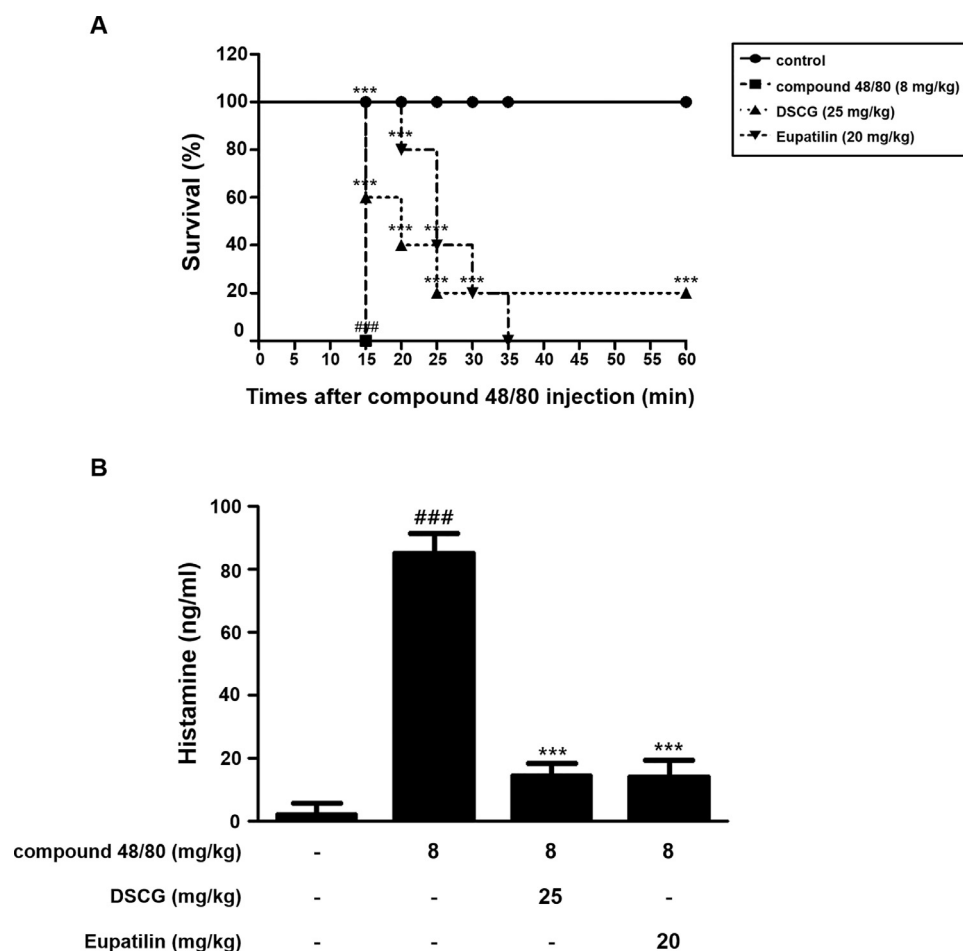


Fig. 4. Effects of eupatilin on allergic hypersensitivity responses in compound 48/80-induced anaphylaxis shock model. Mice were administered with eupatilin, DSCG, and PBS as a vehicle ($n = 6$ per group or total) for 1 h before compound 48/80 injection (8 mg/kg *i.p.*). (A) The survival rates of these mice were monitored for 1 h. (B) Histamine concentrations were measured in mouse serum by EIA. The data shown represent mean $\pm$ SD of three independent experiments. ### $p < 0.001$ vs the control group, ** $p < 0.01$ and *** $p < 0.001$ vs the compound 48/80-treated group.

protein assay reagent (Hercules, CA, USA) according to the manufacturer's instructions. Each protein sample (30 μ g) was electro-blotted and then transferred onto a polyvinylidene fluoride (PVDF) membrane followed by separation using 8–12% sodium dodecyl sulfate polyacrylamide gel electrophoresis. Membranes were incubated for 30 min with blocking solution (2.5 or 5% skim milk) at 25 $^{\circ}$ C, followed by incubation overnight with the primary antibody (1:1000 dilution) at 4 $^{\circ}$ C. Membranes were washed three times with tris-buffered saline/Tween 20 (TBS-T) and incubated with a horseradish peroxidase-conjugated secondary antibody (1:2500 dilution) for 1 h 30 min at room temperature. Membranes were again washed three times with TBS-T and then developed by enhanced chemiluminescence (AbsignalTM, Abclon, Seoul, Republic of Korea).

Preparation of nuclear protein extract

HMC-1 cells were plated in 60 mm dishes (5×10^6 cells/ml) and treated with eupatilin (6.25, 12.5, and 25 μ M) for 30 min and then stimulated with PMACI for 30 min, washed once with PBS, harvested in 1 ml of cold PBS, and pelleted by centrifugation. Nuclear extracts were prepared as previously described (Lee et al., 2015). Cell pellets were resuspended in hypotonic buffer (10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.2 mM PMSF, 0.5 mM DTT, and 10 mg/ml aprotinin) and incubated on ice for 20 min. Cells were then lysed by adding 0.1% Nonidet P-40 and vortexed vigorously for 10 s. Nuclei were pelleted by centrifugation at $12,000 \times g$ for 1 min at 4 $^{\circ}$ C and re-suspended in high salt buffer (20 mM HEPES (pH 7.9), 25% glycerol, 400 mM KCl, 1.5 mM

$MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 1 mM NaF, and 1 mM sodium orthovanadate).

Statistical analysis

All reported values are expressed as the means $\pm$ standard deviation (SD). Data were analyzed using one-way analysis of variance with Dunnett's test, and p -values < 0.05 were considered statistically significant. All statistical analyses were performed using GraphPad Prism (version 5.00 for Windows, San Diego, CA, USA).

Results

Eupatilin suppressed pro-inflammatory cytokines in PMACI-stimulated HMC-1 cells

Pro-inflammatory cytokines, such as IL-1 β , TNF- α , and IL-6, are potent multifunctional cytokines that play a crucial role in the pathogenesis of allergic inflammatory diseases (Amin, 2012). To test the cytotoxic effects of eupatilin, we first performed the MTT assay. Eupatilin was not cytotoxic at concentrations of 6.25–25 μ M (Fig. S1). We examined the effects of eupatilin on the production and mRNA expression of the inflammatory cytokines IL-1 β , TNF- α , and IL-6 in HMC-1 cells by using EIA and qRT-PCR, respectively. Pretreatment with eupatilin suppressed PMACI-stimulated IL-1 β , TNF- α , and IL-6 production (Fig. 1A), as well as their mRNA expression (Fig. 1B) in a dose-dependent manner. These results suggest that eupatilin has anti-inflammatory

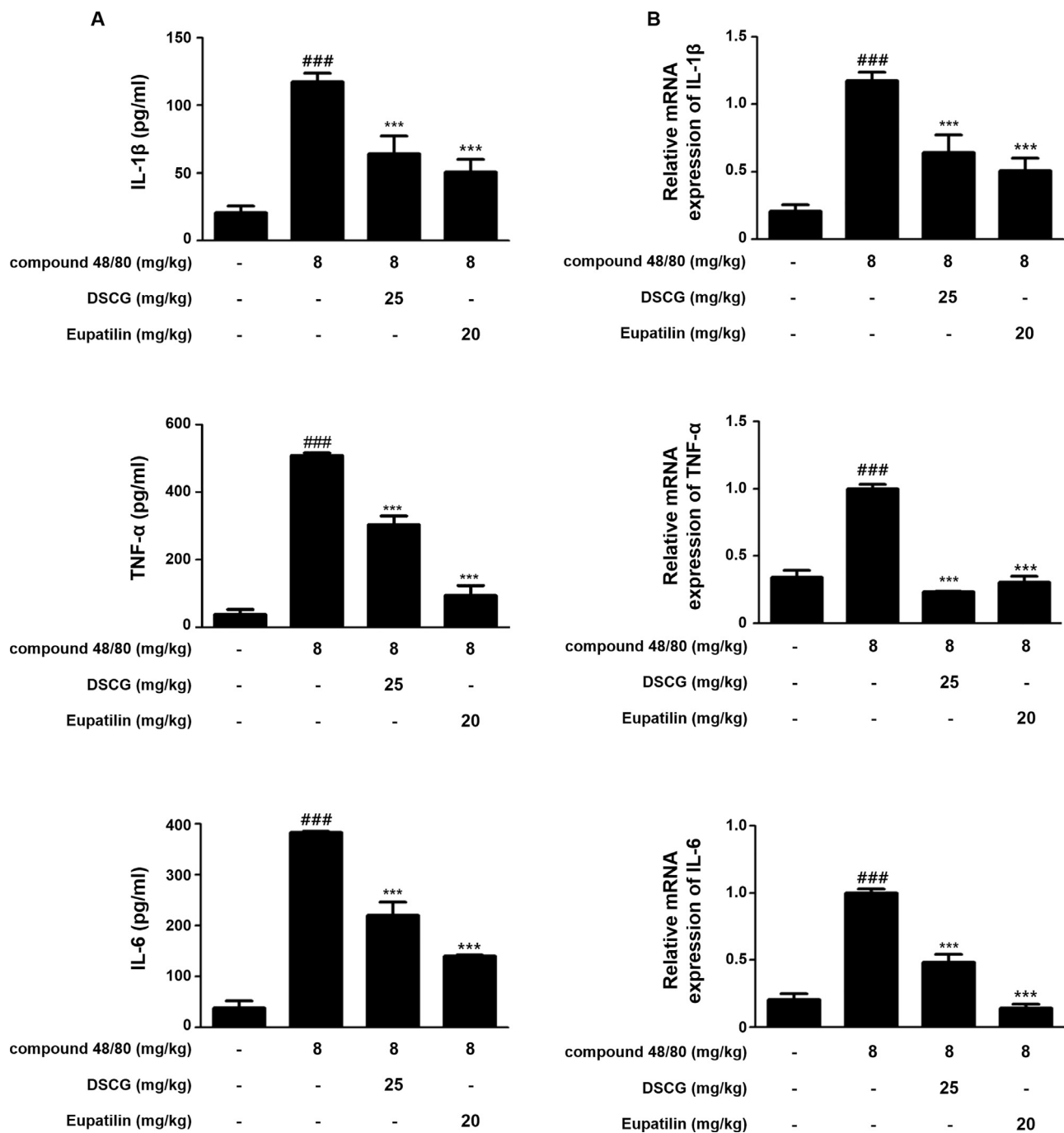


Fig. 5. Effects of eupatilin on the production and mRNA expression level of pro-inflammatory cytokines in compound 48/80-induced anaphylaxis shock model. (A) The production level of IL-1 β , TNF- α and IL 6 were measured in mouse serum using EIA. (B) Total RNA prepared from the liver tissue and mRNA expression level of IL-1 β , TNF- α and IL 6 were determined by real-time PCR. The data shown represent mean $\pm$ SD of three independent experiments. ### $p < 0.001$ vs the control group, *** $p < 0.001$ vs the compound 48/80-treated group.

effects on mast cells by inhibiting the production and mRNA expression of inflammatory cytokines related to allergic reactions.

Eupatilin suppressed NF- κ B activation in PMACI-stimulated HMC-1 cells

NF- κ B is an important regulator of activated mast cell synthesis and release of mediators during allergic inflammation (Perkins, 2007). NF- κ B is translocated into the nucleus via I κ B phosphorylation and degradation, which are regulated by the IKK complex. In addition, Akt promotes IKK-dependent activation of NF- κ B (Dan et al., 2008). To evaluate the intracellular mechanisms responsible for the inhibitory effects of eupatilin on pro-inflammatory cytokine expression, we investigated its effects on the

activation of NF- κ B and phosphorylation of Akt by using western blot analysis in PMACI-stimulated HMC-1 cells. As shown in Fig. 2A, eupatilin suppresses PMACI-stimulated nuclear translocation of the NF- κ B p65 sub-unit, as well as the phosphorylation and degradation of I κ B in HMC-1 cells. In addition, eupatilin attenuated PMACI-stimulated phosphorylation of IKK (α/β) and Akt (ser 273), but did not affect the total amount of IKK(α/β) and Akt in HMC-1 cells (Fig. 2B).

Eupatilin suppressed phosphorylation of MAPKs in PMACI-stimulated HMC-1 cells

MAPKs are well-known mediators of mast cell activation, allergic

inflammation, and cytokine production. MAPKs take part in the activation of NF- κ B in the cytoplasm, as well as in regulation of its nuclear transcription (Dhawan and Richmond, 2002). Thus, to investigate the effects of eupatilin on MAPK signaling pathways in mast cells, the phosphorylation of MAPKs was analyzed using western blot analysis. As shown in Fig. 3, eupatilin attenuated PMACI-induced phosphorylation of p38, ERK, and JNK MAPKs, whereas it did not affect total MAPK expression in HMC-1 cells.

Eupatilin extended survival rates and suppressed production of serum histamine in a compound 48/80-induced anaphylactic shock model

Anaphylactic shock is caused by mast cell activation and degranulation. Anaphylaxis occurs rapidly and systemically releases mediators, such as histamine and cytokines (Metcalfe et al., 2009). To show the effect of eupatilin on allergic hypersensitivity responses, we examined the survival rate in a compound 48/80-induced anaphylactic shock model. After an intraperitoneal injection of compound 48/80, the mice were monitored for 1 h and survival rates were determined. As shown in Fig. 4A, there was 100% mortality in compound 48/80-treated groups 15 min after injection. In contrast, compound 48/80-induced anaphylactic shock was blocked in mice treated with eupatilin (20 mg/kg, *p.o.*) and DSCG (25 mg/kg, *p.o.*). Next, we examined the effects of eupatilin on the release of histamine from mast cells using an EIA assay. Histamine levels significantly increased in the serum of mice with anaphylactic shock, an effect that was attenuated by treatment with eupatilin (Fig. 4B).

Eupatilin suppressed pro-inflammatory cytokines in a compound 48/80-induced anaphylactic shock model

Anaphylactic shock is caused by the release of pro-inflammatory cytokines resulting from the degranulation of mast cells (Kim et al., 2013). To investigate the inhibitory effects of eupatilin on the production and expression of pro-inflammatory cytokines in an anaphylactic shock model, we examined its effect on compound 48/80-induced IL-1 β , TNF- α , and IL-6 production, as well as their mRNA levels using EIA and qRT-PCR, respectively. As shown in Fig. 5A and B, administration of compound 48/80 markedly increased the serum and mRNA levels of IL-1 β , TNF- α , and IL-6; however, pre-treatment with eupatilin (20 mg/kg, *p.o.*) 1 h before administering compound 48/80 significantly decreased the levels of these pro-inflammatory cytokines.

Discussion

Allergic inflammation causes major pathological changes and is characterized by an immediate hypersensitivity reaction induced by the release of preformed mediators from mast cells (Barnes, 2011). Delayed hypersensitivity reactions are the result of pro-inflammatory cytokine production and the recruitment of mast cells to sites of inflammation (Galli and Tsai, 2012). Mast cells are the primary effectors of allergies and are necessary for inflammatory and allergic reactions, such as anaphylaxis (Stone et al., 2010). The release of pro-inflammatory cytokines and histamine following the crosslinking of Fc ϵ RI-bound IgE to antigen in mast cells is considered to be an immediate hypersensitivity reaction (Kemp and Lockey, 2002). HMC-1 cells have been used to study pro-inflammatory cytokine activation pathways in previous studies (Lee et al., 2015; Min et al., 2007). Pro-inflammatory cytokines, such as IL-1 β , TNF- α , and IL-6, play a role in regulating the allergic response (Amin, 2012). IL-1 β is known to upregulate the expression of various genes involved in inflammation and is released into nasal secretions during early or late-phase reactions (Da Silva et al., 2002). TNF- α plays a crucial role in initiating allergic inflammation and promotes inflammation, tissue fibrosis, and leukocyte infiltration (Rasheed et al., 2010). IL-6 is a potent mediator of inflammatory processes that is synthesized by stimulators in mast cells and is essential for

Th2 differentiation (Rincon and Irvin, 2012). These cytokines are generated in mast cells and potentiate inflammatory responses through the subsequent induction of other inflammatory cytokines (Amin, 2012). In this study, we found that eupatilin suppressed the secretion and mRNA levels of IL-1 β , TNF- α , and IL-6 in PMACI-stimulated HMC-1 cells (Fig. 1) and a model of anaphylactic shock (Fig. 5). These data suggest that eupatilin has inhibitory effects on allergic inflammation by suppressing pro-inflammatory cytokines *in vitro* and *in vivo*.

NF- κ B binds specifically to the B site of the immunoglobulin K light chain gene and plays a central role in inflammation by controlling the expression of a network of genes such as IL-1 β , TNF- α , and IL-6 (Barnes, 2011). NF- κ B exists in an inactive form in the cytoplasm and is associated with regulatory proteins called I κ B. Upon stimulation, signal transduction pathways rapidly lead to activation of the IKK complex, which is composed of two subunits (IKK α / β). Activated IKK leads to phosphorylation and proteasomal degradation of I κ B and induces the nuclear translocation of NF- κ B (Israel, 2010). Akt, also called protein kinase B, is a serine/threonine kinase that is implicated in various cellular responses and phosphorylates numerous protein targets that control cell survival, proliferation, and apoptosis (Romashkova and Makarov, 1999). The AKT/IKK(α / β) signaling pathway regulates and integrates NF- κ B by controlling the transcription of genes implicated in the pathogenesis of several diseases, including metabolic disease, cancer, and allergies (Agarwal et al., 2005). In agreement with other reports, our results reveal that eupatilin suppresses PMACI-induced phosphorylation and subsequent degradation of I κ B α and reduces the nuclear translocation of NF- κ B p65 in HMC-1 cells. In addition, eupatilin attenuates PMACI-induced phosphorylation of Akt and IKK(α / β), which control the activation of NF- κ B. These findings suggest that eupatilin inhibits NF- κ B activation by regulating the phosphorylation and degradation of I κ B α via the Akt/IKK(α / β) pathway.

The MAPK cascade is an important signaling pathway in immune responses (Kim et al., 2006). Because of their essential role in intracellular signaling networks, MAPK pathways are appropriate targets for pharmacological treatment of inflammatory disorders (Lewis et al., 1998). MAPK family members include ERK, p38, and JNK, which activate a series of transcript factors such as AP-1, CREB, c-Jun, and STAT1 (Ruimi et al., 2010). In addition, MAPK pathway activation leads to activation of NF- κ B followed by cytosolic I κ B α phosphorylation, which activates transcription factors and translocation of cytosolic NF- κ B p65 into the nucleus. This, in turn, is necessary for directing high-level transcription of many cytokines, adhesion molecules, and other proinflammatory proteins (Gilfillan and Tkaczyk, 2006). In this study, PMACI simultaneously activated all three MAPKs in HMC-1 cells; treatment with eupatilin decreased phosphorylation of p38, ERK, and JNK MAPKs. According to these results, eupatilin inhibits p38, ERK, and JNK MAPK activation in PMACI-stimulated HMC-1 cells. These data suggest that attenuation of MAPK activation is also involved in eupatilin-reduced inflammatory cytokine production. Furthermore, MAPK are involved in the signal transduction pathways that lead to the regulation of pro-inflammatory cytokines (Duan and Wong, 2006). Collectively, these signaling pathways are essential for the degranulation in IgE-mediated mast cells (Rivera and Gilfillan, 2006). Although our findings suggest that eupatilin provided potential protection of degranulation in PMACI-stimulated HMC-1, further studies are required to examine the effect of eupatilin on degranulation of HMC-1.

Anaphylactic shock is an immediate allergic reaction that may cause death. Immediate hypersensitivity is the basis of acute allergic reactions and is caused by molecules released by mast cells when antigens interact with Fc ϵ RI-bound specific IgE (Metcalfe et al., 2009). The release of histamine and pro-inflammatory cytokines from mast cells can be induced in a compound 48/80-induced anaphylactic shock model that been used to evaluate the mechanisms of immediate allergic reactions (Schemann et al., 2012). Histamine is a biogenic amine and is primarily stored within mast cell granules. Histamine is released from activated

mast cells, leading to immediate hypersensitivity (Metcalfe et al., 2009). As shown in Fig. 4, eupatilin blocked anaphylactic shock and suppressed serum histamine levels in a compound 48/80-induced anaphylactic shock model. Additionally, we investigated the effects of eupatilin on the activation of caspase-1 in the anaphylactic shock model. Caspase-1 is a member of the inflammatory caspase family and is responsible for the maturation of pro-IL-1 β . It has been reported that caspase-1 regulates activation of NF- κ B in B cells independently of its enzymatic activity (Lamkanfi et al., 2004). In our supplementary study, eupatilin suppressed caspase-1 activation in the compound 48/80-induced anaphylactic shock model (Fig. S2). These results may be attributable to inhibition of caspase-1 activation by eupatilin, which reduces NF- κ B activation.

Conclusion

In conclusion, our results indicate that eupatilin suppresses anti-allergic inflammation in PMACI-stimulated HMC-1 cells and a compound 48/80-induced anaphylactic shock mouse model. Although previous study reported inhibitory effect of eupatilin in IgE-induced hypersensitivity using RBL-2H3 cell and passive cutaneous anaphylaxis animal model (Lee et al., 2007), we intensively investigated the molecular mechanism of eupatilin, involving the regulation of pro-inflammatory cytokines via NF- κ B activation through Akt/IKK(α / β) and MAPK pathways. These data suggest that eupatilin is a potential candidate for treatment of allergic diseases.

Author contribution

E.H.S., K.S.C and H.J.A. conceived and designed the experiments. E.H.S. performed the experiments and analyzed the data with K.S.C and H.J.A. M.L. and J.H.L contributed reagents, materials, and analysis tools. E.H.S., Y.M.K and K.S.C wrote the paper. All authors read and approved the final manuscript.

Conflict of interest

The authors declare that there is no conflict of interests. The authors declare no competing financial interests

Acknowledgments

This research was supposed by Basic Science Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2014R1A1A2058647).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.phymed.2017.08.027>.

References

- Agarwal, A., Das, K., Lerner, N., Sathe, S., Cicek, M., Casey, G., Sizemore, N., 2005. The Akt/I kappa B kinase pathway promotes angiogenic/metastatic gene expression in colorectal cancer by activating nuclear factor-kappa B and beta-catenin. *Oncogene* 24, 1021–1031.
- Amin, K., 2012. The role of mast cells in allergic inflammation. *Respir. Med.* 106, 9–14.
- Barnes, P.J., 2011. Pathophysiology of allergic inflammation. *Immunol. Rev.* 242, 31–50.
- Cai, M., Phan, P.T., Hong, J.G., Kim, D.H., Kim, J.M., Park, S.J., Liu, X., Han, J.E., Park, H., Choi, J.W., Ryu, J.H., 2012. The neuroprotective effect of eupatilin against ischemia/reperfusion-induced delayed neuronal damage in mice. *Eur. J. Pharmacol.* 689, 104–110.
- Da Silva, C.A., Kassel, O., Mathieu, E., Massard, G., Gasser, B., Frossard, N., 2002.

- Inhibition by glucocorticoids of the interleukin-1beta-enhanced expression of the mast cell growth factor SCF. *Br. J. Pharmacol.* 135, 1634–1640.
- Dan, H.C., Cooper, M.J., Cogswell, P.C., Duncan, J.A., Ting, J.P., Baldwin, A.S., 2008. Akt-dependent regulation of NF-(kappa)B is controlled by mTOR and Raptor in association with IKK. *Genes Dev.* 22, 1490–1500.
- Dhawan, P., Richmond, A., 2002. A novel NF-kappa B-inducing kinase-MAPK signaling pathway up-regulates NF-kappa B activity in melanoma cells. *J. Biol. Chem.* 277, 7920–7928.
- Di Lorenzo, A., Fernandez-Hernando, C., Cirino, G., Sessa, W.C., 2009. Akt1 is critical for acute inflammation and histamine-mediated vascular leakage. *Proc. Natl. Acad. Sci. USA* 106, 14552–14557.
- Duan, W., Wong, W.S., 2006. Targeting mitogen-activated protein kinases for asthma. *Curr. Drug Targets* 7, 691–698.
- Galli, S.J., Tsai, M., 2012. IgE and mast cells in allergic disease. *Nat. Med.* 18, 693–704.
- Gilfillan, A.M., Tkaczuk, C., 2006. Integrated signalling pathways for mast-cell activation. *Nat. Rev. Immunol.* 6, 218–230.
- Hawrylowicz, C.M., O'Garra, A., 2005. Potential role of interleukin-10-secreting regulatory T cells in allergy and asthma. *Nat. Rev. Immunol.* 5, 271–283.
- Holgate, S.T., Polosa, R., 2008. Treatment strategies for allergy and asthma. *Nat. Rev. Immunol.* 8, 218–230.
- Israel, A., 2010. The IKK complex, a central regulator of NF-kappaB activation. *Cold Spring Harb. Perspect. Biol.* 2, a000158.
- Kemp, S.F., Lockey, R.F., 2002. Anaphylaxis: a review of causes and mechanisms. *J. Allergy Clin. Immunol.* 110, 341–348.
- Kim, K.H., Lee, W.R., An, H.J., Kim, J.Y., Chung, H., Han, S.M., Lee, M.L., Lee, K.G., Pak, S.C., Park, K.K., 2013. Bee venom ameliorates compound 48/80-induced atopic dermatitis-related symptoms. *Int. J. Clin. Exp. Pathol.* 6, 2896–2903.
- 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.
- Lamkanfi, M., Kalai, M., Saelens, X., Declercq, W., Vandenaabee, P., 2004. Caspase-1 activates nuclear factor of the kappa-enhancer in B cells independently of its enzymatic activity. *J. Biol. Chem.* 279, 24785–24793.
- Lee, N.Y., Chung, K.S., Jin, J.S., Bang, K.S., Eom, Y.J., Hong, C.H., Nugroho, A., Park, H.J., An, H.J., 2015. Effect of chioric acid on mast cell-mediated allergic inflammation *in vitro* and *in vivo*. *J. Nat. Prod.* 78, 2956–2962.
- Lee, S.H., Bae, E.A., Park, E.K., Shin, Y.W., Baek, N.I., Han, E.J., Chung, H.G., Kim, D.H., 2007. Inhibitory effect of eupatilin and jaceosidin isolated from *Artemisia princeps* in IgE-induced hypersensitivity. *Int. Immunopharmacol.* 7, 1678–1684.
- Lewis, T.S., Shapiro, P.S., Ahn, N.G., 1998. Signal transduction through MAP kinase cascades. *Adv. Cancer Res.* 74, 49–139.
- Madrid, L.V., Wang, C.Y., Guttridge, D.C., Schottelius, A.J., Baldwin Jr., A.S., Mayo, M.W., 2000. Akt suppresses apoptosis by stimulating the transactivation potential of the RelA/p65 subunit of NF-kappaB. *Mol. Cell Biol.* 20, 1626–1638.
- Metcalfe, D.D., Peavy, R.D., Gilfillan, A.M., 2009. Mechanisms of mast cell signaling in anaphylaxis. *J. Allergy Clin. Immunol.* 124, 639–646 quiz 647–638.
- Min, Y.D., Choi, C.H., Bark, H., Son, H.Y., Park, H.H., Lee, S., Park, J.W., Park, E.K., Shin, H.I., Kim, S.H., 2007. Quercetin inhibits expression of inflammatory cytokines through attenuation of NF-kappaB and p38 MAPK in HMC-1 human mast cell line. *Inflamm. Res.* 56, 210–215.
- Pawankar, R., 2014. Allergic diseases and asthma: a global public health concern and a call to action. *World Allergy Organ. J.* 7, 12.
- Perkins, N.D., 2007. Integrating cell-signalling pathways with NF-kappaB and IKK function. *Nat. Rev. Mol. Cell Biol.* 8, 49–62.
- Rasheed, Z., Akhtar, N., Khan, A., Khan, K.A., Haqqi, T.M., 2010. Butrin, isobutrin, and butein from medicinal plant *Butea monosperma* selectively inhibit nuclear factor-kappaB in activated human mast cells: suppression of tumor necrosis factor-alpha, interleukin (IL)-6, and IL-8. *J. Pharmacol. Exp. Ther.* 333, 354–363.
- Rincon, M., Irvin, C.G., 2012. Role of IL-6 in asthma and other inflammatory pulmonary diseases. *Int. J. Biol. Sci.* 8, 1281–1290.
- Rivera, J., Gilfillan, A.M., 2006. Molecular regulation of mast cell activation. *J. Allergy Clin. Immunol.* 117, 1214–1225 quiz 1226.
- Romashkova, J.A., Makarov, S.S., 1999. NF-kappaB is a target of AKT in anti-apoptotic PDGF signalling. *Nature* 401, 86–90.
- Ruimi, N., Rwashdeh, H., Wasser, S., Konkimala, B., Efferth, T., Borgatti, M., Gambari, R., Mahajna, J., 2010. Daedalea gibbosa substances inhibit LPS-induced expression of iNOS by suppression of NF-kappaB and MAPK activities in RAW 264.7 macrophage cells. *Int. J. Mol. Med.* 25, 421–432.
- Schemann, M., Kugler, E.M., Buhner, S., Eastwood, C., Donovan, J., Jiang, W., Grundy, D., 2012. The mast cell degranulator compound 48/80 directly activates neurons. *PLoS One* 7, e52104.
- Seol, S.Y., Kim, M.H., Ryu, J.S., Choi, M.G., Shin, D.W., Ahn, B.O., 2004. DA-9601 for erosive gastritis: results of a double-blind placebo-controlled phase III clinical trial. *World J. Gastroenterol.* 10, 2379–2382.
- Shin, S., 2005. In vitro effects of essential oils from *Ostericum koreanum* against antibiotic-resistant *Salmonella* spp. *Arch. Pharm. Res.* 28, 765–769.
- Stone, K.D., Prussin, C., Metcalfe, D.D., 2010. IgE, mast cells, basophils, and eosinophils. *J. Allergy Clin. Immunol.* 125, S73–S80.
- Turner, M.D., Nedjai, B., Hurst, T., Pennington, D.J., 2014. Cytokines and chemokines: at the crossroads of cell signalling and inflammatory disease. *Biochim. Biophys. Acta* 1843, 2563–2582.

Fig. S1. Effect of eupatilin on cell viability in HMC-1. HMC-1 cells were treated with different concentrations of eupatilin for 24 h, and their viability were determined using MTT assay. The data shown represent mean $\pm$ SD of three independent experiments

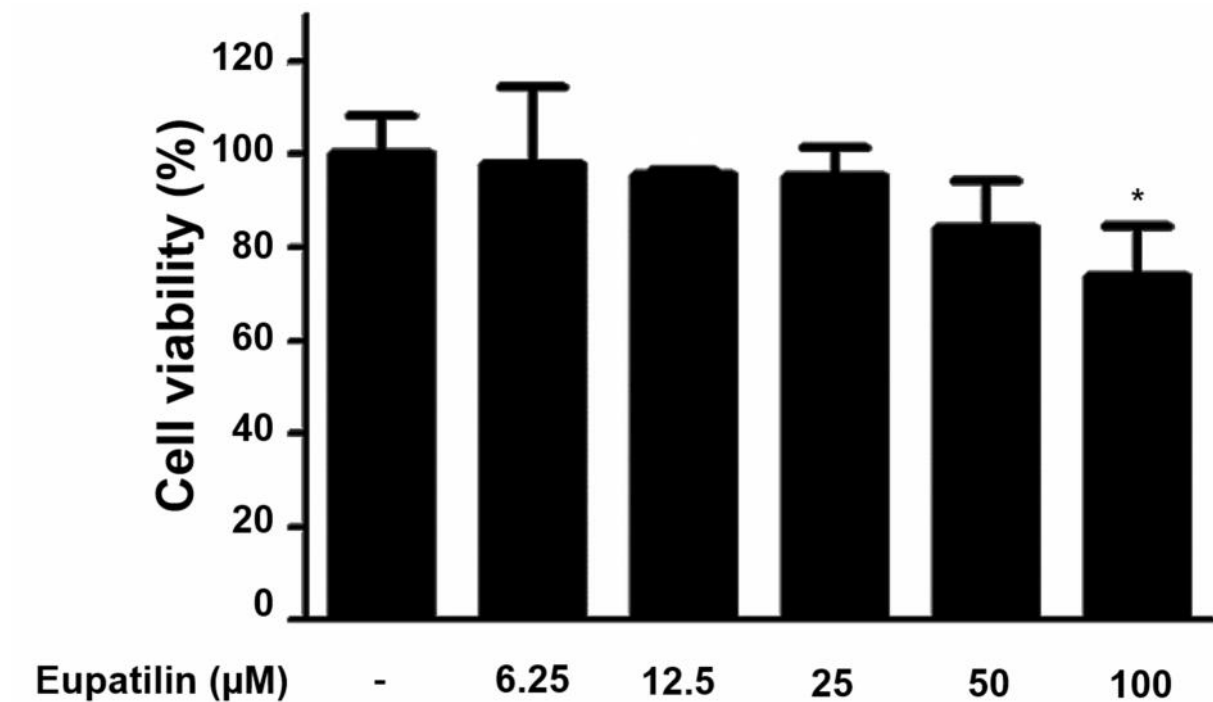


Fig. S2. Effect of eupatilin on caspase-1 activation in compound 48/80-induced anaphylaxis shock model. Expression of procaspase-1 was determined by western blot analysis using specific antibodies. Densitometry analysis of the blots was done using ImageJ software. The data shown represent mean $\pm$ SD of three independent experiments. ### $p < 0.001$ vs the control group, *** $p < 0.001$ vs the compound 48/80-treated group.

compound 48/80 (8 mg/kg)	-	+	+	+
DSCG (mg/kg)	-	-	25	-
Eupatilin (mg/kg)	-	-	-	20

