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# Eupatilin, an activator of PPARα, inhibits the development of oxazolone-induced atopic dermatitis symptoms in Balb/c mice

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ABSTRACT

Eupatilin (5,7-dihydroxy-3′,4′,6-trimethoxyflavone) is the main lipophilic flavonoid obtained from the *Artemisia* species. Eupatilin has been reported to have anti-apoptotic, anti-oxidative and anti-inflammatory activities. Previously, we found that eupatilin increases transcriptional activity and expression of peroxisome proliferator-activated receptor α (PPARα) in a keratinocyte cell line and acts as an agonist of PPARα. PPARα agonists ameliorate atopic dermatitis (AD) and restore the skin barrier function. In this study, we confirmed that the effects of eupatilin improved AD-like symptoms in an oxazolone-induced AD-like mouse model. Furthermore, we found that eupatilin suppressed the levels of serum immunoglobulin E (IgE), interleukin-4 (IL-4), and AD involved cytokines, such as tumor necrosis factor α (TNFα), interferon-γ (IFN-γ), IL-1β, and thymic stromal lymphopoietin (TSLP), IL-33, IL-25 and increased the levels of filaggrin and loricrin in the oxazolone-induced AD-like mouse model. Taken together, our data suggest that eupatilin is a potential candidate for the treatment of AD.

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## 1. Introduction

Atopic dermatitis (AD) is a chronic skin disease characterized by dryness, pruritus, and erythema eczema and the selective accumulation of inflammatory cells. The causes of AD are thought to be a genetic predisposition, environmental causes, immunological abnormalities and abnormalities of the skin barrier. AD involves T lymphocytes, dendritic cells expressing antigen-specific immunoglobulin E (IgE), T helper cell type 1 (Th1) cells and cytokines expressed in Th2 cells. Th1 cells secrete interleukin (IL)-2, interferon γ (IFNγ) and tumor necrosis factor α (TNFα) to activate macrophages, while Th2 cells secrete cytokines such as IL-4, IL-5, IL-6, IL-10 and IL-13 to increase immunoglobulin E (IgE) production and induce mast cell and eosinophil differentiation [1–3]. Under normal conditions, the interaction between Th1 and Th2 cells balances and maintains the immune response. However, in AD, Th2 cells secrete a large number of inflammatory cytokines and trigger an increase in serum IgE, further promoting the inflammatory response [4,5]. Currently, therapeutic agents used for AD are corticosteroids, antihistamines, and antibiotics. However, they have side effects such as skin atrophy and telangiectasia during long-term use.

Eupatilin (5,7-dihydroxy-3′,4′,6-trimethoxyflavone) is the main lipophilic flavonoid obtained from *Artemisia* species. Eupatilin has been reported to have anti-apoptotic, anti-oxidative and anti-inflammatory activities. According to some recent reports, eupatilin can inhibit allergic reactions via affecting mast cells. Moreover, eupatilin suppresses expression of pro-inflammatory cytokines such as IL-4, IL-6 and TNFα through nuclear factor kappa B (NF-κB) [6]. Previously, we found that eupatilin increases the transcriptional activity and expression of PPARα in a keratinocyte cell line and acts as an agonist of PPARα [7,8]. Recently, some reports have shown that PPARα is also expressed in other skin immune cell types, such as T-lymphocytes and macrophages that regulate the inflammatory response [9–11]. PPARα expression is reduced not only in a 2,4-dinitrofluorobenzene (DNFB)-induced allergic contact

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dermatitis-like mouse model but also in humans with AD [12,13]. Wy14643, a PPARα agonist, decreases epidermal hyperplasia and inflammatory cell infiltration by eosinophil and mast cells in an oxazolone-induced AD-like mouse model [14]. Furthermore, wy14643 reduced infiltration of inflammatory cells and serum IgE level in a dust mite-induced NC/Nga mouse model. In addition, the PPARα agonist restored the skin barrier function. Wy14643 also increased the expression of structural proteins such as loricrin, filaggrin and involucrin, the major markers of keratinocyte differentiation, and inhibited epidermal proliferation in mouse epidermis in vivo [15,16].

Previously, we showed that eupatilin can regulate inflammatory responses through PPARα in epidermal keratinocytes. Despite the known biological effects of eupatilin, no study has evaluated the therapeutic effects of eupatilin on an oxazolone-induced AD-like murine models. In this study, we investigated the effects of eupatilin, a PPARα activator, on an oxazolone-induced AD-like mouse model.

## 2. Materials and methods

### 2.1. Reagents

Eupatilin was purchased from Phytochemicals Online (www.phytopurify.com). Oxazolone was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). ELISA kits for IgE and IL-4 were purchased from R&D System, Inc. (Minneapolis, MN, USA). Power SYBR® Green Master Mix was purchased from Applied Biosystems (Foster City, CA, USA).

### 2.2. Animal studies

All of the experiments were performed according to the procedures approved by the KIST's Institutional Animal Care and Use Committee (IRB code No. 2016–011). Six-week-old female Balb/c mice were purchased from Orient Bio, Inc. (Seoul, Korea). The animals were housed under conditions of controlled temperature (23 ± 2 °C), humidity (55 ± 5%), and 12 h light/dark cycles (06:00–18:00 h light, 18:00–06:00 dark). The sensitization and challenge protocol was performed according to the previous method of Shin et al., with slight modifications. Briefly, Balb/c mice were sensitized on day −7 by a single application of 20 μl of 1.0% oxazolone in a mixture of acetone and olive oil (4:1) to the inner and outer surface of both ears. On day 0, the mouse ears were challenged with 20 μl of 0.1% oxazolone at 2-day intervals for 4 weeks post-sensitization. The mice were treated with the indicated concentrations of eupatilin (1.5% or 3.0%) twice a day for 4 weeks. The control group was treated with vehicle alone (acetone and olive oil [4:1]). After 3 weeks, the mice were sacrificed and samples were collected. Ears were stored at −80 °C for RNA isolation and analysis or immediately fixed in 4% formalin for histological analysis.

### 2.3. Real-time quantitative PCR (Q-PCR)

Total RNA was isolated by using the easy-BLUE™ total RNA extraction Kit (iNtRON Biotechnology, Seoul, Korea) according to the manufacturer's instructions. cDNA synthesis and Q-PCR analyses were carried out as described in a previous report. The primer sets were as follows: TNFα, forward 5′- AGC CCC CAG TCT GTA TCC TT -3′ and reverse 5′- CTC CCT TTG CAG AAC TCA GG -3'; TSLP, forward 5′- GCA AAT CGA GGA CTG TGA GAG C -3′ and reverse 5′- TGA GGG CTT CTC TTG TTC TCC G -3'; IL-1β, forward 5′- CAA CCA ACA AGT GAT ATT CTC CAT G -3′ and reverse 5′- GAT CCA CAC TCT CCA GCT GCA -3'; INF-γ, forward 5′- CAA CAG CAA GGC GAA AAA GG -3′ and reverse 5′- CCT GTG GGT TGT TGA CCT CAA -3'; filaggrin, forward 5′- AGA CTG GGA GGC AAG CTA CA -3′ and reverse 5′- CCT GCC TCC TTC AGA GTC AC -3'; loricrin, forward 5′- CTC ATC TTC CCT GGT GCT TC -3′ and reverse 5′- CAG CTA GAG CCT CCT CCA GA -3'; IL-33, forward 5′- CAA TCA GGC GAC GGT GTG GAT GG -3′ and reverse 5′- TCC GGA GGC GAG ACG TCA CC -3'; IL-25, forward 5′- CAG CAA AGA GCA AGA ACC -3′ and reverse 5′- CCC TGT CCA ACT CAT AGC -3'; GAPDH, forward 5′-ACC ACA GTC CAT GCC ATC AC-3′ and reverse 5′-CCA CCA CCC TGT TGC TGT A-3'. Data analyses were performed on 7500 System SDS software version 1.3.1 (Applied Biosystems).

### 2.4. Histological studies

Mouse ears were collected 48 h after the last application of oxazolone and fixed in 4% formalin solution then embedded in paraffin blocks. Tissues were cut into 10 μm slices and stained with hematoxylin-eosin and toluidine blue and then observed under light microscopy (Olympus, Tokyo, Japan). Epidermal thickness was measured as described in previous reports [17].

### 2.5. Immunohistochemistry

Paraffin sections (10 μm thickness) were deparaffinized and immunohistochemically stained for filaggrin (Abcam, Cambridge, MA, USA) or loricrin (Abcam). Immunohistochemical staining was performed according to the manufacturer's guidelines.

### 2.6. Cell culture

RBL-2H3 cells were purchased from the Korean Cell Line Bank (Seoul, Korea). Cells were cultured in DMEM medium supplemented with 10% FBS (HyClone Laboratories Inc., Logan, UT, USA) and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin; Invitrogen, Carlsbad, CA, USA) at 37 °C in a humidified 5% $CO_2$.

### 2.7. β-hexosaminidase release activity

RBL-2H3 cells were seeded into a 24-well plate and cultured overnight. The cells were treated with 0.1 μg/mL IgE and incubated for 4 h at 37 °C in 5% $CO_2$. The IgE-sensitized cells were incubated with the indicated concentrations of eupatilin for 1 h, followed by 30 min incubation with 1 μg/ml DNP-BSA. To measure β-hexosaminidase activity, the culture medium was mixed with 10 mM poly-*N*-acetyl glucosamine (p-NAG) in 0.1 M sodium citrate buffer (pH 4.5) in a 96-well plate and incubated for 1 h at 37 °C. The reaction was terminated by stop buffer. The β-hexosaminidase activity was measured by absorbance at 405 nm.

### 2.8. Statistical analyses

The data are expressed as the means ± SD. Differences between the mean values in the two groups were analyzed using one-way analysis of variance (ANOVA). $P < .05$ was considered statistically significant.

## 3. Results

### 3.1. Effects of eupatilin on AD-like symptoms in oxazolone-induced Balb/c mice

Haptens such as oxazolone and 2,4-dinitrochlorobenzene (DNCB) are used to induce allergic contact dermatitis. Repeated challenges with oxazolone result in Th2-like inflammatory response in mouse models. Recently, some reports have shown that oxazolone reduced the expression of PPARα mRNA [18,19].

In this study, we used an oxazolone-induced animal model to demonstrate the effect of eupatilin on PPARα. First, we investigated the effects of eupatilin, a PPARα agonist in clinical skin conditions, on oxazolone-induced AD-like lesions in Balb/c mice. The group treated with oxazolone showed progressive symptoms of erythema, edema and dryness of the ear skin. In contrast, the group treated with eupatilin exhibited improved erythema, edema and dryness (Fig. 1A).

In addition, eupatilin could restore PPARα mRNA expression in oxazolone-induced Balb/c mice. PPARα mRNA expression was significantly decreased in oxazolone-induced Balb/c mice compared with non-treated control mice. Topical application of eupatilin to oxazolone-induced Balb/c mice markedly increased mRNA expression of PPARα in a dose-dependent manner and showed a 50% reduction in oxazolone-induced ear thickening at day 28 (Fig. 1B and C).

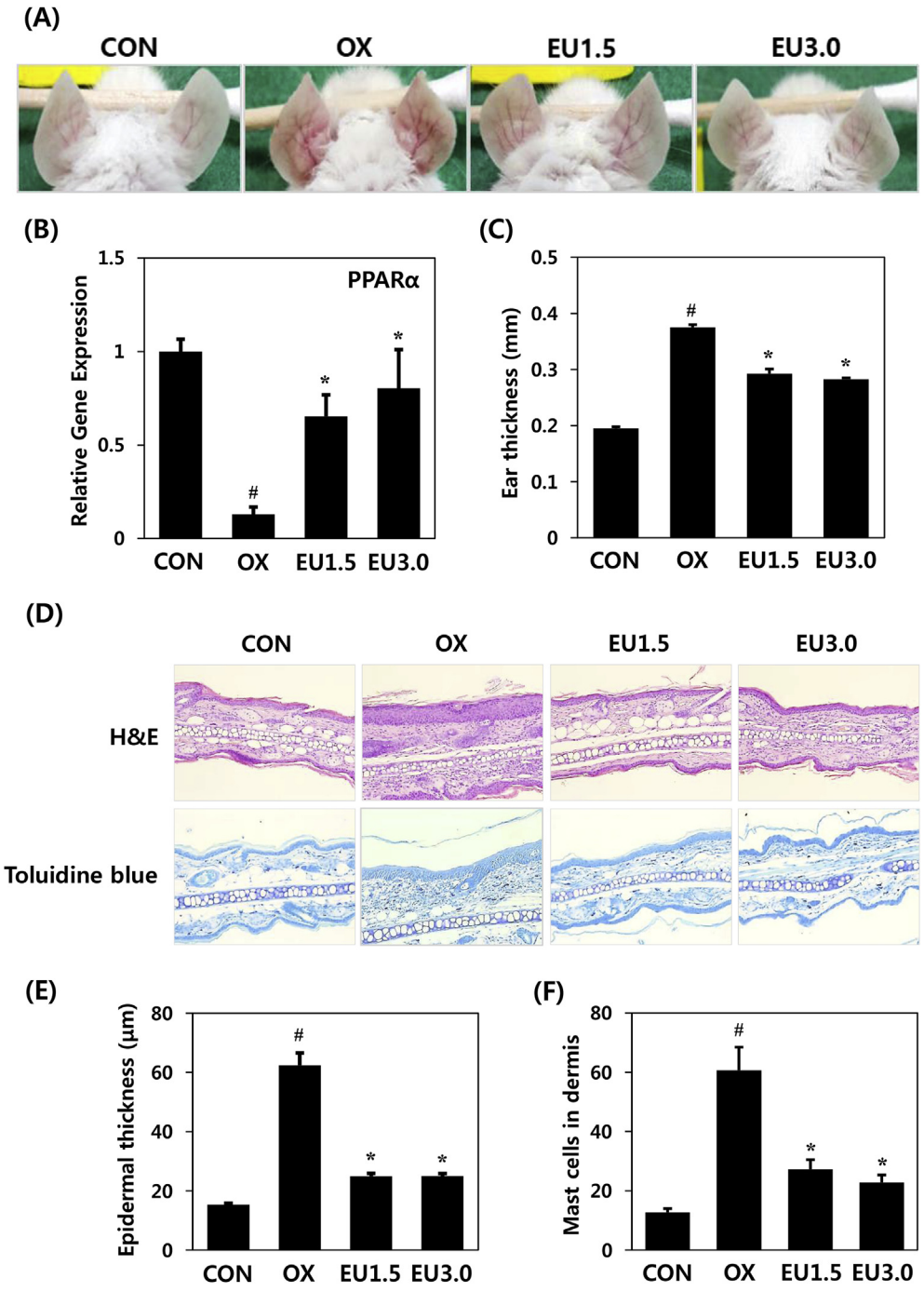



**Fig. 1.** Eupatilin improved atopic dermatitis (AD)-like symptoms in oxazolone-induced Balb/c mice. (A) Clinical features of the skin appearance are shown. (B) Relative gene expression of PPARα was determined by Q-PCR and normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). (C) Ear skin thickness measured using a caliper micrometer. (D) Cross sections of ears were stained with hematoxylin and eosin and toluidine blue. (E) The thickness of the epidermis and (F) the number of mast cells were measured (in pixels) using ImageJ software. Each bar represents the mean ± SD of three independent experiments. #P < .05 vs. control (CON); *P < .05 vs. the oxazolone-induced group (OX). CON, normal control; OX, oxazolone-induced AD; EU1.5, 1.5% eupatilin application; EU3.0, 3.0% eupatilin application.

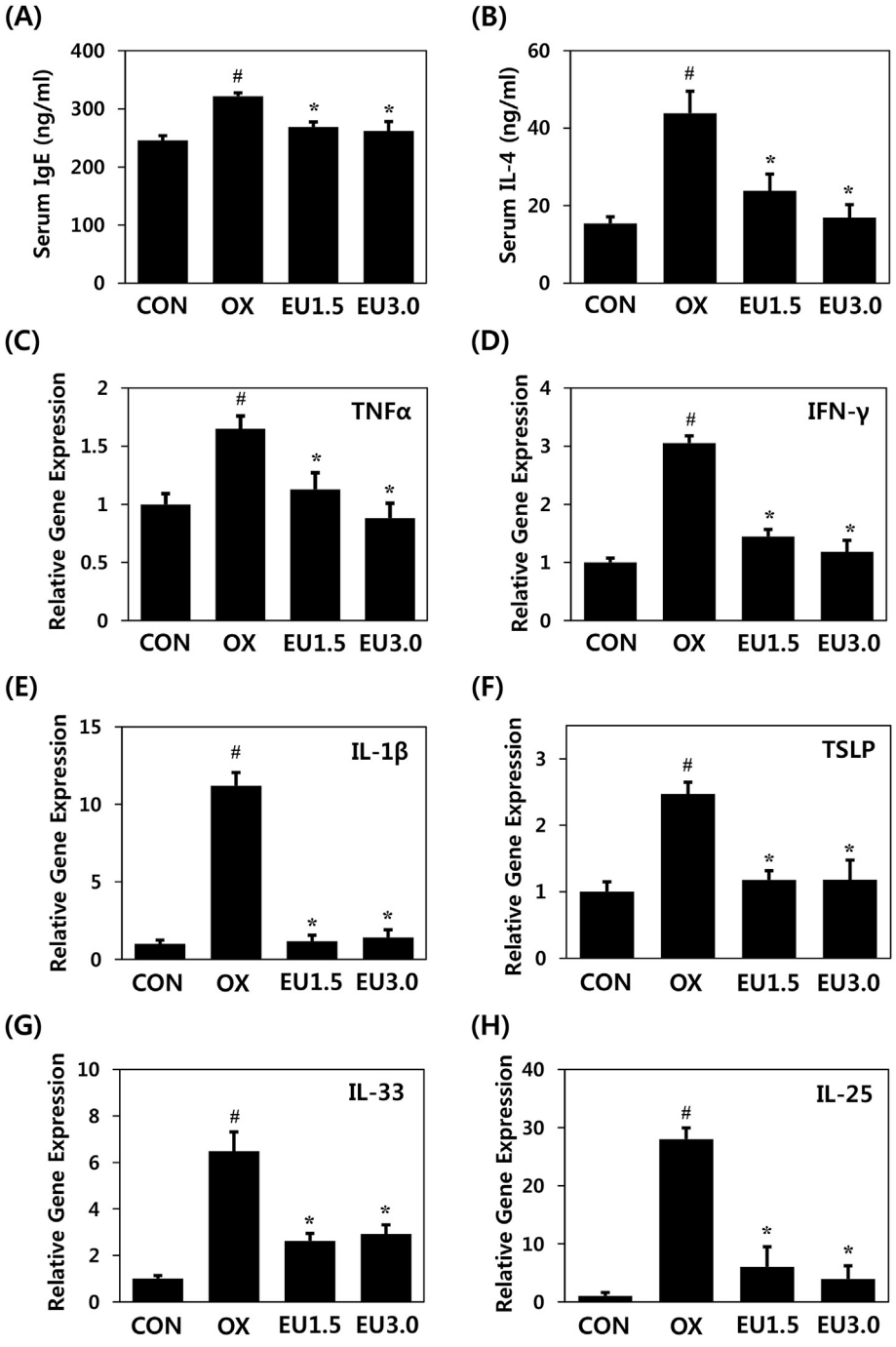



**Fig. 2.** Eupatilin suppressed serum IgE, IL-4 and inflammatory cytokines in oxazolone-induced Balb/c mice. (A) Protein expression levels of serum IgE and (B) serum IL-4 were assessed by ELISA. Relative gene expression of (C) TNFα, (D) IFN-γ, (E) IL-1β, (F) TSLP, (G) IL-33 and (H) IL-25 were determined by Q-PCR and normalized to the expression of GAPDH. Each bar represents the mean ± SD of duplicates. #P < .05 vs. control (CON); *P < .05 vs. oxazolone-induced group (OX). CON, normal control; OX, oxazolone-induced AD; EU1.5, 1.5% eupatilin application; EU3.0, 3.0% eupatilin application.

To further investigate the anti-inflammatory activity, including epidermal hyperplasia, infiltration of inflammatory cells into the skin of oxazolone-induced Balb/c mice treated with eupatilin, skin biopsies were taken and histological observation was performed. As shown in Fig. 1D and E, H&E staining analysis, the eupatilin-treated oxazolone-induced Balb/c mouse showed improvements of epidermal hyperplasia, including lessening of epidermal thickness and markedly reduced infiltration of inflammatory cells. Furthermore, toluidine blue staining analysis in Fig. 1D and F, a method used to specifically identify mast cells, showed that mast cell infiltration into the dermis was significantly decreased in the eupatilin treated group. These results indicate that eupatilin effectively improves the AD-like symptoms in the oxazolone-induced Balb/c mice.

### *3.2. Effects of eupatilin on IgE, IL-4 and inflammatory cytokines in oxazolone-induced Balb/c mice*

To investigate the eupatilin effects on serum IgE and IL-4, general markers of AD, expression changes in oxazolone-induced mice

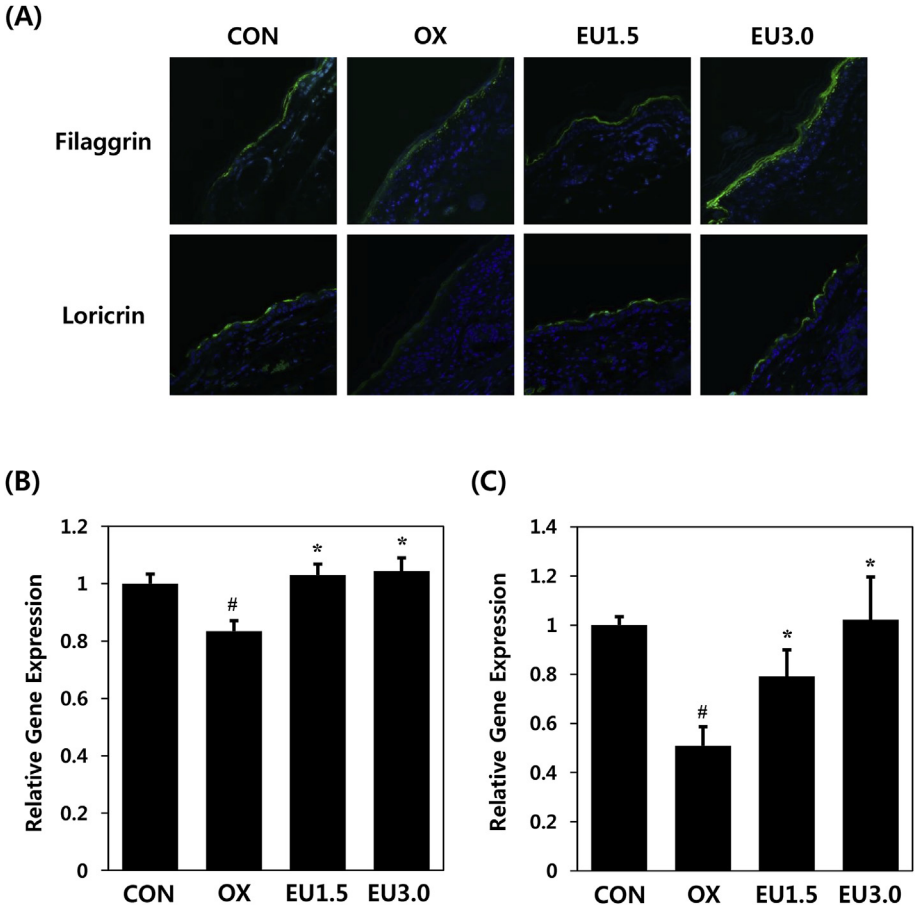



**Fig. 3.** Eupatilin restored filaggrin and loricrin expression in oxazolone-induced Balb/c mice. (A) Representative data of immunohistochemical staining of filaggrin and loricrin protein are shown. (B) Relative gene expression of filaggrin (B) and loricrin (C) were determined by Q-PCR and normalized to the expression of GAPDH. Each bar represents the mean ± SD of duplicates. #P < .05 vs. control (CON); *P < .05 vs. oxazolone-induced group (OX). CON, normal control; OX, oxazolone-induced AD; EU1.5, 1.5% eupatilin application; EU3.0, 3.0% eupatilin application.

were measured by using an ELISA assay kit. The group treated with oxazolone showed increases of serum IgE and IL-4. In contrast, serum IgE and IL-4 levels in the group treated with eupatilin were significantly decreased (Fig. 3A and B). In AD skin lesions, a large number of pro-inflammatory cytokines are secreted by allergens, resulting in increased allergic immune responses. Keratinocytes increase the number of infiltrating eosinophils and lymphocytes by producing pro-inflammatory cytokines such as TNFα and IFNγ, and they secrete a large number of chemokines and increase the production of IgE [20]. Thymic stromal lymphopoietin (TSLP), IL-33 and IL-25 can stimulated to release Th2 cytokines. In addition, keratinocytes are an important source of secretion of TSLP, which activates dendritic cells. Therefore, we investigated the mRNA expression levels of the pro-inflammatory cytokines, TNFα, IFNγ and IL-1β, TSLP, IL-33 and IL-25 by Q-PCR. The group treated with eupatilin had decreased oxazolone-induced TNFα, IFNγ and IL-1β mRNA expression in a dose-dependent manner (Fig. 2C–E). Furthermore, TSLP, IL-33 and IL-25 mRNA expression was also suppressed in mice treated with eupatilin (Fig. 2F–H).

### 3.3. Effects of eupatilin on filaggrin and loricrin expression in oxazolone-induced Balb/c mice

Defects in skin barrier function increase the skin permeability to antigens and irritants in AD, thereby aggravating skin inflammatory responses. Th2 cytokines directly damage the skin barrier and inhibit expression of filaggrin, loricrin, and involucrin, which are the main structural proteins produced after differentiation in keratinocytes. To explain the eupatilin effects on filaggrin and loricrin protein expression in oxazolone-induced mice, we performed immunohistochemistry staining analysis and Q-PCR. Filaggrin and loricrin protein expression were weakened in the cornified layers (stratum corneum), or extended proximally into the suprabasal layers after oxazolone challenges. However, in the group treated with eupatilin, they were strongly expressed in cornified layers (Fig. 3A). Furthermore, eupatilin treatment increased filaggrin and loricrin mRNA expression in oxazolone-induced mice (Fig. 3B and C).

### 3.4. Eupatilin inhibits IL-4 expression and β-hexosaminidase release in RBL-2H3 cells

To determine the anti-allergic properties of eupatilin, we assessed the anti-inflammatory effects of eupatilin. IL-4 mRNA levels were detected in PMA/ionomycin (PI)-induced RBL-2H3 cells. Eupatilin markedly decreased the level of IL-4 mRNA in PI-induced RBL-2H3 cells (Fig. 4A). Then, we tested its ability to inhibit RBL-2H3 basophils' degranulation stimulated by IgE/antigen. Eupatilin significantly inhibited the release of β-hexosaminidase, which is a general biomarker of degranulation and a hallmark characteristic of allergic reactions caused by allergen exposure (Fig. 4B).

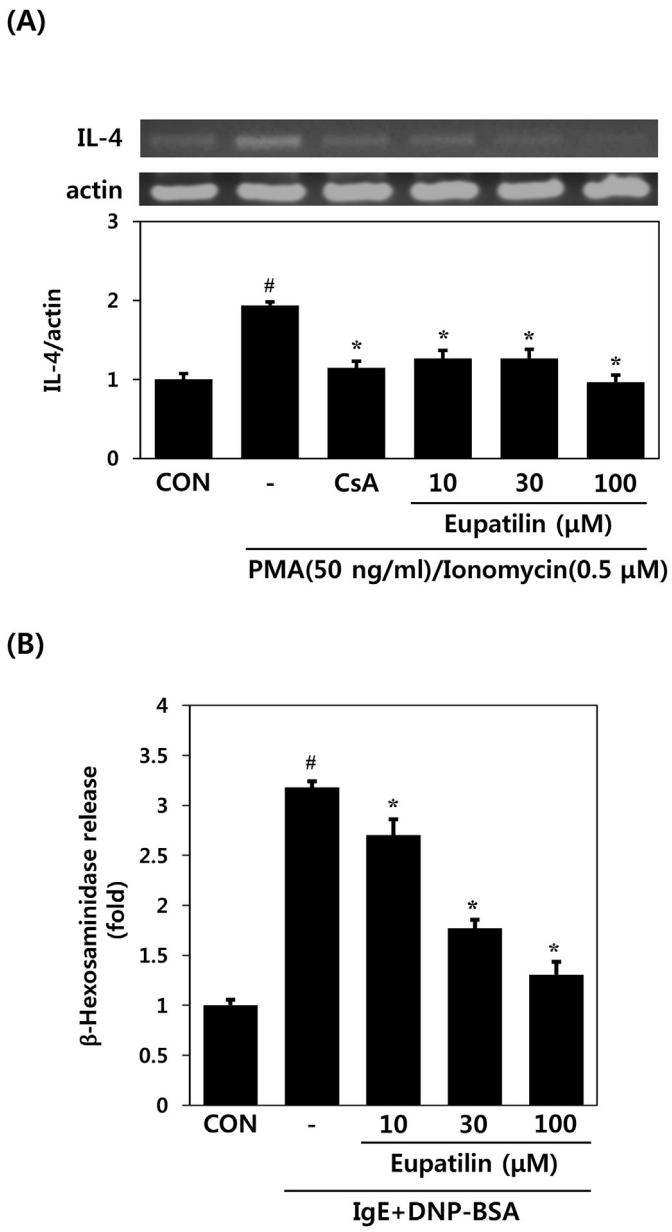



**Fig. 4.** Eupatilin inhibits IL-4 expression and degranulation in RBL-2H3 cells. (A) Representative data of RT-PCR analysis of IL-4 mRNA expression are shown and normalized to GAPDH expression in PMA/ionomycin-induced RBL-2H3 cells. RBL-2H3 cells were treated with eupatilin (10, 30 and 100 μμM) in combination with PMA (50 μg/ml)/ionomycin (0.1 μM) for 8 h to measure IL-4 expression. Each bar represents the mean ± SD of duplicates. #P < .05 vs. control (CON); *P < .05 vs. PMA/ionomycin-induced group. (B) The cells were sensitized with IgE and treated with eupatilin (10, 30, 100 μM). β-hexosaminidase release was detected after stimulation with DNP-BSA. Each bar represents the mean ± SD of 3 experiments. #P < .05 vs. control (CON); *P < .05 vs. the IgE + DNP-BSA-treated group.

## 4. Discussion

AD is a complex inflammatory skin disease that has a great adverse influence on the quality of life of patients. Various animal models have been used to research the mechanism of AD [12,13]. It has been reported that the oxazolone-induced AD mouse model has clinical and immunological characteristics very similar to human AD. Repeated application of oxazolone results in infiltration of T-cell mediated inflammatory cells, skin edema and skin thickening [13]. Clinical features of AD have been reported to be associated with severe pruritus, erythematous and erythema [21]. In this study, we demonstrated the beneficial effects of eupatilin on AD. Here, we found that eupatilin improved the erythema, edema and dryness in an oxazolone-induced Balb/c mouse model (Fig. 1).

The mechanism of AD involves various cells (T lymphocytes, Langerhans cells, eosinophils, keratinocytes) and factors (cytokines, immunoglobulins; especially IgE) [22]. The keratinocytes secrete various cytokines such as IL-4, TNFα, and IFN-γ in response to multiple stimuli and, in turn, are affected by these cytokines [20]. IL-4 is a major cytokine in AD and plays an important role in IgE production and mast cell activity. Allergen-specific IgE acts on high-affinity receptors for IgE (FcεRI) to activate mast cells and basophils. Activated mast cells and basophils are involved in immediate skin reactions (pruritus, erythema) [23]. In this study, eupatilin markedly decreased serum IL-4 and serum IgE levels (Fig. 2A and B).

TNFα and IFN-γ increase production of Monocyte Chemotactic Protein 1 and RANTES (Regulated on Activation, Normal T Cell Expressed and Secreted) by keratinocytes in chronic AD. RANTES is involved in activation and migration of eosinophils and T cells. Patients with both AD and asthma were found to excessively secrete IL-4 and IFN-γ into the blood. TNF-α, which is produced and secreted by mast cells, increases the expression of adhesion molecules of vascular endothelial cells and promotes the entry of eosinophils and T lymphocytes into the target organs [24]. IL-1β increases IgE-mediated mast cell activation such as histamine release and Th2 cytokine secretion, thus affecting the development of IgE-mediated allergic diseases such as AD, asthma and food allergies [25]. Our present results showed that eupatilin suppressed TNFα, IFN-γ and IL-1β mRNA expression.

When the skin barrier is damaged by genetic and various environmental factors in AD, it is easily exposed to various allergens infiltration from the outside. TSLP is secreted in keratinocytes and is called the master switch of allergic inflammatory response because it affects important cells that cause skin inflammation, such as mast cells, basophils, and eosinophils, and induces a Th2 immune response [26]. TSLP also activates dendritic cells, resulting in increased production of Thymus and Activation-regulated Chemokine and Macrophage Derived Chemokine [27]. TSLP is overexpressed in keratinocytes during AD, but it is expressed at normal levels in other forms of dermatitis. Therefore, TSLP is recognized as an important factor in the pathogenesis of AD. And IL-33, IL-25 and TSLP have a key role in murine models of allergic asthma and AD [28]. In this study, eupatilin significantly suppressed TSLP, IL-33 and IL-25 mRNA expression (Fig. 2F–H).

Proliferation, differentiation and death occur sequentially in epidermal cells. Each process is characterized by the expression of specific proteins such as filaggrin, loricrin, and involucrin [29]. IL-4, a Th2 cytokine, inhibits the expression of the epidermal differentiation proteins and reduces antimicrobial peptide production [30]. In addition, TSLP downregulates filaggrin expression in NHEK cells [31]. Recent reports have shown that IL-4 inhibited loricrin transcription by competitively sequestering p300/CBP from the loricrin transcription complex [32]. Our present results showed that eupatilin significantly inhibited filaggrin and loricrin expression (Fig. 3).

Mast cells are known to be key participants in acute and chronic inflammatory diseases in most allergic diseases, including AD [33–35]. Mast cell activation occurs through a high-affinity IgE receptor (FcεRI). FcεRI expression is increased by IL-4 and IL-13 in the cell membrane [36]. We showed that eupatilin suppressed IL-4 expression in PI-induced RBL-2H3 cells. Activation of mast cells by IgE binding to FcεRI promotes degranulation. In this degranulation process, mast cells release histamine, cytokines and chemokines that sustain the inflammatory response. Additionally, as the serum IgE concentration increases, the number of FcεRI increases. We

showed that eupatilin suppressed IgE-induced degranulation in RBL-2H3 cells.

In this study, we found that eupatilin improved AD-like symptoms and inhibited inflammatory reactions in oxazolone-induced Balb/c mice. Mechanistically, eupatilin suppressed oxazolone-induced IgE production by reducing IL-4 expression. In addition, eupatilin inhibited oxazolone-induced inflammatory cytokines expression by inducing TSLP expression. Eupatilin is a potential candidate for the treatment of AD.

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## Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.bbrc.2018.01.098.

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