Biochemical and Biophysical Research Communications 496 (2018) 508–514

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

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

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ARTICLE INFO

Article history:
Received 29 December 2017
Accepted 15 January 2018
Available online 17 January 2018

Keywords:
Eupatilin
PPARα
Atopic dermatitis
IL-4

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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https://doi.org/10.1016/j.bbrc.2018.01.098
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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].

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