Effects of Eupatilin on Insulin-Like Growth Factor 1-Induced Lipogenesis and Inflammation of SZ95 Sebocytes

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Dear Editor:

Acne is an inflammatory disorder of the pilosebaceous unit. Its mechanism is complex, occurring as a result of hyperkeratinization of the pilosebaceous ducts, colonization of *Propionibacterium acnes*, and perifollicular inflammation with an imbalance of sebum production\(^1\). Insulin-like growth factor (IGF)-1 and peroxisome proliferator-activated receptor (PPAR) regulate the growth and differentiation of sebocytes and sebum secretion. Hyperglycemic diets induce IGF-1 production and may aggravate acne\(^2\). Eupatilin [2-(3,4-dimethoxyphenyl)-5,7-dihydroxy-6-methoxychromen-4-one] is the main flavonoid of the *Artemisia* species. Eupatilin exerts various effects including anti-apoptotic, cytoprotective, antioxidant, and anti-inflammatory nature on many different cell lines\(^3\). Eupatilin demonstrates antioxidant activity by suppressing reactive oxygen species and anti-inflammatory activity by inhibiting 5-lipoygenase\(^4\). In addition, eupatilin downregulates the production of inflammatory cytokines such as tumor necrosis factor (TNF)-\(\alpha\), interleukin (IL)-4, IL-6, and IL-18 through nuclear factor kappa B (NF-\(\kappa\)B) in RBL-2H3 cells\(^5\). Eupatilin has been used in an attempt to address inflammatory diseases, but its therapeutic effect on acne and related mechanisms remain unclear. In this study, we aimed to investigate the therapeutic effect of eupatilin on IGF-1-induced inflammation and lipogenesis using human sebocytes.

To examine the effects of eupatilin *in vitro*, we used human SZ95 sebocytes (Supplementary Material). The effects of eupatilin on the proliferation of cultured SZ95 sebocytes were determined with various doses of eupatilin. In MTT assay, the proliferation of SZ95 sebocytes was sequentially decreased according to the concentration (10, 20, 50, or 100 \(\mu\)M of eupatilin) (Supplementary Material). Thus, we determined experimental concentration as 10- and 100- \(\mu\)M of eupatilin. Next, we stained an intracellular lipid droplet formation using Oil Red O to investigate the effects of eupatilin on the lipid synthesis of sebocytes. When SZ95 sebocytes were treated with eupatilin, lipid accumulation in the cytoplasm was significantly reduced (Fig. 1A, B). To elucidate how eupatilin suppressed IGF-1-induced lipogenesis of SZ95 sebocytes, the effects of eupatilin treatment on the IGF-1-induced expression levels of phosphorylated Akt and lipogenesis-related transcription factors (PPAR\(\gamma\) and mature sterol regulatory element-binding protein [SREBP]-1) were measured. In comparison with no treatment, 100 \(\mu\)g/ml of eupatilin significantly reduced the protein levels of phosphorylated Akt, PPAR\(\gamma\), and mature SREBP-1 of the sebocytes, which were increased by IGF-1 pretreatment (Fig. 1C). Likewise, eupatilin also significantly downregulated the mRNA ex-
Fig. 1. Effects of eupatilin on the intracellular lipid synthesis of SZ95 sebocytes. With the exception of the control group, SZ95 sebocytes were pretreated with 50 ng/ml of insulin-like growth factor (IGF)-1 for 48 hours and then with 10 μg/ml or 100 μg/ml of eupatilin for 48 hours. (A) Intracellular lipid droplets of SZ95 sebocytes treated with eupatilin were detected by Oil Red O staining. Bars=20 μm. (B) Supernatant Oil Red O levels (%) were measured by their optical density at 500 nm. (C) Whole-cell lysates were prepared and analyzed by western blotting. Blots were incubated with antibodies specific for total and phosphorylated forms of Akt, peroxisome proliferator-activated receptor (PPAR)-γ, and mature sterol regulatory element-binding protein (SREBP)-1. (D) Quantitative reverse-transcription polymerase chain reaction of PPARγ, SREBP-1a, and SREBP-1c for the evaluation of mRNA expression was performed. Data are presented as the mean±standard error of triplicate assay (n=5). Data were analyzed using the Student’s t-test (*p<0.05, ***p<0.001). RFI: relative fold increase.

pression level of PPARγ, SREBP-1a, and SREBP-1c of the sebocytes (Fig. 1D). These results suggest that eupatilin has an inhibitory effect on IGF-1-induced lipogenesis of sebocytes through suppression of the phosphorylation of Akt, PPARγ, and mature SREBP-1.

To further investigate the anti-inflammatory effects of eupatilin on sebocytes, we analyzed the IGF-1-induced pro-inflammatory cytokines of sebocytes. In the immunofluorescence study, treatment with eupatilin inhibited the translocation of NF-κB p65 induced by 50 ng/ml of IGF-1 (Fig. 2A). This eupatilin treatment also significantly downregulated the mRNA expression levels of (pro)inflammatory cytokines such as TNF-α, IL-6, and IL-8 (Fig. 2B).

In this study, we first showed that the proper concentrations of eupatilin (10 μg/ml and 100 μg/ml) had potent anti-IGF-1 effects on SZ95 sebocytes, as follows: 1) eupatilin suppressed the lipogenesis of sebocytes by interfering with the expression of phosphorylated Akt, PPARγ, and SREBP-1 induced by IGF-1; and 2) eupatilin reduced the inflammatory response of sebocytes by inhibiting NF-κB activation, with IGF-1 able to induce the inflammatory cytokine expression of sebocytes.

SREBP-1 is a major transcription factor that regulates cholesterol/fatty acid metabolism. Smith et al. reported that IGF-1 induced SREBP-1. This activation occurred via a phosphoinositide 3-kinase (PI3K)/Akt pathway. They also showed that IGF-I transmits its lipogenic signal in sebocytes via an Akt pathway. Meanwhile, PPARγ is a potential modulator of lipid production in human sebocytes. PPARs are regulators of lipogenesis and differentiation of keratinocytes as well as sebocytes and have three isoforms (i.e., α, δ, and γ). Notably, PPARγ is important for sebaceous gland development and function. Interestingly, in the present study, the Oil Red O stain showed that eupatilin suppressed lipid synthesis by sebocytes. Although eupatilin from Artemisia plants has been reported as a se-
Inhibition of insulin-like growth factor (IGF)-1–induced (pro)inflammatory cytokines of SZ95 sebocytes by eupatilin. With the exception of the control group, SZ95 sebocytes were treated with 50 ng/ml of IGF-1 for 48 hours and then with 10 μg/ml and 100 μg/ml of eupatilin for 48 hours. (A) Cells were incubated with primary antibody (nuclear factor kappa B [NF-κB] p65) and were finally visualized under a fluorescence microscope. (B) Quantitative Reverse-transcription polymerase chain reaction for the evaluation of mRNA expression of tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-8 was performed. Data are presented as the mean±standard error of triplicate assay (n=5). Data were analyzed using the Student’s t-test (*p<0.05, **p<0.01, ***p<0.001). RFI: Relative fold increase.

ACKNOWLEDGMENT

This work was supported by a National Research Foundation of Korea (NRF) grant funded by the Korean government (MSIP) (no. NRF-2015R1C1A2A01054767 and 2018 R1D1A1B07044100) and by Research Fund of Seoul St. Mary’s Hospital, The Catholic University of Korea. And this research was supported by a grant from the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI) funded by the Ministry of Health & Welfare of the Republic of Korea (no. HI14C2116).

SUPPLEMENTARY MATERIALS

Supplementary data can be found via http://anndermatol.org/src/sm/ad-31-479-s001.pdf.

CONFLICTS OF INTEREST

The authors have nothing to disclose.

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REFERENCES


Can Body Mass Index and/or Waist Circumference Be the Risk Factors of Chronic Spontaneous Urticaria?: A Nationwide Population-Based Study

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Dear Editor:

Several studies have suggested an association between chronic spontaneous urticaria (CSU) and body mass index (BMI)1-3 or metabolic syndrome1,4. In contrast, a French study reported that obesity was not associated with severe CSU5. There is little evidence that waist circumference (WC), another scale which correlates well with visceral obesity, is associated with CSU. We hypothesized that obesity could be associated with increased CSU risk. The aim of our study was to investigate the impact of BMI and/or WC on the risk for CSU in an adult Korean population using a nationwide database. The study was approved by the Institutional Review Board of The Catholic University of Korea (IRB no. KC16EISE0852).

The health check-up database, a sub-dataset of the Korean National Health Insurance Service (NHIS) database (2002 ∼ 2015), was used for data collection. NHIS subscribers are advised to have biannual health check-ups including height, weight, blood pressure, and blood test. The database includes information on height, weight, blood pressure, waist circumference, and other data. The patients included in the analysis were those who had at least one health check-up in the study period.

The study was a retrospective analysis of data from the NHIS database. The study was approved by the Institutional Review Board of The Catholic University of Korea (IRB no. KC16EISE0852). The results of the study are presented in tables and figures.

Received September 13, 2018, Revised November 15, 2018, Accepted for publication January 2, 2019

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Supplementary Materials

MATERIALS AND METHODS

Cells and reagents

Immortalized human SZ95 sebocytes (provided by Prof. Christos C. Zouboulis) were maintained in Sebomed® basal medium (Biochrom GmbH, Berlin, Germany) containing 10% (v/v) fetal bovine serum (Gibco; Invitrogen, Carlsbad, CA, USA), 5 ng/ml of human recombinant epidermal growth factor (Invitrogen, Grand Island, NY, USA), 50 IU/ml of penicillin, and 50 μg/ml of streptomycin (Gibco) in a humidified atmosphere containing 5% CO2 at 37°C (Zouboulis et al., 1999)1. Culture medium was replaced every two days. Eupatilin was provided by Dong-A Pharmaceutical Co., Ltd. (Yongin, Korea) and dissolved in 10% dimethyl sulfoxide. The experimental design is shown in Supplementary Fig. 1.

Determination of cell proliferation by MTT assay

Cell proliferation was analyzed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide MTT assay. SZ95 human sebocytes were seeded (1×10³ cells per well) in triplicate into 96-well plates, incubated overnight, and then treated with eupatilin at the concentrations of 10 μg/ml, 20 μg/ml, 50 μg/ml, and 100 μg/ml (Supplementary Fig. 2) in Sebomed media (Biochrom GmbH) without serum at 37°C in 5% CO2. Subsequently, 100 μl of MTT at 5 mg/ml was added to each well and incubation was continued for 4 hours. Supernatants were removed and formazan crystals resulting from mitochondrial enzymatic activity on the MTT substrate were solubilized with dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO, USA). The absorbance was measured at 540 nm using an enzyme-linked immunosorbent assay reader (VersaMax; Molecular Devices, Sunnyvale, CA, USA).

Oil Red O staining and lipid detection

SZ95 sebocytes were seeded (1×10⁴ cells/ml) on 12-well culture plates, incubated overnight, and then treated with 50 μg/ml of IGF-1 and 10 μg/ml or 100 μg/ml of eupatilin for 48 hours. Cells treated with the vehicle served as controls. At the end of the treatment period, cells were washed with phosphate-buffered saline and fixed through incubation in 10% formalin for one hour at room temperature. Fixed cells were incubated with 60% isopropanol for five minutes and then isopropanol was completely removed by air-dry. Cells were stained for 10 minutes with filtered Oil Red O working solution, prepared immediately before use by making a 6:4 mixture of stock (0.5% Oil Red O from Sigma-Aldrich in 99% isopropanol) and dH2O. Supernatant Oil Red O levels were measured by measuring the optical density at 500 nm.

Western blot analysis

Cells were lysed in lysis buffer containing a protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific, Waltham, MA, USA). Equal amounts (30 μg) of extracted protein were resolved using 6% to 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. Following incubation in blocking solution, the membranes were incubated overnight at 4°C with the appropriate antibodies. Blots were then incubated with peroxidase-conjugated secondary antibodies and visualized by enhanced chemiluminescence substrate (Thermo Fisher Scientific). The following primary antibodies were used: phospho-Akt, Akt, peroxisome proliferator-activated receptor (PPAR) γ, mature sterol regulatory element-binding protein (SREBP)-1 (Cell Signaling Technology, Danvers, MA, USA), and actin (Santa Cruz Biotechnologies, Dallas, TX, USA).

Immunofluorescence

SZ95 sebocytes were grown on cell culture slides (SPL Life Sciences, Pocheon, Korea), fixed through incubation with 4% paraformaldehyde for 20 minutes, and permeabilized through incubation with 0.1% Triton X-100 in phosphate-buffered saline for 10 minutes at room temperature. Cells were then incubated overnight at 4°C with primary antibody (i.e., nuclear factor kappa B [NF-κB] p65) (Santa Cruz Biotechnologies) and then for one hour at room temperature with fluorescein isothiocyanate-conjugated secondary antibody (Santa Cruz Biotechnologies), and were finally visualized under a fluorescence
microscope (Olympus, Tokyo, Japan).

**Reverse-transcription polymerase chain reaction**

To evaluate gene expression, total RNA was isolated using Trizol® Reagent (Invitrogen) according to the manufacturer’s protocols. Equal amounts of RNA (1 μg) were reverse-transcribed into complementary DNA using the Prime Script™ RT reagent Kit with gDNA Eraser® (Takara Bio, Otsu, Japan). Quantitative reverse-transcription polymerase chain reaction (RT-PCR) was performed using a CFX96™ Real-Time PCR Detection System® (Bio-Rad, Hercules, CA, USA) with SYBR® premix EX Taq™ (Takara Bio, Kusatsu, Japan) and specific primers for tumor necrosis factor (TNF-α), interleukin (IL)-6, IL-8, PPAR-γ, SREBP-1a, and SREBP-1c. The cycling conditions consisted of an initialization step for 10 seconds at 95°C followed by two-step PCR for 40 cycles of 95°C for five seconds (denaturation) and 58°C to 60°C for 30 seconds (annealing/extension). Fluorescence intensity was measured in real time using the optical module. Melt curves were used to determine product specificity. Results were normalized to the level of glyceraldehyde 3-phosphate dehydrogenase gene expression. The analysis of relative gene expression data was conducted using the $2^{-\Delta\Delta CT}$ method. All experiments were repeated twice. The primers used are as follows: TNF-α, forward 5’-CCCAGGGACCTCTCTCTAATC-3’ and reverse 5’-ATGGGCTACAGGCTTCTC-3’; IL-6, forward 5’-ACCCCCAATAATAGGACTGGA-3’ and reverse 5’-GAGAAGGC AAATGGACCGAA-3’; IL-8, forward 5’-GGTGCAGTTTTGCCAAGGAG-3’ and reverse 5’-TGGGGTGGAAAGGTTTGGAG-3’; PPAR-γ, forward 5’-GCCCAGGTTTGCTGAATGTG-3’ and reverse 5’-TGAGGACTCAGGGTGGTTCA-3’; and SREBP-1a, SREBP-1c, forward 5’-TCAAATAGGCCAGGGAAGTCA-3’. Statistical analysis

All data were expressed as the mean ± standard error of the mean. One-way analysis of variance followed by Tukey’s multiple comparison test was used for statistical analysis. The Kruskal–Wallis test was used for comparisons of the four groups. Statistical significance was set at $p < 0.05$.

**REFERENCE**

Supplementary Fig. 1. Experimental design. IGF-1: insulin-like growth factor-1.
Supplementary Fig. 2. The effects of eupatilin on cell proliferation by MTT assay. The effect of eupatilin on SZ95 sebocyte proliferation was determined by MTT assay. Cells were treated with various concentrations of eupatilin (10~100 μM). IGF-1: insulin-like growth factor-1.