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Anti-inflammatory effects of Perillae Herba ethanolic extract against TNF-α/IFN-γ-stimulated human keratinocyte HaCaT cells

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ABSTRACT

Ethnopharmacological relevance: Perillae Herba is a perennial plant that is widely distributed throughout Asia. The leaves of Perillae Herba have been widely used to treat various diseases, such as cold due to wind-cold, headache, cough, abdominal fullness, distention, and fish and crab poisoning.

Materials and methods: To assess the anti-inflammatory activity of Perillae Herba leaf ethanolic extract (PHE) in human keratinocytes, we measured the tumor necrosis factor (TNF)-α/interferon (IFN)-γ-induced mRNA expression and production of proinflammatory chemokines such as thymus and activation-regulated chemokines; regulated on activation, normal T cell expressed and secreted; interleukin (IL)-6; and IL-8 in HaCaT cells. We evaluated the ability of PHE to decrease the expression of proinflammatory marker proteins, such as mitogen-activated protein kinase (MAPK), STAT-1, and NK-κB, using western blot analysis and immunocytochemistry.

Results: PHE inhibited activation of p38, ERK, and JNK and suppressed the phosphorylation of STAT-1 and NK-κB in TNF-α/IFN-γ-stimulated HaCaT cells. PHE also suppressed chemokine mRNA and protein levels in TNF-α/IFN-γ-stimulated HaCaT cells. PHE appears to regulate chemokine formation by inhibiting activation of MAPK, as well as the STAT-1 and NK-κB pathways.

Conclusions: PHE suppresses the expression and production of TNF-α/IFN-γ-stimulated proinflammatory chemokines by blocking NF-κB, STAT-1, and MAPK activation.

1. Introduction

Atopic dermatitis (AD) is a chronic, obstinate, pruritic, and multifactorial inflammatory skin disease that is increasing in prevalence worldwide. In early AD progression, pruritus-induced scratching damages the skin barrier. This damage intensifies skin inflammation, activating an immune response that further worsens AD (Leung et al., 2004). Abnormal expression of proinflammatory mediators, such as chemokines and cytokines, is implicated in various inflammatory skin diseases, including AD. Chronic skin inflammation is characterized by increased epidermal thickness and infiltration of inflammatory cells, such as macrophages, mast cells, and eosinophils.

In the epidermis, 90% of the cells are five-layer keratinocytes. These layers include stratum basale, spinosum, granulosum, lucidum, and corneum (Huang et al., 2016). Keratinocytes secrete a unique profile of chemokines and cytokines following exposure to proinflammatory cytokines, such as regulated on activation, normal T cell expressed and secreted (RANTES); thymus and activation-regulated chemokine (TARC); IL-8; and IL-6 (Werfel, 2009; Yang et al., 2015). In chronic AD skin lesions, Th1 cells produce tumor necrosis factor (TNF)-α and interferon (IFN)-γ (Choi et al., 2013).

Perilla frutescens Britton, var. *acuta* Kudo (Perillae Herba) is a perennial herb of the Lamiaceae family and is widely used as herbal medicine in Korea, Japan, and China (Chen et al., 2015a, 2015b). The leaves of Perillae Herba are used to treat various diseases, such as cold due to wind-cold, headache, cough, abdominal fullness, distention, and fish and crab poisoning (Yu et al., 2016).

Several reports have described the effects of Perillae Herba on inflammatory skin diseases. Perillae Herba leaf ethanolic extract (PHE) improved ultraviolet radiation-induced extracellular matrix damage in human dermal fibroblasts and hairless-skin mice (Bae et al., 2017). PHE prevents AD induced by *Dermatophagoides farinae* extract in the NC/Nga AD model (Komatsu et al., 2016).

In addition, the Perillae Herba leaf displays various anti-inflammatory effects on the airway (Chen et al., 2015a, 2015b), colon (Urushima et al., 2015), nose, and eyes (Takano et al., 2004). Moreover, various compounds have been isolated and identified from Perillae Herba, including flavonoids, volatile oils, fatty acids, triterpenes, and

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phenolic compounds. The main active constituents of Perilla leaves are flavonoids, saponins, polysaccharides, amino acids, and trace elements. Rosmarinic acid and luteolin are main active compounds that appear to exert an anti-AD (Jang et al., 2011) (Osakabe et al., 2004) and anti-pruritic effects (Jeon et al., 2014). However, the mechanism of PHE on AD is unclear.

In this study, we examined the anti-inflammatory effects of PHE on human keratinocytes (HaCaT cells) to determine its potential for treating inflammatory skin diseases.

2. Materials and methods

2.1. Cell culture and reagents

The human keratinocyte cell line HaCaT was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 U /ml penicillin and 100 μg/ml streptomycin). All the experiments used a previously described material and method (Yang et al., 2015).

Caffeic acid, rosmarinic acid and luteolin were obtained from Sigma-Aldrichi (St. Louis, MO, USA). Acetonitrile of HPLC grade was obtained from Fisher (Pittsburgh, PA, USA) and trifluoroacetic acid (TFA) obtained from Sigma-Aldrichi (St. Louis, MO, USA). Ultrapure water was prepared by Puris-Evo UP Water system with Evo-UP Dio VFT and Evo-ROP Dico20 (Mirae ST Co., Ltd., Anyang, Gyeonggi-do, Korea). Ultrapure water (UW) was prepared with a resistivity of 18.2 MΩ cm^{-1} (Puris, Esse-UP Water system, Mirae St Co., Anyang, Korea). All other chemicals used analytical reagent grade. All experiments were carried out at least in triplicate.

2.2. Preparation of PHE and standard solution

PHE leaves were obtained from Oriental Herbal Market (Yeongcheon, Korea). All voucher specimens were deposited in the herbal bank of KM-Based Herbal Drug Development Group, KIOM (Daegu, Korea). To prepare the PHE, dried PHE leaves (30.0 g) were extracted with 300 ml of 70% ethanol in a 40 °C shake-incubator for 24 h. The yield was 9.0873%. The freeze-dried extract powder was dissolved in dimethyl sulfoxide (DMSO) and centrifuged at 14,000 rpm for 10 min. The resulting supernatant was filtered through 0.2 mm syringe membrane filter and then stored at 4 °C prior to use.

The standard stock solutions was dissolving accurately weighed in 100% methanol (1 mg/ml). PHE sample was extracted in 100% methanol (25 mg/ml) by ultrasonic for 30 min. All working solutions were filtered (0.2 μm pore size) syringe membrane filter from Whatman Ltd (Maidstone, UK) before injection into the HPLC-DAD system.

2.3. MTT assay

Cell cytotoxicity was detected using a MTT assay. MTT assay performed as described previously (Yang et al., 2016). Briefly, after attaching the cells to the plate, PHE was treated with several concentration (1–200 μg/ml), and incubated for 24 h. MTT solutions were then added to well and the cells were more incubated for 4 h. The formazan produced in living cells was dissolved in DMSO and read at 570 nm using an ELISA reader (SpectraMax® i3, Molecular Devices, CA, USA).

2.4. Cytokine and chemokine analysis

HaCaT cells (1×10^6 cells/well) in 6 well plates were pretreated with PHE at various concentrations (1, 10, 50, and 100 μg/ml) for 1 h, and stimulated with TNF-α/IFN-γ (each 10 ng/ml) for 24 h at 37 °C in a 5% CO_2 incubator. The supernatant was collected and level of RANTES, TARC, IL-6, and IL-8 were determined using ELISA kits according to the manufacturer's instructions. The plates were read at 450 nm, and inhibitory effect of PHE were determined from a standard curve.

2.5. Western blot analysis

Protein expression was evaluated by Western blot analysis according to standard procedures. The cells were pre-treated with PHE and stimulated with TNF-α/IFN-γ during an incubation for the indicated periods at 37 °C. Western blot was performed following a previous method (Yang et al., 2015).

2.6. RT-PCR analysis

Total RNA was prepared by using the RNA-Spin total RNA extraction kit (iNtRoN, Daejeon, Korea), and according to the manufacturer's protocol. Reverse transcription was carried out in a 20 μl reaction with 1 μg of total RNA transformed into cDNA using AccuPower CycleScript RT premix (Bioneer). The PCR conditions for cDNA synthesis were as follows: 12 cycles of primer annealing at 25 °C for 30 s, cDNA synthesis at 45 °C for 4 min, melting of the secondary structure and cDNA synthesis at 55 °C for 30 s, and heat inactivation at 95 °C for 5 min. Then expression of RANTES, TARC, IL-6, IL-8 and β-actin mRNA was measured by real-time PCR. Primer sequences were as follows: RANTES (forward primer; 5′- GGCACGCCTCGCTGTCATCCTCA-3′, reverse primer; 5′-CTTGATGTGGGCACGGGGCAGTG-3′), TARC (forward primer; 5′-AGGGACCTGCACACAGAGAC-3′, reverse primer; 5′-CTCGA GCTGCGT GGATGTGC-3′), IL-6 (forward primer; 5′-ACCTGAACCTT CCAAAGA-3′, reverse primer; 5′-TTCCTCACTACTCTCAAATCT-3′), IL-8 (forward primer; 5′-AGGGTTGTGGAGAAGTTT-3′, reverse primer; 5′-GGCATCTTCACTGATTCTTG-3′), and β-actin (forward primer; 5′- GCTC TTTTCCAGCCTTCCTT-3′, reverse primer; 5′- GAGCCA GAGCAGTGA TCTCC-3′). Gene expression was quantified by real-time PCR using the AccuPower 2× Greenstar qPCR Master (Bioneer) according to the following protocol: pre-denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s, 60 °C for 30 s, and 72 °C for 30 s. Amplifications were carried out using QuantStudio 6 (LifeScience, ABI, USA), The fold change in the expression of the target gene relative to the control was normalized to β-actin using the $2^{-\Delta\Delta Ct}$ method.

2.7. Immunofluorescence assay

HaCaT cells were seeded in a confocal (glass-bottom) dish, incubated without or with PHE for 1 h, and stimulated with TNF-α/IFN-γ (each 10 ng/ml) for 20 min (Kang et al., 2013). After treatment, cells were fixed with 4% paraformaldehyde in PBS. Fixed cell were blocking with 3% goat serum (Gibco, Grand Island, USA) for 1 h. After an overnight incubation at 4 °C with the anti-p65 and anti-STAT-1 antibody (mouse monoclonal antibody; Cell Signaling). The cells were incubated with secondary Alexa-Fluor-488 and 568-labeled antibody (goat-anti mouse; Life Technologies, USA) for 2 h at room temperature. The nuclei were stained using DAPI (4′,6-diamidino-2-phenylindole, Sigma-Aldrich, USA) for 10 min at room temperature. All samples were then observed using an automated live cell imager (LIONHEART™ FX, BioTek, Vermont, USA). Analysis were using by provided software (Gen5 ™ 3.0, BioTek, Vermont, USA).

2.8. Chromatographic conditions and data analysis

The HPLC analysis was performed on a Dionex HPLC system (Dionex Co., Sunnyvale, CA, USA) that consisted of an ultimate 3000 series a binary pump, an auto-sampler, a column oven and a diode array UV/VIS detector (DAD), all piloted by Dionex Chromelon. The chromatographic separation was performed on an Acclaim C_{18} column (Thermo Fisher Scientific Inc., Waltham, MA, USA) using TFA water (0.1%, v/v); solvent A and acetonitrile; solvent B as mobile phase at a flow rate of 1 ml/min. The mobile phase was eluted with following gradient: 0–3 min: 5% B; 3–63 min: 5–70% B; 63–73 min: 70% B. The column was maintained at 40 °C and injection volume was 10 μl, respectively. The programmed wavelength was set at 320 nm and total run time was 73 min.