International Immunopharmacology 65 (2018) 8–15

Contents lists available at ScienceDirect

International Immunopharmacology

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

(*E*)-phenethyl 3-(3,5-dihydroxy-4-isopropylphenyl) acrylate gel improves DNFB-induced allergic contact hypersensitivity via regulating the balance of Th1/Th2/Th17/Treg cell subsets

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ARTICLE INFO

Keywords:
Allergic contact dermatitis
(*E*)-phenethyl
3-(3,5-dihydroxy-4-isopropylphenyl) acrylate
Immunoregulation
Th1/Th2
Th17/Treg

ABSTRACT

(*E*)-phenethyl 3-(3,5-dihydroxy-4-isopropylphenyl) acrylate gels (THCA354) is a novel polyphenols acrylic acid derivative. To investigate the immunoregulatory mechanisms of THCA354, we established a mouse model of 2,4-dinitrofluorobenzene (DNFB)-induced allergic contact dermatitis (ACD). Responses of Th1, Th2, Th17 and regulatory T cells (Tregs) were determined by flow cytometry, reverse-transcriptase polymerase chain reaction (RT-PCR) and enzyme linked immunosorbent assay (ELISA). Our study found that topical application of THCA354 gel could inhibit ear swelling, reduce inflammatory cell infiltration, down-regulate Th1/Th17 responses and enhance Th2/Treg responses. These findings indicated that THCA354 gel exerted its immunotherapeutic effects by modulating the balance of Th1/Th2/Th17/Treg cell subsets, suggesting that THCA354 gel could be used as a promising drug candidate for intervention of ACD.

1. Introduction

As a common skin inflammatory disease, allergic contact dermatitis (ACD) corresponds to a delayed-type hypersensitivity (DTH) response with a skin inflammation mediated by T lymphocytes [1]. It is an inflammatory reaction characterized by the activation of sensitized T lymphocytes in combination with the corresponding antigens and the release of various cytokines, leading to monocyte infiltration as well as tissue degeneration and necrosis. Many studies have shown that DTH responses are mainly mediated by CD4+ T cells [2–4]. CD4+ T cells are subdivided into T-helper Th1 and Th2 cells. The balance between the Th1- and Th2-dominant immunities (Th1/Th2 balance) plays a crucial role in the development and maintenance of various immune disorders [5]. Moreover, the abnormal secretion of cytokines, which is caused by the imbalance of T cell subsets, plays an important role in development of ACD. Recently, Th17 cells, as a novel subset of CD4+ T cells different from Th1 and Th2 cells, have been shown to induce severe autoimmune responses by secreting inflammatory mediator IL-17 [6,7]. IL-17 plays a fundamental role in activating T cells in allergen-specific T-cell-mediated immune responses, and IL-17 efficiently amplifies the allergic reaction by rendering T cells accessible to recruitment in skin inflammation site in ACD [8]. Besides, CD4+ CD25+ regulatory T cells (Tregs) are identified to have a pivotal role in the resolution of DTH responses [9,10]. Treg activity is regulated by a specific transcription factor, the forkhead/winged helix (Foxp3) transcription factor. Since Th17 has pro-inflammatory properties and Tregs have anti-inflammatory effects, regulating the balance between Th17/Tregs may provide a new way to effectively treat ACD.

Up to date, the main drugs for ACD treatment, such as glucocorticoid, H1 receptor antagonist and NSAIDS, have severe adverse reactions and undesirable side effects [11]. Therefore, a new drug, which is effective in dermatitis and has fewer side effects, is badly needed. In an early drug screening study of our team, we have identified the best active pharmaceutical ingredients by anti-inflammatory experiments, and a new type of polyphenol acrylic acid derivatives, (*E*)-phenenthyl-3-(3,5-dihydroxy-4-isopropyl phenyl) acrylate (THCA354) (Fig. 1), has been synthesized.

Existing studies have shown that the phenolic hydroxyl and double bond in polyphenol compound structure have an effect on anti-inflammatory immune adjustment [12,13]. Early pharmacodynamic studies have confirmed that THCA354 gel can effectively inhibit the mouse auricular swelling induced by terephthalic acid (TPA) and xylene, and it can also effectively reduce the expressions of inflammatory factors IL-1β, TNF-α and IL-6 in swelling tissue, suggesting that THCA354 gel has

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https://doi.org/10.1016/j.intimp.2018.09.032
Received 29 March 2018; Received in revised form 7 September 2018; Accepted 22 September 2018

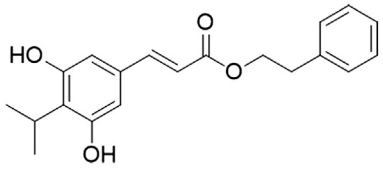

Fig. 1. The structure of THCA354, $C_{20}H_{22}O_4$, MW = 326.14.

significant anti-inflammatory activity [14].

In the present study, we established a mouse model of ACD using 2,4-dinitrofluorobenzene (DNFB), and topical THCA354 gel was used to intervene. By investigating the changes of tissue thickness and the degree of lymphocyte infiltration in mouse ear tissue, as well as by detecting Th1, Th2, Th17, Treg-associated cytokines and specific nuclear transcription factors and the frequency of each cell subsets, it was found that THCA354 gel could alter the immune balance of Th1/Th2/Th17/Treg subsets. These findings indicated that THCA354 gel might be a useful drug candidate for the treatment of ACD.

2. Materials and methods

2.1. Reagents and animals

THCA354 (≥99% purity) was synthesized by our group. THCA354 gels (5%) were produced in our laboratory. 1-Fluoro-2,4-dinitrobenzene (DNFB) was purchased from Sigma. Tissue total protein lysis buffer was obtained from Thermo Fisher. The total protein extraction kit was provided by Beyotime. ELISA kits were supplied from DAKEWE. Trizol kit was purchased from Initrogen. RNA extraction kit was obtained from Tiangen Biotech, Beijing. RNA reverse transcription kit was provided by TaKaRa. Primers for quantitative real-time RT-PCR were synthesized by TaKaRa. Semi-quantitative PCR dye (SYBR Green I) was purchased from TAKARA. Anti-mouse CD3e-FITC, CD4-PE, IFN-γ-APC, IL-4-APC, Foxp3-FITC, CD25-APC and IL-17A-APC were purchased from eBioscience. Ionomycin calcium salt was supplied by from *Streptomyces conglobatus* was purchased from J&K Scientific. Protein transport inhibitor cocktail was obtained from BD Biosciences.

Female BALB/c mice (weighing 16–18 g) were purchased from Beijing HFK Bioscience. Mice were housed under standard conditions (25 °C) with a 12/12-h light/dark cycle and given free access to rodent food and water. All experimental protocols were approved by the institutional animal care and use committee of Third Military Medical University.

2.2. Induction and assessment of contact dermatitis

To induce ACD, mice were sensitized on the shaved ventral abdomen on day 0 by applying 75 μL of 0.5% DNFB (dissolved in a mixture of acetone and olive oil, 4:1) [15]. Four days later (day 4), the inner and outer surfaces of the right ears were challenged with 20 μL 0.5% DNFB, and the left ears were painted with mixture of acetone and olive oil (4:1, v/v) in the same way. On day 5, mice were re-challenged with 20 μL 0.4% DNFB in the right ears, and only mixture of acetone and olive oil (4:1, v/v) was applied in the left ears. Subsequently, ear thickness was determined by a dial thickness gauge (Mitutoyo) before challenge and 24 h after re-challenge. The ear swelling was calculated as the increase in ear thickness.

2.3. Drug treatment

A total of 50 BALB/c mice were randomly and evenly divided into five groups (n = 10) as follows: control group, vehicle group and three THCA354 gel groups. THCA354 (5.0%) was directly added to gel base consisting of carbopol (2.0%), propylene glycoll (5.0%), glycerol (5.0%) and water. Topical treatment was performed once daily for 2 days (days 5 and 6) with 12, 24 and 48 mg THCA354 gel (the amount of THCA354 in the gel was 0.6, 1.2 and 2.4 mg respectively) or vehicle (gel matrix, 48 mg).

2.4. Histopathological examination

Mouse ears were collected and subjected to histological examination. These ears were fixed in 10% formalin solution for 24 h, followed by successive dehydration, embedding, sectioning and dewaxing. Subsequently, paraffin-embedded sections were stained with hematoxylin and eosin (H&E), and examined by Motic digital microscope (Olympus). Images were selected from the appropriate representative areas.

2.5. Flow cytometric analysis

T cells from mouse mandibular lymph nodes (LNs) were collected as previously described [16]. Briefly, cells were surface stained with anti-CD4 and anti-CD25 antibodies in the dark at 4 °C for 30 min. For intracellular staining, cells were permeabilized using a fixed/permeabilized solution and incubated with antibodies against Foxp3. Cells were washed for three times and resuspended in phosphate buffered saline (PBS). The results were expressed as the percentage of each cytokine-producing cell population in a total population of CD25+ cells.

For analysis of intracellular cytokine production, freshly isolated lymphocytes were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS) at a density of 1.2×10^6 cells/mL at 37 °C for 6 h. During 6-h incubation, cells were stimulated with a combination of phorbol 12-myristate 13-acetate (PMA, 10 ng/mL) and ionomycin (1 μg/μL). Then the cells were washed and resuspended in Cell WASH. Cells were directly stained with FITC-conjugated anti-CD4 monoclonal antibody in the dark at 4 °C for 30 min, and fixed with FACS lysing solution for 10 min. Next, cells were washed, pre-incubated with FACS permeabilizing solution for 10 min, and incubated with PE-conjugated monoclonal antibodies against IL-4, IL-17, IFN-γ and their isotype-matched controls in the dark at 4 °C for 30 min. Cells were washed twice and resuspended in phosphate buffered saline (PBS). Flow cytometry was performed using FACS Calibur flow cytometer and Cell Quest software (Beckman Coulter). The results were expressed as the percentage of each cytokine-producing cell population in a total population of CD4+ cells.

2.6. ELISA

Briefly, 1 mL T-PER Tissue protein extraction reagent was added to ear tissue, and then the tissue was homogenized by ultrasound. Tissue homogenates were then centrifuged, and supernatants were collected for cytokine determination. Total protein contents in tissue samples were determined using total protein extraction kit according to the manufacturer's instructions. The contents of inflammatory cytokines were examined using corresponding ELISA kits.

2.7. Semiquantitative RT-PCR

Total RNA was extracted from right ear tissue using tissue RNA extraction kit and reversely transcribed into cDNA using the RNA reverse transcription kit. Expressions of target genes were examined by real-time PCR on a CFX Connect (Bio-Rad) detection system using specific primers (Table 1). Briefly, after an initial denaturation step at 95 °C for 30 s, amplifications were carried out with 42 cycles at a melting temperature of 95 °C for 10 s, an annealing temperature of 60 °C for 20 s and an extension temperature of 72 °C for 20 s.

2.8. Statistical analysis

Data were expressed as means ± standard deviations. Correlation