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Foeniculum vulgare extract and its constituent, *trans*-anethole, inhibit UV-induced melanogenesis *via* ORAI1 channel inhibition

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ABSTRACT

Background: Ultraviolet radiation exposure is the most important cause of extrinsic skin aging (photoaging), which causes skin wrinkling and hyperpigmentation. Although many factors are involved in the photoaging process, calcium release-activated calcium channel protein 1 (ORAI1) has been reported to be involved in UV-induced melanogenesis.
Objective: The aim of the present study was to find inhibitory effects of the extract of *Foeniculum vulgare* (fennel) fruits on ORAI1 ion channels and UV-induced melanogenesis in melanoma cells and to identify its active constituents.
Methods: Active compounds were isolated and quantitatively analyzed. An electrophysiological assay was performed by using the whole-cell patch-clamp technique. Intracellular free calcium concentration was measured by Fura-2. Tyrosinase activity was evaluated by levodopa colorimetry. Effects of the most active compound on cell viability of murine B16F10 melanoma cells and inhibition of melanin content after UVB irradiation were determined.
Results: F. vulgare fruits extract and its hexane fraction strongly blocked ORAI1 currents and tyrosinase activity and significantly inhibited UV-induced melanogenesis. Of the 13 compounds isolated from the hexane fraction, *trans*-anethole (TA) exhibited inhibitory effects on ORAI1 ($IC_{50} = 8.954 \pm 1.36\ \mu M$) and increased cytoplasmic Ca^{2+} concentrations in response. TA inhibited UV-induced melanogenesis without affecting tyrosinase activity.
Conclusion: Our findings suggest that the fruits extract of *F. vulgare* and its active constituent, TA, provide a possible novel approach for treating and preventing UV-induced melanogenesis.

1. Introduction

Melanocytes are located in the basal layer of the epidermis and protect the skin from ultraviolet radiation (UVR) [1]. Melanocytes are surrounded by keratinocytes and communicate with approximately 30–40 keratinocytes via dendritic processes necessary for melanin transfer into keratinocytes [1]. When the skin is exposed to UVR, melanocytes and keratinocytes produce and secrete hormones such as proopiomelanocortin-derived peptides, including α-melanocyte-stimulating hormone (α-MSH) and adrenocorticotropic hormone (ACTH), and endothelin-1 (ET-1), which can indirectly regulate melanocyte proliferation, melanogenesis, and melanocytic dendrite formation [2,3]. Recent publications have reported that opsins, which are G-protein coupled receptors, are expressed in human epidermal melanocytes (HEMs), and that HEMs directly activated by opsins mediate a phototransduction mechanism to increase levels of melanin synthesis at the early time points of UVR exposure [4,5]. Direct and indirect activation of melanocytes by UVR share a common signaling pathway to melanin synthesis and proliferation [6]. Activation of hormones and opsin receptors in turn leads to the activation of a G_q protein-coupled signaling cascade, which induces phospholipase C to produce inositol 1,4,5-trisphosphate ($InsP_3$, IP_3) and diacylglycerol (DAG). IP_3 then binds to IP_3 receptors on the endoplasmic reticulum (ER) and causes depletion of ER Ca^{2+} stores; this subsequently activates store-operated calcium entry (SOCE), which is mediated by the calcium release-activated calcium

Abbreviations: CCE, capacitative calcium entry; ER, endoplasmic reticulum; ET-1, endothelin-1; ORAI1, calcium release-activated calcium channel protein 1; SOCE, store-operated Ca^{2+} entry pathways; STIM1, stromal interaction molecule 1; TA, *trans*-anethole.

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channel protein 1 (ORAI1) [7,8]. This early signaling cascade eventually increases intracellular Ca^{2+} concentrations, which can regulate melanogenesis by diverse signaling pathways. Ca^{2+} can modulate melanin synthesis by activating PKCβ to increase tyrosinase activity, act in the melanosome by synthesis and storage of pigment, and also trigger melanin transfer to the extracelluar surface [6]. Because the importance of intracellular Ca^{2+} signaling in melanogenesis has been recently shown, many pharmaceutical and cosmetic companies have been developing depigmenting agents by targeting the enzyme tyrosinase, which converts L-tyrosine to dopaquinone, a precursor of eumelanin (black-brown) and pheomelanin (yellow-red) [1]. The majority of research on inhibitors of tyrosinase has been conducted after 2000, and many of these studies were performed with tyrosinase inhibitors from natural sources, mostly of Asian origin [9]. Accordingly, this study focused on assessing effective intracellular Ca^{2+} signaling modulation by a natural substance.

Foeniculum vulgare (fennel, Apiaceae) is a highly flavorful herb and has long been used as a spice and vegetable. It is also prescribed by traditional medicine practitioners in Korea and China for the treatment of digestive disorders [10]. Its fruit has been reported to have anti-inflammatory and antioxidant [6], antimicrobial [11], antiulcerative [12], hepatoprotective [13], and antiplatelet effects [14]. To date, many constituents have been isolated from this plant, including monoterpenes, phenylpropanoids, aromatics, and coumarins [15]. As part of our ongoing search for ion channel-inhibiting agents from natural sources [16,17], we investigated whether the fruits of *F. vulgare* can inhibit human ORAI1 (hORAI1) channels, which are known to contribute to UV induced melanogenesis.

Some researchers have focused only on tyrosinase activity; however, in this study, by using whole-cell patch-clamp techniques and by determining intracellular Ca^{2+} concentrations, we evaluated the effects of *F. vulgare* fruits extract, its fractions, and 13 constituents isolated by bioassay-guided fractionation on ORAI1 channel activity. We also confirmed anti-melanogenesis in murine melanoma cells through effective ORAI1 channel inhibition.

2. Materials and methods

2.1. General experimental procedures

The fruits of *F. vulgare* were purchased from a drugstore in Gyeongju, Korea, and identified by Professor Jae-Hyun Lee (College of Oriental Medicine, Dongguk University, Gyeongju, Korea). A voucher specimen (SKKU-Ph-08-18) has been deposited at the Herbarium of the School of Pharmacy, Sungkyunkwan University, Suwon, Korea. All chemicals and drugs were obtained from Sigma-Aldrich (St. Louis, MO, USA), except *N*-[4-[3,5-bis(trifluoromethyl)-1*H*-pyrazol-1-yl]phenyl]-4-methyl-1,2,3-thiadiazole-5-carboxamide (BTP2), which was purchased from Tocris Bioscience (Bristol, UK). Stock solutions were prepared in DMSO at a concentration of 20 mM IP_3, 10 mM BTP2, and were kept at −20 °C. ^1H NMR (500 MHz) spectra were recorded using a Varian Unity INOVA 500 spectrophotometer (Varian, Atlanta, GA, USA). GC–MS data were obtained using a 6890 GC/5973N MSD (Agilent Technologies, Santa Clara, CA, USA) at the Korea Basic Science Institute (Western Seoul Center).

2.2. Extraction and isolation

Air-dried and powdered plant materials (500 g) were chopped and extracted with hot methanol for 3 h to obtain crude extract, which was then consecutively partitioned with *n*-hexane, methylene chloride, ethyl acetate, and *n*-butanol to afford the corresponding fractions. The hexane fraction was subjected to silica gel column chromatographic separation to yield nine subfractions. Subfraction F1 was rechromatographed on a Si column or LiChroprep RP-18 column, affording *trans*-anethole (**1**), α-pinene (**2**), estragole (**3**), myrcene (**4**), and limonene (**5**). Subfractions F5 and F6 were combined, then rechromatographed on a Si column or RP-18 column to yield sabinene (**6**), 1,8-cineole (**7**), γ-terpinene (**8**), β-pinene (**9**), and fenchone (**10**). Finally, subfraction F9 was rechromatographed on an RP-18 column to furnish the aromatic compounds *p*-anisaldehyde (**11**), *p*-anisylacetone (**12**), and *p*-cymene (**13**). All compounds (Fig. 3) were identified by comparison with their previously reported NMR spectral data [18–20]. The purities of all compounds were determined by GC–MS using an HP-5MS capillary column to be 95.8-99.4%. A detailed isolation procedure including compounds purity data have been described in the Supplementary material.

2.3. Quantitative analysis of trans-anethole (TA) by HPLC

The constituents of the hexane fraction have been characterized by RP-HPLC (system: Waters 717 autosampler, detector: Waters 2489 UV, column: Akzo Nobel KR100-5C-18, flow rate: 1 mL/min, injection volumn: 10 μL, temp: 30 °C), exhibiting TA (**1**) (Rt = 41.49 min, 1.70 ± 0.51%), estragole (**3**) (Rt = 40.65 min, 0.23 ± 0.07%), and *p*-anisaldehyde (**11**) (Rt = 26.34 min, 0.15 ± 0.03%) as major compounds which would be selected as marker compounds. The HPLC method for quantitative estimation of *trans*-anethole in the hexane fraction was validated using usual methods: a good linearity was achieved in the linear range of 4.00–100.00 μg/mL with the regression equation of $y = 13710x + 13010$ ($R^2 = 0.9994$) and limit of quantification (LOQ) value of 1.478863 μg/mL.

2.4. Cell culture

HEK293T (American Type Culture Collection) and B16F10 cells (Korean Cell Line Bank) were maintained in Dulbecco's modified Eagle's medium (Life Technologies, Carlsbad, CA, USA) at 37 °C in a humidified incubator containing 70% air, 20% O_2, and 10% CO_2. All media were supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were sub-cultured every 2–3 days.

2.5. Transfection of hORAI1 and STIM1

For the patch-clamp study, the cells were transferred into 25-cm^2 cell culture flasks (Thermo Fisher Scientific, Waltham, MA, USA) one day before transfection. To express ion channels in HEK293T cells, cells were transiently transfected with a mammalian expression vector carrying hORAI1 and human stromal interaction molecule 1 (hSTIM1) using Lipofectamine Plus reagent (Life Technologies) according to the manufacturer's instructions. In all transfection studies, HEK293T cells were co-transfected with pEGFP-N1 plasmid to visualize transfected cells. Average transfection efficiency was approximately 40%, and those cells showed a green fluorescence under a phase-contrast fluorescence microscope (Model Ti-S, Nikon, Tokyo, Japan) from GFP expression. Epifluorescence from cells expressing EGFP was excited by a 130 W xenon lamp using standard fluorescence filters (420–490 nm excitation filter, 510 nm dichroic mirror, 520 nm long pass emission filter) (Nikon). During the whole-cell patch-clamp study, only GFP-positive cells were used. To record hORAI1 currents (I_{ORAI1}), hORAI1, hSTIM1, and pEGFP-N1 were triple-transfected into HEK293T cells at a ratio of 4.5:4.5:1. Cloned genes of hORAI1 and hSTIM1 were generously donated by Dr. Sung Joon Kim, Seoul National University, Korea. Experiments were performed 24–36 h after transfection.

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