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Emodin isolated from *Polygoni Multiflori Ramulus* inhibits melanogenesis through the liver X receptor-mediated pathway

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ABSTRACT

Melanogenesis is a physiological process that results in the synthesis of melanin pigments, which play a crucial protective role against skin photocarcinogenesis. We investigated the effects of a *Polygoni Multiflori Ramulus* extract on melanogenesis and isolated emodin from *Polygoni Multiflori* as an active compound. In addition, the possible mechanisms of action were examined. We found that emodin inhibited both melanin content and tyrosinase activity concentration and time dependently. Tyrosinase, tyrosinase-related protein (TRP)-1, and TRP-2 mRNA levels decreased following emodin treatment. However, while the mRNA levels of microphthalmia-associated transcription factor (MITF) were not affected by emodin, emodin reduced MITF protein levels. Furthermore, expression of the liver X-receptor (LXR) α gene, but not the LXR β gene was upregulated by emodin. Moreover, emodin regulated melanogenesis by promoting degradation of the MITF protein by upregulating the LXR α gene. The emodin effects on MITF was found to be mediated by phosphorylation of p42/44 MAPK. Taken together, these findings indicate that the inhibition of melanogenesis by emodin occurs through reduced MITF protein expression, which is mediated by upregulation of the LXR α gene and suggest that emodin may be useful as a hyperpigmentation inhibitor.

1. Introduction

Melanogenesis is the melanin pigment synthetic process and plays a critical protective role against skin UV irradiation and oxidative stressors. However, an abnormally excessive accumulation of melanin is closely related to psychosocial and cosmetic problems. Hyperpigmentation, which is represented as melasma, freckles and dark spots, is caused by various triggers, including ultraviolet irradiation, inflammation, abnormal release of α-melanocyte stimulating hormone, and rubbing of the skin [6,11,15,31].

Liver X receptors (LXRs) are ligand-activated nuclear receptors and include two forms, such as LXR α and LXR β. LXR α is highly expressed in the liver, whereas LXR β is more ubiquitous [26]. LXRs play pivotal roles in lipid metabolism and cholesterol homeostasis and are involved in suppressing inflammatory reactions [3,1].

LXRs are expressed in skin tissues, such as sebaceous glands, hair follicles, epidermal keratinocytes, and fibroblasts [2,7,27]. Activating LXR exerts diverse activities, including induction of keratinocyte differentiation [16], lipid synthesis and differentiation into sebocytes, and inhibition of hair growth [27,8]. LXRs are highly expressed in the melanocytes from perilesional skin of patients with vitiligo [17]. In addition, Lee et al. (2013) demonstrated that activating LXR inhibits melanogenesis, suggesting that LXRs are

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involved in the regulation of melanin production [19].

A cell-based, compound library screen that was intentionally biased to select compounds with relatively low toxicity and high activity was conducted to determine how to mitigate hyperpigmented symptoms. A LXRE (liver X receptors responsive element)-luciferase reporter assay was used as the screening tool to evaluate the depigmenting effects. During this screening, *Polygoni Multiflori Ramulus* and its isolated compound, emodin, were selected as candidate depigmenting agents.

Emodin (3-methyl-1,6,8-trihydroxyanthraquinone) is an anthraquinone derivative that possesses vasorelaxative, immunosuppressive, hepatoprotective, and anti-tumor activities [5,9,18,24,28]. Emodin is a tyrosine kinase inhibitor that restricts the activities of the $p56^{lck}$, *HER*-2/neu, and *ras*-oncogenes, all of which are involved in cell proliferation, transformation, and differentiation signaling pathways [4,12,32,33]. Emodin also induces apoptosis in several cancer cell types [14,20,29] and inhibits tumor necrosis factor-α-induced matrix metalloproteinase-1 expression by suppressing activator protein-1 [21]. Additionally, emodin inhibits melanogenesis in human melanocytes by suppressing Kit signaling [22]. Therefore, this prompted us to investigate the effects of emodin on melanogenesis and the signaling pathways involved in melanogenesis.

In the present study, we present evidence that emodin inhibits melanogenesis by promoting degradation of the microphthalmia-associated transcription factor (MITF) protein by upregulating LXR α expression.

2. Materials and methods

2.1. Materials

TRIzol reagent, random primers, and Moloney murine leukemia virus reverse transcriptase were obtained from Invitrogen (Carlsbad, CA, USA). All TaqMan reverse transcription polymerase chain reaction (RT-PCR) reagents, including the primers and probes, were purchased from Applied Biosystems (Carlsbad, CA, USA). Anti-LXRα and anti-LXRβ were purchased from Abcam (Cambridge, MA, USA). Emodin (99%) was isolated and purified from *Polygoni Multiflori Ramulus* and then dissolved in dimethyl sulfoxide (DMSO). Anti-β-actin, anti-tyrosinase, TO901317, and forskolin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-tyrosinase-related protein (TRP)-1, anti-TRP-2, and anti-MITF were purchased from NeoMarkers (Fremont, CA, USA). Anti-DDK monoclonal antibody and LXRα (NR1H3 human cDNA ORF clone) were purchased from OriGene Technologies Inc (Rockville, MD, USA).

2.2. Preparation of the Polygoni Multiflori Ramulus extract and emodin

2.2.1. Preparation of the Polygoni Multiflori Ramulus extract

The air-dried powdered roots (1 kg) of *Polygoni Multiflori Ramulus* were extracted with 70% aqueous ethanol at room temperature. The 70% ethanol extract was concentrated under reduced pressure, lyophilized to yield 120 g of extract, and stored at −20 °C. The *Polygoni Multiflori Ramulus* extract was dissolved in 10% DMSO and used.

2.2.2. Isolation and identification of emodin

The *Polygoni Multiflori Ramulus* extract was suspended in H_2O and fractionated successively with equal volumes of *n*-hexane, $CHCl_3$, EtOAc, and *n*-BuOH. The EtOAc fraction was evaporated to give 4.5 g of dry residue. The EtOAc fraction was subjected to vacuum liquid chromatography (silica-gel, 7.0 × 15.0 cm) and eluted with a $CHCl_3$/MeOH gradient system to afford 10 fractions. Subfraction 4 (1.19 g) was isolated by medium-pressure liquid chromatography (RediSep, C18, 130 g, UV at 280 nm; flow rate, 24 mL/min) eluted with MeOH/H_2O gradient system to afford seven fractions. Subfraction 4 (120.2 mg) was purified successively on a Sephadex LH-20 column (Pharmacia, Uppsala, Sweden) eluted with chloroform: methanol (6: 4, v/v) to give emodin (20.1 mg).

The chemical structure was determined by comparing ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) spectra with trimethylsilane as an internal standard (Varian Inova NMR, 400 MHz; Varian Technologies, Palo Alto, CA, USA).

The compound was identified as emodin (C15H10O5, MW: 270.24, yellow powder). IR (KBr) υ_{max} 3440 (OH), 1652, 1630(CO), 1560, 1539, 1425, 1371, 1298, 1060, 850 cm^{-1}; 1H NMR (400 MHz, CD3OD): δ 2.41 (3H, s, H-11), δ 6.56 (1H, d, $J = 2.4$ Hz, H-7), δ 7.10 (1H, d, $J = 1.4$ Hz, H-2), δ 7.18 (1H, d, $J = 2.4$ Hz, H-5), δ 7.48 (1H, br s, $J = 1.4$ Hz, H-4); 13C-NMR (100 MHz, CD3OD); δ 22.1 (C-11), δ 108.7 (C-7), δ 109.8 (C-5), δ 110.0 (C-9a), δ 114.3 (C-8a), δ 121.2 (C-4), δ 124.9 (C-2), δ 133.8 (C-4a), δ 136.3 (C-10a), δ 149.1 (C-3), δ 162.1 (C-8), δ 165.6 (C-1), δ 167.0 (C-6), δ 182.3 (C-10), δ 191.1 (C-9).

2.3. Cell culture

Human epidermal melanocytes were obtained from Cascade Biologics (Portland, OR, USA) and maintained in Medium 154 (Cascade Biologics) supplemented with 0.2% (v/v) bovine pituitary extract, 0.5% (v/v) fetal bovine serum, 5 μg/ml bovine insulin, 5 μg/ml bovine transferrin, 3 ng/ml basic fibroblast growth factor, 0.18 μg/ml hydrocortisone, 3 μg/ml heparin, and 10 ng/ml phorbol 12-myristate 13-acetate at 37 °C in a humidified atmosphere containing 95% air/5% CO_2.

2.4. Melanin content assay

Melanin content in the cultured melanocytes was measured based on the method described by Huh et al. (2010) [10]. Briefly, cell pellets were solubilized in boiled 1 N NaOH (60 °C) for 1 h, and color was analyzed at 475 nm. Melanin content was represented by the absorbance/μg protein in the cell extract.

2.5. Measurements of cellular tyrosinase activity

Cellular tyrosinase activity was determined as described previously [25], with slight modifications. Briefly, Human melanocytes were treated with emodin and the cells were washed twice with phosphate-buffered saline (PBS) and homogenized with 50 mM sodium phosphate (pH 6.8) buffer containing 1% Triton X-100, and 1 mM PMSF at 4 °C with 30 repeated strokes in a Dounce homogenizer. Detergent was used to release the membrane-bound tyrosinase from melanosomes. The lysates were centrifuged at 15,000 rpm for 15 min to obtain the supernatant as the source of crude cellular tyrosinase. The protein content in the supernatant was determined using a Bradford assay with BSA as the protein standard. Intracellular tyrosinase activity was then determined as follows: 200 μl of the reaction mixture contained 50 mM sodium phosphate buffer (pH 6.8), 2 mM L-DOPA and 100 μg of supernatant protein, and was incubated at 37 °C for 15 min, following which the dopachrome formation was monitored by measuring absorbance at a wavelength of 470 nm.

2.6. Cell viability assay

General viability of the cultured cells was determined by reducing 3-(4,5-dimetylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan. After emodin treatment, the cells were incubated at 37 °C under 5% CO_2. A MTT solution (1 mg/ml in