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Scopoletin from *Cirsium setidens* Increases Melanin Synthesis via CREB Phosphorylation in B16F10 Cells

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In this study, we isolated scopoletin from *Cirsium setidens* Nakai (Compositae) and tested its effects on melanogenesis. Scopoletin was not toxic to cells at concentrations less than 50 μM and increased melanin synthesis in a dose-dependent manner. As melanin synthesis increased, scopoletin stimulated the total tyrosinase activity, the rate-limiting enzyme of melanogenesis. In a cell-free system, however, scopoletin did not increase tyrosinase activity, indicating that scopoletin is not a direct activator of tyrosinase. Furthermore, Western blot analysis showed that scopoletin stimulated the production of microphthalmia-associated transcription factor (MITF) and tyrosinase expression via cAMP response element-binding protein (CREB) phosphorylation in a dose-dependent manner. Based on these results, preclinical and clinical studies are needed to assess the use of scopoletin for the treatment of vitiligo.

Key Words: *Cirsium setidens*, CREB, MITF, Scopoletin, Tyrosinase

INTRODUCTION

Skin color is determined by the amount and distribution of melanin pigment produced in melanocytes of the skin. Melanin helps protect skin against UV radiation [1,2]. Vitiligo is a skin disease characterized by depigmented sections of skin, and occurs when cutaneous melanocytes die or become non-functional [3].

Cirsium setidens Nakai (Compositae) has been widely used as a traditional medicine to treat edema, hemorrhage, and diabetes [4]; however, the effects of *C. setidens* on melanogenesis have not been investigated. Recently, we isolated pectolinarin, scopoletin, acacetin, cirsimarin, and cirsimaritin from *C. setidens*. Through preliminary screening, we found that scopoletin increased melanin synthesis in B16F10 cells. Scopoletin, 7-hydroxy-6-methoxychromen-2-one, is a naturally-derived coumarin and a phytoalexin. It has been isolated from many different plant families including Gramineae, Liliaceae, Musaceae, Compositae, Convolvulaceae, and Loasaceae [5,6]. Scopoletin has been reported to inhibit acetylcholinesterase [7], to have antioxidant properties [8] and anti-inflammatory effects [9], and to reduce insulin resistance [10]; however, the effects of scopoletin on melanin synthesis have yet to be reported.

Melanin production is regulated by three melanocyte-specific enzymes: tyrosinase, tyrosinase related protein-1 (TRP-1) and tyrosinase related protein-2 (TRP-2). Tyrosinase is the rate-limiting enzyme in melanogenesis and catalyzes the hydroxylation of tyrosine to 3,4-hydroxyphenylalanine (DOPA) as well as the oxidation of DOPA to dopaquinone [11]. Due to these various functions, tyrosinase plays an important role in regulation of melanin synthesis [1]. The expression of tyrosinase is controlled by a specific transcription factor called microphthalmia-associated transcription factor (MITF) [12,13]. MITF expression is in turn regulated by the phosphorylation of cAMP-responsive element binding protein (CREB) [14].

Because vitiligo is a hypopigmentary disorder, scopoletin may be a therapeutic option to increase melanin synthesis. In the present study, we tested the effects of scopoletin isolated from *C. setidens* on melanogenesis and found that scopoletin increased melanin synthesis. Hence, the purpose of this study was to examine the potential of scopoletin as a therapy for vitiligo and to achieve a mechanistic understanding of its actions.

METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM), antibiotic

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ABBREVIATIONS: CREB, cAMP-response element binding protein; α-MSH, α-melanocyte-stimulating hormone; MITF, microphthalmia-associated transcription factor; TRP, tyrosinase-related protein.

(penicillin, streptomycin), and trypsin-EDTA were purchased from WelGENE (Daegu, Korea). Fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT, USA). Kojic acid, α-melanocyte-stimulating hormone (α-MSH), mushroom tyrosinase, and 3,4-dihydroxy-L-phenylalanine (L-DOPA) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Antibody specific for phospho-CREB (ser133, #9198) and total-CREB (#9197) was from Cell Signaling Technology (Beverly, MA, USA). Antibodies specific for tyrosinase (C-19) and actin (I-19) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), and microphthalmia (MITF) Ab-1 (C5, MS-771-P0) was obtained from NeoMarkers (Fremont, CA, USA). Secondary antibodies specific for anti-goat IgG (PI-9500), anti-mouse IgG (PI-2000), and anti-rabbit IgG (PI-1000) were purchased from Vector Laboratories (Burlingame, CA, USA).

Cell culture

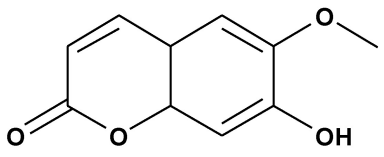
B16F10 murine melanoma cells were obtained from the Korean Cell Line Bank (Seoul, Korea). The cells were maintained in DMEM supplemented with 10% (v/v) FBS, 50 μg/ml of streptomycin, and 50 μg/ml of penicillin in 5% CO_2 at 37°C.

Preparation of C. setidens extracts and isolation of scopoletin

The dried aerial parts of *C. setidens* were extracted exhaustively with 80% ethanol. The crude syrup was separated into five fractions corresponding to n-hexane, chloroform, ethyl acetate, butanol, and water layers, respectively. Scopoletin was isolated from the ethyl acetate layer. Colorless needles crystallized from the ethyl acetate layer and had a mp of 204~205°C. NMR analysis confirmed that the compound was scopoletin (Fig. 1) [15]. 1H-NMR: 600 MHz-CD_3OD, δ 7.90 (H, d, J=9.4 Hz, H-4), 7.20 (H, s, H-8), 6.75 (H, s, H-5), 6.21 (H, d, J=9.5 Hz, H-3), 3.80 (3H, s, OCH_3); ^{13}C-NMR: 150 MHz, CD_3OD, δ ppm.

Cell viability assay

Cell viability was determined using a crystal violet assay. After incubating cells with scopoletin for 24 h in serum-free media, the media was removed, and the cells were stained with 0.1% crystal violet in 10% ethanol for 5 min at room temperature. The cells were then rinsed four times with distilled water, and the crystal violet retained by adherent cells was extracted with 95% ethanol. Absorbance was determined at 590 nm using an ELISA reader (VERSAMax; Molecular Devices, Sunnyvale, CA, USA).



Scopoletin

Fig. 1. Structure of scopoletin.

Measurement of melanin content

Extracellular melanin release was measured as described previously [16], with a slight modification. Briefly, B16F10 cells were incubated at a density of 5×10^4 cells in 6-well plates overnight. Cells were treated with increasing concentrations of scopoletin in phenol red-free DMEM for 3 days and α-MSH (1 μM) was used as a positive control. Two hundred μl aliquots of media were then placed in 96-well plates and the optical density (OD) of each culture well was measured using an ELISA reader at 400 nm. The number of cells was then counted using a hemocytometer. Melanin production was expressed as a percentage of the control.

Tyrosinase activity

Tyrosinase activity was assayed as DOPA oxidase activity. B16F10 cells were incubated at a density of 5×10^4 cells in 6-well plates, and incubated with scopoletin in DMEM for 3 days. Cells were washed with PBS and lysed with lysis buffer (0.1 M phosphate buffer [pH 6.8] containing 1% Triton X-100). Cells were then disrupted by freeze-thawing, and lysates were clarified by centrifugation at 13,000 rpm for 30 min. After quantifying protein content using a protein assay kit (Bio-Rad, Hercules, CA, USA), the cell lysates were adjusted with lysis buffer to the same protein concentration, 90 μl of each lysate was pipetted into the wells of a 96-well plate, and 10 μl of 10 mM L-DOPA was added. Control wells contained 90 μl of lysis buffer and 10 μl of 10 mM L-DOPA. After incubation at 37°C for 20 min, DOPAchrome formation was monitored by measuring absorbance at 475 nm using an ELISA reader.

A cell-free assay system was used to determine the direct effect of scopoletin on tyrosinase activity. Seventy microliters of phosphate buffer containing scopoletin was mixed with 20 μl of 53.7 units/ml mushroom tyrosinase, and 10 μl L-DOPA was then added. Following incubation at 37°C for 20 min, the absorbance was measured at 475 nm.

Western blot analysis

Cells were lysed in cell lysis buffer (62.5 mM Tris-HCl [pH 6.8], 2% SDS, 5% β-mercaptoethanol, 2 mM phenylmethylsulfonyl fluoride, protease inhibitors [Complete™; Roche, Mannheim, Germany], 1 mM Na_3VO_4, 50 mM NaF and 10 mM EDTA). Twenty micrograms of protein per lane was separated by SDS-polyacrylamide gel electrophoresis and blotted onto polyvinylidene fluoride (PVDF) membranes, which were then blocked with 5% dried milk in Tris-buffered saline containing 0.5% Tween 20. Blots were then incubated with the appropriate primary antibodies at a dilution of 1 : 1000, and further incubated with horseradish peroxidase-conjugated secondary antibody. Bound antibodies were detected using an enhanced chemiluminescence plus kit (Thermo Scientific Inc., Bremen, Germany). Images of the blotted membranes were obtained using a LAS-1000 luminescent image analyzer (Fuji Film, Tokyo, Japan).

Statistics

Statistical significance of the differences between groups was assessed using analysis of variance (ANOVA), followed by the Student's *t*-test. p values < 0.01 were considered significant.

RESULTS

Effects of scopoletin on cell viability

A compound was isolated from *C. setidens*, and the structure was identified as scopoletin by NMR analysis (Fig. 1) [15]. To investigate the effects of scopoletin on cell viability, B16F10 cells were treated with various concentrations of scopoletin for 24 h. Scopoletin was not cytotoxic to B16F10 cells at concentrations ≤50 M (Fig. 2). Therefore, concentrations up to 50 μM were used in the following experiments.

Effects of scopoletin on melanin synthesis and tyrosinase activity

To investigate the effects of scopoletin on melanogenesis, melanin content and tyrosinase activity were measured. A potent melanogenic hormone, α-MSH was used as a positive control. Scopoletin significantly increased melanin synthesis in a concentration-dependent manner (Fig. 3). Melanin production increased approximately 3-fold after 50 μM scopoletin treatment. In accordance with melanin content, tyrosinase activity was also increased by scopoletin treatment in a dose-dependent manner (Fig. 4). The melanogenic effect of 50 μM scopoletin was comparable to that of 1 μM α-MSH. Using a cell-free system, we determined that scopoletin did not have a direct effect on tyrosinase activity (Fig. 5). Based on these results, we suggest that scopoletin may increase tyrosinase expression, but does not directly activate tyrosinase.

Effects of scopoletin on MITF and tyrosinase protein levels

Tyrosinase is regulated by the expression of MITF. Hence, we examined the effects of scopoletin on the expression of MITF and tyrosinase after treatment with 0～50 μM scopoletin. As expected from previous experiments, scopoletin induced the expression of both MITF and tyrosinase in a dose-dependent manner (Fig. 6).

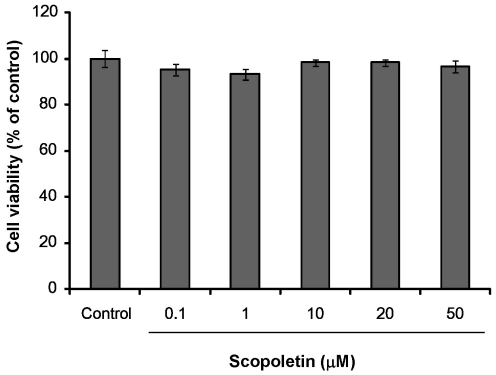


Fig. 2. Effect of scopoletin on B16F10 cell viability. B16F10 cells (2×10^4 cells/well) were incubated for 24 h in serum-free DMEM, and then treated with 0.1, 1, 10, 20, or 50 μM scopoletin. After another 24 h, cell viability was determined by crystal violet assay. Results were expressed as % of the control. Each determination was made in triplicate and data shown represent the mean±S.D.

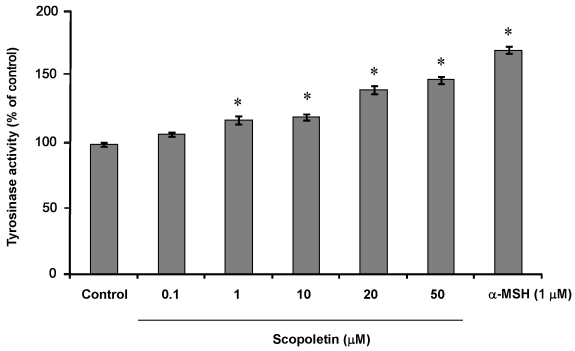


Fig. 4. Effect of scopoletin on tyrosinase activity in B16F10 cells. B16F10 cells (5×10^4 cells/well) were incubated with scopoletin (0.1, 1, 10, 20, or 50 μM) for 72 h. α-MSH (1 μM) was used as a positive control. Tyrosinase activity was measured as described in the materials and methods section. Each determination was made in triplicate and data shown represent the mean±S.D. *$p<0.01$ compared to the untreated control.

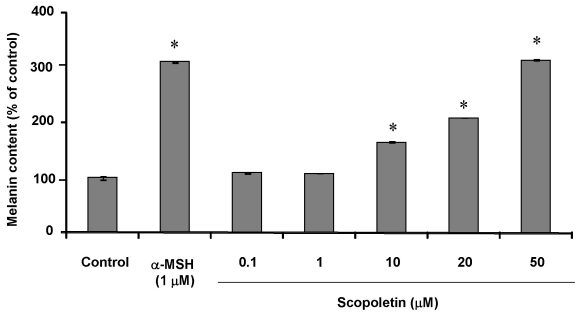


Fig. 3. Effect of scopoletin on melanin content. B16F10 cells (5×10^4 cells/well) were incubated with scopoletin (0.1, 1, 10, 20, or 50 μM) for 72 h. α-MSH (1 μM) was used as a positive control. Melanin released into the cell culture medium was measured as described in the materials and methods section. Each determination was made in triplicate and data shown represent the mean±S.D. *$p<0.01$ compared to the untreated control.

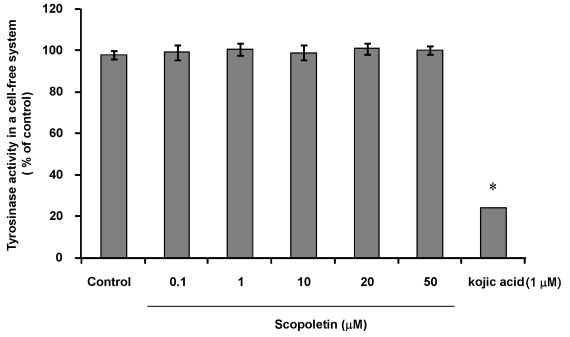


Fig. 5. Effect of scopoletin on tyrosinase activity in a cell-free system. To test the direct effect of scopoletin on tyrosinase, tyrosinase activity was measured in a cell-free system as described in the materials and methods section. Each determination was made in triplicate and data shown represent the mean±S.D. *$p<0.01$ compared to the untreated control.

Effects of scopoletin on CREB phosphorylation

CREB phosphorylation strongly stimulates MITF and tyrosinase expression. We investigated whether scopoletin affects CREB phosphorylation in a time-course experiment. Our results showed that scopoletin increased phospho-CREB levels in a dose- and time-dependent manner (Fig. 7). CREB was phosphorylated after 60 min of scopoletin treatment.

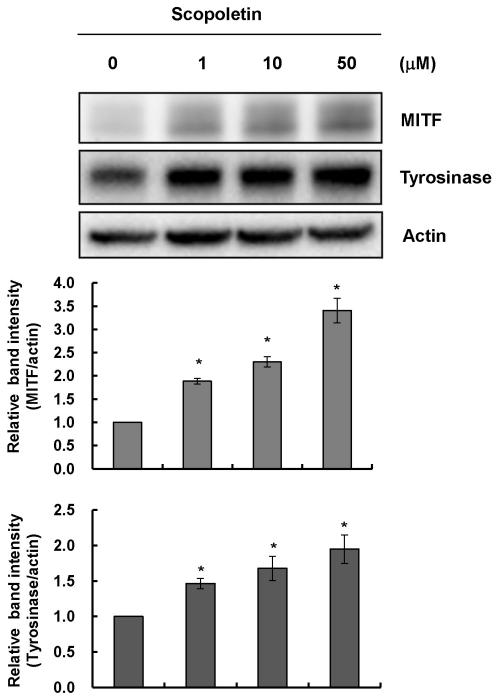


Fig. 6. Effect of scopoletin on MITF and tyrosinase expression in B16F10 cells. Cells were exposed to 0~50 μM scopoletin for 72 h. MITF and tyrosinase expression were examined by Western blot analysis. Equal protein loading was confirmed by β-actin expression. Band intensity relative to control was determined by densitometric analysis. Values are expressed as mean±S.D. (n=3). *p< 0.01 compared to untreated control.

DISCUSSION

The present study established that scopoletin, a principal component of *C. setidens*, increases melanin production in B16F10 cells. Many skin whitening agents, such as kojic acid and arbutin, directly inhibit tyrosinase activity [17]. However, experiments conducted using a cell-free system containing mushroom tyrosinase established that scopoletin does not directly affect tyrosinase activity (Fig. 5). Previous reports have shown that the hypopigmentary effects of some agents are due to decreased MITF and tyrosinase expression [13,18]. In contrast, increased MITF and tyrosinase may induce melanogenesis. Therefore, we investigated MITF and tyrosinase protein expression in an attempt to clarify the mechanisms behind scopoletin's induction of hyperpigmentation. Western blot analysis suggested that scopoletin increased MITF and tyrosinase protein production in a dose-dependent manner (Fig. 6). These results indicate that MITF and tyrosinase play major roles in regulating melanogenesis.

The synthesis and secretion of α-MSH occur in keratinocytes of the skin. α-MSH binds to the melanocortin 1 receptor (MC1R) on the surface of melanocytes [19]. This binding leads to the activation of adenylate cyclase, which synthesizes cyclic adenosine monophosphate (cAMP). An increased level of cAMP results in phosphorylation and activation of CREB. Phosphorylated CREB mediates MITF promoter activation, resulting in increased expression of tyrosinase, the rate-limiting enzyme of melanin synthesis [14,19]. Because scopoletin stimulated melanin production via increased expression of MITF and tyrosinase, we investigated the effect of scopoletin on the cAMP pathway. Scopoletin was found to increase levels of phosphorylated CREB in a dose- and time-dependent manner (Fig. 7). In contrast, scopoletin inhibits CREB phosphorylation in fibroblast-like synoviocytes [20]. Thus, scopoletin-induced CREB phosphorylation may be cell type-specific. These results suggest that scopoletin stimulates melanin synthesis in melanocytes by increasing CREB phosphorylation and upregulating expression of MITF and, ultimately, tyrosinase.

Melanin pigment is indispensible for skin protection and homeostasis, and alteration in melanin production occurs

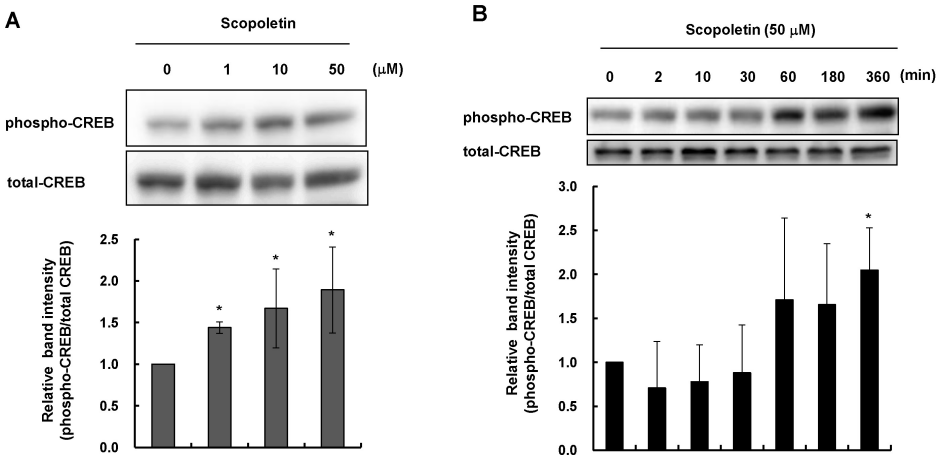


Fig. 7. Effect of scopoletin on the phosphorylation of CREB in B16F10 cells. Cells were treated with 1~50 μM scopoletin for 60 min (A), or exposed to 50 μM scopoletin for 0, 2, 10, 30, 60, 120, or 360 min (B). The phosphorylation of CREB was examined by Western blot analysis. Equal protein loading was confirmed by total-CREB expression. Band intensity relative to the control was determined by densitometric analysis. Values are expressed as mean± S.D. (n=3). *p< 0.01 compared to untreated control.

in many pigmentation disorders. Therefore, the proper production of melanin is very important for skin health [1]. Recent studies have demonstrated that vitiligo patients possess defects in the melanocortin system [3,21,22], and afamelanotide, an analogue of α-MSH, is currently undergoing clinical trials for the treatment of vitiligo [22]. Since it is a synthetic peptide, afamelanotide is given via subcutaneous injections, which are inconvenient. In contrast, scopoletin has a relatively simple structure and could be applied to the skin in the form of a cream, lotion, or ointment. This potentially makes scopoletin a better option for the treatment of vitiligo.

The present study determined that scopoletin from *C. setidens* increases melanin synthesis. Furthermore, scopoletin was found to increase tyrosinase and MITF through CREB phosphorylation. Taken together, these results suggest that scopoletin could be used for the treatment of vitiligo and for sunless tanning.

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