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Myriocin, a serine palmitoyltransferase inhibitor, increases melanin synthesis in Mel-Ab cells and a skin equivalent model

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The purpose of this study was to investigate effects of myriocin, an inhibitor of serine palmitoyltransferase, on melanogenesis. It was found that myriocin increased melanin synthesis in a concentration-dependent manner. Moreover, myriocin up-regulated microphthalmia-associated transcription factor (MITF) and tyrosinase expression via phosphorylation of CREB, but it did not directly activate tyrosinase, a rate-limiting melanogenic enzyme. Furthermore, we demonstrated increased melanin synthesis with myriocin on a pigmented skin equivalent model established using *Cervi cornus Colla* (deer antler glue). One and 5 μM of myriocin darkened the color of the skin equivalent. These results suggest that myriocin may have potential effects for the treatment of hypopigmentary skin diseases like vitiligo or for sunless tanning.

1. Introduction

After the term of "melanin" was first used by Berzelius for dark animal pigments in 1840 (d'Ischia et al. 2013), many studies have been performed to elucidate the regulation of melanogenesis. However, melanin is still considered one of the most enigmatic pigments and has not been well characterized (Niki et al. 2011). Melanin synthesis is an important process for skin pigmentation and occurs in melanosomes, the specialized organelles in melanocytes. Synthesis is largely regulated by tyrosinase, a rate-limiting melanogenic enzyme, tyrosinase-relate protein 1 (TRP-1), and TRP-2 (Garcia-Borron and Solano 2002). Microphthalmia-associated transcription factor (MITF) is well known as a specific transcription factor for tyrosinase (Kim and Lee 2013). Moreover, phosphorylation of cAMP-responsive element binding protein (CREB) is the key mechanism in melanogenesis, leading to promotion of MITF expression (Goding 2000).

Melanin is synthesized in melanocytes and transferred to keratinocytes of the skin. It is distributed not only to the skin, but also to the eye, brain, hair, and inner ear (Simon and Peles 2010). Human skin is often exposed to UV irradiation, which can cause DNA damage. Melanin, however, protects the skin from such harmful environmental factors (Nestle et al. 2009). If there is not enough melanin or melanin transfer to keratinocytes, human skin will be damaged by UV irradiation and result in hypopigmentary disorders like vitiligo.

Vitiligo is a common dermatological disorder and manifests as skin depigmentation due to abnormal melanocyte function resulting from mutation or loss of melanocytes (Wang et al. 2011). Although a number of treatments for vitiligo are known, many of them are unsatisfactory. Hence, the purpose of this study was to examine the effects of myriocin on melanin synthesis.

Myriocin is isolated from *Myriococcum albomyces* and has antifungal effects (Kluepfel et al. 1972). It is an inhibitor of serine palmitoyltransferase, the first step in ceramide biosynthesis, and is known to extensively deplete sphingolipids from cells, for example, ceramide and sphingosine-1-phosphate (S1P) (Meyer et al. 2012). In previous studies, we have demonstrated that ceramide and S1P decreased melanin synthesis in human and mouse melanocytes, respectively (Kim et al. 2003; Kim et al. 2002; Kim et al. 2011). Therefore, we hypothesized that myriocin could increase melanin synthesis as an inhibitor of ceramide synthesis.

In the present study, we investigated the effects of myriocin on melanin synthesis both in Mel-Ab cells and in a skin equivalent model containing Mel-Ab cells. Furthermore, related signal transduction pathways were examined.

Abbreviations: CREB, cAMP-responsive element binding protein; DOPA, 3,4-dihydroxyphenylalanine; GSK3β, glycogen synthase kinase 3β; MITF, microphthalmia-associated transcription factor; PVDF, polyvinylidene fluoride; S1P, sphingosine-1-phosphate; TRP, tyrosinase-related protein; UV, ultraviolet.

2. Investigations and results

2.1. Effects of myriocin on cell viability

Mel-Ab cells were treated with 0.01–10 μM of myriocin to determine cytotoxicity, and cell viability was determined using a crystal violet assay. Myriocin had no cytotoxic effect on Mel-Ab cells at concentrations less than 10 μM (Fig. 1A).

2.2. Effects of myriocin on tyrosinase activity and melanin synthesis

Next, the effects of myriocin on melanogenesis were assessed through melanin content and tyrosinase activity assays. Mel-Ab cells were treated with 0.01–10 μM of myriocin for 3 d.

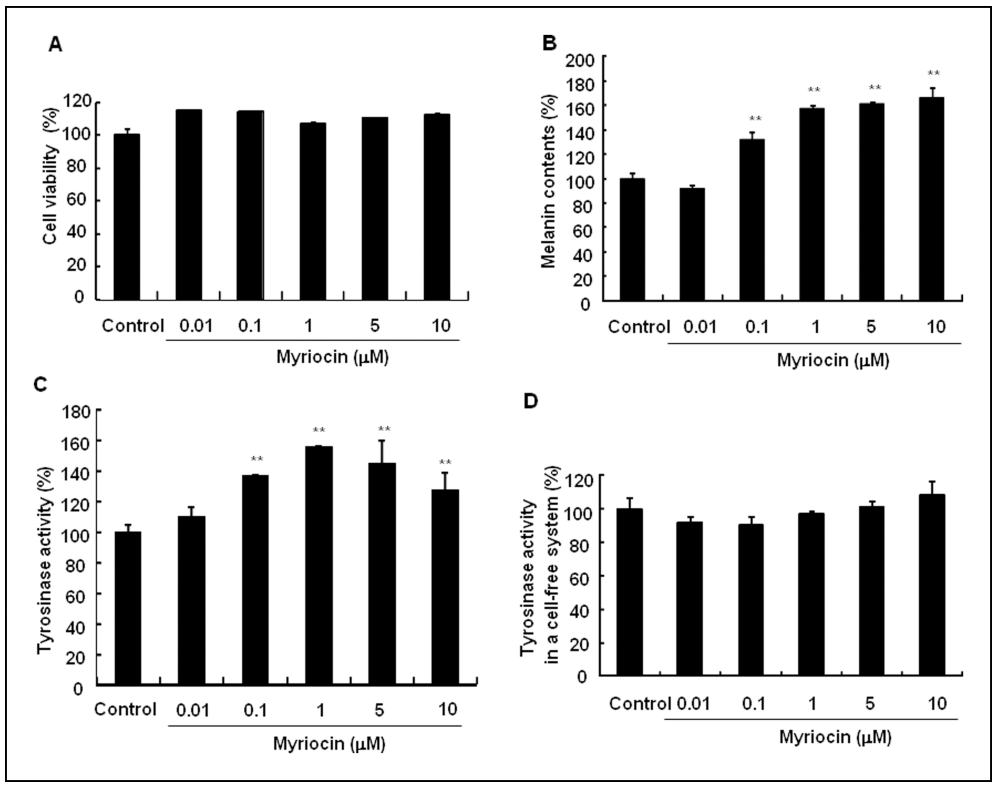

Fig. 1: Effects of myriocin on melanogenesis. After serum starvation for 24 h, Mel-Ab cells were incubated in serum-free media with various concentrations (0.01–10 μM) of myriocin for 24 h. (A) The viability of the cells was determined by crystal violet assay. Mel-Ab cells were cultured with 0.01–10 μM myriocin for 3 d, and melanin content (B) and tyrosinase activity (C) were measured. (D) The direct effect on tyrosinase was measured in a cell-free system. Each measurement was made in triplicate, and data represent the mean ± SD.

Myriocin significantly increased melanin synthesis and tyrosinase activity in a concentration-dependent manner (Figs. 1B and C). In addition, mushroom tyrosinase was used to determine if myriocin would directly affect tyrosinase. However, myriocin showed no direct effect on tyrosinase (Fig. 1D). From these results, it was proposed that myriocin increases melanogenesis through increasing tyrosinase activity rather than direct activation of tyrosinase.

2.3. Effects of myriocin on MITF and tyrosinase protein levels

CREB is one of the key molecules of melanogenesis and is well known as an MITF activator. As shown in Fig. 2A, CREB was phosphorylated at 24 h. Corresponding expression of MITF was increased at 48 h. Finally, tyrosinase level was increased by the activation of MITF production after treatment with 1 μM myriocin at 72 h (Fig. 2A). However, no change on β-catenin expression was observed.

2.4. Effects of myriocin on the melanogenesis-related signaling pathways

To determine which of the pathways for hyperpigmentation is affected by myriocin treatment, CREB, Akt, GSK-3β, and p38 phosphorylation and β-catenin level were examined by Western blotting assay in a time-course experiment. Importantly, only phospho-CREB was increased time-dependently, while Akt,

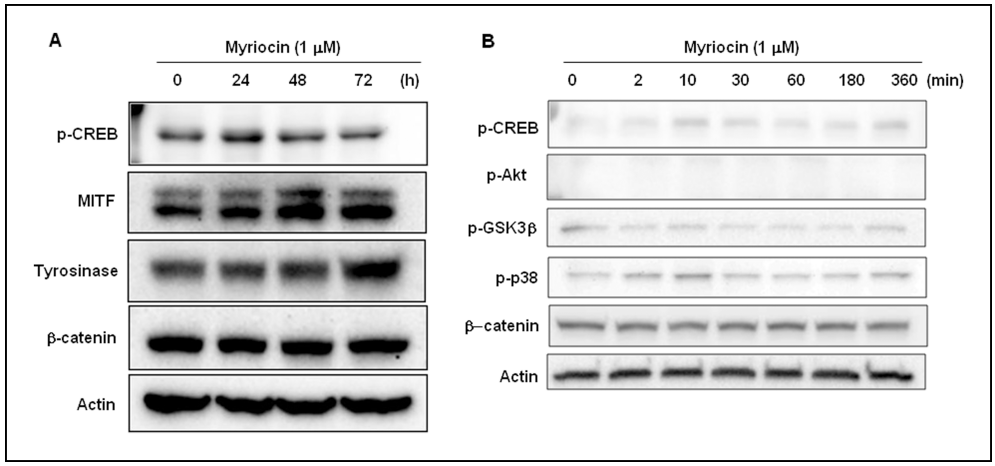

Fig. 2: Effects of myriocin on melanogenesis-related signaling pathways. (A) Mel-Ab cells were treated with 1 μM of myriocin for 24–72 h. Phospho-CREB, MITF, tyrosinase, and β-catenin expression was measured by Western blot analysis. To examine signal transduction pathways, Mel-Ab cells were treated with 1 μM of myriocin for the time points indicated. Phospho-CREB, phospho-Akt, phospho-GSK3β, phospho-p38, and β-catenin level were detected by Western blot analysis. Equal protein loading was assured by reaction with anti-actin antibody.

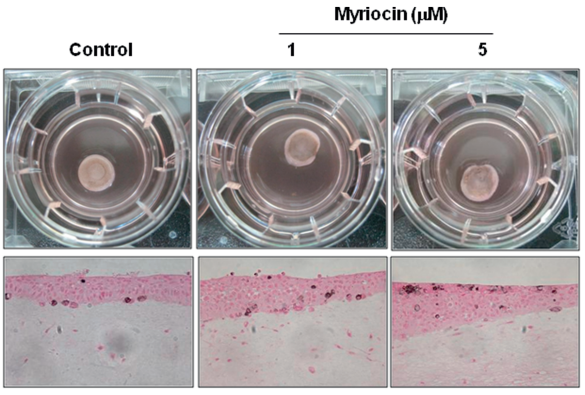

Fig. 3: Effects of myriocin on skin equivalents. Pigmented skin equivalents were made and treated with 1 and 5 μM of myriocin as described in the text. (A) Photographs of skin equivalents in 24 mm trans-well culture after 5 d of 1 and 5 μM myriocin treatment. (B) Melanin in the pigmented skin equivalents was stained by Fontana-Masson.

GSK-3β, and p38 were not phosphorylated and β-catenin level was not changed (Fig. 2B).

2.5. Effects of myriocin on skin equivalent model

As shown in Fig. 3, the skin equivalent model treated with 1 and 5 μM of myriocin was darker than the untreated control. Moreover, the skin equivalent section was stained by Fontana-Masson to detect melanin. Melanin pigment was detected at a higher level in the myriocin-treated skin equivalent than in the untreated control.

3. Discussion

Melanin plays protects the skin against UV irradiation (d'Ischia et al. 2013). Thus, the lack of melanin evokes more sensitive reactions of skin to UV light. Deficiency of melanocytes and melanin can cause hypopigmentary skin diseases such as vitiligo (Lotti et al. 2008). There are many treatment options for vitiligo, including narrowband UVB light to stop the death of melanocytes and to restore pigmentation (Westerhof and d'Ischia 2007). Alternatively, an agent increasing melanin synthesis should be helpful to treat vitiligo. In the present study, we found that myriocin significantly increased melanin synthesis in Mel-Ab cells.

Tyrosinase is the rate-limiting enzyme of melanogenesis (Le-Thi-Thu et al. 2011). As shown in Fig. 1, myriocin dramatically increased melanin content and tyrosinase activity but did not directly activate tyrosinase. In addition, the expression of tyrosinase increased after treatment with myriocin at 72 h, while MITF increased at 48 h (Fig. 2A). These results suggest that myriocin activates the signal transduction pathways for the expression of tyrosinase. It was also found that CREB, an upstream regulator for MITF, was phosphorylated time-dependently (Fig. 2B). From this result, we suggested that the primary mechanism of increasing melanin by myriocin was phosphorylation of CREB. In previous studies, we showed that ceramide inhibited melanogenesis in human and mouse melanocytes (Kim et al. 2002; Kim et al. 2001). Moreover, we also reported that an artificial ceramide analog, PC102, decreased melanin synthesis (Jeong et al. 2013). These results suggest that myriocin may increase melanin synthesis through inhibition of ceramide synthesis. Indeed, our results showed that myriocin increased melanogenesis. On the other hand, it has been reported that ceramide inhibits the proliferation of melanocytes *via* inactivation of Akt (Kim et al. 2001). Furthermore, ceramide is well-known to induce apoptosis in a variety of cell types (Aflaki et al. 2012; Mullen and Obeid 2012; Zhang et al. 2012). On the basis of the previous results, myriocin may have proliferative and cytoprotective effects on melanocytes. As mentioned, vitiligo results from the death of melanocytes. Therefore, myriocin treatment may be helpful against vitiligo.

To date, animal experiments have been limited for the development of new cosmetics and medicines. Therefore, skin equivalents have been used to replace some animal experiments (Faller et al. 2002). We previously established skin equivalents using *Cervi cornus Colla* (deer antler glue) (Kim et al. 2013). In the present study, myriocin showed darkening effects in a pigmented-skin equivalent model. Although our model should be further developed to make skin equivalents more representative of real human skin, it successfully expressed dark pigmentation, and melanin pigment increased after treatment with myriocin.

In conclusion, myriocin promoted melanogenesis through phosphorylation of CREB and increased MITF and tyrosinase levels. Furthermore, the pigment-inducing effect of myriocin was confirmed in a skin equivalent model containing Mel-Ab cells. Therefore, we suggest that myriocin could be used for the treatment of vitiligo or sunless tanning.

4. Experimental

4.1. Materials

Myriocin, Dulbecco's Modified Eagle's Medium (DMEM), Nutrient Mixture F-12 Ham, sodium bicarbonate, HEPES, formaldehyde, insulin, L-ascorbic acid, isoproterenol, hydrocortisone, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), cholera toxin (CT), synthetic melanin, L-DOPA, and mushroom tyrosinase were obtained from Sigma (St Louis, MO, USA). DMEM/Nutrient Mixture F-12, 3:1 Mixture (DMEM/F-12), antibiotic-antimycotic (penicillin, streptomycin), trypsin-EDTA, and sodium hydroxide were purchased from WelGENE (Dalseogu, Daegu, South Korea). *Cervi cornu Colla* (CCC) was obtained from Siwon Herbal Medicine Co. (Jincheon-gun, South Korea); hyalruronic acid (HA) was obtained from LG Life Sciences, Ltd. (South Korea); and fetal bovine serum (FBS) was obtained from Hyclone (Logan, UT, USA). Recombinant human epidermal growth factor (EGF) was obtained from Invitrogen Co. (Gibco, Camarillo, CA). Antibodies specific for phospho-CREB (ser133, #9198), phospho-Akt (Ser473, #9271), phospho-GSK3β (#9336), phospho-p38 MAPK (#9211), and β-catenin (#9581) were from Cell Signaling Technology (Beverly, MA, USA). Antibodies specific for tyrosinase (C-19) and actin (I-19) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Microphthalmia Ab-1 (C5, MS-771-P0) was 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).

4.2. Cell culture

The Mel-Ab cell line is a mouse-derived spontaneously immortalized melanocyte cell line that produces large amounts of melanin (Dooley et al. 1994). Mel-Ab cells were incubated in DMEM supplemented with 10% (v/v) FBS, 100 nM TPA, 1 nM CT, 50 μg/ml of streptomycin, and 50 μg/ml of penicillin at 37 °C in 5% CO_2. CCD-25Sk human fibroblasts were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA), and HaCaT human keratinocytes were purchased from Cell Lines Service (Eppelheim, Germany). The cells were grown in DMEM supplemented with 10% (v/v) FBS, 50 μg/ml streptomycin, and 50 μg/ml penicillin at 37 °C in 5% CO_2.

4.3. Cell viability assay

Cell viability was determined using a crystal violet assay. After incubating Mel-Ab cells with myriocin for 24 h, the media were 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).

4.4. Measurement of melanin content

Melanin content was measured as previously described, with slight modifications (Tsuboi et al. 1998). Cells were treated with various concentrations (0.01–10 μM) of myriocin in DMEM containing 10% FBS for 3 d. Cell pellets were dissolved in 550 μl of 1 N NaOH at 100 °C for 30 min and centrifuged at 13,000 × g for 5 min. Supernatants were analyzed at 400 nm using an ELISA reader.

4.5. Tyrosinase activity

Tyrosinase activity was assayed as DOPA oxidase activity. Mel-Ab cells (1×10^5) were seeded in six-well plates and incubated with various concentrations (0.01–10 μM) of myriocin in DMEM for 3 d. The cells were washed with phosphate buffered saline and lysed with lysis buffer (0.1 M phosphate buffer pH 6.8 containing 1% Triton X-100). They were then disrupted by freeze-thawing, and the lysates were clarified by centrifugation at 15,000 rpm for 30 min. After quantification of the protein content using a protein assay kit (Bio-Rad, Hercules, CA), the protein concentrations were adjusted with lysis buffer. Then 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 the absorbance at 475 nm using an ELISA reader. A cell-free assay system was used to determine the direct effect of myriocin on tyrosinase activity. Phosphate buffer (70 μL) containing myriocin was mixed with 20 μL (53.7 units/mL) of mushroom tyrosinase, and 10 μL of 10 mM L-DOPA was added. Following incubation at 37 °C for 20 min, the absorbance was measured at 475 nm.

4.6. Western blot analysis

Mel-Ab cells were lysed in a cell lysis buffer (62.5 mM Tris-HCL [pH 6.8], 2% SDS, 5% β-mercaptoethanol, 2 mM phenylmethylsulfonyl fluoride, and protease inhibitors [Complete^{TM}; Roche, Mannheim, Germany], 1 mM Na_3VO_4, 50 mM NaF and 10 mM EDTA). Proteins in the total cell lysates were separated by SDS-polyacrylamide gel electrophoresis using 20 μg of protein per lane, blotted onto polyvinylide fluoride (PVDF) membranes, and 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 1:1,000 and then re-incubated with horseradish peroxidase-conjugated secondary antibody. Bound antibody was identified using an enhanced chemiluminescence testing kit (Thermo Scientific Inc., Bremen, Germany). All images of the immunoblots were obtained using a LAS-1000 lumino-image analyzer (Fuji Film, Tokyo, Japan).

4.7. Preparation of dermal equivalents

Dermal equivalents were made as described previously with some modifications (Auger et al. 1995). In brief, type 1 collagen, which was extracted from rat tail tendons, was dissolved by stirring in 1/1000 glacial acetic acid at 4 °C for 48 h. Dermal equivalents were made by mixing the following solutions; 1% type 1 collagen solution, 10 × media (DMEM: Nutrient Mixture F-12 Han = 3: 1), 10 × reconstitution buffer (0.05 N NaOH, 0.26 mM $NaHCO_3$, 200 mM HEPES), 5 mg/ml hyaluronic acid (HA), and 0.33 g/ml CCC. Dermal equivalents including CCC were prepared based on the composition described in a previous study (Kim et al. 2013). Dermal equivalents contained 3×10^5 fibroblasts. Three milliliters of this mixture was poured into 24 mm transwell inserts with 3.0 μm pore polycarbonate membranes (Corning, Inc., Tewksbury, MA, USA), and then the inserts were placed in an incubator at 37 °C for gelation.

4.8. Reconstruction of pigmented skin equivalents

To reconstruct pigmented skin equivalents, Mel-Ab cells were seeded onto dermal equivalents at a density of 1×10^5 cells after treatment with 0.03 mg/ml collagen type IV, and then HaCaT cells were seeded onto dermal equivalents at a density of 1×10^6 cells and cultured in a submerged condition for 1 d and then at the air-liquid interface for 4 d. The growth medium was composed of DMEM/F-12 supplemented with 5% FBS, 0.4 μg/ml hydrocortisone, 1 M isoproterenol, 25 μg/ml ascorbic acid, and 5 μg/ml insulin. For the submerged culture, 1 ng/ml epidermal growth factor (EGF) was added in the growth medium, and for the air-liquid interface culture, 10 ng/ml EGF also was added. One or 5 μM of myriocin was added for 4 d.

4.9. Fontana-Masson stain assay

After 5 d, skin equivalents were fixed in 10% formaldehyde for 1 d and processed for conventional paraffin embedment. Four- to 6-μm-thick sections were then prepared. To detect melanin, a Fontana-Masson stain kit (ScyTek Laboratories, Logan, UT, USA) was used according to the manufacturer's protocol.

4.10. Statistics

The statistical significance of intergroup differences was assessed by analysis of variance (ANOVA) followed by Student's t-test. P-values < 0.01 were considered significant.

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