Asiaticoside, a component of *Centella asiatica*, inhibits melanogenesis in B16F10 mouse melanoma

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Abstract. Melanogenesis is the process of generating pigmentation via melanin synthesis and delivery. Three key enzymes, tyrosinase, tyrosinase-related protein 1 (TRP1) and TRP2, metabolize melanin from L-tyrosine. Melanin synthesizing enzymes are regulated by microphthalmia-associated transcription factor (MITF). The titrated extract of *Centella asiatica* (TECA) contains the major components asiatic acid, asiaticoside and madecassic acid. The present study revealed that TECA reduces the melanin content in melanocytes. Moreover, the asiaticoside contained in TECA modulated melanogenesis by inhibiting tyrosinase mRNA expression. The decrease in tyrosinase mRNA levels was mediated through MITF. Uniquely, asiaticoside inhibited MITF by decreasing its DNA binding affinity. In conclusion, the results of the present study indicate that asiaticoside treatment may have beneficial effects in hyperpigmentation diseases or for skin whitening.

Introduction

Melanogenesis is the process of melanin synthesis and delivery through various enzymes (1). In particular, tyrosinase, tyrosinase-related protein 1 (TRP1), and tyrosinase related protein 2 (TRP2) are key enzymes in melanin synthesis (2,3). The melanin synthesizing enzymes transfer L-tyrosine to melanin though a multistep transformation (4). Microphthalmia-associated transcription factor (MITF) transcriptionally regulates these key enzymes in melanocytes (5). Therefore, the regulation of MITF is central for hyper- or hypo-pigmentation diseases and for skin whitening for cosmetic purposes (6,7).

Centella asiatica is a medicinal plant widely used in South Asia. *C. asiatica* extracts have therapeutic applications, particularly in neuroprotection and wound healing (8-11). In addition, its extracts are widely used for the treatment of inflammatory skin disorders, including leprosy, lupus, varicose ulcers, eczema, atopic dermatitis and psoriasis (12). The *C. asiatica* extract is mainly administered as a titrated extract. Titrated extract of *C. asiatica* (TECA) contains asiatic acid, asiaticoside and madecassic acid (12,13). In dermatology, these components are used to achieve preventive and therapeutic effects. Asiaticoside prevents ultraviolet A-dependent photoaging by suppressing ultraviolet A-induced reactive oxygen species production (14). In addition, asiatic acid, madecassic acid and asiaticoside induce collagen I synthesis (15).

Recently, Saraf *et al* (16) indicated that a cosmetic formulation containing *C. asiatica* may possess de-melanogenic potential (16). However, the mechanism behind the activity of *C. asiatica* in melanocytes has yet to be elucidated. Therefore, a detailed understanding of the changes in melanogenesis is important for elucidating the mechanism of asiaticoside-dependent hypopigmentation. The present study aimed to determine how *C. asiatica*, particularly asiaticoside, affects melanogenesis.

Materials and methods

Materials and cell culture. TECA was obtained from Bayer (Leverkusen, Germany). Asiatic acid, madecassic acid, asiaticoside and 1-methyl-3-(2-methylpropyl)-7H-purine-2,6-dione (IBMX) were purchased from Sigma Aldrich (St. Louis, MO, USA). B16F10 cells were purchased from Korea Cell Line Bank (Seoul, Korea) and were cultured in Dulbecco's modified Eagle's medium (Sigma Aldrich) supplemented with 10% fetal bovine serum (Sigma Aldrich) and 1% penicillin-streptomycin (Gibco®, Life Technologies, Carlsbad, CA, USA). Cultures were maintained at 37˚C with 5% CO_2.

Viability assays. The B16F10 cells were seeded in 96-well plates at a density of $4x10^3$ cells per well. The cells were cultured with TECA, asiatic acid, madecassic acid or

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asiaticoside. After 24 or 48 h, the cells were incubated with 0.5 mg/ml MTT for 2 h. Formazan crystals were dissolved in dimethyl sulfoxide. The mean absorbance at 405 nm was assessed using an EL800 Absorbance Microplate Reader (Molecular Devices, Sunnyvale, CA, USA).

Assessment of melanin content. The melanin content was assessed using the method previously described by Hosoi *et al* with several modifications (17). The B16F10 cells were seeded at a density of $2x10^5$ cells in 60-mm culture dishes. The cells were cultured with TECA, asiatic acid, madecassic acid or asiaticoside. Following incubation, the melanocytes were washed in phosphate-buffered saline and lysed in 1 N NaOH. The mean absorbance at 405 nm was detected using an EL800 Absorbance Microplate Reader (Molecular Devices).

Western blot analysis. The B16F10 cells were cultured with TECA, asiatic acid, madecassic acid or asiaticoside. Following incubation, the cells were harvested and lysed in radioimmunoprecipitation assay buffer [50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid and 0.1% sodium dodecyl sulfate]. The total protein concentration of the cell lysates was determined using a protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of protein were separated with 12 or 10% SDS-PAGE and transferred to nitrocellulose membranes (Whatman, Dassel, Germany). The membrane was blocked in 5% skimmed milk. Anti-tyrosinase goat polyclonal IgG, anti-TRP1 goat polyclonal IgG, anti-MITF rabbit monoclonal IgG and anti-β-actin mouse monoclonal IgG antibodies were used to detect specific proteins and were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The membranes were visualized using SuperSignal West Pico (Pierce Biotechnology, Inc., Rockford, IL, USA) and Las-4000 (Fujifilm, Minato-ku, Japan).

Quantitative polymerase chain reaction (qPCR). Total RNA from the B16F10 cells was isolated using RiboEX (Geneall, Seoul, Korea) according to the manufacturer's instructions. cDNA was synthesized using reverse transcriptase (Bioneer Corp., Daejeon, Korea) and used as template for the PCR. The PCR mixtures contained template, 0.5 μM primers and HOT FIREPol Eva Green® qPCR Mix Plus (Solis Biodyne, Tartu, Estonia) from a SYBR green I-based qPCR kit. Reactions were run in LinegeneK (BioER, Hangzhou, China) using the following program: 5 min denaturation at 95˚C, followed by 40 cycles of 15 sec denaturation at 95˚C, 15 sec annealing at 58˚C and 15 sec polymerization at 72˚C. The sequences of the primers used were as follows: Tyrosinase forward, 5'-ACACACTGGAAGGATTTGCC-3' and reverse, 5'-GAGCGGTATGAAAGGAACCA-3'; and β-actin forward, 5'-CGACAGGATGCAGAAGGAG-3' and reverse, 5'-ACATCTGCTGGAAGGTGGA-3'. The relative quantity of all samples was calculated using a serial dilution standard curve.

Chromatin immunoprecipitation (ChIP). Following treatment with TECA and TECA components, the cells were washed in ice-cold phosphate-buffered saline and cross-linked for 15 min at 25˚C with 1% formaldehyde. The cross-linked cells were harvested and resuspended in ChIP lysis buffer [50 mM HEPES (pH 7.5), 140 mM NaCl, 1% Triton X100]. The cells were sonicated and immunoprecipitated with the anti-MITF antibody (Santa Cruz Biotechnology, Inc.). Following immunoprecipitation, cross-linked proteins were degraded using protease K, and DNA was collected using the PCR purification kit (Geneall). The tyrosinase promoter site was detected using PCR. The following primer sequences for the tyrosinase promoter were used: Forward, 5'-AGTCATGTGCTTTGCAGAAGAT-3' and reverse, 5'-CAGCCAAGAACATTTTCTCCTT-3'.

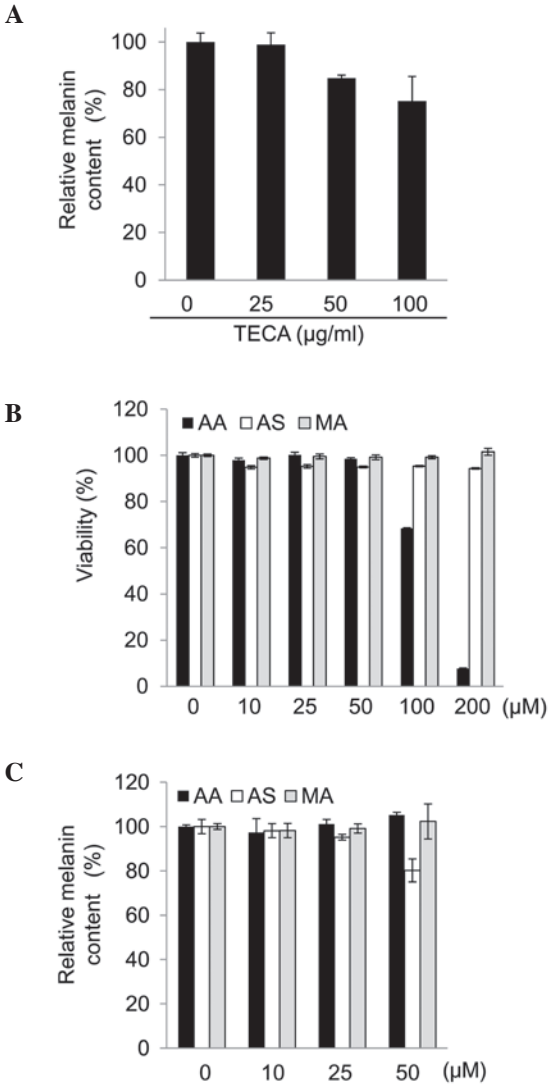

Figure 1. Inhibitory effect of TECA and TECA components in melanogenesis. B16F10 cells were incubated with the indicated concentrations of TECA or TECA components (asiatic acid, asiaticoside and madecassic acid). (A) The melanin content of TECA-treated B16F10 cells was assessed via the optical density (405 nm). Optical density results are expressed as the relative difference from the control. (B) B16F10 cells were cultured with the indicated concentrations of asiatic acid, asiaticoside and madecassic acid for 48 h. Cell viability was measured using MTT assays. Cytotoxicity was calculated using untreated B16F10 cells as a control. Data are expressed as the mean ± standard deviation from three experiments. (C) Following 48 h of incubation, cell lysates were analyzed by measuring the optical density at 405 nm. Data are presented as the mean ± standard deviation from three experiments. TECA, titrated extract of *Centella asiatica*; AA, asiatic acid; AS, asiaticoside; MA, madecassic acid.

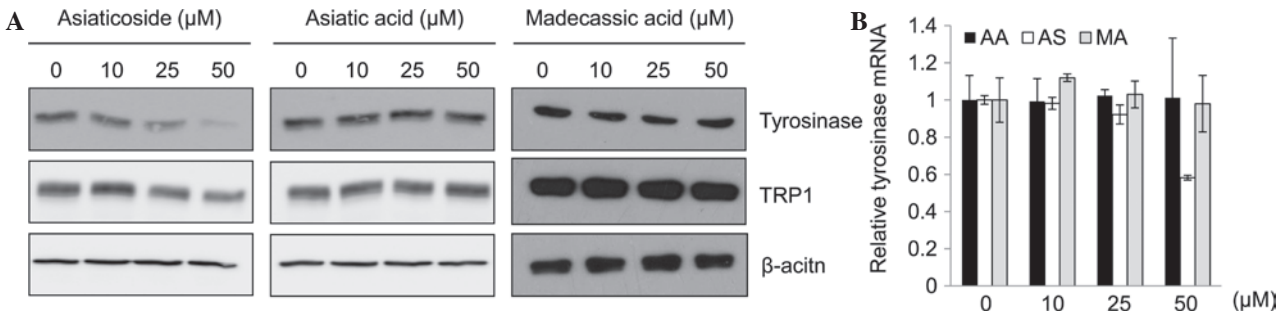

Figure 2. Effect of asiaticoside on tyrosinase expression. B16F10 cells were incubated with the indicated concentrations of TECA or TECA components (asiatic acid, asiaticoside and madecassic acid). (A) Following treatment with asiatic acid, asiaticoside and madecassic acid, the expression levels of tyrosinase, TRP1, and TRP2 were analyzed using SYBR Green I-based quantitative polymerase chain reaction (qPCR) assays. (B) The protein levels of tyrosinase were analyzed by western blot analysis. β-actin was used as a loading control. TECA, titrated extract of *Centella asiatica*; AA, asiatic acid; AS, asiaticoside; MA, madecassic acid; TRP, tyrosinase-related protein.

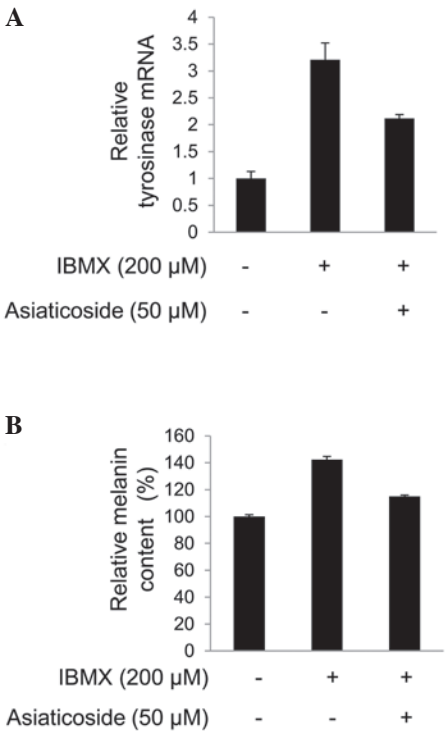

Figure 3. Effect of asiaticoside on IBMX-treated B16F10 cells. (A) B16F10 cells were incubated with the indicated concentrations of asiaticoside and IBMX. Following treatment, the expression levels of tyrosinase were analyzed using SYBR Green I-based quantitative polymerase chain reaction (qPCR). (B) The melanin content of the asiticoside-treated B16F10 cells was assessed via the optical density (405 nm). Optical density results are expressed as the relative difference from the control. IBMX, 1-methyl-3-(2-methylpropyl)-7H-purine-2,6-dione.

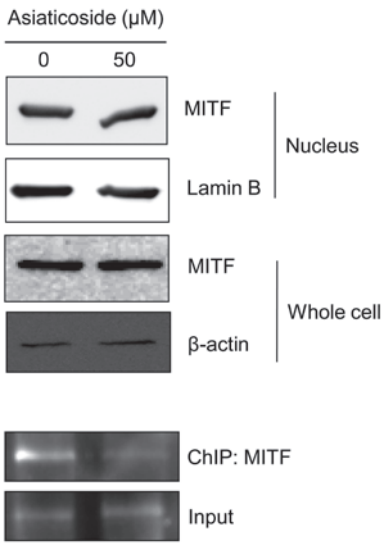

Figure 4. Inhibition of MITF activity in B16F10 cells incubated with asiaticoside at the indicated concentrations. Following treatment, the cells were divided into nuclear and cytosolic fractions. MITF protein levels were assessed using western blot analysis (top panel). Lamin B and β-actin were used as a loading control and a fraction positive control, respectively. The binding affinity and interaction between the tyrosinase promoter and MITF was assessed using ChIP (bottom panel). MITF, microphthalmia-associated transcription factor; ChIP, chromatin immunoprecipitation.

Results

TECA represses melanogenesis in B16F10 cells. To examine whether TECA possesses cytotoxic activity, the B16F10 cells were treated with TECA at various concentrations (0, 10, 25, 50, 100 and 200 μg/ml) for 48 h. After 48 h, no significant changes were observed in B16F10 cell viability with 10 to 100 μg/ml TECA (data not shown). Therefore, <100 μg/ml TECA was used in the following experiments. As shown in Fig. 1A, TECA decreased the melanin content in the B16F10 cells in a concentration-dependent manner.

Asiaticoside, the major component of TECA, represses melanogenesis in B16F10 cells. As melanogenesis was repressed by TECA, the present study also investigated which of its components contributed to the repression of this process. The cytotoxic effects of the major components of TECA (asiaticoside, asiatic acid and madecassic acid) were assessed in the B16F10 cells using MTT assays. The dose-response curves to the TECA components in the B16F10 cells are shown in Fig. 1. Asiaticoside and madecassic acid (0-200 μM) had no effect on cell viability (Fig. 1B). However, asiatic acid induced cytotoxicity at concentrations of >100 μM in the B16F10 cells. Therefore, the melanin content was assessed following incubation with 50 μM asiatic acid. Under non-toxic conditions, the melanin content was decreased by asiaticoside, but not by asiatic acid or madecassic acid (Fig. 1C). As tyrosinase has a significant role in melanogenesis, the effects of asiaticoside on tyrosinase protein and mRNA expression

in the B16F10 cells were assessed. Fig. 2A and B shows that asiaticoside suppressed tyrosinase mRNA and protein expression in the B16F10 cells.

In addition, asiaticoside repressed the melanogenesis induced by IBMX by reducing the level of tyrosinase mRNA (Fig. 3A). Consistent with this, the melanin content was decreased by asiaticoside in the B16F10 cells undergoing IBMX-induced melanogenesis (Fig. 3B). The decrease in tyrosinase mRNA is common in MITF-repressed melanocytes, as MITF transcriptionally regulates tyrosinase (5). Furthermore, IBMX, a cyclic adenosine monophosphate (cAMP) phosphodiesterase inhibitor, induces the level of expression in MITF target genes by increasing the level of cAMP (18). Therefore, MITF activity was assessed in the present study.

Asiaticoside decreases tyrosinase mRNA expression by repressing the DNA binding affinity of MITF. As described in the introduction, MITF is a key regulator of melanogenesis and a possible target for the treatment of hyperpigmentation diseases (6,7). Therefore, in the present study, it was hypothesized that the asiaticoside-dependent inhibition of melanogenesis could be regulated by MITF transactivation activity. To determine whether asiaticoside regulates MITF, the translocation and protein expression of MITF in asiaticoside-treated B16F10 cells was monitored. Asiaticoside did not regulate MITF expression in the whole cell lysates. In addition, the nuclear translocation of MITF was unchanged following incubation with asiaticoside. However, asiaticoside treatment altered the DNA binding affinity of MITF in the B16F10 cells (Fig. 4).

Discussion

C. asiatica extracts are used for treating psoriasis and wounds of the skin (19,20). *C. asiatica* extracts promote wound healing through production of collagen (21). In a previous study, Saraf *et al* (16) reported that hydroalcoholic extracts of *Curcuma caesia* (rhizome), *Areca catechu* (seeds), *Centella asiatica* (leaves), *Cinnamon zeylanicum* (dried bark), and *Tamarindus indica* (fruit pulp) reduce melanin content, as measured by mexameter. However, the study indicated that herbal extracts enhance photoprotection by increasing the sun protection factor value (16). Therefore, the present study evaluated the effects of *C. asiatica* extracts on melanogenesis in melanocytes.

As shown in Fig. 1, TECA decreased the melanin content in the B16F10 cells in a concentration-dependent manner. Melanin is produced by a multi-step metabolic conversion of L-tyrosine to melanin (22). The metabolic synthesis of melanin is performed by enzymes such as tyrosinase, TRP1 and TRP2 (23). Tyrosinase is the major enzyme involved in the melanin metabolic pathway (23). In the present study, asiaticoside inhibited melanogenesis by the inhibition of tyrosinase protein and mRNA expression (Fig. 2A and B). In IBMX-induced melanogenesis, tyrosinase transcription is regulated by MITF (24). Therefore, to determine the effect of asiaticoside on MITF, the expression levels, translocation and DNA binding affinity of MITF protein were assessed in the present study. Notably, as shown Fig. 4, asiaticoside inhibits melanogenesis by interfering with the DNA binding of MITF. Similarly, Um *et al* (25) reported that {1-[2-(4-chloro-phenoxy)-ethyl]-1H-benzoimidazol-2-ylsulfanyl}-acetic acid inhibits melanogenesis by interfering with direct MITF binding to E-box DNA. In addition, MITF DNA binding is inhibited by PIAS3, a protein inhibitor of activated signal transduction and activator of transcription 3 (26).

In conclusion, these results indicate that TECA, and in particular, asiaticoside, inhibited melanogenesis by regulating the DNA binding affinity of MITF. Furthermore, the melanin content was reduced by decreasing the level of expression in tyrosinase and MITF target genes. Therefore, the present study revealed that asiaticoside is a novel candidate for melanogenesis inhibition through repression of DNA binding to MITF.

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