The effects of Caffeoylserotonin on inhibition of melanogenesis through the downregulation of MITF via the reduction of intracellular cAMP and acceleration of ERK activation in B16 murine melanoma cells

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INTRODUCTION

The overproduction and accumulation of melanin in the skin may lead to pigmentary disorders, such as melasma, freckles, post-inflammatory melanoderma and solar lentigo. In mammals, pigmentation results from the synthesis and distribution of melanin in the skin, hair bulbs, and eyes. Three melanocyte-specific enzymes, tyrosinase (TYR), tyrosinase-related protein (TRP)-1, and TRP-2 catalyze melanin biosynthesis (1, 2). Tyrosinase, the rate-limiting enzyme in melanin biosynthesis, catalyses the hydroxylation of tyrosine to form 3,4-dihydroxy-phenylalaine (DOPA) and the oxidation of DOPA to produce DOPA-quinone. Tyrosinase-related protein-2 acts as a dopachrome tautomerase and catalyses the rearrangement of dopachrome to form 5, 6-dihydroxyindole-2-carboxylic acid (DHICA).

Tyrosinase-related protein-1 oxidizes DHICA to produce carboxylated indole-quinone (3). Thus, TRP-1 and TRP-2 function in the biosynthesis of melanin downstream of TYR. The expression of these three enzymes is strongly regulated by microphthalmia-associated transcription factor (MITF) (4). Upregulation of the cyclic AMP (cAMP) pathway by forskolin or alpha-melanocyte-stimulating hormone (α-MSH) induces stimulation of melanin synthesis in correlation to the protein levels of TYR, TRP-1, and TRP-2 via an increase in MITF expression (5). The α-MSH stimulates melanogenesis via an increase in intracellular cAMP levels through the activation of protein kinase A (PKA) and cAMP-responsive element binding protein (CREB). The CREB binds with the cAMP response element (CRE) motif of the MITF promoter and activates MITF gene transcription, which in turn stimulates TYR gene expression. Thus, the cAMP pathway mediates the regulation of TYR expression and melanogenesis via MITF (6).

Extracellular signal-regulated kinase (ERK) signaling is reportedly also involved in the regulation of melanogenesis via stimulation of MITF degradation (7). When ERK signaling is activated, phosphorylated ERK (p-ERK) induces the phosphorylation of MITF and triggers its degradation leading to the inhibition of melanogenesis. Inhibition of the ERK pathway has been reported to enhance TYR promoter activity and increase melanogenesis. Thus, it is well documented that the ERK pathway is closely associated with MITF-mediated melanogenesis (8).

Caffeoylsertotonin (CaS) belongs to a class of phenylpropanoid amides found at low levels in a wide range of plant species along with other various serotonin derivatives (9). Serotonin derivatives function in the defense mechanism against pathogen attack (10) and exhibit various health-supporting activities (11).
Our previous report demonstrates strong radical scavenging and antioxidant activity of CaS (12). CaS showed strong radical scavenging and protective activity against oxidative stress-induced cell death, the inhibition of intracellular ROS generation, prevention of lipid peroxidation, and reduction of H2AX phosphorylation in HepG2 and HaCaT cells. The melanogenesis-inhibiting effects of serotonin derivatives, along with mushroom TYR inhibition by CaS in vitro, have recently been reported (13). Although CaS is important as a part of a new class of potent TYR inhibitors, to the best of our knowledge there are no detailed reports on the effects of CaS on mediating intracellular mechanism of melanogenesis.

In this study, we investigated the effects of CaS on melanogenesis in mouse B16 melanoma cells and confirmed that α-MSH-induced depigmentation was associated with the inhibition of TYR, TRP-1, and MITF at the transcriptional level. Moreover, the results suggest that the inhibition of melanogenesis by CaS treatment is related to the downregulation of MITF via the reduction of intracellular cAMP levels and ERK activation.

RESULTS

Inhibitory effects of CaS on melanin formation and cellular TYR activity

To analyze the effects of CaS on cell proliferation, B16F10 melanoma cells were treated with CaS at concentrations ranging from 1 to 100 μM for 24 h. As shown in Fig. 1B, treatment with CaS did not affect cell proliferation up to 20 μM; however, at concentrations of 50 and 100 μM, strong cytotoxicity was observed. There was an approximately 60 and 72% decrease in cell viability at 50 and 100 μM, respectively. Thus, CaS at a concentration <20 μM was used to determine its effect on melanogenesis in B16F10 cells. To evaluate the effects of CaS on melanogenesis in B16 melanoma cells, B16F10 cells were stimulated with α-MSH and subsequently treated with various concentrations of CaS (1, 5, 10, and 20 μM) for 24 h. A remarkable decrease in the melanin content in the supernatant and cell pellet was observed in a dose-dependent manner (Fig. 1C). The TYR activity using L-DOPA as a substrate was also significantly reduced in the cells in a dose-dependent manner and reached to maximum reduction at a concentration of 20 μM (Fig. 1D). These results demonstrate that CaS at a concentration of 20 μM inhibited TYR activity and decreased melanin content without influencing melanocyte viability.

Effects of CaS on melanogenic enzyme expression

To determine whether CaS influences the mRNA and protein expression of melanogenic enzymes such as TYR, TRP-1, TRP-2, and MITF, we performed semi-RT-PCR and Western blotting analysis. As shown in Fig. 2A and B, mRNA and protein levels of TYR and MITF were markedly decreased by treatment with CaS in a dose-dependent manner; TRP-1 expression levels were decreased at 20 μM CaS. However, there was no significant change in TRP-2 expression with CaS treatment.

Effects of CaS on ERK activation

The ERK signaling is involved in the regulation of melanogenesis via stimulation of MITF degradation (7). Thus, to investigate the effects of CaS on ERK activation, ERK phosphorylation was ana-
Fig. 2. Effects of CaS on the expression of melanogenesis-related genes. The B16F10 cells were incubated in the presence of α-MSH and then treated with different concentrations of CaS (1, 5, 10, and 20 μM) for 24 h. (A) Total RNA was extracted and analyzed by RT-PCR for the expression of MITF, TYR, TRP-1, and TRP-2. PCR product levels were measured by densitometry, with the values from CaS-treated cells being quantified relative to the α-MSH-treated control. (B) Intracellular TYR, TRP-2, TRP-1, and MITF levels were measured by Western blotting. Protein levels were quantified by densitometry relative to those of the α-MSH-treated control (after normalization relative to β-actin, shown just below the gel data). *P < 0.01, **P < 0.001, and ***P < 0.0001 vs. the α-MSH-treated control.

Fig. 3. Effects of CaS on the phosphorylation of ERK in B16F10 cells. (A) Melanocytes were treated with α-MSH and 20 μM CaS for the indicated times. (B) Cells were treated with α-MSH and 20 μM CaS for the indicated times in the presence or absence of PD98059 (50 μM). Whole-cell lysates were then subjected to Western blotting using antibodies against ERK, p-ERK, MITF, and TYR. Equal protein loading was confirmed by reaction with β-actin.

ERK phosphorylation and the melanogenic genes MITF and TYR. The CaS rapidly responded to the increase in intracellular cAMP levels induced by α-MSH in melanocytes and further accelerated the activation of ERK via phosphorylation and subsequently in-
Disruption of the induction of melanogenic enzymes.

Discussion

It has previously been reported that CaS possess inhibitory effects on mushroom TYR and cell-free TYR activity (14). However, there are no detailed reports available on the molecular mechanism of melanogenesis at the cellular level. This is a critical issue because most of the depigmentation candidates do not show a whitening effect in vivo, even though they have strong inhibitory effects on TYR activity in vitro. Herein, we demonstrated the molecular mechanism of CaS, a hydroxycinnamic acid amide of serotonin, on hypopigmentary effects through the downregulation of melanin synthesis. Specifically, CaS inhibited melanin synthesis through downregulation of cAMP, MITF expression, and its associated genes. In addition, CaS reduced the increase in intracellular cAMP levels and accelerated ERK phosphorylation.

The α-MSH is known to activate MC1R, a receptor expressed in melanocytes, and this receptor-ligand interaction leads to G-protein-dependent activation of the enzyme, adenylate cyclase (AC) followed by increased intracellular cAMP levels, which activate the enzyme cAMP-dependent protein kinase (PKA) and CREB transcription factor. The PKA and CREB induce MITF transcription and MITF in turn upregulates the levels of several melanogenic enzymes, including TYR, thus promoting the synthesis of eumelanin (15, 16). The cAMP has been reported to inhibit the MAP kinase pathway in many cell types. However, it has been demonstrated that cAMP upregulation activates MAPK (ERK1/2) in B16 melanoma cells and normal human melanocytes (5). The ERK-dependent phosphorylation of MITF could lead to the stimulation of melanogenesis through regulation of the transcriptional co-activator, p300/CBP (17). However, melanoma cells treated with PD98059, a specific inhibitor of MEK inhibitor, or the overexpression of dominant negative mutants of Ras and MEK induce melanogenesis due to an increase in TYR expression (8). Thus, sustained ERK activation inhibits melanogenesis. Several studies have found that ERK activation induces the phosphorylation of serine 73 of MITF, which causes its ubiquitination and subsequent proteasome-mediated degradation (18). To explain the physiological role of such an inhibition, it was suggested that cAMP activation of this signaling pathway could constitute a fine regulatory mechanism of cAMP retrocontrol to prevent over-melanin production that could be noxious for melanocytes. As shown in Fig. 4A, CaS nearly suppressed the increase in intracellular cAMP levels upon induction by α-MSH within 5 min. The ERK phosphorylation was induced after 2 h in α-MSH-treated melanocytes and sustained for 18 h (Fig. 4B). However, CaS increased ERK phosphorylation over 5-fold (8.9 vs. 1.6) within 1 h as compared with the α-MSH-treated control. The ERK phosphorylation was rapidly decreased to the α-MSH-treated state within 3 h and induced the degradation of MITF and TYR. Thus, the effect of CaS on ERK activation was correlated with the inhibition of the increase in intracellular cAMP levels.

To confirm whether increases in ERK phosphorylation are responsible for the anti-melanogenesis effect of CaS, a MEK/ERK inhibitor was used and the expression levels of MITF and TYR were analyzed. The MEK1 inhibitor, PD98059 strongly recovered MITF and TYR expression reduced by CaS (Fig. 3B). These results suggest that CaS-stimulated ERK signaling plays a primary role in anti-melanogenesis in B16F10 cells. In this study, we could not analyze whether CaS activates MC1R or AC. However, we suggest that CaS may inhibit the activation of MC1R or AC, block the signaling between MC1R and AC via a heterotrimeric G protein, or serve as an inhibitor that acts downstream of AC. To clarify the inhibitory effects of CaS on melanogenesis, further studies using radiolabeled CaS or in vitro assays with AC are needed.

In conclusion, we analyzed the inhibitory effects of CaS on melanogenesis via the measurement of melanin production and an analysis of the expression of pigmentation-related genes, in-
including TYR, TRP-2, TRP-1, and MITF. The CaS inhibited cellular melanin production and melanogenic gene activity in B16F10 cells by inhibiting the increase in intracellular cAMP levels as well as accelerating ERK phosphorylation. Thus, these results suggest that CaS may be useful in cosmetic whitening or as a therapeutic agent for use in the treatment of hyperpigmentation.

MATERIALS AND METHODS

Chemicals and reagents
L-DOPA, melanin, and α-MSH were purchased from Sigma (St. Louis, MO). Anti-TYR (H-109), TRP-1 (H-90), TRP-2 (H-130), MITF (H-30), and anti-β-actin antibody (sc-61615) were from Santa Cruz Biotechnology (Santa Cruz, CA). Phospho-ERK1/2 and total ERK1/2 were from Cell Signaling Technology (Beverly, MA). The secondary antibodies used were an anti-goat IgG (sc-2033) and anti-rabbit IgG (sc-2004). Dulbecco’s Modified Eagle Medium (DMEM), fetal bovine serum (FBS), and penicillin/streptomycin were purchased from Thermo scientific Hyclone (Logan, Utah). The CaS was synthesized by a reaction of these activated esters of hydroxycinnamic acids with serotonin hydrochloride in an alkaline solution as described elsewhere (14).

Cell viability assay
The B16F10 cell line was purchased from the Korean Cell Line Bank (KCLB, Seoul, Korea) and cultured in DMEM supplemented with 10% FBS, and penicillin/streptomycin at 37°C in a humid atmosphere of 5% CO2. Cells suspended in the culture medium containing 10% FBS were split into flat-bottomed 96-well plate and after the cells were attached to the plate, they were treated with various concentrations (1-100 μM) of CaS for 24 h. Proliferation of adherent cells was determined by a colorimetric method based on 2, 3-bis (2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide inner salt (XTT) (WelGene, Korea) as described elsewhere (2). Cell viability was quantified as a fold as compared to the untreated control.

Measurement of the melanin content
Melanin content was measured using a slightly modified version of the method developed by Tsuboi et al. (19). Briefly, cells were treated with CaS at the indicated concentrations in the presence or absence of α-MSH for 24 h in phenol red-free DMEM. After treatment, the supernatant was transferred to a fresh tube and read directly at 420 nm with an ELISA plate reader. Next, the cells were harvested and solubilized in 2 N NaOH at 80°C for 2 h then centrifuged for 10 min at full speed. The optical density (OD) of the sample was then measured at 420 nm. The total melanin content was calculated as the sum of the melanin content from the supernatant and its cell pellet for each sample.

TYR activity assay
The TYR activity was determined with respect to its DOPA oxidase activity using the method described by Takahashi et al. (20) with slight modifications. Briefly, B16F10 cells were seeded on a 48-well plate (2 × 104 cells per well) and cultured with different concentrations of CaS (1, 5, 10, and 20 μM) in the presence of α-MSH. After 24 h, the cells were washed twice with cold phosphate-buffered saline (PBS) and lysed with 0.1 M sodium phosphate buffer (pH 6.8) containing 1% Triton X-100 and a protease inhibitor cocktail (Sigma, St. Louis, MO). The cells were disrupted by freeze-thawing, and the lysates were clarified by centrifugation at 10,000 × g for 10 min. After quantification of the protein levels by BCA protein assay (Pierce, Rockford, IL) and adjustment of the concentration with lysis buffer, 100 μl of each lysate was placed in a well of a 96-well plate and 100 μl of substrate solution (1 mM L-DOPA in PBS, pH 6.8) was added. After incubation for 30 min at 37°C, TYR activity was analyzed spectrophotometrically by following the oxidation of DOPA to dopachrome at 450 nm using an ELISA plate reader.

Western blotting
Cells were lysed in cold RIPA buffer (pH 7.4) containing protease and protease inhibitor cocktail. The whole-cell lysates, which contained 20 μg of protein per lane, were separated by SDS-PAGE and detected with antibodies. Bound antibodies were detected using an enhanced chemiluminescence kit (Amersham Biosciences, Little Chalfont, UK). Equal loading was assessed using anti-β-actin antibody to normalize the amounts of total protein.

Semi-quantitative reverse transcription polymerase chain reaction (semi-RT-PCR)
To determine the effects of CaS on melanogenesis-related gene expression, semi RT-PCR was carried out. The B16F10 cells were stimulated with 100 nM of α-MSH in the presence or absence of CaS. For analysis of the TYR, TRP-1, TRP-2 and MITF mRNA levels, total cellular RNA was prepared using TRI reagent (MRC Inc., Cincinnati, OH) following the manufacturer’s instructions. Reverse transcription and cDNA amplification were carried out with 25 ng of isolated total RNA using a maxime RT-PCR Premix (iNIRON, Sungnam, Korea). The reaction was cycled 40 times for TYR, TRP-1, TRP-2, and MITF for 30 s at 94°C, 30 s at 58°C, and 60 s at 72°C. The reaction for β-actin was cycled 30 times for 30 s at 94°C, 30 s at 55°C, and 60 s at 72°C. The primers used for RT-PCR were as follows: for TYR (284 bp) 5'-AGTTGGCTCCGAAAGCCACCGT-3' (forward) and 5'-TGCCACGGGCTGAGGA-3' (reverse); for TRP-1 (308 bp) 5'-TCACGGGCCTCGCTCAATTG-3' (forward) and 5'-GGGCTCAATGGCCCAAG-3' (reverse); for TRP-2 (289 bp) 5'-AGCCCCGTGTTGGGCTGTT-3' (forward) and 5'-TGCACCTGAGGTCGCGTGGGATTCC-3' (reverse); for MITF (258 bp) 5'-AGGCCGGCAACCGGGCATTG-3' (forward) and 5'-TGCGCTCAATGGCCACCTCGGC-3' (reverse); and for β-actin (243 bp) 5'-GGCCCTAGGCGCAGAAATG-3' (forward) and 5'-GGGACGCTGTTGGCGTACAGG-3' (reverse).

Cyclic AMP analysis
Intracellular cyclic AMP (cAMP) levels were determined using a Cyclic AMP XPlSM Assay kit (#4339) (Cell Signaling, Danvers,
The B16F10 cells were stimulated with 100 nM of α-MSH in the presence or absence of 20 μM CaS for 5-180 min. Cyclic AMP concentration in the sample was determined based on a standard curve by following manufacturer’s instructions and expressed as nmol per each sample.

**Statistical analysis**

Values were expressed as mean ± SD for three independent experiments. Statistical differences were determined with ANOVA using SPSS 12.0 K (Chicago, IL). The P values < 0.05 were considered significant.

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