Beta-carboline alkaloids harmaline and harmalol induce melanogenesis through p38 mitogen-activated protein kinase in B16F10 mouse melanoma cells

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INTRODUCTION

The synthesis of melanin pigments or melanogenesis has many important physiological functions that include photoprotection of the human skin from ultraviolet (UV) irradiation (1). Melanogenesis is a complex pathway involving melanin synthesis, melanin transport, and melanosomal release (2). Melanin synthesis is stimulated by various effects such as α-melanocyte-stimulating hormone (α-MSH); cyclic AMP (cAMP) elevating hormones including forskolin, glycyrrhizin, and isobutylmethylxanthine; UV-B radiation; and the placental total lipid fraction (3-5). In addition, melanin synthesis occurs in melanocytes and melanoma cells through an enzymatic process catalyzed by tyrosinase, tyrosinase related protein-1 (TRP-1), and tyrosinase related protein-2 (TRP-2), which converts tyrosine to 3,4-dihydroxyphenylalanine (DOPA) and catalyzes the oxidation of DOPA into DOPAquinone (6). Dopaquinone is converted to dopachrome that is in turn converted to dihydroxyindole or dihydroxyindole-2-carboxylic acid (DHICA) to form eumelanin. The cascade of enzymatic reactions in melanin synthesis is related in tyrosinase, TRP-1 (dopachrome tautomerase) and TRP-2 (DHICA oxidase) (7).

Microphthalmia-associated transcription factor (MITF) is the most important transcription factor in the regulation of tyrosinase and expression of the genes for TRP-1 and TRP-2, as tyrosinase, TRP-1, and TRP-2 harbor the MITF binding site, thereby leading to the regulation of activation of melanocyte differentiation (8). cAMP response element binding protein (CREB) is also one of the major transcription factors of MITF and, thus, plays a central role in melanogenesis (9). The CREB binding site is present in the MITF promoter region; as a consequence, CREB binds to and activates the MITF promoter, which leads to the indirect activation of melanogenesis (10).

Mitogen-activated protein kinases (MAPKs) are a highly conserved family of protein serine/threonine kinases that include extracellularly responsive kinases (ERK1/2), c-jun N-terminal or stress-regulated protein kinases (JNK/SAPK), and p38 MAPKs. They are involved in a diversity of cellular activities and have an important regulatory role in melanogenesis (11). p38 MAPK activation is related to an increase in melanin synthesis and is involved in the expression of melanogenesis related molecules (12). In addition, p38 MAPK activation is involved in α-MSH-induced melanogenesis, such as activation of MITF expression and activation of tyrosinase transcription (13), whereas the extracellular signal-regulated kinase (ERK1/2 and c-Jun NH2-terminal kinase/stress-activated protein kinase (JNK/SAPK) pathways are related with the down-regulation of melanogenesis (14). Accordingly, ERK signaling inactivation or p38 MAPK signaling activation stimulates melanogenesis by increasing MITF expre-
β-carbolines are component of some medicinal plants, such as Passiflora edulis, Passiflora incarnate and Peganum harmala. These plants have been used for anti-jaundice, anti-lumbago and anti-inflammation agent in oriental medicine. Recently, the mechanism responsible for traditional herbal medicines variable effects has not been fully clarified yet. β-carboline alkaloids are widely distributed in environments including several plant families, well-cooked foods, tobacco smoke, and alcoholic beverages (15). Additionally, they are endogenous in mammalian tissues. β-carboline alkaloids have a reported wide range of pharmacological, neurophysiologic, and biochemical actions (16, 17); are both mutagenic and carcinogenic (18); and act as a neurotoxins in some neurodegenerative diseases. In contrast, the β-carboline alkaloids harmaline and harmalol (Fig. 1A) inhibit monoamine oxidase and do not induce neurotoxicity (19). In addition, β-carbolines possess effective antioxidant and radical scavenging properties, protect from oxidative neuronal damage, and inhibit the ion-mediated cytotoxic effect of 1-methyl-4-phenylpyridinium (MMP+) and cytotoxic effects on tumor cells (17). Furthermore, plants containing β-carboline alkaloids have long been used as traditional medicines for the treatment of various diseases including cancer, jaundice, malaria, and asthma (19, 20). Recently, it was reported that β-carbolines induce apoptosis by caspase-8 activation in carcinoma cells and function as an anti-inflammatory compound that suppresses lipopolysaccharide (LPS)-induced nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) through the nuclear factor (NF)-κB and IκBα decay (18, 19). However, it is not known whether β-carbolines directly contribute to melanogenesis dysfunction. Therefore, using mouse B16F10 melanoma cells, we attempted to determine the effects and mechanisms of the β-carbolines harmaline and harmalol for 72 h. Cellular melanin content was assessed by determination of intracellular melanin, expressed as a percentage related to the total protein. As shown in Fig. 1A, harmaline and harmalol treatment increased the melanin content, consistent with enhanced melanin synthesis in response to serial concentrations of harmaline and harmalol. Of note, augmentation of intracellular melanin content by harmaline appeared more sustained than that evoked by harmalol. The cell growth effect of harmaline and harmalol were investigated in cells treated with various concentrations (1-20 μM) of harmaline and harmalol for 72 h (data not shown). Cell growth arrest was induced by 10 μM harmaline and 20 μM harmalol, while treatment with either β-carboline alkaloid did not result in cell necrosis and detachment from the culture plates (data not shown).

RESULTS

Harmaline and harmalol increase cellular melanin content in B16F10 melanoma cells

Cells were treated with various concentrations of harmaline and harmalol for 72 h. Cellular melanin content was assessed by determination of intracellular melanin, expressed as a percentage related to the total protein. As shown in Fig. 1A, harmaline and harmalol treatment increased the melanin content, consistent with enhanced melanin synthesis in response to serial concentrations of harmaline and harmalol. Of note, augmentation of intracellular melanin content by harmaline appeared more sustained than that evoked by harmalol. The cell growth effect of harmaline and harmalol were investigated in cells treated with various concentrations (1-20 μM) of harmaline and harmalol for 72 h (data not shown). Cell growth arrest was induced by 10 μM harmaline and 20 μM harmalol, while treatment with either β-carboline alkaloid did not result in cell necrosis and detachment from the culture plates (data not shown).

Harmaline and harmalol induce tyrosinase activity and melanogenesis-related proteins

To verify if the effect of harmaline and harmalol on melanin synthesis and tyrosinase activity occurred in a time-dependent
manner, cells were exposed to 5 μM harmaline, 20 μM harmalol, or, as a positive control, 1 μM α-MSH. Tyrosinase is a key enzyme in melanin biosynthesis, in which melanin is formed from the tyrosinase-catalyzed oxidation of L-tyrosine. Thus, melanogenesis is regulated by the activity of tyrosinase, TRP-1, and TRP-2. An increase of cellular tyrosinase activity and melanin synthesis was evident in cells exposed to harmaline and harmalol, consistent with harmaline and harmalol accumulation of melanin biosynthesis by B16F10 cells via enhancement of tyrosinase activity. In order to clarify further the mechanism of tyrosinase activation by harmaline and harmalol, the levels of melanogenesis related proteins including tyrosinase, TRP-1, and TRP-2 were determined in B16F10 cells exposed to 5 μM harmaline and 20 μM harmalol by Western blot analysis. Tyrosinase, TRP-1, and TRP-2 expression were enhanced in harmaline- and harmalol-treated cells (Fig. 1E). The results were consistent with a harmaline- and harmalol-induced melanin synthesis mediated by the activation of the expression of tyrosinase, TRP-1, and TRP-2.

Harmaline and harmalol increase MITF expression and CREB activation

MITF plays an important role in melanogenesis as the transcription factor that regulates tyrosinase, TRP-1, and TRP-2 gene expression (7). The mechanism by which MITF regulates melanogenesis involves the p38 MAPK signaling pathway (12), which also induces the activation of melanogenesis by α-MSH (4). Furthermore, phosphorylation of CREB binds the CRE consensus sequence of the MITF promoter to activate the expression of MITF (14). Since earlier experiments in this study demonstrated that harmaline and harmalol increased melanin synthesis and melanogenesis related gene expression, we further hypothesized that these harmaline and harmalol could affect the expression of MITF and CREB activation, which transcriptionally activate melanogenesis. To investigate this hypothesis, the effect of harmaline and harmalol on MITF expression and CREB activation was examined in B16F10 cells by immunofluorescence and Western blotting analyses. Immunofluorescence studies with monoclonal anti-MITF and anti-phospho-CREB antibodies detected increased fluorescence intensity in cells treated with harmaline and harmalol compared with untreated cells (Fig. 2A). Western blotting analysis using the antibodies detected increased MITF expression and CREB activation in harmaline and harmalol treated cells (Fig. 2B). Stimulation of MITF expression and CREB activation by harmaline and harmalol reached its maximum after 24 h of treatment.

Harmaline and harmalol induces phosphorylation of p38 MAPK in B16F10 melanoma cells

p38 MAPK induces melanogenesis (12). In order to demonstrate the mechanisms of harmaline and harmalol-induced melanogenesis, p38 MAPK phosphorylation was evaluated using a Western blotting assay with B16F10 cells treated for various times with harmaline or harmalol. p38 MAPK was phosphorylated in a time-dependent fashion (Fig. 3A) with p38 MAPK activation induced 4 h after the treatments, indicating that the activation of melanogenesis by harmaline and harmalol is related to the activation of p38 MAPK. To directly demonstrate the involvement of p38 MAPK signaling in harmaline and harmalol-induced melanogenesis, B16F10 cells were treated with the p38 MAPK inhibitor SB203580 for 1 h before harmaline and harmalol treatment, and the cells were treated with harmaline and harmalol for 72 h. Melanin synthesis and tyrosinase activity were induced in harmaline- and harmalol-treated cells; these inductions were abrogated by SB203580 (Fig. 3B, C). Melanogenesis related protein was also assessed.
after SB203580 treatment. As expected, harmaline- and harmalol-induced upregulation of tyrosinase and TRP-1 were reduced by SB203580. Therefore, the p38 pathway specific inhibitor arrested the melanogenesis induced by harmaline and harmalol, and reduced the harmaline and harmalol mediated induction of MITF and CREB phosphorylation (Fig. 3D, E). The results were consistent with the suggestion that harmaline- and harmalol-induced melanogenesis may be mediated by the p38 MAPK signaling pathway.

DISCUSSION

Recent investigations of the roles of food and natural compounds in signal transduction and cell regulation have shed new light on the mechanisms of melanogenesis. Most studies on β-carbolines have focused on their possible involvement in neuropharmacological, antioxidant, antimutagenic, and anti-cancer treatment activities. Until now, the effects of β-carbolines on pigmentation have not been demonstrated. The present results show that harmaline and harmalol induce increased intracellular melanin content and tyrosinase activity through p38 MAPK-dependent MITF and CREB activation in B16F10 cells.

Harmaline and harmalol induced melanin content and tyrosinase activity in concentration- and time-dependent manners, which correlated with tyrosinase, TRP-1, and TRP-2 induction. Therefore, harmaline- and harmalol-induced MITF and CREB requires the activation of melanogenesis related proteins, and p38 MAPK activation is responsible for both harmaline- and harmalol-induced melanogenesis related protein expression, and harmaline- and harmalol-induced MITF and CREB activation.

Melanins, which are formed from the amino acid precursor L-tyrosine within melanocytes, play a crucial protective role from skin damage caused by UV radiation and other sources (23). Melanogenesis, which is characterized by an excessive biosynthesis of melanin pigments, induces various related pigment disorders (2). It is stimulated by several factors, including UV-B radiation and cAMP-elevating agents including α-MSH, forskolin, and isobutylmethylxanthine (3,4). These melanogenesis inducers are the major signaling pathways in melanogenesis, such as p38 MAPK signaling and protein kinase C and cAMP-mediated pathways. Activation of p38 MAPK induces MITF expression and CREB phosphorylation, as well as promoting the transcription of melanogenesis related genes. As a result, p38 MAPK signaling pathway activation leads to the stimulation of melanogenesis.

Treatment of B16F10 cells with 1-10 μM harmaline and 5-20 μM harmalol increased the intracellular melanin content in a concentration dependent manner for 72 h. Indeed, the intracellular melanin content was increased by 5 μM harmaline and 20 μM harmalol by 2.3-fold and 2.1-fold, respectively, compared to control levels. In addition, intracellular tyrosinase activity was also increased by 5 μM harmaline and 20 μM harmalol in a time-dependent manner. Tyrosinase activity was increased by 5 μM harmaline and 20 μM harmalol to 1.8-fold and 1.6-fold, respectively, compared to control levels at 72 h, while treatment with 1 μM α-MSH significantly increased the activity by 3.1-fold. In addition, an in situ tyrosinase activity assay also revealed an upregulation of tyrosinase activity in response to harmaline and harmalol treatment of B16F10 cells. MITF and CREB are important transcription factors in the regulation of melanogenesis related protein (14). To demonstrate
the mechanism of harmaline- and harmalol-induced melanogenesis, B16F10 cells were treated with 5 μM harmaline and 20 μM harmalol for various times. As shown in Fig. 3, treatment of B16F10 cells with harmaline and harmalol induced MITF expression and CREB phosphorylation. cAMP is an important factor in the melanogenesis-related signal transduction pathways, regulating the activation of protein kinase A following CREB phosphorylation. We measured the intracellular cAMP levels in B16F10 cells and observed no increase when cells were treated with harmaline and harmalol (data not shown). These results indicate that harmaline and harmalol do not stimulate melanogenesis via cAMP levels.

Activation of p38 MAPK stimulates melanogenesis (12). We confirmed that the increased synthesis of melanin induced by harmaline and harmalol is mediated by p38 MAPK activation; measurement of the level of p38 MAPK phosphorylation various lengths of times revealed that p38 MAPK phosphorylation increased when cells were treated with harmaline or harmalol. As both compounds activate the p38 MAPK signaling pathway, we expected, and demonstrated, the suppressive activity of harmaline- and harmalol-induced melanogenesis by SB203580. The melanin content and tyrosinase activity of cells treated with harmaline and harmalol decreased following treatment with SB203580. In addition, this treatment decreased expression of harmaline- and harmalol-induced melanogenesis-related proteins.

In summary, the β-carbolines harmaline and harmalol induce cellular melanin biosynthesis and tyrosinase activity in B16F10 cells, up-regulating CREB phosphorylation and expression of MITF, tyrosinase, TRP-1 and TRP-2, and phosphorylation of p38 MAPK. These consistent results suggest that β-carbolines might be useful for treatment of hypopigmentation-related disorders such as vitiligo.

MATERIALS AND METHODS

Materials

Harmaline and harmalol, α-MSH, L-DOPA, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and all other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). SB203580 was purchased from A.G. Scientific (San Diego, CA, USA). Antibodies recognizing phospho-p38, phospho-CREB, and CREB were obtained from Cell Signaling Technology (Beverley, MA, USA). Antibodies to p38, tyrosinase, TRP1, TRP2, and MITF were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture

Cells of the B16-F10 murine melanoma cell line obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA) were grown as monolayers in Dulbecco’s modified Eagle’s medium (DMEM; GIBCO BRL, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; GIBCO BRL). The cells were incubated at 37°C in a humidified atmosphere composed of 5% CO₂ and 95% air. To avoid changes in cell characteristics produced by extended cell culture periods, cells were used between passages 15 and 25. Each cell suspension was split every 2 days to maintain exponential growth.

Cell viability assay

The cells were incubated in wells of a 24-well plate at a density of 4 × 10⁴ cells/well. MTT solution (50 μg/ml) was added to each well. The plates were then incubated for an additional 3 h at 37°C in a 5% CO₂ atmosphere, after which the supernatant was removed and the formazan crystals that had formed in the viable cells were solubilized with dimethylsulfoxide (DMSO). The absorbance of each well was then read at 570 nm using an enzyme-linked immunosorbant assay (ELISA) reader (Wallace, Boston, MA, USA).

Determination of melanin content

Data are expressed as mean ± standard error (SE). Each experiment was repeated at least three times. Statistical analysis was performed with SPSS, version 16.0 software to determine significant differences. We used either one- or two-way ANOVA followed by Dunn’s post hoc tests for analyses. Values of *P < 0.05 and **P < 0.01 were considered statistically significant.

B16 F10 cell tyrosinase activity assay

Tyrosinase activity was determined by measuring the rate of dopachrome formation of L-DOPA. Cells were treated with harmaline and harmalol for 72 h, after which the cells were washed in ice-cold PBS and lysed in PBS containing 1% (w/v) Triton X-100. The tyrosinase substrate, L-DOPA (2 mg/ml), was prepared in the same phosphate lysis buffer. Each extract was placed in wells of a 96-well plate and the enzymatic assay was commenced by adding L-DOPA. After incubation, dopachrome formation was assayed by measuring absorbance at 405 nm using a microplate reader. The value of each measurement was expressed as percentage change from the control. In-situ L-DOPA reactivity of B16F10 cells was assessed using cultures fixed in 3.5% paraformaldehyde in PBS for 10 min at room temperature, after which they were permeabilized with 0.01% methanol for 10 min. Cells were incubated in L-DOPA (2 mg/ml) for 4 h at 37°C prior to photography using an Axiovert 40CFL inverted microscope (Carl Zeiss, Jena, Germany) equipped with an Infinity CAPTURE application version 4.6.0 digital video camera system (Lumenera, Ottawa, Ontario, Canada).

Western blot analysis

Cells were harvested in ice-cold lysis buffer consisting of 1% Triton X-100, 1% deoxycholate, and 0.1% sodium dodecyl sulfate (SDS). The protein content of the cell lysates was then determined using Bradford reagent (Bio-Rad; Hercules, CA, USA). Protein in each sample (50 μg total protein) were resolved by 7.5% SDS-polyacrylamide gel electrophoresis (SDS-
REFERENCES

The authors have declared that no conflict of interest exists.

was performed with SPSS, version 16.0 software to determine the experiment was repeated at least three times. Statistical analysis

... tometry was performed using ImageQuant TL software (Amersham Biosciences).

Immunofluorescence confocal microscopy

B16F10 cells cultured directly on glass cover-slips were fixed with 3.5% paraformaldehyde in PBS for 10 min at room temperature, after which they were permeabilized with 100% methanol for 10 min. To evaluate MITF expression and CREB phosphorylation, the cells were treated with a 1: 1,000 dilution in PBS of polyclonal antibody against MITF or phospho-CREB overnight. Next, the cells were extensively washed with PBS and incubated with a 1: 500 dilution in PBS of secondary fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit IgG antibody for 1 h at room temperature. Finally, the nuclei were stained with 1 g/ml of 4',6-diamidino-2-phenylindole (DAPI) and then analyzed by confocal microscopy using a Zeiss LSM 510 Meta apparatus.

Statistical analysis

The data is expressed as mean ± standard error (SE). Each experiment was repeated at least three times. Statistical analysis was performed with SPSS, version 16.0 software to determine significant differences. We used either one- or two-way ANOVA followed by Dunn's post hoc tests for analyses. Values of *P < 0.05 was considered statistically significant

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