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

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Melanin synthesis is regulated by melanocyte specific enzymes and related transcription factors. β-carboline alkaloids including harmaline and harmalol are widely distributed in the environment including several plant families and alcoholic beverages. Presently, melanin content and tyrosinase activity were increased in melanoma cells by harmaline and harmalol in concentration- and time-dependent manners. Increased protein levels of tyrosinase, tyrosinase-related protein-1 (TRP-1), and TRP-2 were also evident. In addition, immunofluorescence and Western blot analyses revealed harmaline and harmalol increased cAMP response element binding protein phosphorylation and microphthalmia-associated transcription factor expression. In addition to studying the signaling that leads to melanogenesis, roles of the p38 MAPK pathways by the harmaline and harmalol were investigated. Harmaline and harmalol induced time-dependent phosphorylation of p38 MAPK. Harmaline and harmalol stimulated melanin synthesis and tyrosinase activity, as well as expression of tyrosinase and TRP-1 and TRP-2 indicating that these harmaline and harmalol induce melanogenesis through p38 MAPK signaling. [BMB reports 2010; 43(12): 824-829]

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 melanosome release (2). Melanin synthesis is stimulated by various effects such as α-melanocyte-stimulating hormone (α-MSH); cyclic AMP (cAMP) elevating agents 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 tryrosinase, 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 dihyroxyindole or dihydroxyindole-2-carbxylic 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 melanogeneis, such as activation of MITF expression and activation of tyrosinase transcription (13), whereas the extracellular signal-regulated kinase (ERK)1/2 and c-Jun NH_2-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-

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ssion and tyrosinase activity.

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 inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) through the nuclear factor (NF)-kappaB and I-kappaB kinase (IKK) signaling pathways (21, 22). 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 on melanogenesis, specifically whether these β-carbolines are affected by melanogenesis, and characterized the signal transduction pathway involved.

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

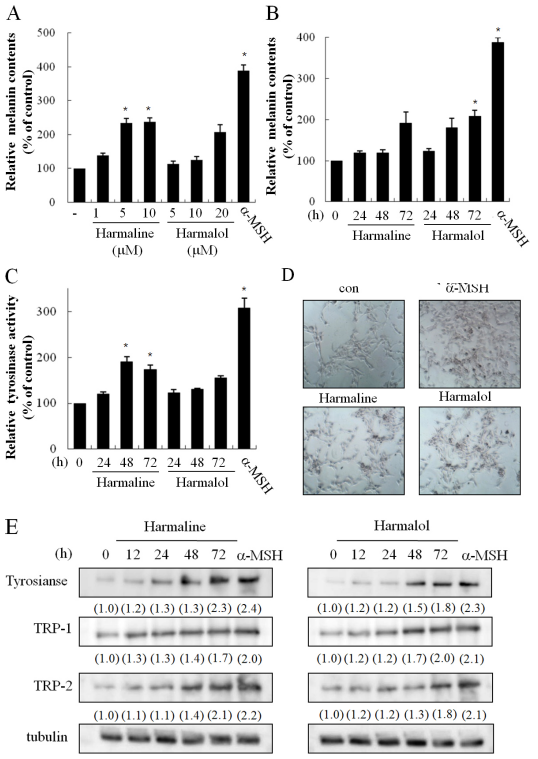

Fig. 1. Effect of harmaline and harmalol on melanin synthesis, tyrosinase activity and melanogenesis related proteins in B16F10 cells. (A) B16 cells treated for 72 h with harmaline (1-10 μM) and harmalol (5-20 μM) were analyzed for their melanin content. The melanin content was corrected based on the amount of protein concentration. α-MSH (1 μM) served as the positive control for 72 h. Cells treated with harmaline (5 μM) and harmalol (20 μM) for determined times were analyzed for their melanin content (B) and tyrosinase activity (C). α-MSH (1 μM) was the positive control for 72 h. The cellular tyrosinase activity was determined and is reported as a percentage reported relative to that in the control cells. Values are means ± SE of three independent experiments. *P < 0.05 versus untreated control. *In situ* tyrosinase activity determined by incubation of cells in L-DOPA. Images were captured under identical conditions using phase contrast microscopy and are representative of three independent experiments (D). (E) Cells were incubated for determined times with harmaline and harmalol. The cellular extracts were then subjected to Western blot analysis using tyrosinase, TRP-1, and TRP-2 antibody. Equal protein loading was confirmed by α-tubulin expression. Numbers at the bottom are expressed as relative intensity of band (fold of control) estimated by using ImageQuant TL software.

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 bisosynthesis, in which melanin is formed from the tyrosinase-catalyzed oxidation of L-tyrosinase. 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 expressions 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 fluoresecence 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

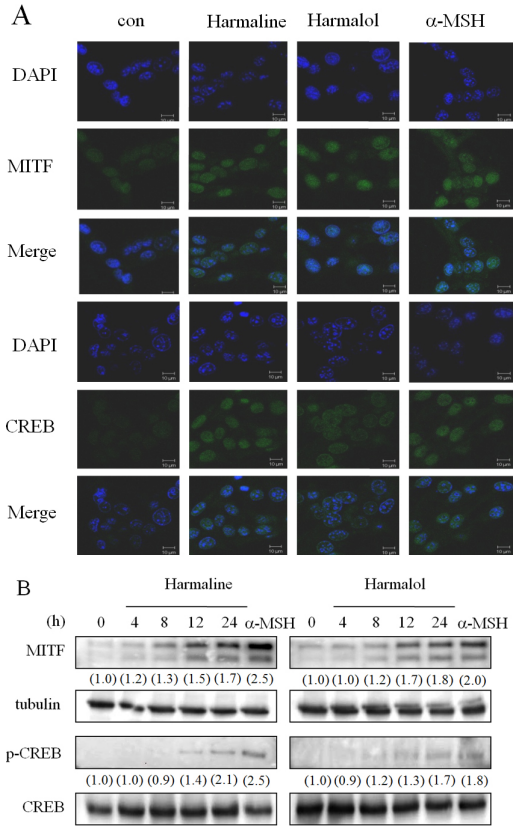

Fig. 2. Effects of harmaline and harmalol on MITF and phospho-CREB expression. B16F10 cells were grown on glass cover slips and then treated with harmaline (5 μM) and harmalol (20 μM). α-MSH (1 μM) served as the positive control for 24 h. At 24 h, the cells were fixed and analyzed by immunofluorescence labeling with a rabbit monoclonal anti-MITF and anti-phospho-CREB antibody, followed by incubation with Alex Fluor 488-conjugated goat anti-rabbit IgG. Nuclei were labeled with DAPI. Original magnification, ×100. (A). Cellular protein extracts were analyzed by Western blotting with anti-MITF and anti-phospho-CREB (B). Equal protein loading was confirmed by α-tubulin and CREB expression. Numbers at the bottom are expressed as relative intensity of band (fold of control) estimated by using Image Quant TL software.

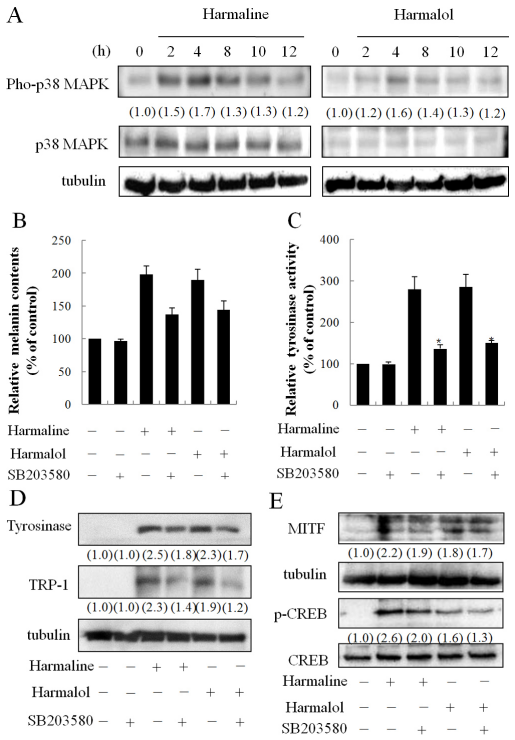

Fig. 3. Effects of harmaline and harmalol on phosphorylation of p38 MAPK in B16F10 melanoma cells. (A) Cells were treated with harmaline (5 μM) and harmalol (20 μM) for the indicated times. The cells were then harvested, after which the level of phosphorylated p38 was determined by Western blot analysis; detection of non-phosphorylated p38 was estimated protein-loading control for each lane. Numbers at the bottom are expressed as relative intensity of band (fold of control) estimated by using ImageQuant TL software. Cells were either untreated or pretreated with SB203580 (20 μM) for 1 h before harmaline (5 μM) and harmalol (20 μM) were applied for 72 h, and then analyzed by melanin content assay (B), tyrosinase activity assay (C) and Western blotting for anti-tyrosianse and anti-TRP-1 (D). Cells were exposed to harmaline (5 μM) and harmalol (20 μM) in the presence of SB203580 (20 μM) for 24 h. The expression levels of the MITF and CREB phosphorylation were examined by western blotting (E). Each percentage in the treated cells is reported relative to that in the control cells. Values are means ± SE of three independent experiments. *P < 0.05 compared with harmaline or harmalol and SB203580 co-treated one. Numbers at the bottom are expressed as relative intensity of band (fold of control) estimated by using ImageQuant TL software.

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 phosphrylation (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 realated 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 SB 203580, a selective inhibitor of p38 MAPK. The melanin content and tyrosinase activity of cells treated with harmaline and harmalol decreased following treatment with SB23580. 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-cabolines 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-dimetylthiazol-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% CO_2 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 4×10^4 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% CO_2 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 100% 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-

PAGE), transferred to a polyvinylidene difluoride (PVDF) membrane and exposed to the appropriate antibodies. The proteins were visualized by the enhanced chemiluminescence detection system (Amersham Biosciences, Piscataway, NJ, USA) using horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies. Images were acquired using an ImageQuant 350 analyzer (Amersham Biosciences) and densitometry 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 phosph-CREB overnight. Next, the cells were extensively washed with PBS and incubated with a 1 : 500 dilution in PBS of secondary fluorescein isothiocynate (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

Conflict of interest

The authors have declared that no conflict of interest exists.

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