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Partially purified components of *Nardostachys chinensis* suppress melanin synthesis through ERK and Akt signaling pathway with cAMP down-regulation in B16F10 cells

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ABSTRACT

Ethnopharmacological relevance: Nardostachys chinensis has been used in folk medicine to treat melasma and lentigines in Korea. We investigated the inhibitory activities of *Nardostachys chinensis* in melanogenesis and its related signaling pathway.
Materials and methods: Bioassay-guided fractionation of *Nardostachys chinensis* using solvent partitioning and purification with octadecylsilane open-column chromatography resulted in partial purification. The active 20% methanol chromatographic fraction from the ethyl acetate layer (PPNC) was used to investigate melanogenesis by melanin synthesis, tyrosinase activity assay, cAMP assay, Western blot and flow cytometric analyses in B16F10 mouse melanoma cells.
Results: PPNC markedly inhibits melanin synthesis and tyrosinase activity in a concentration-dependent manner. We also found that PPNC decreases microphthalmia-associated transcription factor (MITF), tyrosinase, tyrosinase-related protein (TRP)-1, and dopachrome tautomerase (Dct) protein expressions and MITF and tyrosinase mRNA levels. Moreover, PPNC reduces intracellular cAMP levels and activates mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) and phosphatidylinositol 3-kinase (PI3K)/Akt expression in B16F10 cells. The specific MEK/ERK inhibitor PD98059 and PI3K/Akt inhibitor LY294002, block the PPNC-induced hypopigmentation effect, and abrogate the PPNC-suppressed expression of melanogenic proteins such as MITF, tyrosinase, TRP-1, and Dct. Using flow cytometry, we elucidated whether PPNC directly induces ERK phosphorylation at the level of an intact single cell. PPNC shows marked expression of phosphorylated ERK in live B16F10 cells and abrogates PPNC-induced phosphorylated ERK by PD98059 treatment.
Conclusions: PPNC stimulates MEK/ERK phosphorylation and PI3K/Akt signaling with suppressing cAMP levels and subsequently stimulating MITF and TRPs down-regulation, resulting in melanin synthesis suppression.

1. Introduction

Pigmentation plays an important evolutionary role in camouflage, animal mimicry, and protection against harmful UV radiation in humans (Slominski et al., 2004; Costin and Hearing, 2007). Melanin is synthesized in the skin within specialized organelles named melanosomes; these melanin-containing melanosomes are then transferred to neighboring keratinocytes (Singh et al., 2008; Park et al., 2009). Melanin synthesis is stimulated by several signal transduction pathways, including the critical cAMP-mediated pathway, which plays an important role in melanogenesis regulation (Busca and Ballotti, 2000). In response to UV radiation, which is a potent cAMP activator, ligands bind to the MC1 receptor in the human epidermis, and this binding activates

Abbreviations: CREB, cAMP response element-binding protein; Dct, dopachrome tautomerase; ERK, extracellular signal-related kinase; MAPK, mitogen-activated protein kinase; MC1, melanocortin 1; MEK, mitogen-activated protein kinase kinase; MITF, microphthalmia-associated transcription factor; MSH, melanocyte-stimulating hormone; *N. chinensis*, *Nardostachys chinensis*; PI3K, phosphatidylinositol 3-kinase; PKA, protein kinase A; PKC, protein kinase C; PPNC, partially purified components of *Nardostachys chinensis*; TRP-1, tyrosinase-related protein-1; UV, ultraviolet.

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cAMP-dependent protein kinase A (PKA) and other regulatory proteins (Busca and Ballotti, 2000). PKA phosphorylates the cAMP response element-binding protein (CREB), which is known to be an activator of microphthalmia-associated transcription factor (MITF) gene expression. Activated CREB induces MITF transcription, which up-regulates the expressions of tyrosinase, tyrosinase-related protein (TRP)-1, and dopachrome tautomerase (Dct), thereby promoting eumelanin synthesis (Bertolotto et al., 1998a,b,c).

Many approaches have been used to help clarify the specific mechanism controlling melanin biosynthesis via tyrosinase regulation. Recent studies suggest that the mitogen-activated protein kinase (MAPK) pathway, and particularly the extracellular signal-related kinase1/2 (ERK), are involved in MITF regulation (Kawano et al., 2007; Alesiani et al., 2009). ERK phosphorylates MITF, which promotes its degradation, thereby leading to decreased melanin production (Kim et al., 2010). Another signaling pathway involved in negative melanogenesis regulation includes phosphatidylinositol 3-kinase (PI3K)/Akt signaling, which phosphorylates MITF and inhibits its activation, leading to melanogenesis inhibition (Oka et al., 2000; Khaled et al., 2003).

Nardostachys chinensis belongs to the Valerianaceae family, a widely used sedative herb (Lu and Chen, 1986; Takemoto et al., 2008). In the West, since ancient times, its oil has been used in the form of incense for psychological stability; it is also used as an ingredient in ointments. Rhizomes and roots of *Nardostachys* have been used in medicine against fungal infections, cardiac problems, and mental disorders (Kumar et al., 2006; Subashini et al., 2006; Mukherjee et al., 2007). In Korea, *Nardostachys chinensis* has been used in folk medicine to treat melasma and lentigines (Choi et al., 2001). However, the effects of *Nardostachys chinensis* on melanogenesis are not fully understood. In this study, we investigated the inhibitory effect of partially purified *Nardostachys chinensis* components against melanin biosynthesis and its molecular mechanism in B16F10 cells.

2. Materials and methods

2.1. Preparation of PPNC

The roots of *Nardostachys chinensis* Batalin were purchased from a local herbal store, Kwang-Myung-Dang (Busan, South Korea). The *Nardostachys* rhizome was confirmed and authenticated by Professor Y.T. Lee, College of Oriental Medicine, Dongeui University. A voucher specimen (WC35) was deposited at the School of Korean Medicine, Pusan National University.

Dry powdered *Nardostachys chinensis* roots (500 g) were immersed in 11 l of distilled water and boiled under reflux for 150 min. The resultant extract was centrifuged (2000 × *g* for 20 min at 4 °C) and filtered through a 0.2-μm filter. The filtrate was then evaporated to dryness under reduced pressure. Next, the dried extract (96.9 g) was dissolved in water and further fractionated in succession with ethyl acetate (2 l 5×), *n*-butanol, and distilled water, which produced fractions of 19.9 g, 24.1 g, and 51.2 g, respectively. The ethyl acetate fraction was concentrated by rotary vacuum evaporation (Eyela, Tokyo, Japan). The 7.7-g residue was then subjected to octadesylsilane (ODS) open-column chromatography using a 50 mm × 183 mm YMC-GEL (Kyoto, Japan) and eluted with the following incrementally increasing solvent system involving methanol (MeOH): 100% distilled water, 20% MeOH, 40% MeOH, 60% MeOH, 80% MeOH, 100% MeOH, and 100% dichloromethane. Seven fractions were obtained; these fractions were concentrated under reduced pressure and freeze-dried. The active 20% MeOH fraction (1764.9 mg) was used as the PPNC, and a voucher specimen (WC35-2-2) was deposited at the School of Korean Medicine, Pusan National University.

2.2. Chemicals and antibodies

Melanin, 3,4-dihydroxyphenylalanine (L-DOPA), 3-(4,5-dimetylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), PD98059, LY294002, bovine serum albumin (BSA), paraformaldehyde, and β-actin antibody were purchased from Sigma (St. Louis, MO, USA). A monoclonal antibody to MITF (#MAB3747) was obtained from Millipore (Bedford, MA, USA). Tyrosinase (C-19), TRP-1 (G-17), Dct (D-18), phospho-MEK (pMEK, ser218/ser222, sc-7995), MEK (12-B), phospho-ERK (pERK, tyr204, E-4), ERK (K-23), phospho-Akt (pAkt, ser473, sc-7985-R), Akt (C-20), phospho-CREB (pCREB, ser133, sc-7978-R), CREB (sc-58), phospho-p38 (pp38, Tyr182, D-8), and p38 (H-147) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Fluorescein isothiocyanate (FITC)-labeled anti-rabbit IgG (#4412) and FITC-labeled anti-mouse IgG (#4408) antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). The cAMP assay kit was purchased from R&D Systems (Minneapolis, MN, USA).

2.3. Cell culture

B16F10 mouse melanoma cells (CRL 6323) were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco Life Technologies, France) supplemented with 10% fetal calf serum and 1% penicillin/streptomycin in a 5% CO_2 humidified incubator at 37 °C.

2.4. Cell viability assay

The general viability of the cultured cells was determined through the reduction of MTT to formazan. After PPNC treatment, the cells were incubated for 48 h at 37 °C in an atmosphere containing 5% CO_2. MTT (1 mg/ml in PBS) was then added to each well at one-tenth the media volume. Cells were incubated at 37 °C for 3 h, and dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals. Absorbance was measured at 570 nm by using a spectrophotometer (Spectra MAX 190; Molecular Devices, Sunnyvale, CA, USA).

2.5. Measurement of cellular melanin contents

B16F10 cells were treated with PPNC for 5 days, were washed with PBS, and were then dissolved in 1 N NaOH in 10% DMSO at 80 °C for 1 h. The relative melanin content was determined by measuring the absorbance at 475 nm using an enzyme-linked immunosorbent assay (ELISA) reader.

2.6. Tyrosinase activity assay

After PPNC treatment for 3 days, tyrosinase activity was estimated by measuring the rate of L-DOPA. The cells were lysed by incubation at 37 °C for 30 min in RIPA buffer (0.1 M sodium phosphate, pH 7.0, 1% Triton X-100, 0.1 mM PMSF, and 1 mM NaF). Lysates were then centrifuged at 10,000 × *g* for 20 min to obtain the supernatant as the crude tyrosinase extract for the activity assay. The reaction mixture contained 0.1 M sodium phosphate (pH 7.0), 0.05% L-DOPA, and supernatant (tyrosinase source). After incubation at 37 °C for 1 h, the dopachrome was monitored by measuring the absorbance at 405 nm using an ELISA reader. The value of each measurement is expressed as percentage change from the control.

2.7. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was prepared from B16F10 cells treated with PPNC using TRIZOL reagent™ (Invitrogen, Paisley, UK)