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Vasoactive intestinal peptide stimulates melanogenesis in B16F10 mouse melanoma cells via CREB/MITF/tyrosinase signaling

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ABSTRACT

Vasoactive intestinal peptide (VIP), one of the major skin neuropeptides, has been suggested to have active roles in the pathogenesis of inflammatory skin disorders such as atopic dermatitis and psoriasis, which can commonly cause post-inflammatory hyperpigmentation. However, the effect of VIP on melanogenesis remains unknown. In this study, we showed that the melanin contents, tyrosinase activity, and gene expression of tyrosinase and microphthalmia-associated transcription factor (MITF) were significantly increased by treatment with VIP in B16F10 mouse melanoma cells and the stimulatory melanogenic effect was further examined in human epidermal melanocytes (HEMns). In addition, phosphorylated levels of CRE-binding protein (CREB) and protein kinase A (PKA) were markedly increased after VIP treatment, but not p38 mitogen-activated protein kinase (p38 MAPK), extracellular signal-regulated kinase (ERK), or Akt, indicating the possible PKA-CREB signaling pathway involved in VIP-induced melanogenesis. This result was further verified by the fact that VIP induced increased melanin synthesis, and protein levels of phosphorylated CREB, MITF, tyrosinase were significantly attenuated by H89 (a specific PKA inhibitor). These data suggest that VIP-induced upregulation of tyrosinase through the CREB-MITF signaling pathway plays an important role in finding new treatment strategy for skin inflammatory diseases related pigmentation disorders.

1. Introduction

Vasoactive intestinal peptide (VIP) is a major skin neuropeptide secreted from sensory and autonomic nerve endings [1] distributed in the epidermis [2], basal layer, dermis, sweat glands [3], and hair follicles [4] in a variety of biological conditions. VIP belongs to the gastrin/glucagon/secretin family of secretory peptides, is composed of 28 amino acids, and binds to structurally distinct G protein-coupled receptors, VIP receptor 1 (VIPR1) and VIPR2 [5,6]. Activation of the VIP/VIPR system mediates various physiological processes, including mast cell degranulation, plasma extravasation, vasodilatation, and immunomodulation [7,8]. Previous studies suggest important roles of VIP in the pathogenesis of inflammatory skin diseases such as psoriasis and atopic dermatitis [2,9].

Melanin plays important roles in human skin color regulation and in photo-protection of skin cells from potent damage caused by UV radiation [10]. However, abnormal melanin accumulation is responsible for pigmented disorders such as post-inflammatory hyperpigmentation (PIH), which is extremely common in inflammatory associated skin disorders, including psoriasis and atopic dermatitis [11–13].

The second messenger cyclic AMP (cAMP) leads to protein

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kinase A (PKA) activation and then consequent cAMP responsive element-binding protein (CREB) phosphorylation, which is well known as an important index in the upregulation of microphthalmia-associated transcription factor (MITF), a basic helix-loop-helix transcriptional factor critical for melanogenesis [14–16]. In turn, MITF critically regulates melanogenesis related genes, such as tyrosinase, tyrosinase-related protein-1 (TRP-1) and tyrosinase-related protein-2 (TRP-2) [17]. In addition, p38 mitogen-activated protein kinase (p38 MAPK), extracellular signal-regulated kinase (ERK), and Akt [18–20] signalings are also known as active pathways regulating melanogenesis in melanoma cells and melanocytes.

In the present study, we examined the effect of VIP on melanogenesis in B16F10 mouse melanoma cells and human epidermal melanocytes. The aim of this study was to investigate the role of VIP on melanogenesis through the determination of melanin synthesis and the underlying molecular events.

2. Materials and methods

2.1. Antibodies and reagents

VIP was obtained from R&D Systems (Minneapolis, MN).

Forskolin, L-DOPA, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), H89 were from Sigma (St. Louis, MO, USA). Antibody against MITF (C5, MS-771-P0) was obtained from Neo Markers (Fremont, CA, USA). Antibodies specific to β-actin, TRP-1, TRP-2, and tyrosinase were from Santa Cruz (Santa Cruz, CA, USA). Specific antibodies for phospho-ERK1/2 (Thr202/Tyr204, #9101S), phospho-Akt (Ser473, #9271S), phospho-P38 MAPK (T180/Y182, #9211S), phospho-CREB (Ser133, #9198S), total CREB (#9197S) and phospho-PKA C (T197, #4781S), were from Cell Signaling Technology (Beverly, MA, USA).

2.2. Cell culture

B16F10 mouse melanoma cells obtained from the Korean Cell Line Bank (Seoul, Korea) were cultured in phenol red-free Dulbecco's modified Eagle's medium (DMEM, Gibco, Rockville, MD) with 10% fetal bovine serum (FBS) and antibiotics. Cells were incubated at 37 °C under a humidified atmosphere condition with 5% CO2. Primary human epidermal melanocytes (HEMns) were obtained from human adult foreskin. HEMns were cultured in Medium 254 (M254, Life Technologies) supplemented with human melanocyte growth supplement (Life Technologies). HEMns at passages of 4–8 were used for the experiments.

2.3. Cell viability assay

MTT assay was used for measuring cell viability. B16F10 cells were incubated for 24 h, and then treated at the indicated concentrations of VIP for 48 h, followed by addition of MTT (5 mg/ml dissolved in PBS). After incubation for an additional 4 h, the supernatant was removed and the formazan crystals were dissolved with DMSO. The absorbance was measured at 570 nm with a spectrophotometer (Molecular Devices, Sunnyvale, CA, USA).

2.4. Melanin content measurement

Melanin contents were determined as described previously [21,22], with some modifications. B16F10 cells and HEMns were incubated overnight, then treated with various concentrations of VIP for 3 and 6 days, respectively. Following treatment, the absorbance of culture media was measured using a spectrophotometer at 405 nm. Cells were then lysed and the supernatants were determined for the concentration of protein. Cell pellets were dissolved in 100 μl of 1 N NaOH at 90 °C for 15 min. The absorbance was measured spectrophotometrically at 405 nm. Extracellular and intracellular melanin contents were normalized to the protein concentrations.

2.5. Tyrosinase activity assay

B16F10 cells were incubated with VIP at different time points. Cells were lysed with PBS containing 1% Triton X-100 after washing twice with ice-cold PBS. Each cell lysate for 15 μg protein was adjusted with lysis buffer for total volume of 90 μl, and transferred to a 96-well plate with addition of 10 μl L-DOPA (10 mmol/l). The plate was incubated at 37 °C for 15 min, and then the absorbance was measured at 475 nm.

2.6. Reverse transcription and real-time quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA) and the same amount of total RNA was converted to cDNA using First Strand cDNA Synthesis kit (MBI Fermentas, Vilnius, Lithuania) according to the manufacturer's instructions. The cDNAs of tyrosinase, MITF, and endogenous reference 36B4 were quantitatively evaluated by a 7500 Real-time PCR system (Applied Biosystems, Foster City, CA, USA) using the SYBR Premix Ex Taq™ (Takara Bio Inc., Shiga, Japan). The primer sequences were as follows: mouse tyrosinase sense oligonucleotide, 5′-CCTCCTGGCAGATCATTTGT-3′, and antisense oligonucleotide 5′-GGCAAATCCTTCCAGTGTGT-3′; mouse MITF sense oligonucleotide, 5′-ATGCTGGAAATGCTAGAATACAGT-3′, and antisense oligonucleotide 5′-ATCATCCATCTGCATGCAC-3′; mouse 36B4 sense oligonucleotide, 5′-TGGGCTCCAAGCAGATGC-3′, and antisense oligonucleotide 5′-GGCTTCGCTGGCTCCCAC-3′. Data were analyzed using the comparative ΔΔCt method, and presented as fold changes in gene expression normalized to 36B4.

2.7. Western blotting

Cells were scraped at different time points, and then lysed in RIPA buffer (Merck Millipore, Billerica, MA) containing phosphatase inhibitor (Sigma-Aldrich, St. Louis, MO) and protease inhibitor (Roche Applied Science, Rockford, IL). Equal amounts (20 μg) of protein were loaded on 10% SDS-polyacrylamide gel, separated by electrophoresis and transferred onto polyvinylidene difluoride membranes (Roche Applied Science). Membranes were then blocked with Tris-buffered saline containing 0.1% Tween 20 and 5% skim milk, which were subsequently incubated with primary antibodies specific to each protein for 18 h at 4 °C, followed by additional incubation with horseradish peroxidase-conjugated secondary antibodies. Target proteins were developed by enhanced chemiluminescence (ECL, Amersham, Buckinghamshire, England). For some experiments, signal intensity was quantified using a densitometric program (Image J, W. Rasband, NIH, USA).

2.8. Statistical analysis

Data was presented as the mean ± standard deviation. Statistical significance was analyzed with the Student's t-test. Differences were determined statistically significant at a P-value of less than 0.05.