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Cross-talk between 5-hydroxytryptamine and substance P in the melanogensis and apoptosis of B16F10 melanoma cells

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ABSTRACT

Skin pigmentation is a complex process controlled by many different factors. Substance P (SP) regulates many biological functions, including melanogenesis and stress. Our previous study indicated that regulation of SP on melanocyte function was mediated by neurokinin 1 receptor (NK_1 receptor). Substantial evidence has accumulated that psychological stress can be associated with skin pigmentation, so that the impact of 5-hydroxytryptamine (5-HT), one of the important factors participating in stress process, on melanogenesis has also been concerned. It has been reported that 5-HT induces melanin synthesis via 5-HT_{2A} receptor. Furthermore, 5-HT_{2A} receptor and NK_1 receptor are G-protein coupled receptors (GPCRs) and both expressed on melanocyte, the present study was designed to investigate whether SP has influence on the adjustment function of 5-HT. Our data demonstrated that, SP inhibited 5-HT_{2A} receptor expression to neutralize the pro-melanogenesis effect of 5-HT on B16F10 cells. The up-regulation of NK_1 receptor expression was simultaneous with the down-regulation of 5-HT_{2A} receptor treated by SP. This inhibition of 5-HT_{2A} receptor expression by SP could be reversed by NK_1 receptor antagonist Spantide I. Our studies indicated that SP could directly induce B16F10 cells apoptosis in vitro. 5-HT and 5-HT_{2A} receptor agonist could mitigate this apoptotic effect of SP. It is the strong evidence of possible cross-talk between GPCRs and giving enlightenments when screening desirable drugs for target receptors.

1. Introduction

Skin is the largest organ in human body, which plays a role as biologically active barrier to the external environment. It acts not only as a target for neuroendocrine signals but also a source of hormones and neurotransmitters, particularly the epidermis (Slominski and Wortsman, 2000). Melanocytes are an intraepidermal population of dendritic cells responsible for the pigmentation of skin and hair, and thereby contribute to the appearance of skin and provide photoprotection and thermoregulation by producing melanin (Lin and Fisher, 2007). Melanin biosynthesis process is under a complex control mediated by enzymatic cascade, such as tyrosinase (TYR), tyrosinase-related protein 1 (TRP-1) and dopachrome tautomerase (DCT) (Fang et al., 2001; Slominski et al., 2004b). The tyrosinase family genes TYR, TRP-1, DCT responsible for pigmentation are transcriptionally regulated by microphthalmia associated transcription factor (MITF) (Grill et al., 2013; Levy et al., 2006). Skin cells can produce hormones, neurotransmitters and neuropeptides and corresponding functional receptors, owing to which constitute the cutaneous equivalent of the hypothalamic-pituitary-adrenal (HPA) axis (Slominski, 2005; Arck et al., 2006). It has recently uncovered that cutaneous expression of the biochemical machinery involved in the sequential transformation of L-tryptophan to serotonin and melatonin (Slominski et al., 2002, 2005). Serotonin (5-hydroxytryptamine, 5-HT) is a neurotransmitter whose actions are mediated via an interaction with receptors, including seven families (5-HT_{1-7} receptors) with at least 21 subtypes. Skin cells express mRNA encoding 5-HT receptors, including 5-$HT_{1A, -1B, -2A, -2B, -2C \text{ and } -7}$ receptors (Slominski et al., 2004a, 2003b). 5-HT_{2A} receptor was reported participating in the regulatory processes of 5-HT on melanogenesis (Lee et al., 2011). Our preliminary study also found that 5-HT_{1A} receptor took a vital part in 'brain-skin connection' to regulate skin pigmentation (Wu et al., 2014). 5-HT receptors (except 5-HT_3 receptor) belong to G-protein

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coupled receptors (GPCRs).

Substance P (SP) is an undecapeptide that belongs to the tachykinin family of peptides. Today it is fully recognized that SP is released from both central nervous system (CNS) and the peripheral nervous system (PNS) and functions as a neurotransmitter (Harrison and Geppetti, 2001). The cutaneous pruritus, dermatitis, and other skin inflammatory diseases are said to be induced by stress (Kimyai-Asadi and Usman, 2001). The skin disease pathology is mostly attributed to central activation of the HPA axis or the sympathetic nervous system (Slominski and Wortsman, 2000). There is abundant evidence proved that stress can induce the release of SP and other neuromediators from peripheral nerve endings in the skin resulting in neurogenic inflammation (Arck et al., 2001, 2003). SP is released under stress conditions and accumulates around wounds. The released SP in skin can initiate inflammation via the accumulation of immune factors (Katsanos et al., 2008). SP can as well as evoke uncomfortable skin sensations such as numbness, itching (Andoh et al., 1998), sensitivity, and tingling (Summer et al., 2007; Wei et al., 2009). SP is also involved in stress-induced hair loss (Peters et al., 2007; Liu et al., 2013). Calcitonin gene-related peptide cooperates with substance P to inhibit melanogenesis and induces apoptosis of B16F10 cells (Zhou et al., 2015). It is conjectured that SP and its related signaling pathways may be a potent target for depigmentation under stress or wound conditions in which SP naturally accumulates (Park et al., 2015). SP binds to at least three types of G protein–coupled receptors (the neurokinin receptors,NK_1 receptor, NK_2 receptor, NK_3 receptor) to regulate biological functions. Among the receptors, NK_1 receptor shows the highest affinity for SP (De Swert and Joos, 2006). It is demonstrated that SP and NK_1 receptor both play a role in skin pigment abnormalities (Falabella et al., 2003; Park et al., 2015; Ping et al., 2012).

Some studies indicated that antagonism of NK_1 receptor in rats increases 5-HT transmission in the hippocampus (Blier et al., 2004). Since the SP–NK_1 receptor and 5-HT–5-HT_{2A} receptor are involved in melanin biosynthesis, we investigated whether there exists some cross-talk between 5-HT and SP and the impact of this cross-talk on melanocyte functions.

2. Materials and methods

2.1. Cell culture and materials

Murine B16F10 melanoma cell line (B16F10) was obtained from CAS (Chinese Academy of Sciences, China). The B16F10 cells were grown in DMEM medium (GIBICO, USA) supplemented with 10% heat-inactivated fetal bovine serum (GIBICO, USA), 100 U/ml penicillin and 100 μg/ml streptomycin (GIBCO, USA) in a humidified atmosphere with 5% CO_2 at 37 °C. SP, NK_1 receptor antagonists (spantide I), 5-HT, 5-HT_{2A} receptor agonist (DOI) and 5-HT_{2A} receptor antagonist (ketanserin) were purchased from TOCRIS Bioscience (United Kingdom). β-actin antibodies, and horseradish peroxidase-conjugated secondary antibodies were purchased from Sigma-Aldrich (USA). Tyr (C-19) and TRP-1antibody were from Santa Cruz Biotechnology (USA). MITF antibody was purchased from Bioworld Technology (USA). DCT, NK_1 receptor and 5-HT_{2A} receptor antibodies were from Abcam Technology (UK). ProLong gold anti-fade reagent with DAPI were from Life Technologies Corporation (USA). Enhanced BCA protein assay kit, phenylmethylsulfonyl fluoride (PMSF) and cell lysis buffer for Western and IP, and One Step TUNEL Apoptosis Assay Kit were from Beyotime Institute of Biotechnology (China).

2.2. Melanin measurement and tyrosinase assay

The tyrosinase activity and melanin content of the cultured melanoma cells were determined as described previously (Zhou et al., 2014). In brief, B16F10 cells were treated for 48 h, washed with ice-cold PBS, lysed by incubation in cell lysis buffer [1 mM PMSF] at 4 °C for 20 min, and then lysates were centrifuged at 15,000*g* for 15 min to obtain the supernatant for activity assay and centrifugation for melanin contents assay. Protein concentrations were determined by BCA Kit. 100 μl of supernatant containing the same 30 μg total proteins was added to each well in 96-well plate, and then mixed with 100 μl 0.1% L-DOPA in PBS (pH 6.8). After incubation at 37 °C for 45 min, the dopachrome was monitored by measuring the absorbance at 475 nm.

Total melanin in the cell pellet was dissolved in 100 μl of 1 N NaOH/10% DMSO for 1 h at 80 °C, and solubilized melanin was measured at 405 nm. Melanin content was calculated from a standard curve using synthetic melanin.

2.3. Western blot analysis

The protein suspension was obtained as the method mentioned above.Western blot was performed as described previously (Zhou et al., 2014). The primary antibodies used were Mitf (BS1550), TYR (SC7833), TRP-1 (SC10443), DCT (ab74073), NK_1 receptor (ab75516), 5-HT_{2A} receptor (ab66049).

2.4. TUNEL staining

Apoptotic cells were detected using a TUNEL assay (C1090). Briefly, a cell smears were washed by PBS, and fixed with 4% paraformaldehyde solution for 60 min. Then washed by PBS and added PBS containing 0.1%Triton X-100 for 2 min in the ice bath. And the labelling reaction was performed using a labelling solution containing terminal deoxynucleotidyl transferase, its buffer, and fluorescein dUTP at 37 °C for 60 min in a humidity chamber. Following incubation, excess labelling solution is washed off with PBS and the cell smears are mounted on coverslips with Anti-fade Fluorescence Mounting Medium, and the nuclei were stained with DAPI. Images were captured using a CCD camera (Olympus CKX41, Tokyo, Japan).

2.5. Statistical analysis

All data were expressed as mean ± S.D. Statistical analysis was performed with one-way ANOVA followed by Tukey's *post-hoc* test for multiple comparisons tests. Significant differences were accepted when $P < 0.05$.

3. Results

3.1. Effect of 5-HT, SP and their cross-talk on melanogenesisin B16F10 cells

To investigate the effect of 5-HT,SP and their cross-talk on melanogenesis, B16F10 cells were divided into 4 groups, treated respectively with culture medium only, 5-HT (10,010 μM), SP (10 nM) and (100 μM 5-HT ± 10 nM SP) together. As shown in Fig. 1A, 100 μM of 5-HT increased tyrosinase activity significantly while 10 nM of SP made decreasing effect after treatments for 48 h. 5-HT up-regulated the level of melanin synthesis and SP down-regulated melanogenesisin B16F10 cell (Fig. 1B). When the B16F10 cells were treated with 100 μM 5-HT and 10 nM SP simultaneously, the neutralizing efficacy was got, both on tyrosinase activity and melanin content (Fig. 1A and B).