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Citrus flavanone naringenin enhances melanogenesis through the activation of Wnt/β-catenin signalling in mouse melanoma cells

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ABSTRACT

Citrus fruits are the major source of flavonoids for humans, and flavanones are the main flavonoids in the *Citrus* species. Among the *Citrus* flavanones, the glycoside derivatives of naringenin, naringin and narirutin, are the most abundant in grapefruit. The present study aimed to investigate the molecular events of melanogenesis induced by naringenin in murine B16-F10 melanoma cells. Melanin content, tyrosinase activity and Western blot analysis were performed to elucidate the possible underlying mechanisms. Exposure of melanoma cells to naringenin resulted in morphological changes accompanied by the induction of melanocyte differentiation-related markers, such as melanin synthesis, tyrosinase activity, and the expression of tyrosinase and microphthalmia-associated transcription factor (MITF). We also observed an increase in the intracellular accumulation of β-catenin as well as the phosphorylation of glycogen synthase kinase-3β (GSK3β) protein after treatment with naringenin. Moreover, the activity of phosphatidylinositol 3-kinase (PI3K) was up-regulated by naringenin since the phosphorylated level of downstream Akt protein was enhanced. Based on these results, we concluded that naringenin induced melanogenesis through the Wnt-β-catenin-signalling pathway.

Introduction

The colour of mammalian skin and hair is determined by a number of factors, the most obvious phenotypical characteristics of which is the distribution of the melanin pigment. Melanin synthesis is a complex process that occurs within specialized intracellular organelles named melanosomes in melanocytes. Melanogenesis can be stimulated by stress, including UV radiation, inflammation, and hormones (Costin and Hearing 2007). Furthermore, α-melanocyte-stimulating hormone (α-MSH) and cAMP-elevating agents, such as forskolin and IBMX, through the activation of protein kinase A (PKA) and cAMP-related element binding protein (CREB) transcription factor, promote an increase in the expression of microphthalmia-associated transcription factor (MITF), a master regulator of the development and differentiation of melanocytes. MITF transcriptional regulates the expression of tyrosinase and tyrosinase-related protein that control the conversion of tyrosine to melanin pigments (Vachtenheim and Borovanský 2010). Previous papers have linked up-regulated melanogenesis to melanoma, and this viewpoint enhances the importance of further melanogenetic studies.

The Wnt-signalling pathways play an important role in melanocyte development, melanoma genesis and pigment cell formation (O'Connell and Weeraratna 2009). Glycogen synthase kinase 3 (GSK3) is one of the few signalling mediators that play a central role in a diverse range of signalling pathways, including those activated by Wnts, growth factors, and G protein-coupled ligands (Wu and Pan 2010). When WNT proteins bind to their receptors, they inactivate GSK3β, an enzyme that phosphorylates β-catenin and specifically targets its destruction in the proteasome (Bienz 2005). Then, β-catenin accumulates in the cytoplasm and translocates to the nucleus. Increased levels of nuclear β-catenin increase the expression of MITF, and in turn increase the survival and proliferation of melanoma cells (Miller and Mihm 2006). However, GSK3β which is a negative regulator of Wnt signalling, is capable of activating MITF function through phosphorylation at Ser 298 (Takeda et al. 2000a). A recent study has demonstrated that inhibiting GSK3β increases melanogenesis both in murine B16 cells and human melanocytes (Bellei et al. 2008). It is therefore possible that GSK3β contributes to maintaining the levels of MITF in melanogenesis.

Flavanones occur almost exclusively in citrus fruits. *Citrus* flavanones exhibit wide ranges of biological activities, such as antioxidant, anti-inflammatory and anti-tumour activities, which indicate that these compounds may exert beneficial effects against cardiovascular diseases or cancers (Benavente-García and Castillo 2008). The main flavonoids in grapefruit are naringin

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(naringenin-7-neohesperoside) (70%) and narirutin (naringenin-7-rutinoside) (20%) (Kawaii et al. 1999). Most studies on naringenin have reported its possible roles in grapefruit juice-drug interactions (Fuhr 1998). Recently, several reports have focused on the potential use of flavonoids for preventing oxidative skin damage (Mortimer 1997; Proteggente et al. 2003). Bioflavonoids with flavanone structures, such as hesperidin, have been found to inhibit tyrosinase activity in human primary melanocytes (Zhu and Gao 2008). In a previous study, it has been shown that naringenin increases the melanin content and tyrosinase activity by increasing the expression of melanogenic enzymes (Ohguchi et al. 2006). The mechanisms underlying the activities of naringenin and its glycosides on melanogenesis have not yet been well elucidated. The present study aimed to evaluate whether the flavanones in grapefruit juice affect melanogenesis in melanoma cells and to elucidate the possible underlying related signalling.

Materials and methods

Materials

Naringenin, L-DOPA, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), melanin and IBMX were obtained from Sigma–Aldrich (St. Louis, MO, USA). Naringin and narirutin were purchased from Acros Organics and ECHO Chemical Co., respectively. Antibodies for tyrosinase, β-catenin, and phospho-Akt (Ser 473) were obtained from Epitomics (Burlingame, CA, USA). Actin and phospho-GSK3β (Ser 9) antibodies were supplied by Millipore (Temecula, CA, USA). Anti-MITF antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Naringenin, narirutin, or naringin was dissolved in dimethylsulfoxide (DMSO) and further diluted in culture medium. The final DMSO concentration in the medium was 0.1% and did not affect cellular function or the assay systems used in this study.

Cell culture

The B16-F10 murine melanoma cells were purchased from the Bioresource Collection and Research Center (Hsinchu, Taiwan) and maintained in Dulbecco's modified Eagle's medium (DMEM; Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (Biological Industries, Kibbutz Beit Haemek, Israel), 50 U/ml penicillin, and 50 μg/ml streptomycin in a humidified incubator at 37 °C in 5% CO_2/air.

Cell viability assay

Briefly, cells were seeded at a density of 7×10^4/ml on 96-well plates and cultured overnight as described above. The medium was then replaced with fresh medium containing flavanones at various concentrations. After incubation for 48 h at 37 °C in 5% CO_2/air, MTT (final concentration, 0.5 mg/ml) was added, and the cells were then incubated at 37 °C for 2 h. Finally, the cells were lysed and absorbance was detected at 550 nm. For cell number determination, a standard correlation between the known cell numbers and the absorbance density values was constructed for measuring the cell number from various detected absorbance density values.

Melanin content determination

The melanin content was measured by a previously described method (Kim et al. 2005) with slight modifications. Cells were seeded at a density of 9×10^5/ml in 60-mm dishes and cultured as described above. After overnight incubation, cells were then cultured for 48 h with or without flavanones in either the absence or presence of LY294002. The medium was then removed, and the cells were washed twice with phosphate-buffered saline (PBS) and harvested by trypsinisation using 0.05% trypsin/0.02% EDTA. The harvested cells were centrifuged, and the pellet was dissolved by adding 1 N NaOH, followed by incubation at 60 °C for 1 h. The amount of melanin in the solution was determined by measuring the absorbance at 470 nm using the microplate reader (BioTek, Synergy HT). The total melanin content was estimated using the standard curve of synthetic melanin.

Tyrosinase assay

Tyrosinase enzyme activity was estimated spectrophotometrically as described earlier (Bellei et al. 2008), using L-DOPA as the substrate. B16-F10 cells cultured with or without naringenin for 48 h were solubilised with 0.1 M sodium phosphate buffer (pH 6.8) containing 1% Triton X-100. The cells were then disrupted, and centrifuged at $10{,}000 \times g$ for 30 min. After protein quantification and adjustment, 90 μl of cell lysate (each containing the same amount of protein) was incubated in duplicate with 10 μl of 10 mM L-DOPA at 37 °C for 90 min. The absorbance was then monitored at 475 nm. In order to assess the direct activity of tyrosinase, naringenin was added to cell lysate at the highest concentration and incubated for 5 min at room temperature. The cell lysates were then mixed with L-DOPA solution and incubated at 37 °C for 2 h, as described above.

Western blot analysis

Whole cell lysates were prepared using RIPA buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 1 mM PMSF, 1 mM Na_3VO_4, 1 mM NaF, 1 μg/ml aprotinin, 1 μg/ml pepstatin, and 1 μg/ml leupeptin). Aliquots of cell lysates were separated by electrophoresis on sodium dodecyl sulphate-polyacrylamide gels and transferred onto polyvinylidene difluoride membranes and then blotted with the appropriate antibodies. Finally, the proteins were detected using an enhanced chemiluminescence kit (Amersham Biosciences, Buckinghamshire, UK). Quantitative analysis was performed using ImageQuant analysis software (GE Healthcare).

Statistical evaluation

Data are expressed as mean ± S.E.M. of the indicated number of separate experiments. A one-way analysis of variance was performed for multiple comparisons, and if there was significant variation between treatment groups, the mean values were compared with the respective control using Student's *t*-test. *P* values less than 0.05 were considered significant.

Results and discussion

As a first step towards determining the effects of naringenin and its related glycosides on melanogenesis, we measured the cell viability and melanin content in B16-F10 melanoma cells. Cells treated with various concentrations of the flavanones (3–100 μM) were estimated using the mitochondria MTT reduction assay. The results demonstrated that the three structure-related flavanones – naringenin, naringin and narirutin – had no cytotoxic effects at concentrations ranging from 3 to 50 μM (data not shown). The cells were then exposed to the flavanones (50 μM) for 48 h, and cellular melanin contents were examined. As shown in Fig. 1a, naringenin increased melanin synthesis apparently. Narirutin or naringin, the rutinose or neohesperidose glycoside of naringenin, did not show the melanogenic effects as potential as naringenin did. Although narirutin also enhanced the melanin production, it simultaneously increased the cell number, indicating that the effects of narirutin on melanogenesis may occur due to cell growth. The cell proliferation and membrane integrity of 50 μM naringenin was also evaluated by a trypan blue exclusion assay after 48 h treatment. Naringenin did not exert the proliferative effect in B16-F10 cells since no statistically significant differences were observed between naringenin-treated cells and DMSO-treated control cells (data not shown). Here, we confirmed that only naringenin enhanced melanin synthesis in B16-F10 cells, but not its derivatives