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Short communication

Hesperetin induces melanin production in adult human epidermal melanocytes

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ABSTRACT

One of the major sources of flavonoids for humans are citrus fruits, hesperidin being the predominant flavonoid. Hesperetin (HSP), the aglycon of hesperidin, has been reported to provide health benefits such as antioxidant, anti-inflammatory and anticarcinogenic effects. However, the effect of HSP on skin pigmentation is not clear. Some authors have found that HSP induces melanogenesis in murine B16-F10 melanoma cells, which, if extrapolated to *in vivo* conditions, might protect skin against photodamage. Since the effect of HSP on normal melanocytes could be different to that observed on melanoma cells, the described effect of HSP on murine melanoma cells has been compared to the effect obtained using normal human melanocytes. HSP concentrations of 25 and 50 μM induced melanin synthesis and tyrosinase activity in human melanocytes in a concentration-dependent manner. Compared to control melanocytes, 25 μM HSP increased melanin production and tyrosinase activity 1.4-fold ($p < 0.01$) and 1.1-fold ($p < 0.01$), respectively, and the corresponding increases in the case of 50 μM HSP were 1.9-fold ($p < 0.001$) and 1.3-fold ($p < 0.001$). Therefore, HSP could be considered a valuable photoprotective substance if its capacity to increase melanin production in human melanocyte cultures could be reproduced on human skin.

1. Introduction

Human skin pigmentation has many valuable functions, the most important being its role in photoprotection due to its ability to absorb ultraviolet radiation (Costin and Hearing, 2007; Lin and Fisher, 2007; Park et al., 2009). Melanogenesis is the process responsible for cutaneous synthesis and the distribution of the pigment melanin, which is stimulated by many intrinsic and extrinsic regulation factors, such as ultraviolet radiation and the melanocyte-stimulating hormone (α-MSH). Melanin is synthetized by melanocytes via an enzymatic cascade leading to the conversion of tyrosine to melanin pigment, and one of the key enzymes in this process is tyrosinase, which is considered the rate-limiting enzyme for controlling the production of melanin. Enzymatic components of melanosomes include tyrosinase, tyrosinase-related protein 1 and DOPAchrome tautomerase. Phenotypic diversity of skin pigmentation is closely related to the variation in the number, size, composition and distribution of melanosomes, whereas melanocyte numbers typically remain relatively constant (Lin and Fisher, 2007).

A great deal of research has recently focused on the potential use of flavonoids to prevent photodamage. Hesperetin (HSP, 3′,5,7-trihydroxy-4′-methoxyflavanone) belongs to the flavanone subclass of flavonoids, one of the largest groups of phenolic plant constituents. HSP is the aglycone of hesperidin, the predominant flavonoid found in citrus fruits (Nielsen et al., 2006). The chemical structure, molecular formula and molecular weight of HSP is shown in Fig. 1. HSP has been reported to possess many beneficial effects due to its antioxidant (Choi, 2008; Kim et al., 2004; Pollard et al., 2006), anticarcinogenic (Aranganathan and Nalini, 2009; Li et al., 2013; Yang et al., 2013), anti-inflammatory (Hirata et al., 2005), neuroprotective (Huang et al., 2012a) and cardioprotective (Trivedi et al., 2011) properties. Moreover, HSP is hypolipidemic (Morin et al., 2008), platelet-aggregation-inhibitory (Jin et al., 2007) and effective in attenuating airway hyperresponsiveness (Shih et al., 2012). However, the effect of HSP on skin pigmentation is not clear. Some authors have described a whitening effect of HSP when applied on the skin of laboratory animals (Huang et al., 2010; Tsai et al., 2010), while others have found that HSP induces melanogenesis in murine melanoma cells when added to the culture medium (Huang

Abbreviations: α-MSH, melanocyte-stimulating hormone; DMSO, dimethylsulfoxide; HEMa, Hightly pigmented adult human epidermal melanocytes; HSP, Hesperetin; L-DOPA, 3,4-dihydroxy-L-phenyalanine; MITF, microphthalmia-associated transcription factor; PBS, phosphate-buffered saline.

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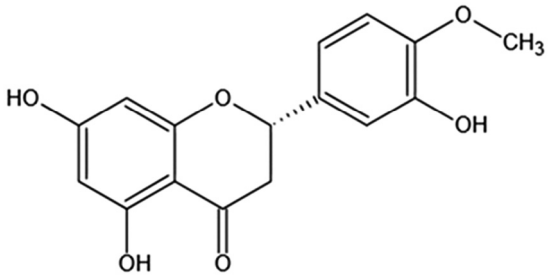

Hesperetin
$C_{16}H_{14}O_6$
MW: 302.28 g/mol

Fig. 1. Chemical structure, molecular formula and molecular weight of hesperetin.

et al., 2012b), which, if extrapolated to *in vivo* conditions, would lead to a tanned skin. As the effect of HSP on tumoral cells might be different to that obtained on normal melanocytes, it was decided to reproduce the described effect of HSP on B16-F10 melanoma cells and to compare it to the effect obtained using normal human melanocytes.

2. Materials and methods

2.1. Chemicals and reagents

Hesperetin (purity ≥ 98.0%), melanin, L-DOPA (3,4-dihydroxy-L-phenyalanine), NaOH and Triton X-100 were all obtained from Sigma–Aldrich (St. Louis, MO, USA).

HSP was dissolved in dimethylsulfoxide (DMSO) to obtain a stock solution (66 mM), which was conveniently diluted in culture medium for the biological assays. The final DMSO concentration in the medium was 0.075% and did not affect cellular functions or the assay systems used in this study.

2.2. Cell culture

The murine B16-F10 melanoma cell line was maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% gentamicin/amphothericin in a humidified incubator at 37 °C with 5% CO_2. All reagents were purchased from Gibco (Life Technologies, Grand Island, USA).

Hightly pigmented adult human epidermal melanocytes (HEMa) were purchased from Gibco C-024-5C. Cells were maintained in Medium 254 with the addition of Human Melanocyte Growth Supplement-2, PMA-Free (HMGS-2) and 1% gentamicin/amphothericin, all from Gibco (Life Technologies) in a humidified incubator at 37 °C with 5% CO_2.

The B16-F10 and HEMa cells were seeded at a density of 4×10^5 cells/mL and 1.5×10^5 cells/mL, respectively, on 100 mm dishes for melanin assays, or on 6-well plates for tyrosinase determination, and cultured as described above. B16-F10 cells were cultured overnight, while HEMa cells were incubated for 48 h, thereafter the medium was replaced with the corresponding fresh medium containing or not the different hesperetin solutions. Fresh medium was added with or without hesperetin every other day.

2.3. Cell viability

Cell viability was determined using a Coulter Particle counter (Beckman COULTER). After incubation, as described above, of B16-F10 for two days or for five days in the case of HEMa with hesperetin at 37 °C with 5% CO_2, the medium was removed, and the cells were washed twice with phosphate-buffered saline (PBS) and harvested by trypsinization using 0.05% trypsin/0.02% EDTA. The cell solutions were counted in a Coulter Counter to assess viability.

Cell images of the culture were taken at different days with a Nikon eclipse TE 2000-S microscope using a 20× or 40× phase-contrast objective attached to a Nikon digital camera.

2.4. Melanin content determination

Melanin content was measured spectrophotometrically. Briefly, cells were cultured as described above on 100 mm dishes. After incubation as described above of B16-F10 for two days or for five days in the case of HEMa with hesperetin at 37 °C with 5% CO_2, the medium was removed, and the cells were washed twice with PBS and harvested by trypsinization using 0.05% trypsin/0.02% EDTA. The harvested cells were centrifuged, the pellet was dissolved by adding 1 N NaOH, and the mixture was incubated at 60 °C for 1 h. The amount of melanin in the solution was determined by measuring the absorbance at 470 nm using a spectrophotometer (Hitachi U-2900). The total melanin content was estimated using a standard curve of synthetic melanin. The melanin content was calculated by normalizing the melanin contents to total cellular protein (mg of melanin/mg of protein) and reported as a control percentage. The protein content was determined using the Lowry method as described below.

2.5. Total cellular protein determination

The protein content was determined using the method previously described by Lowry et al. (1951). Briefly, 0.1 mL of cell solution was added to 2 mL of Lowry reagent and the solution mixture was allowed to stand for 15 min. Protein concentration was estimated by measuring the absorbance of the solution at 750 nm after adding 0.2 mL of 1N Folin–Ciocalteu's phenol reagent to the above solution. Protein content was calculated using bovine serum albumin as standard.

2.6. Tyrosinase assay

Tyrosinase enzyme activity was estimated spectrophotometrically, using L-DOPA as substrate. HEMa cells were cultured on 6-well plates with or without hesperetin for five days and at the end-point were solubilized with 0.1 M sodium phosphate buffer (pH 6.8) containing 1% Triton X-100. The cells were then disrupted by freezing at −20 °C for 30 minutes and thawing, and the lysates were clarified by centrifugation at 10,000 × *g* for 15 min. After protein quantification, as described above, and adjusting protein concentrations, 90 μL of each cell lysate (each sample containing the same amount of protein) were aliquoted into each well of a 96-well plate, and 10 μL of 10 mM L-DOPA was then added to each well. Following a 90 min incubation at 37 °C in the dark, the end-point absorbance was measured spectrophotometrically at 475 nm by a microplate reader (Thermo Electron Corporation "Multiskan EX").

2.7. Statistics

The data were expressed as mean ± standard deviation (SD). All statistical comparisons were performed with IBM SPSS Statistic 19 (SPSS Inc, Chicago, IL, USA). A one-way analysis of variance was performed for multiple comparisons. In the event of significant variation among treatment groups, the mean values were compared to the respective control using Dunnett's test. P-values below 0.05 were considered statistically significant.

3. Results

3.1. Hesperetin induces melanogenesis in the murine B16-F10 melanoma cell line

The murine melanoma cell line was used to estimate the potential effects of hesperetin on melanogenesis. The cells were exposed to hesperetin for 2 days. After 50 μM HSP treatment, and as a first step toward determining the effects of hesperetin on melanogenesis, we observed the morphology of the B16-F10 cells. Melanocyte differentiation is normally characterized as an increase in melanocyte dendrite production (Busca and Ballotti, 2000). B16-F10 cells treated with hesperetin did not exhibit a significant change in the morphology compared to control cells (Fig. 2a). Then, the cellular melanin content was examined (Fig. 2b) and it was found that hesperetin increased melanin synthesis in B16-F10 cells 1.7-fold ($p < 0.001$), compared to the value of the DMSO-treated control. Moreover, no cytotoxic effects were observed as cell viability was unaffected and no statistically significant differences were found (Fig. 2c). These results are expressed as percentage relative to the DMSO-treated control.

3.2. Hesperetin induces melanogenesis in human epidermal melanocytes

Given the role of hesperetin in the melanoma cell line, we investigated if hesperetin could induce melanin synthesis in human epidermal melanocytes. Therefore, HEMa were used to estimate the potential effects of hesperetin on melanogenesis. Thus, melanocytes were exposed to different HSP concentrations for 5 days. Fig. 3