BIOMOLECULES & THERAPEUTICS

Original Article
Biomol Ther 21(5), 343-348 (2013)

Hesperidin Suppresses Melanosome Transport by Blocking the Interaction of Rab27A-Melanophilin

Bora Kim[1], Jee-Young Lee[2], Ha-Yeon Lee[3], Ky-Youb Nam[2], Jongll Park[3], Su Min Lee[1], Jin Eun Kim[1], Joo Dong Lee[1] and Jae Sung Hwang[3,*]

[1]Enprani Co., Ltd. R&D Center of Skin Science and Cosmetics, Incheon 400-103,
[2]Bioinformatics & Molecular Design Research Center, Seoul 120-749,
[3]Department of Genetic Engineering, Graduate School of Biotechnology and Skin Biotechnology Center, Kyung Hee University, 446-701 Yongin, Republic of Korea

Abstract

We investigated the inhibitory effects of hesperidin on melanogenesis. To find melanosome transport inhibitor from natural products, we collected the structural information of natural products from Korea Food and Drug Administration (KFDA) and performed pharmacophore-based *in silico* screening for Rab27A and melanophilin (MLPH). Hesperidin did not inhibit melanin production in B16F10 murine melanoma cells stimulated with α-melanocyte stimulating hormone (α-MSH), and also did not affect the catalytic activity of tyrosinase. But, hesperidin inhibited melanosome transport in melanocyte and showed skin lightening effect in pigmented reconstructed epidermis model. Therefore, we suggest that hesperidin is a useful inhibitor of melanosome transport and it might be applied to whitening agent.

Key Words: Hesperidin, Melanosome transport, Rab27A, Melanophilin, Pigmented reconstructed epidermis model

INTRODUCTION

Melanosomes are specialized organelles that synthesize and store melanin pigments in pigment cells. Pigmentation of mammalian hair and skin requires proper formation and transport of melanosomes along two cytoskeletal elements, namely microtubules and actin filaments. During recent years, pigment granules, or melanosomes, within pigment cells have provided an excellent model for understanding the molecular mechanisms by which motor proteins associate with and move intracellular organelles (Hume and Seabra, 2011). A tripartite protein complex composed of the small GTPase Rab27A, its specific effector Slac2a (also called melanophilin, MLPH) and the actin-based motor myosin Va, has been shown to mediate actin-based melanosome transport in mammalian epidermal melanocytes and the Rab-myosin transport system now appears to be a fundamental mechanism of organelle transport in many cell types (Fukuda *et al.*, 2002; Kuroda *et al.*, 2005; Hume and Seabra, 2011).

Specially, human skin hyperpigmentation disorders such as melansma, occur when the synthesis and/or distribution of melanin increases. The distribution of melanin in the skin is completed by melanosome transport and transfer. The transport of melanosomes, the organelles where melanin is synthesized, in a melanocyte precedes the transfer of the melanosomes to a keratinocyte (Boissy, 2003). Therefore, hyperpigmentation can be regulated by decreasing melanosome transport and transfer (Hakozaki *et al.*, 2002; Chang *et al.*, 2012).

The well known availability and safety of natural products are the reasons they widely used in medicine, cosmetics, and other products (Rajnarayana *et al.*, 2001; Cazarolli *et al.*, 2008). To identify natural melanosome transport suppressor as binding blocker of Rab27A- Melanophilin, we collected the structural information of natural products from herbal medicine information systems of Korea Food & Drug Administration (KFDA, http://www.mfds.go.kr/herbmed/index.do) and performed docking simulation with Melanophilin. Among hits, hesperidin only can be purchased.

Hesperidin is one of the citrus flavonoid shown to be active against various oxidative stress mediated diseases. Hesperidin found in orange peel is a flavanone glycoside consisting

Open Access **http://dx.doi.org/10.4062/biomolther.2013.032**

Received Apr 17, 2013 **Revised** Aug 8, 2013 **Accepted** Aug 9, 2013

***Corresponding Author**
E-mail: jshwang@khu.ac.kr
Tel: +82-31-888-6179, Fax: +82-31-888-6173

of the flavone hesperidin bound to the disaccharide rutinose. The sugar group makes hesperidin more water-soluble than hesperitin, another compound in orange peel (Vallejo *et al.*, 2010). Exogenous hesperidin has been shown to influence a wide variety of biological functions. For example, hesperidin induces apoptosis and suppresses proliferation in human cancer cells (Ghorbani *et al.*, 2012), inhibition of tumor development in various tissues including the skin (Tanaka *et al.*, 2012; Saiprasad *et al.*, 2013). Many of these beneficial effects of hesperidin can be attributed to its antioxidant activity (Elavarasan *et al.*, 2012). Topical hesperidin improves epidermal permeability barrier function and epidermal differentiation in normal murine skin (Hou *et al.*, 2012). To search for new skin-lightening agents, we investigated the effect of hesperidin on depigmenting mechanisms.

MATERIALS AND METHODS

Docking study

We performed an automated docking to determine the binding model between natural products and MLPH. We collected the structural information of 273 natural products from KFDA and used it for our docking study. Computations were performed on a Windows platform using DS modeling/CDOCKER (Accelrys Inc., San Diego, USA) with the CHARMM force field (Vieth *et al.*, 1998; Wu *et al.*, 2003). The molecular dynamics (MD) simulated annealing process is performed using a flexible ligand docking. The final minimization step is applied to ligand's docking pose. The minimization consists of 1,000 steps of steepest descent using an energy tolerance of 0.001 kcal/mol.

Cell culture

B16F10 murine melanoma cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% penicillin-streptomycin (10,000 U and 10,000 μg/ml) in 5% CO_2 at 37°C. Melan-a cells were maintained in RPMI1640 supplemented with 10% (v/v) FBS, PMA to 200 nmol and 1% (v/v) penicillin-streptomycin in 10% CO_2 at 37°C.

Cell viability assay

B16F10 murine melanoma cells were seeded onto a 96-well plate at 4×10^3 cells/well. After incubation with various concentration of hesperidin, 50 μl of 2 mg/ml MTT, which is converted to a formazan product by metabolically active cells, was added to each well. After 3 h of incubation, the supernatant was removed and the formazan crystals were dissolved in dimethyl sulphoxide. The optical densities at 540 nm were measured using an ELISA reader.

Measurement of melanin secretion in B16F10 melanoma cells

Extracellular melanin release was measured as previously described (Siegrist and Eberle, 1986). Briefly, B16F10 cells were incubated at a density of 1.5×10^5 cells in six-well plates overnight. α-MSH (5 nM) was then added and cells were treated with increasing concentrations of hesperidin (10-250 μM) in phenol red free DMEM for 3 days. 200 μl aliquots of media were then placed in 96-well plates and optical densities (OD) were measured at 405 nm using an ELISA reader. Melanin productions were expressed as percentages of those of untreated controls.

Tyrosinase activity assay

The tyrosinase activity assay was performed with mushroom tyrosinase because of its ready availability. Each sample was dissolved in DMSO and used for the experiment at 100 times dilution. 0.1 M potassium phosphate buffer (pH 6.8), 3 mM L-tyrosine solution with or without a sample chemical and 2,000 units/ml tyrosinase in aqueous solution were mixed. The mixture incubated at 37°C for 10 min and the reaction was monitored at 475 nm. A control reaction was conducted with DMSO alone. The percentage of activity of tyrosinase was calculated as follows: *100-[(A-B)/A×100]*, where *A* represents the difference in the absorbance of the control sample, and B represents the difference in the absorbance of the test sample. Kojic acid was used as references which are well-known tyrosinase inhibitor.

Melanosome aggregation measurement

Melan-a cells were incubated at a density of $1x10^5$ cells in 24-well plates overnight. After washing in DPBS, melan-a was treated with diluted sample in RPMI1640 with 2% FBS for 3 days. Cells were observed at bright field using an Olympus CKX41 culture microscope (Olympus, Japan) and images were photographed using DMCe camera (INS Industry, Korea) and DMC advanced software associated with the microscope. Evaluation of melanosome aggregation was performed by counting perinuclear melanosome aggregated cells in three random microscopic fields per well at ×200 magnification. Values represent the mean ± SD.

Pigmented reconstructed skin model

Reconstructed human epidermis MEL-300-B (MatTek, Ashland, MA, USA) consisted of normal human-derived epidermal keratinocytes and NHEM that had been cultured to form a multilayered, highly differentiated model. Reconstructed epidermis was placed in 6-well plates and incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. The tissue was incubated for 14 days and fed with 5 ml of fresh medium every other day. At indicated time points the samples photographed. Color reflectance of photos has been used to extract the separate components of skin color histogram that occur due to the presence of melanin by image analysis program (Adobe Photoshop CS; Adobe Systems, USA). The larger L value means that color of epidermis is brighter. The percentage of inhibition of L value was calculated as follows: (A/B ×100)-100, where *A* represents the L value of sample (hesperidin), and *B* represents control (0.1% DMSO).

RESULTS

Docking study

We collected the chemical information of 273 natural products that related on the inflammation activity with no harmful activity of normal cells. The information of activity of natural compounds is manually extracted from the Database of Standardization of Herbal Medicines (http://www.herbdb.co.kr). This database includes the pharmaceutical activity and biological origin of each natural compound from various species. We calculated docking score (Dock Score) and selected 10 top ranked natural compounds. As shown in Table 1, the dock

score of 10 natural compounds show small variations and 10 hits form good docking models with MLPH. The structures of 10 hits are represented in Fig. 1A. Among those, hesperidin and Neohesperidin dihydro-chalcone were registered at ICID (International Cosmetic Ingredient Dictionary), which means it can be included in cosmetic formulations.

Table 1. The docking score for 10 hits

Compound name	Dock Score
Acetoside	6.45
Cimicifugoside H-1	6.11
Albiflorin	6.02
23-O-acetylshengmanol 3-xyloside	5.90
3,5-Di-O-caffeoylquinic acid	5.77
Hesperidin	5.72
Forsythiaside	5.56
Neohesperidin dihydro-chalcone	5.54
Loniceroside A	5.32
Saikosaponin C	5.20

Among two compounds, we further investigated the effect of hesperidin which is registered at ICID (International Cosmetic Ingredient Dictionary) and could be obtained at affordable price for industrialization with higher docking score.

Hesperidin, a flavanone glycoside, has a disaccharide rutinose moiety that found abundantly in citrus and its aglycone form is hesperetin (Fig. 1B). It binds to MLPH at the interaction site of Rab27A with complex hydrogen bonding interactions. E32 of MLPH participated in the hydrogen-bonding interaction with Y6 of Rab27A and it is important to binding activity of Rab27A-MLPH (Fukuda, 2002). This hydrogen bond is represented in our docking model. Two hydroxyl groups of rutinose of hesperidin formed hydrogen bonds with side chain of E32 (Fig. 2). Additionally, the rest hydroxyl groups of rutinose also participated in hydrogen bonds with side chains of R29, D25 and Y122 of MLPH. These interactions may contribute to stabilizing the binding between hesperidin and MLPH and therefore the disaccharide rutinose of hesperidin is important structural constituent for hydrogen bonding interactions with

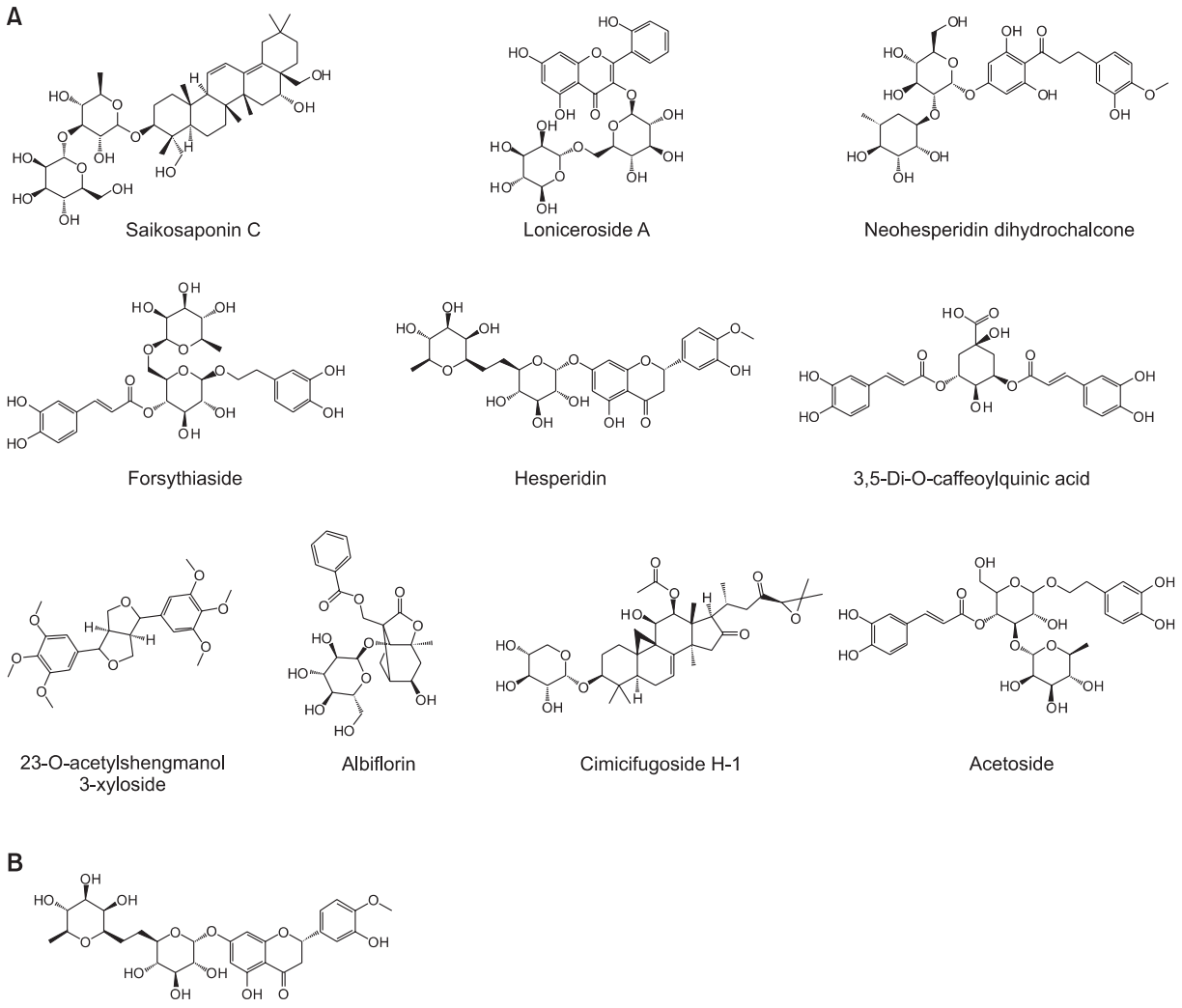

Fig. 1. The structure of hit compounds. (A) Structure of 10 hits (B) Structure of hesperidin.

MLPH. 3'-hydroxyl moiety of hesperidin was also formed a hydrogen bond with backbone amide of G133. The non-polar residues, V21, L28 and F129 on MLPH, formed hydrophobic contacts with hesperidin. It is known that V21 is important residue which essential in recognition and binding of Rab27A-MLPH. From these results, we confirmed that hesperidin could be blocker of Rab27A-MLPH with strong binding interactions by complex hydrogen bonding interactions.

Effect of hesperidin on tyrosinase activity and melanogenesis in B16F10 melanoma cells

We also investigated the effect of hesperidin on tyrosinase activity using mushroom tyrosinase (Fig. 3A). Hesperidin had no inhibitory effect on tyrosinase activity, whereas kojic acid (a direct inhibitor of tyrosinase) had a strong inhibitory effect. In addition, we treated B16F10 melanoma cells with hesperidin to determine whether it has a cytotoxic effect; cell viability was determined using MTT assay. Hesperidin didn't showed cytotoxicity (5-20 μM) (Fig. 3B). Cells were exposed to hesperidin in the presence of α-MSH (5 nM) for 3 days and extracellular melanin release was measured. As shown in Fig. 1B, hesperidin has no inhibitory effect on melanin synthesis in B16F10 cells (Fig. 3B).

Effect of hesperidin on melanosome transport in melanocyte

In order to determine the inhibition activity of melanosome transport, hesperidin was assayed using melan-a cells (Fig. 4). Hesperidin induced the aggregation of melanosome and identified as active blockers to reduce melanosome transport.

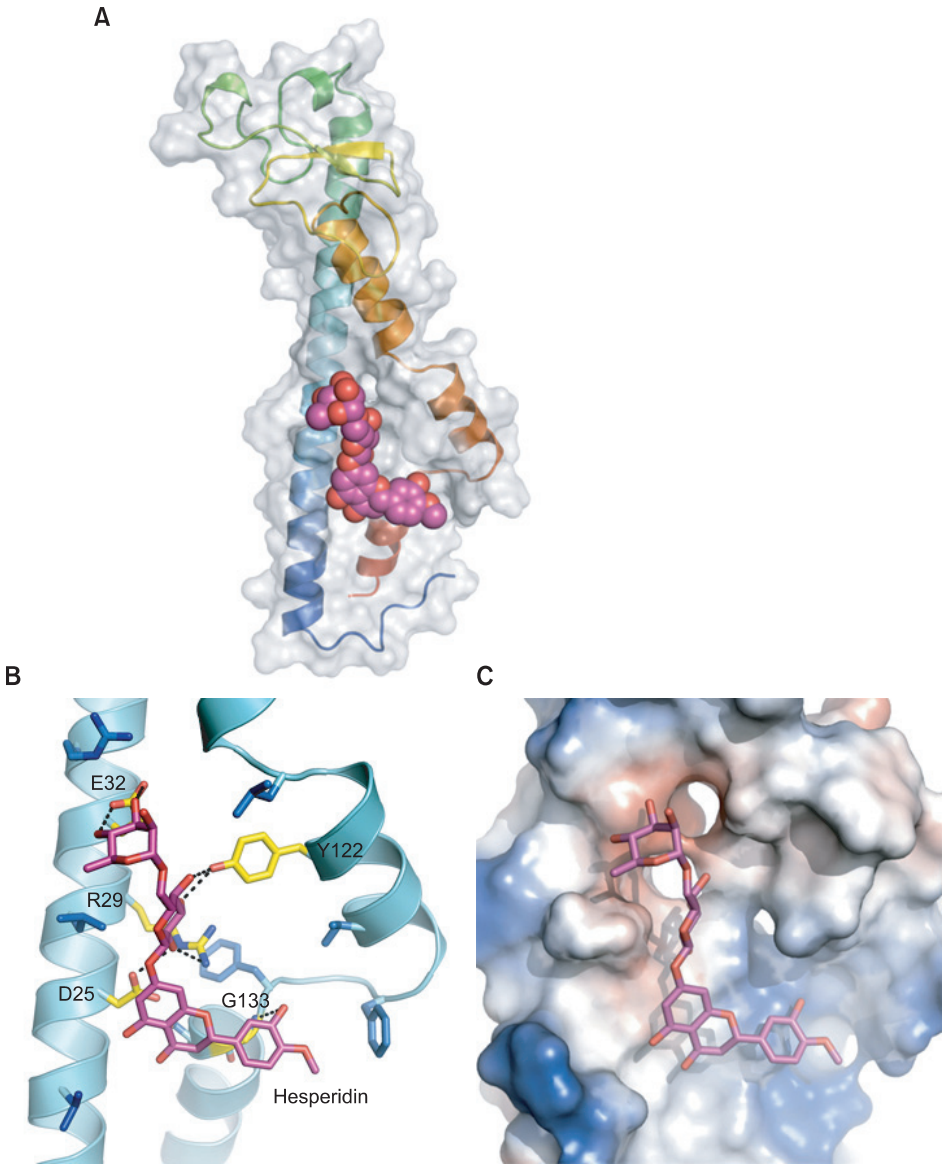

Fig. 2. Results of Docking study. (A) Overall structure of hesperidin and MLPH (B) Interaction model between hesperidin and MLPH. Yellow residues participated hydrogen bonding interactions with hesperidin. Black dashed lines are depicted hydrogen bonds (C) Surface model of hesperidin and MLPH.

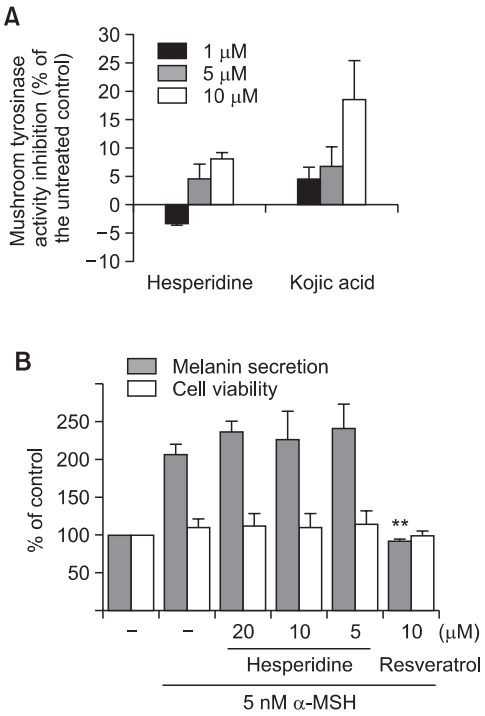

Fig. 3. Measurement of the inhibitory effect on mushroom tyrosinase and inhibitory effect on melanogenesis in B16F10 melanoma cells (A) The tyrosinase activity assay was performed with mushroom tyrosinase. (B) Cells were treated with various concentrations (5-20 μM) of Hesperidin for a total of 7 days. Melanin content of cells was determined. The data shown represent the mean ± SD derived from three determinations. Kojic acid and resveratrol were used as a positive control. ***p*<0.01 indicated statistically significant differences from the α-MSH treated group.

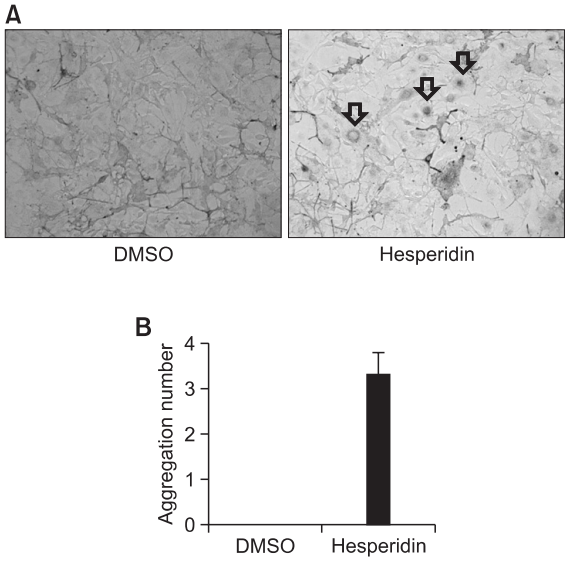

Fig. 4. Aggregation of melanosome (A) and quantification of the number of aggregated cells (B) treated with DMSO or 10 μM hesperidin for 72 hours. The number of aggregated cells was counted as described material and method.

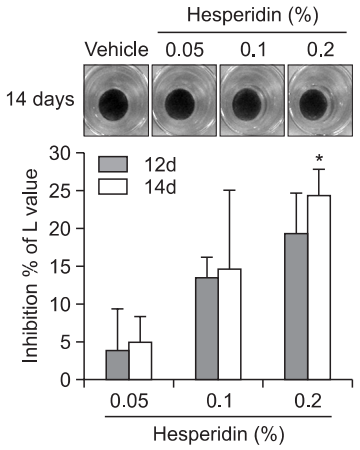

Fig. 5. Macroscopic views of Pigmented reconstructed epidermis model treated with hesperidin. Different concentrations of hesperidin, and the vehicle (0.1% DMSO) were applied topically on the reconstructed skin and tissues were monitored for up to 14 days. **p*<0.05 indicated statistically significant differences from the vehicle. L values are data for skin lightness using semi-quantitative image analysis program.

Effect of hesperidin on melanogenesis in cultured human skin model

To identify the effects of hesperidin on skin pigmentation, we used three-dimensional cultured human skin models (MelanoDerm™). Different concentrations of hesperidin and the vehicle emulsion were applied topically on the reconstructed skin and tissues were monitored for up to 14 days post-application. 0.2% Hesperidin indicated statistically significant differences from the vehicle (*p*<0.05). Photographs of skin specimens show reduced pigmentation in hesperidin-treated tissues (Fig. 5).

DISCUSSION

Skin pigmentation is determined by: (a) the migration of melanoblasts to that tissue during development, (b) their survival and differentiation to melanocytes, (c) the density of melanocytes, (d) the expression/function of enzymatic and structural constituents of melanosomes (e) the synthesis of different types of melanin (eu- and pheomelanin), (f) the transport of melanosomes to dendrites, (g) the transfer of melanosomes to keratinocytes, and finally (h) the distribution of melanin in suprabasal layers of the skin (Yamaguchi *et al.*, 2007). Important factors that regulate human skin pigmentation include melanocytes in the epidermis that synthesize the melanin and neighboring keratinocytes that receive and distribute it in the upper layers of the skin (Ando *et al.*, 2012). Keratinocytes in fair skin tend to cluster their poorly pigmented melanosomes above the nuclei, whereas in dark skin the heavily pigmented melanosomes are distributed individually in keratinocytes (Thong *et al.*, 2003).

Over recent years, pigment granules, or melanosomes, within pigment cells have provided an excellent model for understanding the molecular mechanisms by which motor proteins associate with and move intracellular organelles (Hume and Seabra, 2011). Melanosomes move from the perinuclear

area of melanocytes where they are produced toward the plasma membrane as they become more melanized due to the functions of microtubules, actin filaments, and myosin, which is similar to the movement of other organelles in other types of cells (Hirokawa and Noda, 2008).

Rab27A, melanophilin, and myosin Va make a complex to link melanosomes to the F-actin based motors. Rab27A links synaptoagmin-like protein2-a (Slp2-a) with phosphatidylserine, thereby docking melanosomes at the plasma membrane, which suggests the role of Slp2-a as a regulator of melanosome exocytosis (Kuroda and Fukuda, 2004). Darkly-pigmented melanocytes with significantly higher RAB27A expression were found to transfer significantly more melanosomes to keratinocytes than lightly-pigmented melanocytes in co-culture and *in vivo* (Yoshida-Amano *et al.*, 2012).

The regulation of human skin pigmentation has been a longstanding goal for cosmetic and pharmaceutical applications. It has implications regarding social standing, cosmetic appearance and of course photoprotection of the skin against cancer and photoaging (Yamaguchi *et al.*, 2007). In this study, we report the depigmenting effect of hesperidin by blocking the Rab27A - melanophilin interaction. Virtual docking study showed that hesperidin could be blocker of Rab27A-MLPH and this assumption was proved by melanosome aggregation assay in melan-a cells. Although hesperidin did not inhibit tyrosinase directly and did not inhibit melanin production in α-MSH-stimulated B16F10 melanoma cells but hesperidin containing emulsion reduced skin pigmentation in reconstructed human epidermal skin. Photographs of skin specimens show slightly reduced pigmentation in hesperidin-treated tissues. From these results, we conclude that hesperidin could be a useful inhibitor of melanosome transport and it might be applied for skin whitening cosmetics.

ACKNOWLEDGMENTS

This study was supported by a grant of the Korea Healthcare technology R&D Project, Ministry of Health & Welfare, Republic of Korea (Grand No.: A103017) and by a Interrelated Development Program (R-0000451) of Inter-Economic Regions, Ministry for Knowledge Economy, Republic of Korea.

REFERENCES

Ando, H., Niki, Y., Ito, M., Akiyama, K., Matsui, M. S., Yarosh, D. B. and Ichihashi, M. (2012) Melanosomes are transferred from melanocytes to keratinocytes through the processes of packaging, release, uptake, and dispersion. *J. Invest. Dermatol.* **132**, 1222-1229.

Boissy, R. E. (2003) Melanosome transfer to and translocation in the keratinocyte. *Exp. Dermatol.* **12** Suppl 2, 5-12.

Cazarolli, L. H., Zanatta, L., Alberton, E. H., Figueiredo, M. S., Folador, P., Damazio, R. G., Pizzolatti, M. G. and Silva, F. R. (2008) Flavonoids: prospective drug candidates. *Mini Rev. Med. Chem.* **8**, 1429-1440.

Chang, H., Choi, H., Joo, K. M., Kim, D. and Lee, T. R. (2012) Manassantin B inhibits melanosome transport in melanocytes by disrupting the melanophilin-myosin Va interaction. *Pigment Cell Melanoma Res.* **25**, 765-772.

Elavarasan, J., Velusamy, P., Ganesan, T., Ramakrishnan, S. K., Rajasekaran, D. and Periandavan, K. (2012) Hesperidin-mediated expression of Nrf2 and upregulation of antioxidant status in senescent rat heart. *J. Pharm. Pharmacol.* **64**, 1472-1482.

Fukuda, M. (2002) Synaptotagmin-like protein (Slp) homology domain 1 of Slac2-a/melanophilin is a critical determinant of GTP-dependent specific binding to Rab27A. *J. Biol. Chem.* **277**, 40118-40124.

Fukuda, M., Kuroda, T. S. and Mikoshiba, K. (2002) Slac2-a/melanophilin, the missing link between Rab27 and myosin Va: implications of a tripartite protein complex for melanosome transport. *J. Biol. Chem.* **277**, 12432-12436.

Ghorbani, A., Nazari, M., Jeddi-Tehrani, M. and Zand, H. (2012) The citrus flavonoid hesperidin induces p53 and inhibits NF-kappaB activation in order to trigger apoptosis in NALM-6 cells: involvement of PPARgamma-dependent mechanism. *Eur. J. Nutr.* **51**, 39-46.

Hakozaki, T., Minwalla, L., Zhuang, J., Chhoa, M., Matsubara, A., Miyamoto, K., Greatens, A., Hillebrand, G. G., Bissett, D. L. and Boissy, R. E. (2002) The effect of niacinamide on reducing cutaneous pigmentation and suppression of melanosome transfer. *Br. J. Dermatol.* **147**, 20-31.

Hirokawa, N. and Noda, Y. (2008) Intracellular transport and kinesin superfamily proteins, KIFs: structure, function, and dynamics. *Physiol. Rev.* **88**, 1089-1118.

Hou, M., Man, M., Man, W., Zhu, W., Hupe, M., Park, K., Crumrine, D., Elias, P. M. and Man, M. Q. (2012) Topical hesperidin improves epidermal permeability barrier function and epidermal differentiation in normal murine skin. *Exp. Dermatol.* **21**, 337-340.

Hume, A. N. and Seabra, M. C. (2011) Melanosomes on the move: a model to understand organelle dynamics. *Biochem. Soc. Trans.* **39**, 1191-1196.

Kuroda, T. S. and Fukuda, M. (2004) Rab27A-binding protein Slp2-a is required for peripheral melanosome distribution and elongated cell shape in melanocytes. *Nat. Cell. Biol.* **6**, 1195-1203.

Kuroda, T. S., Itoh, T. and Fukuda, M. (2005) Functional analysis of slac2-a/melanophilin as a linker protein between Rab27A and myosin Va in melanosome transport. *Methods Enzymol.* **403**, 419-431.

Rajnarayana, K., Prabhakar, M. C. and Krishna, D. R. (2001) Influence of rice bran oil on serum lipid peroxides and lipids in human subjects. *Indian J. Physiol. Pharmacol.* **45**, 442-444.

Saiprasad, G., Chitra, P., Manikandan, R. and Sudhandiran, G. (2013) Hesperidin alleviates oxidative stress and downregulates the expressions of proliferative and inflammatory markers in azoxymethane-induced experimental colon carcinogenesis in mice. *Inflamm. Res.* **62**, 425-440.

Siegrist, W. and Eberle, A. N. (1986) In situ melanin assay for MSH using mouse B16 melanoma cells in culture. *Anal. Biochem.* **159**, 191-197.

Tanaka, T., Tanaka, T., Tanaka, M. and Kuno, T. (2012) Cancer chemoprevention by citrus pulp and juices containing high amounts of beta-cryptoxanthin and hesperidin. *J. Biomed. Biotechnol.* **2012**, 516981.

Thong, H. Y., Jee, S. H., Sun, C. C. and Boissy, R. E. (2003) The patterns of melanosome distribution in keratinocytes of human skin as one determining factor of skin colour. *Br. J. Dermatol.* **149**, 498-505.

Vallejo, F., Larrosa, M., Escudero, E., Zafrilla, M. P., Cerda, B., Boza, J., Garcia-Conesa, M. T., Espin, J. C. and Tomas-Barberan, F. A. (2010) Concentration and solubility of flavanones in orange beverages affect their bioavailability in humans. *J. Agric. Food Chem.* **58**, 6516-6524.

Vieth, M., Hirst, J. D. and Brooks, C. L. 3rd (1998) Do active site conformations of small ligands correspond to low free-energy solution structures? *J. Comput. Aided Mol. Des.* **12**, 563-572.

Wu, G., Robertson, D. H., Brooks, C. L. 3rd and Vieth, M. (2003) Detailed analysis of grid-based molecular docking: A case study of CDOCKER-A CHARMm-based MD docking algorithm. *J. Comput. Chem.* **24**, 1549-1562.

Yamaguchi, Y., Brenner, M. and Hearing, V. J. (2007) The regulation of skin pigmentation. *J. Biol. Chem.* **282**, 27557-27561.

Yoshida-Amano, Y., Hachiya, A., Ohuchi, A., Kobinger, G. P., Kitahara, T., Takema, Y. and Fukuda, M. (2012) Essential role of RAB27A in determining constitutive human skin color. *PLoS One* **7**, e41160.