Inhibitory Effects of Resveratrol on Melanin Synthesis in Ultraviolet B-Induced Pigmentation in Guinea Pig Skin

Taek Hwan Lee[1], Jae Ok Seo[2], So-Hyeon Baek[3] and Sun Yeou Kim[2,4,5,]*

[1]College of Pharmacy, Yonsei University, Incheon 406-840,
[2]College of Pharmacy, Gachon University, Incheon 406-799,
[3]National Institute of Crop Science, Rural Development Administration, Iksan 570-080,
[4]Gachon Institute of Pharmaceutical Science, Gachon University, Incheon 406-799,
[5]Gachon Medical Research Institute, Gil Medical Center, Inchon 405-760, Republic of Korea

Abstract

Resveratrol is a polyphenolic compound found in various natural products such as grapes and berries and possesses anti-cancer, anti-hyperlipidemia, and anti-aging properties. Recently, it has been reported that resveratrol inhibits α-melanocyte-stimulating hormone signaling, viability, and migration in melanoma cells. However, these effects have not been confirmed *in vivo*, specifically brownish guinea pigs. To evaluate the potential of resveratrol as a regulator of melanin for hyperpigmentation therapy, the influence of resveratrol on pigmentation was investigated by ultraviolet B-induced hyperpigmentation in brownish guinea pig skin. We found that resveratrol reduced the expression of melanogenesis-related proteins tyrosinase, tyrosinase-related proteins 1 and 2, and microphthalmia-associated transcription factor in melanoma cells. Furthermore, topical application of resveratrol was demonstrated to significantly decrease hyperpigmentation on ultraviolet B-stimulated guinea pig skin *in vivo*. Based on our histological data, resveratrol inhibits melanin synthesis via a reduction in tyrosinase-related protein 2 among the melanogenic enzymes. This study is the first to provide evidence supporting resveratrol as a depigmentation agent, along with further clinical investigation of resveratrol in ultraviolet B-induced skin disorders such as hyperpigmentation and skin photoaging.

Key Words: Resveratrol, Depigmentation, Hyperpigmentation, Melanogenesis, Guinea pig, Ultraviolet B

INTRODUCTION

Melanin is the predominant contributing to skin pigmentation, and is also found in the hair, eyes, ears, and brain. A primary function of melanin is to counteract skin damage caused due to ultraviolet radiation (UVR) (Moon *et al*., 2012). Since UVR exposure causes intense and extensive damage to the skin, the function of melanin is very important. Melanin acts as a shield for our skin yet its abundance in the body can be carcinogenic and could potentially lead to malignancy. In recent years, resolving problems caused by UV irradiation has become imperative.

Melanin is produced by melanocytes in the basal layer of the epidermis. Melanocytes contain granules within the cytoplasm called melanosomes. These melanosomes have melanin pigments, which can be transferred out to adjacent keratinocytes in the epidermis (Riley, 1997). Melanin synthesis, or

melanogenesis, is affected by many enzymes and is induced by the α-melanocyte-stimulating hormone (α-MSH) and UVR. Tyrosinase is the predominant enzyme in melanogenesis, as it is involved in two melanin synthesis processes. The first step is the conversion of tyrosine to 3,4-dihydroxyphenylalanine (L-DOPA), which is subsequently converted to dopaquinone (Gillbro and Olsson, 2011). Melanin is a polymer synthesized by tyrosinase-related protein (TRP)-2 that converts DOPA chrome to 5,6-dihydroxyindole 2-carboxylic acid (DHICA). One of another enzymes, TRP-1, can oxidize DHICA to indole-5-6-quinone carboxylic acid. Thus, they regulate the synthesis of brownish 5,6-dihydroxyindole-2-carboxylic acid-rich melanins. These proteins are activated by microphthalmia-associated transcription factor (MITF), the key transcriptional regulator in melanogenesis (Bertolotto *et al*., 1998; Lin *et al*., 2002). In this way, the melanin synthesis-related enzyme tyrosinase, TRP-1, TRP-2 and its transcription factor MITF are necessary for

*Corresponding Author
E-mail: sunnykim@gachon.ac.kr
Tel: +82-32-899-6411, Fax: +82-32-899-6591

www.biomolther.org

regulating melanogenesis. Therefore, efforts have increased to find the regulatory agents of these melanogenic proteins (Kobayashi *et al.*, 1994).

Resveratrol (3',4',5'-trihydroxy-trans-stilbene) is a polyphenolic compound found in various natural products such as grapes and berries and has been reported to possess biological activities such as antioxidation (Goldberg, 1996), anticancer (Song *et al.*, 2010; Del Follo-Martinez *et al.*, 2013), anti-inflammation (Gatson *et al.*, 2013), cardioprotection (Osman *et al.*, 2013), and anti-aging (Marchal *et al.*, 2013). Moreover, depigmentation effects of resveratrol has recently also been demonstrated in human melanocytes by transcriptional regulation of melanogenic genes (Newton *et al.*, 2007). Notably, resveratrol inhibits mRNA expression of tyrosinase, TRP-1, MITF, and DCT in human melanocytes. However, it is unknown whether topical application of resveratrol to the dorsal skin of brownish guinea pigs *in vivo* prevents ultraviolet B (UVB)-induced hyperpigmentation. To make sure the whitening effects of resveratrol, our study was conducted to focus on animal experiments using UVB-irradiated brownish guinea pigs *in vivo* models. In addition, the whitening efficacy of resveratrol was confirmed in α-MSH-stimulated B16 melanoma cell line.

MATERIALS AND METHODS

Cell culture

B16F10 mouse melanoma cells were cultured in Dulbecco's modified eagle medium (DMEM) with 10% fetal bovine serum and penicillin/streptomycin in air containing 5% CO_2 at 37°C.

Measurement of melanin contents

The melanin content was determined according to the modified methods of Hosoi *et al.* (1985) (Hosoi *et al.*, 1985). B16F10 cells were cultured at 5×10^4 cells/well in 48-well plates. After 24 h, the cells were stimulated by α-MSH 100 ng/ml. At the same time, various concentrations of resveratrol (1, 10, and 100 μM) treated for 48 h. After washing with phosphate buffered saline (PBS), the cells were harvested by trypsinization. The cell pellet was solubilized in 200 μl of 1 N NaOH. The absorbance of each well was measured at 405 nm using a spectrophotometer.

Measurement of cell viability

The cell proliferation assay was carried out using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). B16-F10 cells were cultured in 5×10^4 cells/well in 48-well plates. After 24 h, the cells were treated with various concentrations of samples (1, 10, and 100 μM) for 48 h. At the end of the incubation, 50 μl of MTT solution (1 mg/ml in PBS) was added to each well. After incubation at 37°C for 1 h, the medium was gently removed and 150 μl of dimethyl sulfoxide (DMSO) was added. The absorbance of each well was measured at 570 nm using a spectrophotometer.

Animals

Five-week-old male guinea pigs (KIWA:A1) (weight, 272-297 g; n=4) were obtained from Japan Kiwa Laboratory Animals Co., Ltd. (Wakayama, Japan). The experimental protocol for this study was approved by the Institutional Animal Care and Use Committee of Korea Conformity Laboratories (IA13-
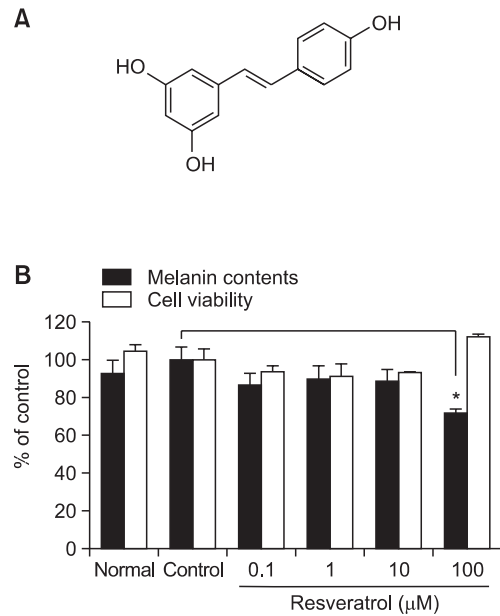
Fig. 1. Resveratrol effect on melanogenesis in α-MSH-stimulated B16F10 cells. The chemical structure of resveratrol (A). Melanin contents and cell viability were determined in B16F10 cells treated with 1, 10, and 100 μM resveratrol at the same time as α-MSH 100 ng/ml except in the normal group for 48 h (B). The results are the averages of the three independent experiments, and the data are expressed as means ± SD (*p<0.05).

00229). The guinea pigs were housed in a temperature- and humidity-controlled room (22 ± 1°C, 60 ± 5% humidity) with 12 h light/dark cycles. After 1 week of quarantine, the guinea pigs were acclimated to individual cages. During the experimental period, food and water were given ad libitum.

### Ultraviolet B irradiation-induced hyperpigmentation

The UV source was supplied by a closely spaced array of five Sankyo Denki sun lamps, which have peak irradiance at 310 nm (Kanagawa, Japan). Bulbs were positioned 15 cm above the guinea pigs. The irradiation (0.1 mW/cm^2) was measured with an IL1700 Research Radiometer (International Light, Inc., Newburyport, MA, USA) equipped with a UVB sensor. The guinea pigs were exposed to 390 mJ/cm^2 UVB radiation three times per week for two weeks. UVB irradiation was applied to the dorsal skin of the guinea pigs after hair removal.

Sample treatment in guinea pigs

Resveratrol was dissolved in a mixture of ethanol and propylene glycol (3:7, v/v). The sample solution was applied topically to the dorsal skin once a day for 12 days after the final UVB treatment. Post treatment, 1% resveratrol (200 μl; 10 mg/ml) was applied to 2 cm^2 of skin.

Fontana-Masson stain

To analyze changes in the melanin content of the skin tissue, the guinea pigs were sacrificed after the final treatment and biopsies were obtained from the dorsal skin, which were fixed in 4% paraformaldehyde for 24 h and embedded in paraffin. Sections, approximately 4 μm thick, were stained with Fontana-Masson staining solution (IHC WORLD, GA, USA). The stained slides were examined under a light microscope.
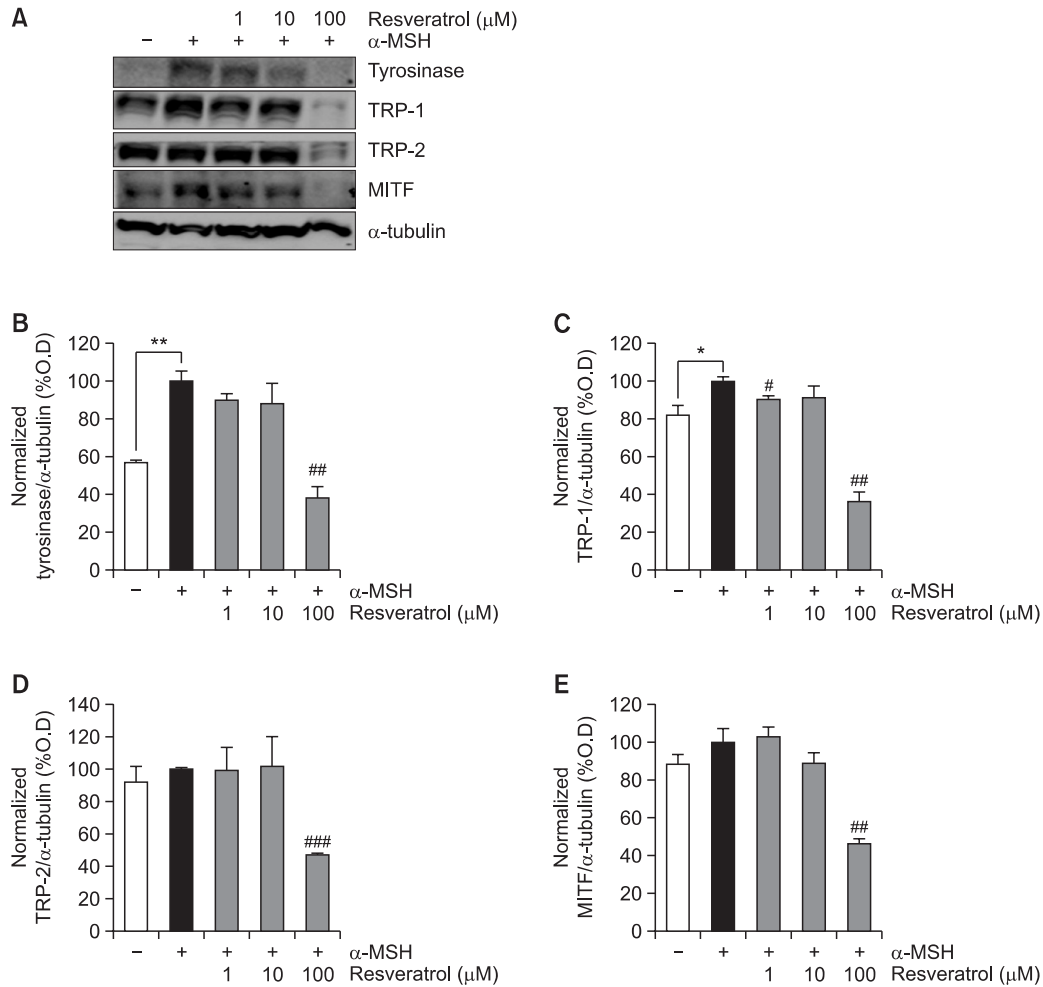
Fig. 2. Effects of resveratrol on the expression of melanogenesis-related proteins in B16F10 cells. B16F10 cells were treated with 1, 10, and 100 μM resveratrol for 48 h. All groups except the normal group stimulated melanogenesis by α-MSH. Forty micrograms of total protein from the cell lysates were used for Western blot analysis (A). The graph of protein expression, tyrosinase (B), TRP-1 (C), TRP-2 (D) and MITF (E) were normalized to the band corresponding to α-tubulin. Student's t-test was used for statistical analyses, $*p<0.05$ and $**p<0.01$ indicate statistically significant differences compared to the normal. $^{\#}p<0.05$, $^{\#\#}p<0.01$ and $^{\#\#\#}p<0.001$ indicate statistically significant differences compared to the control.

Western Blot analysis

Changes in the levels of proteins related to melanin production, including tyrosinase, TRP-1, TRP-2, and MITF in the B16F10 cells and skin of guinea pigs were evaluated by Western blotting. Prepared samples were homogenized and lysed in ice-cold radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitors and was centrifuged at 14,000 rpm for 20 min at 4°C. The supernatant was collected and assayed for protein concentration using the protein assay kit (Bio-Rad, CA, USA). Lysates containing 40 μl of protein were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) with 10% resolving and 3% acrylamide stacking gel and were transferred to nitrocellulose sheets. Blocking was performed in tris-buffered saline containing 5% skim milk powder and 0.1% Tween-20. The primary antibodies used for Western blotting include mouse anti-a-tubulin antibody, goat anti-tyrosinase, and goat anti-TRP-1 and -TRP-2 (Santa Cruz Biotechnology Inc., CA, USA), and mouse anti-MITF (Neo-markers, CA, USA). Finally, immune complexes were detected

with a ChemiDocXRS+ imaging system (Bio-Rad, CA, USA).

Statistical analysis

Data were expressed as mean ± SD from at least three independent experiments. Student's t-test was used for statistical analyses, and only p values<0.05 were reported as significant. $*p<0.05$, $**p<0.01$, and $***p<0.001$ indicate statistically significant differences from the control group.

RESULTS

Resveratrol decreases melanin production in cultured B16F10 cells

In Fig. 1B, the melanin contents were increased in α-MSH (100 ng/ml)-stimulated control cells as compared to the non-stimulated normal cells. However, resveratrol treatment significantly decreased melanin production by 28.2 ± 1.9% without cell toxicity at 100 μM after 48 h treatment in α-MSH stimu-

A

B
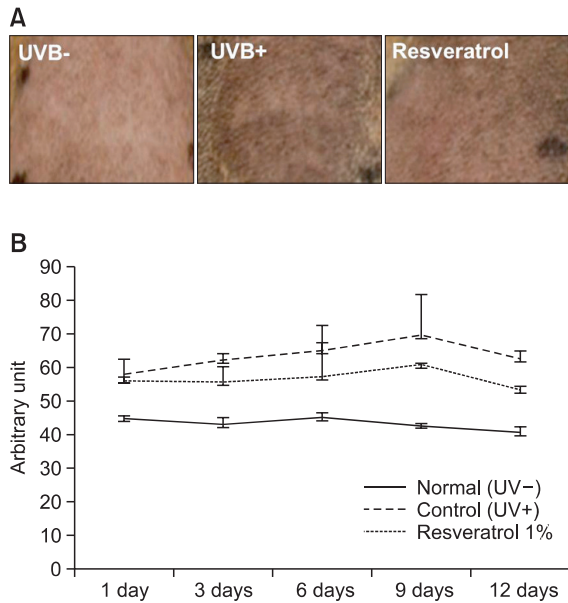
Fig. 3. Inhibitory effects of resveratrol on melanin synthesis in UVB-induced skin pigmentation of brown guinea pigs. Representative features of the dorsal skin of brown guinea pigs were obtained after administration of 1% resveratrol for 2 weeks (A). Skin colors were measured using a Derma lab combo system (B).

lated B16F10 cells.

Resveratrol down-regulates the expression of melanogenic proteins in B16F10 cells

Chen *et al*. reported that resveratrol inhibits melanogenesis in B16 melanoma cells (Chen *et al*., 2013). However, this paper focused on regulation of skin cancer such as melanoma rather than melanogenesis. They studied effects of resveratrol on α-MSH signal transduction involving Wnt/β-catenin, c-Kit, and MITF. Melanogenesis mainly depends on regulation of melanogenic proteins such as tyrosinase, TRP-1, and TRP-2. Therefore, we performed Western blot analysis to determine whether the inhibitory effects of resveratrol are related to the regulation of melanogenesis-related proteins. As shown in Fig. 2, the protein expression of tyrosinase, TRP-1, TRP-2, and MITF were increased by α-MSH treatment. But, resveratrol led to a significant decrease in α-MSH-induced melanogenic protein levels at 100 μM. Based on data shown in Fig. 2, we confirmed the inhibitory effects of resveratrol on melanin synthesis via regulation of melanogenic proteins such as tyrosinase, TRP-1, TRP-2, and MITF expression in B16F10 cells.

Resveratrol regulates UVB-induced pigmentation *in vivo*

Inhibitory effects of resveratrol have not yet been confirmed in an UVB-induced brownish guinea pigs *in vivo* model. Therefore, we induced hyperpigmentation of the dorsal skin of guinea pigs with UVB irradiation for 2 weeks. After inducing pigmentation, dorsal skins of guinea pigs were administered with 1% resveratrol for 2 weeks, namely a 1% resveratrol-containing solution (200 μl) every day for 14 days. As a result, topical application of resveratrol decreased UVB-induced skin hyperpigmentation (Fig. 3A) with a visual score. In addition, we measured a pigment index, which indicates change of skin

color using a Derma lab combo system. While UVB exposure increased the pigment index from 40.7 ± 1.6 in the normal group to 62.6 ± 2.3 in the control group, the pigment index was decreased to 53.4 ± 1.0 by treatment of resveratrol as compared to the control group (Fig. 3B).

To investigate the mechanism of resveratrol on depigmentation, we determined the protein levels of melanogenesis-regulated proteins including tyrosinase, TRP-1, TRP-2, and MITF by Western blot analysis in skin tissue. As seen in Fig. 4, the expression of tyrosinase, TRP-1, TRP-2, and MITF were increased by UVB exposure. However, the protein levels of tyrosinase, TRP-1, TRP-2, and MITF were decreased by resveratrol treatment. Thus, we confirmed that resveratrol regulated melanogenesis-related protein in the dorsal skin of UVB-irradiated guinea pigs. In addition, we assessed melanin production in the skin tissues by the Fontana-Masson staining technique. Melanin synthesis of skin epidermis was increased by UVB exposure. Based on the histological study in Fig. 5, resveratrol treatment decreased melanin production, but not to the levels observed for the normal group.

DISCUSSION

Recently, interest in the various activities of resveratrol has risen along with the well-being trend. Resveratrol is widely present in natural sources such as grapes, berries, and nuts and possesses various activities including anticancer properties, extension of lifespan (Baur and Sinclair, 2006), and anti-hyperpigmentation (Wu *et al*., 2012). Depigmentation agents commonly have similar phenol-based structures, which can inhibit tyrosinase activity (Newton *et al*., 2007). Resveratrol also has a phenol-based structure, and several *in vitro* studies have reported that resveratrol originated from various natural products, which inhibit melanin synthesis (Ohguchi *et al*., 2003; Yanagihara *et al*., 2012). Shin *et al*. reported that oxyresveratrol, found in natural sources such as *Morus alba* L., suppressed the dopa oxidase activity of tyrosinase (Shin *et al*., 1998). Moreover, various resveratrol analogs demonstrated tyrosinase inhibitory activities (Franco *et al*., 2012). In addition, resveratrol suppresses not only melanin production but also cell viability and invasiveness of melanoma (Chen *et al*., 2013). However, Chen *et al*. focused on inhibition of skin cancer yet this study did not investigate changes in melanongenesis-related proteins such as tyrosinase, TRP-1, and TRP-2. Therefore, in this study we investigated the effects of resveratrol on melanongenesis-related proteins by Western blot analysis. Our study shows that resveratrol significantly suppresses expression of all melanogenic protein such tyrosinase, TRP-1, TRP-2, and MITF in α-MSH stimulated B16F10 melanoma cells. Although it has been previously reported that resveratrol inhibits overexpressed MITF level by α-MSH stimulation, we additionally confirmed that resveratrol reduced the expression of tyrosinase, TRP-1, and TRP-2, which are typical proteins involved in melanogenesis. We found that this process involves a decrease in intracellular melanogenic enzymes at the protein level.

Although previous studies have reported the inhibitory role of resveratrol *in vitro*, depigmentation effects of resveratrol *in vivo*, UVB-induced brownish guinea pigs model, one of the most authentic animal models, have not yet been determined. In addition, there is no report on the effect of resveratrol on the
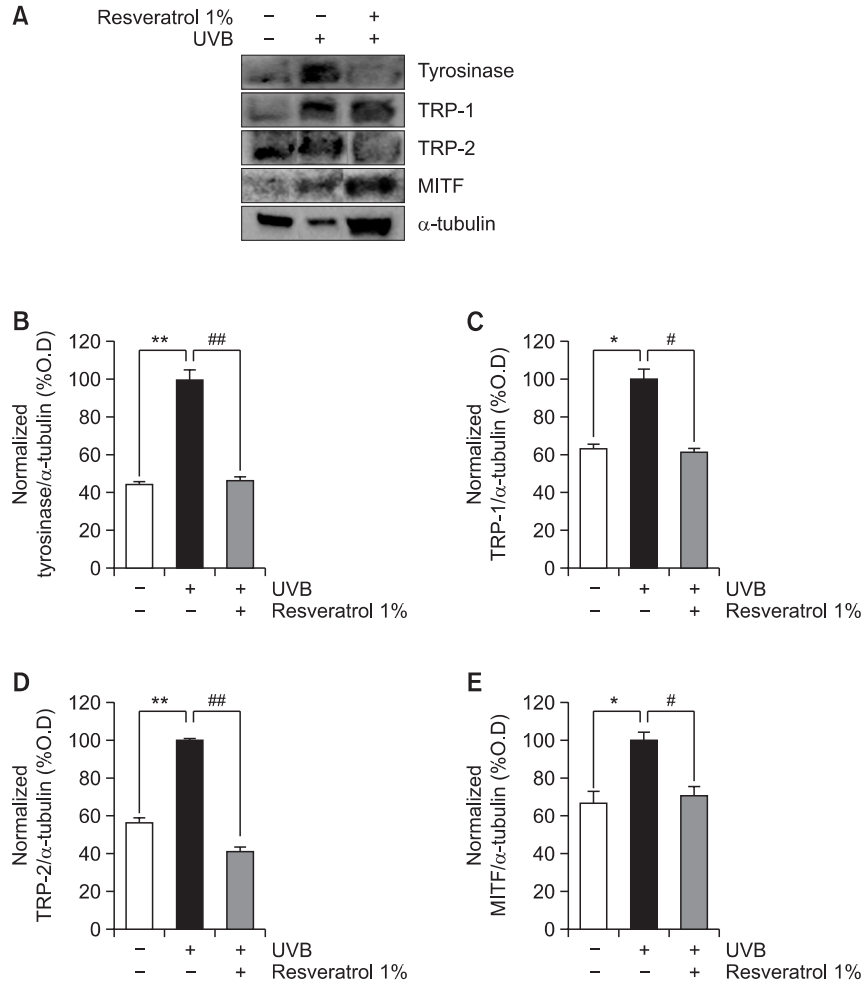
Fig. 4. Effects of resveratrol on the expression of melanogenesis-related proteins in the skin of guinea pigs. Forty micrograms of total protein from the skin tissue lysates were used for Western blot analysis (A). For Western blot analysis, the data are represented as the relative density of tyrosinase (B), TRP-1 (C), TRP-2 (D) and MITF (E) bands normalized to α-tubulin.
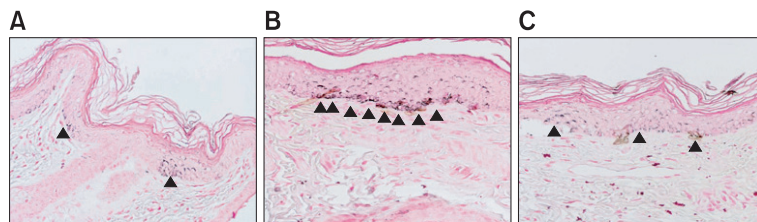
Fig. 5. Results of Fontana-Masson stain for melanin in brown guinea pig skins. (A) UVB negative group, (B) UVB positive group, (C) UVB positive and 1% resveratrol treated group. Arrow heads indicate melanin.

expression of melanogenesis-related proteins in the dorsal skin tissue by western blotting analysis. In this study, UVB-induced dorsal skin of guinea pigs visually changed from black to brown color, and pigmentation values were increased from 44.9 ± 0.8 to 58.1 ± 4.4 after the final tanning treatment. After UVB exposure, the color change value of the dorsal skin was gradually increased for up to 9 days, possibly because UVB stimulated-melanocytes continued to produce melanin (Fig. 3B). However, melanin production was steadily suppressed by resveratrol treatment. Moreover, there were no side effects such as erythema, edema, and itching during all the experiment. As shown *in vitro*, the molecular mechanisms of resveratrol for depigmentation involve inhibition of the expression of melanogenesis-related proteins. Melanin production was also decreased in the skin tissue, as shown by a histological study (Fig. 5). These findings seem that resveratrol directly affects melanocytes located in the epidermis after penetration. Given the *in vivo* efficacy of resveratrol, although resveratrol

is relatively polar substance, it seems that resveratrol is somewhat absorbed by the skin. All data indicate that resveratrol potentially decreases melanin synthesis via regulation of expression of melanogenic proteins in the skin exposed to UVB irradiation by Western analysis.

Our study suggests that resveratrol significantly inhibits melanin synthesis *in vitro* and *in vivo*. These results also support the fact that resveratrol may be a potential skin whitening agent for pharmaceutical and cosmetic use. Further studies are required for clinical and toxicological investigation of resveratrol in the field of skin depigmentation.

ACKNOWLEDGMENTS

REFERENCES

Baur, J. A. and Sinclair, D. A. (2006) Therapeutic potential of resveratrol: the *in vivo* evidence. *Nat. Rev. Drug Discov.* **5**, 493-506.

Bertolotto, C., Busca, R., Abbe, P., Bille, K., Aberdam, E., Ortonne, J. P. and Ballotti, R. (1998) Different cis-acting elements are involved in the regulation of TRP1 and TRP2 promoter activities by cyclic AMP: pivotal role of M boxes (GTCATGTGCT) and of microphthalmia. *Mol. Cell. Biol.* **18**, 694-702.

Chen, Y. J., Chen, Y. Y., Lin, Y. F., Hu, H. Y. and Liao, H. F. (2013) Resveratrol inhibits alpha-melanocyte-stimulating hormone signaling, viability, and invasiveness in melanoma cells. *Evid. Based Complement. Alternat. Med.* **2013**, 632121.

Del Follo-Martinez A., Banerjee, N., Li, X., Safe, S. and Mertens-Talcott, S. (2013) Resveratrol and quercetin in combination have anticancer activity in colon cancer cells and repress oncogenic microRNA-27a. *Nutr. Cancer* **65**, 494-504.

Franco, D. C., de Carvalho, G. S., Rocha, P. R., da Silva, Teixeira R., da Silva, A. D and Raposo, N. R. (2012) Inhibitory effects of resveratrol analogs on mushroom tyrosinase activity. *Molecules* **17**, 11816-11825.

Gatson, J. W., Liu, M. M., Abdelfattah, K., Wigginton, J. G., Smith, S., Wolf, S. and Minei, J. P. (2013) Resveratrol decreases inflammation in the brain of mice with mild traumatic brain injury. *J. Trauma Acute Care Surg.* **74**, 470-474.

Gillbro, J. M. and Olsson, M. J. (2011) The melanogenesis and mechanisms of skin-lightening agents--existing and new approaches. *Int. J. Cosmet. Sci.* **33**, 210-221.

Goldberg, D. M. (1996) More on antioxidant activity of resveratrol in red wine. *Clin. Chem.* **42**, 113-114.

Hosoi, J., Abe, E., Suda, T. and Kuroki, T. (1985) Regulation of melanin synthesis of B16 mouse melanoma cells by 1 alpha, 25-dihydroxyvitamin D3 and retinoic acid. *Cancer Res.* **45**, 1474-1478.

Kobayashi, T., Urabe, K., Winder, A., Jimenez-Cervantes, C., Imokawa, G., Brewington, T., Solano, F., Garcia-Borron, J. C. and Hearing, V. J. (1994) Tyrosinase related protein 1 (TRP1) functions as a DHICA oxidase in melanin biosynthesis. *EMBO J.* **13**, 5818-5825.

Lin, C. B., Babiarz, L., Liebel, F., Roydon, P. E, Kizoulis, M., Gendimenico, G. J, Fisher, D. E. and Seiberg, M. (2002) Modulation of microphthalmia-associated transcription factor gene expression alters skin pigmentation. *J. Invest. Dermatol.* **119**, 1330-1340.

Marchal, J., Pifferi, F. and Aujard, F. (2013) Resveratrol in mammals: effects on aging biomarkers, age-related diseases, and life span. *Ann. N. Y. Acad. Sci.* **1290**, 67-73.

Moon, E. J, Kim, A. J. and Kim, S. Y. (2012) Sarsasapogen inincreases melanin synthesis via induction of tyrosinase and microphthalmia-associated transcription Factor Expression in melan-a cells. *Biomol. Ther.* **20**, 340-345.

Newton, R. A., Cook, A. L., Roberts, D. W., Leonard, J. H., Sturm, R. A. (2007) Post-transcriptional regulation of melanin biosynthetic enzymes by cAMP and resveratrol in human melanocytes. *J. Invest. Dermatol.* **127**, 2216-2227.

Ohguchi, K., Tanaka, T., Ito, T., Iinuma, M., Matsumoto, K., Akao, Y. and Nozawa, Y. (2003) Inhibitory effects of resveratrol derivatives from dipterocarpaceae plants on tyrosinase activity. *Biosci. Biotechnol. Biochem.* **67**, 1587-1589.

Osman, A. M., Al-Harthi, S. E., Alarabi, O. M., Elshal, M. F., Ramadan, W. S., Alaama, M. N., Al-Kreathy, H. M., Damanhouri, Z. A. and Osman, O. H. (2013) Chemosensetizing and cardioprotective effects of resveratrol in doxorubicin- treated animals. *Cancer Cell Int.* **13**, 52.

Riley, P. A. (1997) Melanin. *Int. J. Biochem. Cell Biol.* **29**, 1235-1239.

Shin, N. H., Ryu, S. Y., Choi, E. J., Kang, S. H., Chang, I. M., Min, K. R. and Kim, Y. (1998) Oxyresveratrol as the potent inhibitor on dopa oxidase activity of mushroom tyrosinase. *Biochem. Biophys. Res. Commun.* **243**, 801-803.

Song, S. H., Min, H. Y. and Lee, S. K. (2010) Suppression of prostaglandin E2-mediated cell proliferation and signal transduction by resveratrol in human colon cancer cells. *Biomol. Ther.* **18**, 402-410.

Wu, Y., Jia, L. L., Zheng, Y. N., Xu, X. G., Luo, Y. J., Wang, B., Chen, J. Z., Gao, X. H., Chen, H. D., Matsui, M. and Li, Y. H. (2012) Resveratrate protects human skin from damage due to repetitive ultraviolet irradiation. *J. Eur. Acad. Dermatol. Venereol.* **27**, 345-350

Yanagihara, M., Yoshimatsu, M., Inoue, A., Kanno, T., Tatefuji, T. and Hashimoto, K. (2012) Inhibitory effect of gnetin C, a resveratrol dimer from melinjo (Gnetum gnemon), on tyrosinase activity and melanin biosynthesis. *Biol. Pharm. Bull.* **35**, 993-996.