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Hypopigmentary effects of 4-*n*-butylresorcinol and resveratrol in combination

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In the present study, the effects of 4-*n*-butylresorcinol and/or resveratrol on melanogenesis were studied. To achieve synergistic effects and avoid potential adverse effects, combinations of the agents in low concentrations were investigated. Our results show that 1 μM of 4-*n*-butylresorcinol and 1 μM of resveratrol did not individually inhibit melanin synthesis. However, the combination of 4-*n*-butylresorcinol (1 μM) and resveratrol (1 μM) significantly reduced melanin synthesis. Furthermore, 4-*n*-butylresorcinol (10 μM) and resveratrol (10 μM) decreased melanogenesis much stronger. 4-*n*-Butylresorcinol is reported to directly inhibit tyrosinase, the rate-limiting melanogenic enzyme, without changing tyrosinase levels. Our results also showed that resveratrol did not directly inhibit tyrosinase at 0.1–10 μM. Literature has reported that resveratrol led to post-transcriptional regulation of tyrosinase. However, Western blot analysis showed that neither 4-*n*-butylresorcinol nor resveratrol alone decreased tyrosinase protein levels. Surprisingly, the combination of 4-*n*-butylresorcinol and resveratrol reduced tyrosinase levels. Therefore, these results indicate that the synergistic hypopigmentary effect of 4-*n*-butylresorcinol and resveratrol results from a decreased level of tyrosinase possibly resulting from synergistic action of 4-*n*-butylresorcinol on tyrosinase alteration by resveratrol.

1. Introduction

The regulation of melanin pigments determines skin color. Thus, increased production of melanin synthesis could induce cosmetic problems. Tyrosinase plays a key role in melanogenesis, because it catalyses the rate-limiting reaction of the melanogenic process (Hearing and Jimenez 1989; Kobayashi et al. 1994; Yokoyama et al. 1994), and melanogenesis is mostly regulated by the expression and activation of tyrosinase (Hearing and Tsukamoto 1991). Therefore, many studies have focused on potent tyrosinase inhibitors. These inhibitors often require unacceptably high doses and thus have adverse effects, such as skin irritation or permanent depigmentation. Thus, a combined therapy using a safe low dose is needed to treat hyperpigmentary disorders.

Resorcinol derivatives were reported to inhibit tyrosinase activity (Chen et al. 2004; Tasaka et al. 1998). In addition, it has been reported that compounds with a 4-substituted resorcinol skeleton also inhibited tyrosinase activity (Shimizu et al. 2000). We reported that KI-063, a resorcinol derivative, showed strong tyrosinase inhibitory activity (Kim et al. 2008). Furthermore, we found that 4-*n*-butylresorcinol directly inhibited tyrosinase and strongly inhibited melanogenesis without affecting the major signal transduction pathways related to melanogenesis (Kim et al. 2005). Thus, 4-*n*-butylresorcinol is widely used as a treatment for melasma (Khemis et al. 2007).

Resveratrol is a hydroxystilbene compound found in red wine. Recently, it has been reported that resveratrol inhibits the activity of microphthalmia-associated transcription factor (MITF) promoter (Lin et al. 2002). MITF is known to stimulate tyrosinase expression, and it is a major regulator of melanogenesis (Bentley et al. 1994; Bertolotto et al. 1998). Thus, treatment with resveratrol reduced UVB-induced pigment deposition in Yucatan swine (Lin et al. 2002). On the other hand, it was reported that tyrosinase inhibition by resveratrol did not result from MITF alterations (Newton et al. 2007). Instead, resveratrol inhibits tyrosinase directly and induces a post-transcriptional change of tyrosinase (Newton et al. 2007).

In this study, we used low doses of 4-*n*-butylresorcinol and resveratrol to achieve synergistic effects while avoiding the potential adverse effects, and investigated the combined hypopigmentary effects on melanin synthesis in a spontaneously immortalized mouse melanocyte cell line, Mel-Ab.

2. Investigations and results

2.1. Effects of resveratrol on tyrosinase activity

To determine whether or not resveratrol can be safely used as a skin-whitening agent, the Mel-Ab cells were treated 0–100 μM resveratrol, and cell viability was measured using a crystal violet assay. Resveratrol showed no cytotoxic effect on Mel-Ab cells at concentrations ≤ 10 μM (Fig. 1A). Accordingly, cells were exposed to 0–10 μM resveratrol for the following experiments. In addition, tyrosinase activity was also measured in a cell-free system using mushroom tyrosinase, as described in the

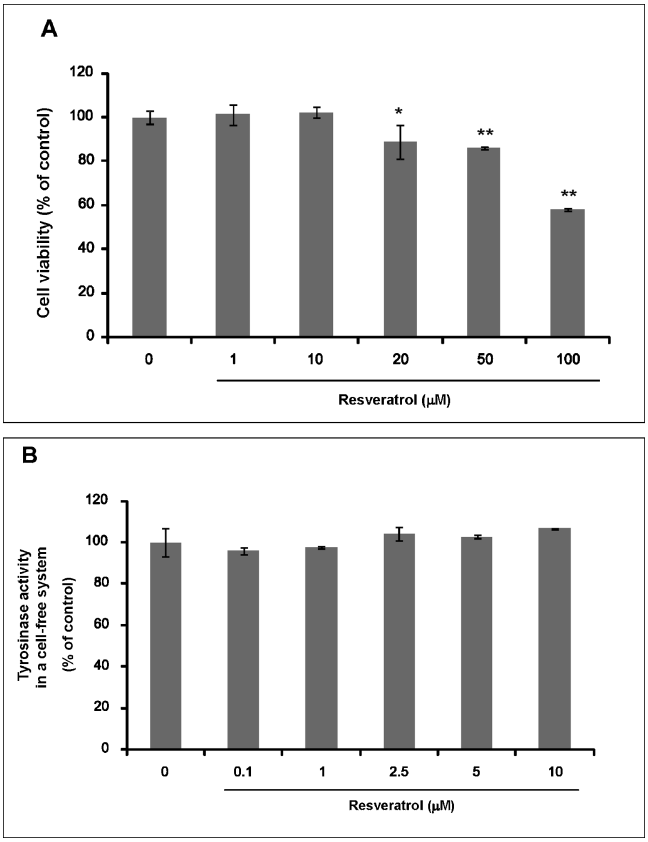

Fig. 1: Effects of resveratrol on tyrosinase activity. (A) Cells were serum-starved for 24 h and treated with resveratrol (1–100 μM) in serum-free media for 24 h. Cell viabilities were determined by crystal violet assay. (B) To test the direct effects of resveratrol on tyrosinase, tyrosinase activity was measured in a cell-free system. Resveratrol (0.1–10 μM) was added to each well. Each determination was made in triplicate and data shown are means ± SD. * $P < 0.05$ and ** $P < 0.01$ compared to untreated control

Experimental section, to determine whether resveratrol directly inhibits the tyrosinase activity. However, resveratrol showed no direct effect on tyrosinase activity at concentrations ≤ 10 μM (Fig. 1B).

2.2. The combination of 4-n-butylresorcinol with resveratrol revealed a synergistic effect on melanogenesis inhibition

Previously, we reported that 4-*n*-butylresorcinol showed a strong inhibitory effect on tyrosinase activity (Kim et al. 2005). Therefore, we next investigated the combined effects of 4-*n*-butylresorcinol and resveratrol on melanogenesis. Cells were exposed to 0–10 μM 4-*n*-butylresorcinol and 0–10 μM resveratrol for 3 days and then photographed under a phase contrast microscope (Fig. 2A). Our results showed that exposure to 10 μM 4-*n*-butylresorcinol or 10 μM resveratrol individually resulted in less pigmented cells than the control, and that the combined treatment with 4-*n*-butylresorcinol and resveratrol resulted in markedly less pigmented cells. We also measured the melanin contents of the Mel-Ab cells after 4-*n*-butylresorcinol and/or resveratrol treatment. 4-*n*-Butylresorcinol treatment inhibited melanin synthesis at a concentration of 10 μM. Resveratrol also reduced melanin synthesis at a concentration of 10 μM. Furthermore, the combination of 4-*n*-butylresorcinol and resveratrol did show a significant additive effect on melanogenesis inhibition (Fig. 2B). Interestingly, melanin downregulation by 4-*n*-butylresorcinol was significantly potentiated by 1 μM resveratrol treatment, although 1 μM resveratrol showed no melanin-decreasing effects (Fig. 2B). These results indicate that low concentrations of 4-*n*-butylresorcinol and resveratrol work synergistically, because they may regulate melanogenesis via different mechanisms.

2.3. The combination of 4-n-butylresorcinol with resveratrol decreased tyrosinase expression

It was recently reported that resveratrol reduces the level of melanin synthesis by a post-transcriptional change of tyrosinase (Newton et al. 2007). Thus, the tyrosinase activity was measured after a 3-day-treatment with 4-*n*-butylresorcinol and resveratrol. As shown in Fig. 3A, resveratrol (10 μM) significantly inhibited tyrosinase activity.

Furthermore, the protein levels of tyrosinase were examined after 4-*n*-butylresorcinol and/or resveratrol treatment. Treatment with 4-*n*-butylresorcinol or resveratrol alone had no influence on tyrosinase levels. However, the treatment with 4-*n*-butylresorcinol and resveratrol together for 3 days reduced the protein level of tyrosinase, whereas MITF levels were not significantly changed (Fig. 3B). Thus, reduced tyrosinase protein levels may be responsible for the hypopigmentary effects of the combination of 4-*n*-butylresorcinol and resveratrol.

3. Discussion

Previously, we reported that 4-*n*-butylresorcinol directly inhibits tyrosinase activity and suppressed melanin synthesis (Kim et al. 2005). Resveratrol is a major active constituent of red wine. In addition to antioxidative effects (Kairisalo et al. 2011), resveratrol has been reported to inhibit tyrosinase activity, which is known to regulate the rate-limiting step for melanin synthesis (Kim et al. 2002). In this study, the IC_{50} for resveratrol-mediated inhibition of tyrosinase activity was >100 μM for murine tyrosinase and 54.6 μM for mushroom tyrosinase. In another study, resveratrol inhibited mushroom tyrosinase activity with an IC_{50} value of 55.6 μM (Ha et al. 2007). In the present study, however, we found that resveratrol showed a significant cytotoxic effect on Mel-Ab cells at concentrations ≥ 20 μM. To guarantee the safety of resveratrol, it should be used at concentrations of ≤ 10 μM, and we therefore performed the tyrosinase inhibition assay at such concentrations. Although high doses of resveratrol showed tyrosinase inhibitory activity, low doses of resveratrol did not inhibit tyrosinase (Fig. 1B).

As shown in Fig. 2B, resveratrol (10 μM) significantly inhibited melanin synthesis, although it did not demonstrate direct tyrosinase inhibition at the same concentration. Thus, it was hypothesized that other mechanisms are involved in resveratrol-induced hypopigmentation. It has been reported that resveratrol (30 μg/mL) reduced MITF and tyrosinase promoter activities in B16 murine melanoma cells (Lin et al. 2002). However, our Western blot analysis showed that 10 μM resveratrol did not reduce MITF and tyrosinase protein levels. The concentration of 30 μg/mL resveratrol is equivalent to ~100 μM resveratrol. Thus, high concentrations of resveratrol could reduce MITF and tyrosinase levels. In agreement with our results, it was reported that tyrosinase inhibition by resveratrol did not result from MITF alterations (Newton et al. 2007). Instead, resveratrol-treated cells showed a post-transcriptional change of tyrosinase, namely ER-retained, immature tyrosinase, whereas normal melanocytes mainly contained the Golgi-processed form (Newton et al. 2007). Therefore, the detected tyrosinase in Western blot analysis may be an immature form, although resveratrol did not reduce tyrosinase levels in our experiment.

In the present study, the combination of 4-*n*-butylresorcinol and resveratrol acted in a synergistic manner, indicating that the strong tyrosinase inhibitory activity of 4-*n*-butylresorcinol

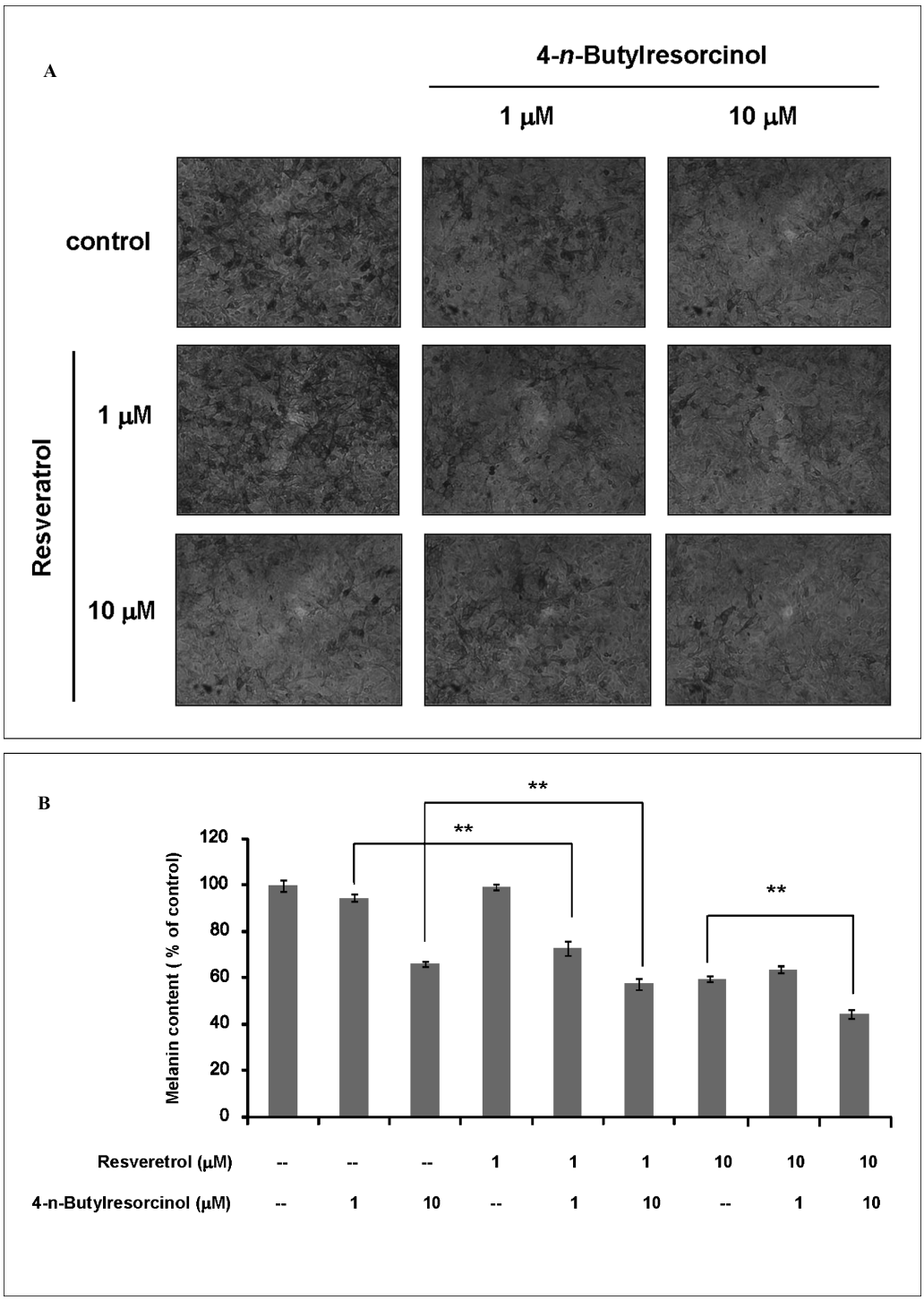

Fig. 2: Combined effects of 4-*n*-butylresorcinol and resveratrol on melanin synthesis in Mel-Ab cells. Cells were cultured with 1 or 10 μM of 4-*n*-butylresorcinol and/or resveratrol for 3 days. (A) Phase contrast photomicrographs were taken using a digital video camera, and (B) melanin contents were measured as described. The results shown are the averages of triplicate experiments ± SD. ** $P < 0.01$ between the groups

works in combination with the tyrosinase alteration induced by resveratrol. It is interesting that the combination of 4-*n*-butylresorcinol and resveratrol reduced tyrosinase levels without a change in MITF levels, although 4-*n*-butylresorcinol or resveratrol alone had no influence on it. Because 4-*n*-butylresorcinol strongly inhibits tyrosinase, the high-affinity interaction between 4-*n*-butylresorcinol and tyrosinase could help resveratrol to decrease tyrosinase levels.

In summary, this study demonstrates that the combined treatment using 4-*n*-butylresorcinol and resveratrol synergistically reduced melanin synthesis in Mel-Ab cells. In addition, the synergistic hypopigmentary effect of 4-*n*-butylresorcinol and resveratrol results from decreased levels of tyrosinase. Therefore, the low dose combination of 4-*n*-butylresorcinol and resveratrol provides a powerful tool to maximize hypopigmentary effects with few adverse effects.

4. Experimental

4.1. Materials

4-*n*-Butylresorcinol was synthesized by SK chemicals (Suwon, Korea). Resveratrol, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), cholera toxin (CT), synthetic melanin, L-DOPA, and mushroom tyrosinase were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Antibodies specific for

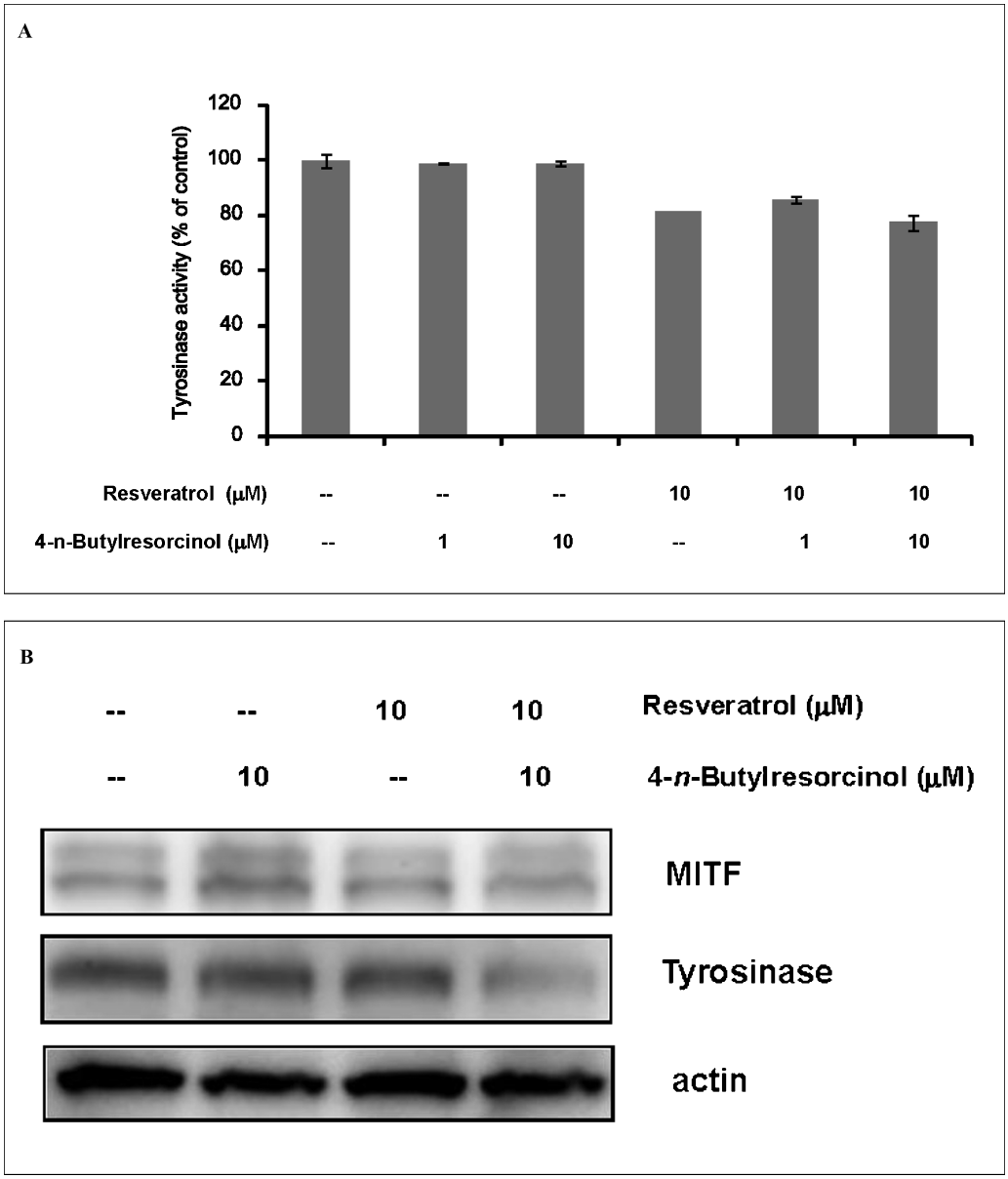

Fig. 3: Combined effects of 4-*n*-butylresorcinol and resveratrol on tyrosinase in Mel-Ab cells. Cells were cultured with 4-*n*-butylresorcinol (1–10 μM) and/or resveratrol (10 μM) for 3 days. (A) Tyrosinase activity was measured as described. The results shown are the averages of triplicate experiments ± SD. (B) Whole cell lysates were then subjected to Western-blot analysis using antibodies against MITF and tyrosinase. Equal protein loadings were confirmed using anti-actin antibody

tyrosinase (C-19) and actin (I-19) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), and microphthalmia Ab-1 (C5, MS-771-P0) was obtained from NeoMarkers (Fremont, CA, USA). Secondary antibodies specific for anti-goat IgG (PI-9500), anti-mouse IgG (PI-2000), and anti-rabbit IgG (PI-1000) were purchased from Vector Laboratories (Burlingame, CA, USA).

4.2. Cell cultures

The Mel-Ab cell line is a mouse-derived spontaneously immortalized melanocyte cell line that produces large amounts of melanin (Dooley et al. 1994). Mel-Ab cells were incubated in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS; Hyclone, Logan, UT, USA), 100 nM TPA, 1 nM CT, 50 μg/ml of streptomycin, and 50 μg/ml of penicillin (Hyclone) at 37 °C in 5% CO_2.

4.3. Cell viability assay

Cell viability was determined using a crystal violet assay. After incubating cells with resveratrol for 24 h, the media were removed, and the cells were stained with 0.1% crystal violet in 10% ethanol for 5 min at room temperature. The cells were then rinsed 4 times with distilled water, and the crystal violet retained by adherent cells was extracted with 95% ethanol. Absorbance was determined at 590 nm using an ELISA reader (VERSAMax; Molecular Devices, Sunnyvale, CA, USA).

4.4. Measurement of melanin content and microscopy

Melanin content was measured as previously described, with slight modifications (Tsuboi et al. 1998). Cells were treated with various concentrations (1–10 μM) of resveratrol in DMEM containing 10% FBS for 3 days. Cell pellets were dissolved in 550 μl of 1 N NaOH at 100 °C for 30 min and centrifuged at 13,000 × *g* for 5 min. Supernatants were analyzed at 400 nm using an ELISA reader. Before melanin content was measured, the cells were observed under a phase contrast microscope (Olympus IX50, Tokyo, Japan) and photographed using a DCM300 digital camera for a microscope (Scopetek, Inc., Hangzhou, China), which was supported by ScopePhoto software (Scopetek, Inc.).

4.5. Tyrosinase activity

Tyrosinase activity was assayed as DOPA oxidase activity. Mel-Ab cells were incubated at a density of 2×10^5 cells in 6-well plates and incubated with test substances in DMEM for 3 days. Cells were washed with PBS and lysed with lysis buffer (0.1 M phosphate buffer [pH 6.8] containing 1% Triton X-100). They were then disrupted by freeze-thawing, and lysates were clarified by centrifugation at 13,000 rpm for 30 min. After quantifying protein content using a protein assay kit (Bio-Rad, Hercules, CA, USA), the protein concentrations were adjusted with lysis buffer. Then, 90 μL of each lysate was pipetted into the wells of a 96-well plate, and 10 μL of 10 mM L-DOPA was added.

Control wells contained 90 μL of lysis buffer and 10 μL of 10 mM L-DOPA. After incubation at 37 °C for 20 min, dopachrome formation was monitored by measuring absorbance at 475 nm using an ELISA reader.
A cell-free assay system was used to determine the direct effect of resveratrol on tyrosinase activity. Phosphate buffer (70 uL) containing resveratrol was mixed with 20 μL (53.7 units/mL) mushroom tyrosinase, and 10 μL of 10 mM L-DOPA was then added. Following incubation at 37 °C for 20 min, the absorbance was measured at 475 nm.

4.6. Western blot analysis

Cells were lysed in cell lysis buffer (62.5 mM Tris-HCl [pH 6.8], 2% SDS, 5% β-mercaptoethanol, 2 mM phenylmethylsulfonyl fluoride, and protease inhibitors [Complete^{TM}; Roche, Mannheim, Germany], 1 mM Na_3VO_4, 50 mM NaF, and 10 mM EDTA). Proteins (20 μg per lane) were separated by SDS-polyacrylamide gel electrophoresis and blotted onto polyvinylidene fluoride (PVDF) membranes, which were then saturated with 5% dried milk in Tris-buffered saline containing 0.5% Tween 20. Blots were then incubated with the appropriate primary antibodies at a dilution of 1:1000, and further incubated with horseradish peroxidase-conjugated secondary antibody. Bound antibodies were detected using an enhanced chemiluminescence plus kit (Amersham International, Little Chalfont, UK). The images of the blotted membranes were obtained using a LAS-1000 lumino-image analyzer (Fuji Film, Tokyo, Japan).

4.7. Statistics

Differences between results were assessed for significance using the Student's t-test. *P* values of <0.05 were regarded as significant.

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