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Research Paper

Acteoside inhibits melanogenesis in B16F10 cells through ERK activation and tyrosinase down-regulation

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Abstract

Objectives Acteoside is a phenylpropanoid glycoside extracted from the leaves of *Rehmannia glutinosa* that displays various biological activities. In this study, we tested the effects of acteoside on tyrosinase activity and melanin biosynthesis in B16F10 melanoma cells. We also explored molecular mechanisms for the inhibition of melanogenesis observed, focusing on the signalling pathway of extracellular signal-regulated kinase (ERK).

Methods The effects of acteoside were determined using several cell-free assay systems and B16F10 melanoma cells for melanin content and tyrosinase activity. To investigate effects on melanogenic regulatory factors we performed reverse transcription polymerase chain reaction, cAMP assay and Western blot analyses.

Key findings Acteoside showed an inhibitory effect on tyrosinase activity and melanin synthesis in both cell-free assay systems and cultured B16F10 melanoma cells. Acteoside decreased levels of tyrosinase, tyrosinase-related protein-1 (TRP-1) and microphthalmia-associated transcription factor (MITF) proteins, whereas it increased ERK phosphorylation. A specific ERK inhibitor, PD98059, abolished the acteoside-induced down-regulation of MITF, tyrosinase and TRP-1 proteins. The ERK inhibitor increased tyrosinase activity and melanin production and reversed the acteoside-induced decrease in tyrosinase activity and melanin content. In addition, acteoside suppressed melanogenesis induced by α-melanocyte-stimulating hormone and showed UV-absorbing effects.

Conclusions Acteoside decreased tyrosinase activity and melanin biosynthesis in B16F10 cells by activating ERK signalling, which down-regulated MITF, tyrosinase and TRP-1 production.

Keywords acteoside; extracellular signal-regulated kinase (ERK); melanogenesis; microphthalmia-associated transcription factor (MITF); tyrosinase

Introduction

Melanogenesis is the physiological process that protects the skin from solar ultraviolet (UV) light.[1,2] However, abnormal activation of melanin synthesis may cause aesthetic problems and promote malignant melanoma. The biosynthesis of melanin pigments takes place in melanocytes under the regulation of several melanogenic enzymes, including tyrosinase, tyrosinase-related protein-1 (TRP-1) and TRP-2.[3] Tyrosinase, a copper-containing enzyme, initiates melanogenesis by hydroxylating L-tyrosine to form 3,4-dihydroxyphenyl-L-alanine (L-DOPA), and oxidizing L-DOPA to dopaquinone, which in the absence of thiols forms DOPAchrome.[4,5] TRPs participate in the conversion of DOPAchrome to indole 5,6-quinone-2-carboxylic acid (DHICA) and in DHICA oxidation.[6] Inhibitors specific for tyrosinase and TRPs are sought for cosmetic and medicinal applications.[7–9]

Microphthalmia-associated transcription factor (MITF) is a strong inducer of the melanocyte-specific enzymes[3] and MITF mutations cause abnormal melanin pigmentation.[10] α-Melanocyte stimulating hormone (α-MSH), which increases intracellular cAMP, promotes melanin synthesis through the induction of MITF expression.[11,12] Mitogen-activated protein kinases (MAPKs) also influence melanogenesis. Inhibition of the extracellular signal-regulated kinase (ERK) pathway increases tyrosinase activity in B16 melanoma cells, whereas sustained ERK activation reduces melanin synthesis through

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MITF degradation and subsequent down-regulation of tyrosinase and TRP-1.[13] Surprisingly, activation of the p38 MAPK pathway is reported to increase melanin synthesis by stimulating MITF expression and tyrosinase transcription.[14] This illustrates the complexity of MAPK involvement in melanogenesis.

Much attention has been focused on the development of melanogenic inhibitors from natural resources. We previously isolated a water-soluble phenylpropanoid glycoside, acteoside, from the leaves of *Rehmannia glutinosa*.[15] Acteoside is reported to have antiviral, hepatoprotective, anti-inflammatory and possibly other beneficial properties.[16–19] We previously observed that it has antioxidant and immune-stimulating effects, and inhibits matrix metalloproteinase (MMP) activity.[15] A recent report shows that acteoside also inhibits α-MSH-induced melanin production in murine melanoma cells by inhibiting adenylyl cyclase activity.[20] These reports led us to suggest that acteoside has the capacity to prevent abnormal pigmentation. However, the mechanisms by which acteoside regulates melanogenesis and melanocyte pigmentation are unclear. In this study, we explored mechanisms for the inhibition of melanogenesis which we observed, focusing on the ERK-signalling pathway. We demonstrate the cellular mechanisms involved in acteoside-mediated anti-melanogenesis in the presence and absence of α-MSH, where ERK and/or cAMP-induced MITF down-regulation and tyrosinase activity inhibition are critical events.

Materials and Methods

Mice, chemicals, and laboratory wares

Female C57BL/6 mice, 5–6 weeks old, were purchased from Damul Science (Yoosung, South Korea). The Chonbuk National University Committee on Ethics in the Care and Use of Laboratory Animals approved our protocol for animal care and use (approved number: CBU 2010–0040). Acteoside (3,4-dihydroxy-β-phenethyl-*O*-α-rhamnopyranosyl-(1→3)-4-*O*-caffeoyl-β-D-glucopyranoside; $C_{29}H_{36}O_{15}$) (Figure 1a) was isolated from the fresh leaves of *Rehmannia glutinosa* according to the method reported by Chun *et al*.,[21] where 0.36 g of acteoside (>90% purity) was finally isolated from 500 g of the leaves. Acteoside was dissolved in distilled water immediately before use. The ERK inhibitor PD98059 was obtained from TOCRIS (Bristol, UK). In this study, arbutin and kojic acid were used as positive controls. Unless otherwise specified, chemicals and laboratory wares were obtained from Sigma

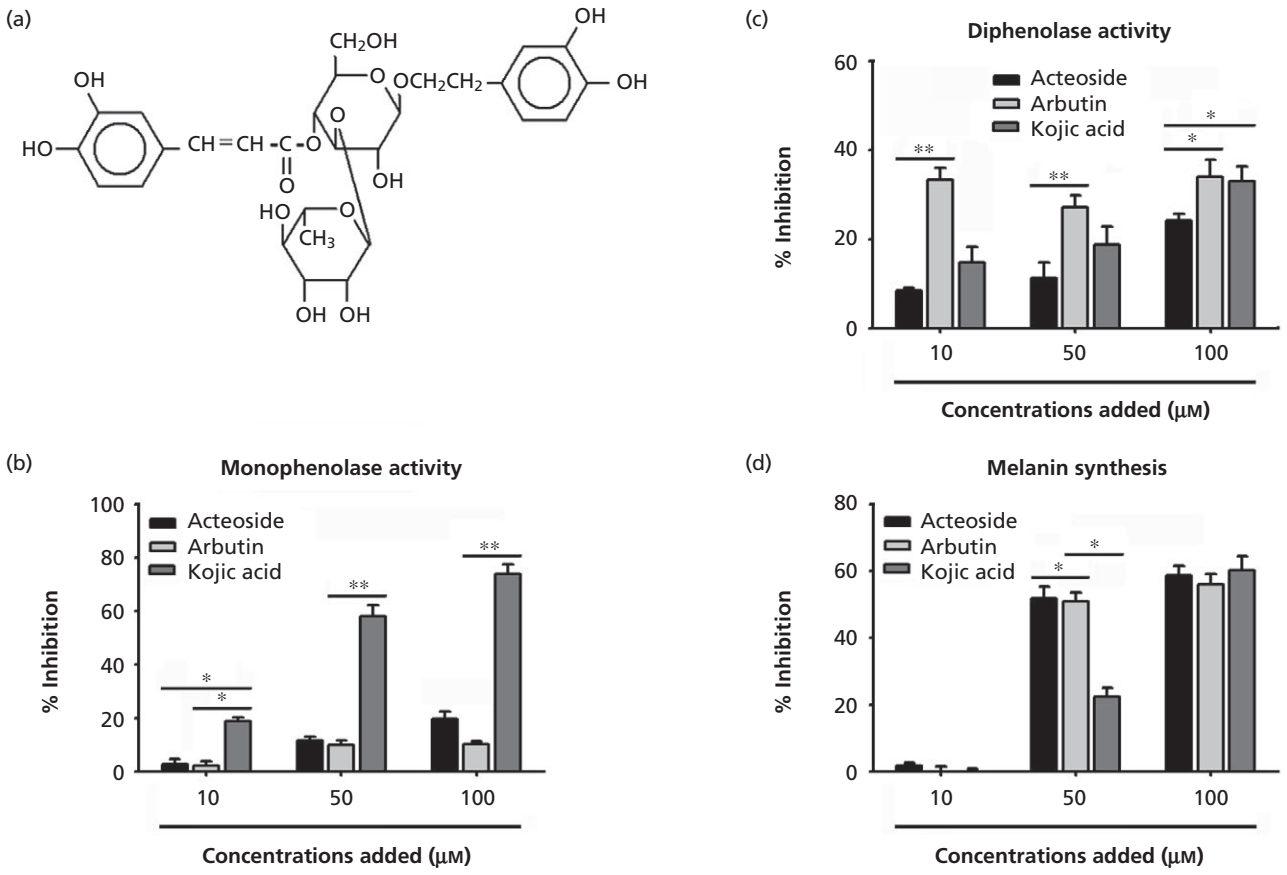

Figure 1 Chemical structure of acteoside (a) and effects of acteoside on tyrosinase activity and melanin synthesis in cell-free assay systems (b,c,d). To verify that acteoside directly inhibits tyrosinase activity the monophenolase (b) and diphenolase (c) activity of mushroom tyrosinase was determined by measuring the formation of dopachrome. (d) The inhibitory effect of acteoside on melanin biosynthesis was measured in a cell-free system as described in 'Materials and Methods'. $^*P < 0.05$ and $^{**}P < 0.01$ indicate significant differences between the experiments.

Chemical Co. (St Louis, USA) and SPL Life Sciences (Pochun, South Korea), respectively.

Cell cultures and treatments

B16F10 melanoma cells were gifted kindly from Dr Suhnyoung Im (Chonnam National University, Gwangju, South Korea). Cells were cultured in Dulbecco's minimal essential medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS; HyClone, Logan, USA) and antibiotics (100 U/ml of penicillin and 100 µg/ml of streptomycin). The B16F10 cell suspension (10^7 cells/ml) was subcutaneously injected into C57BL/6 mice (100 µl/mouse) to stimulate the melanogenic potential of the cells and melanomas 15 mm in diameter were collected to prepare a single-cell suspension by an enzymatic digestion. A single-cell suspension (1×10^5 cells/ml) of melanoma cells was distributed in volumes of either 2 ml or 100 µl to 6-well or 96-well flat-bottomed plates, respectively. Two days later, the culture medium was replaced with fresh medium containing graded concentrations (0–500 µM) of acteoside with and without 20 µM PD98059. In addition, a sample of B16F10 cell suspension was directly divided into culture plates before in-vivo injection and processed for the analyses of melanin content, tyrosinase activity and cAMP level at various times after treatment with acteoside and/or α-MSH.

Cell-free enzymatic assays for tyrosinase activity and melanin synthesis

To test the direct inhibitory effects of acteoside on tyrosinase activity and melanin synthesis, we performed cell-free enzymatic assays. For tyrosinase activity, both the monophenolase and diphenolase activity of mushroom tyrosinase was determined by measuring formation of dopachrome (2-carboxy-2,3-dihydro-indole-5,6-quinone). To assay the monophenolase, 40 µl of 1.5 mM tyrosine solution and 230 µl of 50 mM Tris-HCl (pH 7.5) were mixed and incubated at 37°C for 10 min. Twenty microlitres of mushroom tyrosinase (2000 U/ml) and 20 µl of a solution containing 0–100 µM acteoside were added to the mixture. Absorbance was measured at 490 nm using a SpectraCount ELISA reader (Packard Instrument Co., Downers Grove, USA). For the diphenolase assay, L-DOPA was used as the substrate. Each reaction contained 95 µl of 20 mM L-DOPA, 875 µl of 50 mM Tris-HCl (pH 7.5) and 20 µl of a solution containing 0–100 µM acteoside. A 50-µl volume of mushroom tyrosinase (2000 U/ml) was added to the mixture after 5 min of incubation at 37°C, and the absorbance was measured at 475 nm using a spectrophotometer (Beckman DU 530, Krefeld, Germany).

The inhibitory effect of acteoside on melanin biosynthesis was also measured using a cell-free assay system. Each assay contained 40 µl of 1.5 mM tyrosine, 240 µl of 0.1 M phosphate buffer (pH 6.5), 6 µl of a solution containing 0–100 µM acteoside and 20 µl of 2000 U/ml mushroom tyrosinase. After 10 min of incubation at 37°C, the assay was initiated by adding 20 µl of 0.03% L-DOPA and allowed to continue for 60 min at 37°C. The assay mixture was centrifuged at 12 000*g* for 20 min and the pellet was incubated at 60°C for 1 min with 200 µl of 1 N NaOH. Finally, the absorbance was measured at 490 nm using ELISA reader.

Assay of cellular tyrosinase activity

Acteoside at various concentrations (0–500 µM) was added to B16F10 cells at 70–80% confluence and incubation continued for two days. The cells were then harvested and incubated for 30 min at 4°C in 150 µl of a lysis buffer containing 0.1 M sodium phosphate (pH 6.8), 1% Triton X-100, and 0.1 mM phenylmethylsulfonyl fluoride. The lysates were centrifuged at 12 000*g* for 30 min. After quantifying protein levels and adjusting concentrations with lysis buffer, the supernatants (50 µl of each lysate) were mixed with 100 µl of 0.1 M sodium phosphate buffer (pH 6.8) and 40 µl of 1.5 mM tyrosine in a 96-well plate. Absorbance was measured at 405 nm every 3 min for 30 h at 37°C using an ELISA plate reader.

Tyrosinase zymography

Cellular tyrosinase activity was also determined by zymography. Lysates were made from B16F10 cells after two days of acteoside treatment, as described above, and a sample of each lysate (50 µg protein) was mixed with a loading buffer not containing β-mercaptoethanol and applied without boiling to a 12% polyacrylamide gel containing sodium dodecyl sulfate (SDS). After electrophoresis, the gels were allowed to equilibrate in 100 mM sodium phosphate buffer (pH 6.8) for 1 h and then incubated in 30 ml of 5 mM L-DOPA until colour developed in bands corresponding to tyrosinase. These bands appeared as dark-yellow zones against a colourless background, with intensity proportional to tyrosinase activity. Band intensities were evaluated using a Gel-Print System (Core Bio Corporation, Seoul, South Korea).

Measurement of cellular melanin content

B16F10 melanoma cells cultured in six-well plates were treated with various concentrations (0–500 µM) of acteoside for two days or with 200 µM acteoside for 0–5 days. Cells were harvested, washed twice with phosphate-buffered saline (PBS), and then dried at 60°C for 30 min. Each dried sample was incubated in 500 µl of 1 N NaOH for 1 h at 80°C and then centrifuged at 12 000*g* for 30 min. Optical densities of the supernatants were measured at 405 nm using an ELISA plate reader. The melanin content was calculated by comparison of the sample OD_{405} to a standard curve for synthetic melanin and expressed as µg/mg protein. In addition, the pigmentation of each cell lysate was photographed using a digital camera just before the melanin determination.

Western blot analysis

B16F10 melanoma cells were resuspended in a nonidet P (NP)-40 lysis buffer (30 mM Tris-Cl, pH 7.5, 1 mM EDTA, 150 mM NaCl, and 1% NP-40) for 20 min, and the protein content of each lysate was quantified using the Bradford method.[22] Samples containing equal amounts of protein (40 µg/sample) were analysed by 15% SDS-PAGE and blotted onto PVDF membranes. The blots were probed with primary antibodies and then incubated with a horseradish peroxidase-conjugated anti-IgG in blocking buffer for 1 h. After washing, the blots were developed with enhanced chemiluminescence (Santa Cruz Biotechnology Inc., Santa Cruz, USA) and exposed to X-ray film (Eastman-Kodak, Rochester, USA). The polyclonal antibodies specific to

tyrosinase (sc-15341), TRP-1 (sc-25543), TRP-2 (sc-25544), MITF (sc-25386) and ERK (sc-94), and the monoclonal antibody specific to p-ERK (sc-7383) were purchased from Santa Cruz Biotechnology, Inc. The monoclonal antibody specific to α-tubulin was purchased from Sigma-Aldrich.

RNA preparation and polymerase chain reaction

Total RNA was isolated from B16F10 cells using the SV Total RNA Isolation System (Promega, Madison, USA) according to the manufacturer's instructions. Reverse transcription and polymerase chain reaction (PCR) amplification were performed using an Access RT-PCR System (Promega) as recommended by the manufacturer. The primer sequences used, designed according to Kim *et al*.[23] were as follows: tyrosinase, 5′-GGCCAG CTT TCA GGC AGA GGT-3′ (forward), 5′-TGG TGC TTC ATG GGC AAA ATC-3′ (reverse); and β-actin, 5′-CGA GCG GGA AAT CGT GCG TGA CAT TAA GGA GA-3′ (forward), 5′-CGT CAT ACT CCT GCT TGC TGA TCC ACA TCT GC-3′ (reverse). PCR was performed for 26–30 cycles of 94°C for 1 min, 55–58°C for 1 min, and 72°C for 1 min in a DNA thermal cycler (model PTC-100, Waltham, USA). PCR products were analysed on 1.5% agarose gels and visualized using ethidium bromide staining. Band intensity was calculated using a gel imaging system (model F1-F2 Fuses type T2A; BIO-RAD, Segrate, Italy).

cAMP assay

B16F10 melanoma cells were stimulated with 1 μM α-MSH for 1 h at 37°C in the presence and absence of acteoside (0–500 μM). Concentrations of cellular cAMP were determined using a cAMP immunoassay kit (Cayman Chemical Company, Ann Arbor, USA) according to the manufacturer's instructions and expressed as a percentage of the value for untreated cells.

Absorption of UV-A and UV-B

The UV spectrum of acteoside (250 μM) was measured as previously described,[24] using an Agilent 8453 UV-visible spectrophotometer (Agilent Technologies, Inc., Santa Clara, USA).

Statistical analyses

Unless otherwise specified, data are expressed as the mean ± standard deviation (SD) as performed in triplicate or more replicate experiments. The Kruskal–Wallis test followed by Dunn's multiple comparison test was used for the analyses of statistical significances among the groups using the GraphPad Prism program (version 4.0.) (GraphPad Software, San Diego, USA). $P < 0.05$ was considered statistically significant. In addition, all the figures shown were obtained from at least triplicate experiments with similar results.

Results

Acteoside inhibits tyrosinase activity and melanin synthesis in cell-free assay systems

We initially examined the effects of acteoside on viability and proliferation of B16F10 melanoma cells. Acteoside did not cause cytotoxic effects at the concentrations examined (0–500 μM), whereas kojic acid at 100 μM increased slightly the number of trypan blue-positive cells (data not shown). Subsequently, acteoside was tested for inhibitory effects on the monophenolase and diphenolase activity of tyrosinase. Acteoside inhibited monophenolase activity in a dose-dependent manner, with 11.6 and 19.7% inhibition at 50 and 100 μM, respectively (Figure 1b). Unlike acteoside and arbutin, kojic acid strongly inhibited the monophenolase activity of tyrosinase. Thus kojic acid at 100 μM inhibited tyrosinase activity by 73.9%. Acteoside, arbutin and kojic acid also inhibited the diphenolase activity, while arbutin showed the most strong activity at the same concentrations, showing approximate 30% inhibition, independent of concentration between 10 and 100 μM (Figure 1c). All of the inhibitors exerted strong dose-dependent effects on melanin synthesis (Figure 1d). Acteoside and arbutin showed approximately two-fold greater inhibition than kojic acid at 50 μM. These results suggested that acteoside directly inhibited oxidation of L-tyrosine to L-DOPA, and of L-DOPA to dopaquinone, and also inhibited melanin synthesis.

Concentration-dependent inhibition of tyrosinase activity in acteoside-treated melanoma cells

Acteoside induced a dose-dependent reduction in tyrosinase activity in B16F10 cells, which was significant at concentrations higher than 100 μM (Figure 2a). The results from tyrosinase zymography supported this finding, in that both the mature and immature forms of tyrosinase were reduced in proportion to the acteoside concentration (Figure 2b). With the addition of 200 and 500 μM acteoside, the band corresponding to mature tyrosinase decreased in intensity to 32 and 18%, respectively, as compared with the band in untreated cells. Consistent with this result, both the protein and mRNA levels of tyrosinase in B16F10 cells diminished with acteoside in proportion to dose (Figure 2c and 2d). These results suggested that acteoside down-regulates cellular tyrosinase activity in melanoma cells at the level of transcription and possibly, translation of tyrosinase mRNA.

Acteoside inhibits melanin pigmentation in B16F10 melanoma cells in a dose-dependent manner

A naked-eye inspection of cell pellets suggested that acteoside-treated cells contained less melanin than untreated control cells (data not shown). The anti-melanogenic effect of acteoside was confirmed by the analysis of absorbance at 405 nm using the ELISA reader, which showed a concentration-dependent decrease in the melanin content of acteoside-treated melanoma cells (Figure 3a). Acteoside at 200 μM showed similar potency to that of kojic acid at 500 μM in the reduction of melanin pigmentation. To confirm the inhibitory effect of acteoside on melanin synthesis, cells were exposed to 200 μM acteoside and assayed for melanin content at various times (0–5 days) (Figure 3b). In the untreated control cells, pigment content ranged from 43 to 54 μg/mg protein during the experiment. A significant decrease in melanin content in acteoside-treated cells became apparent

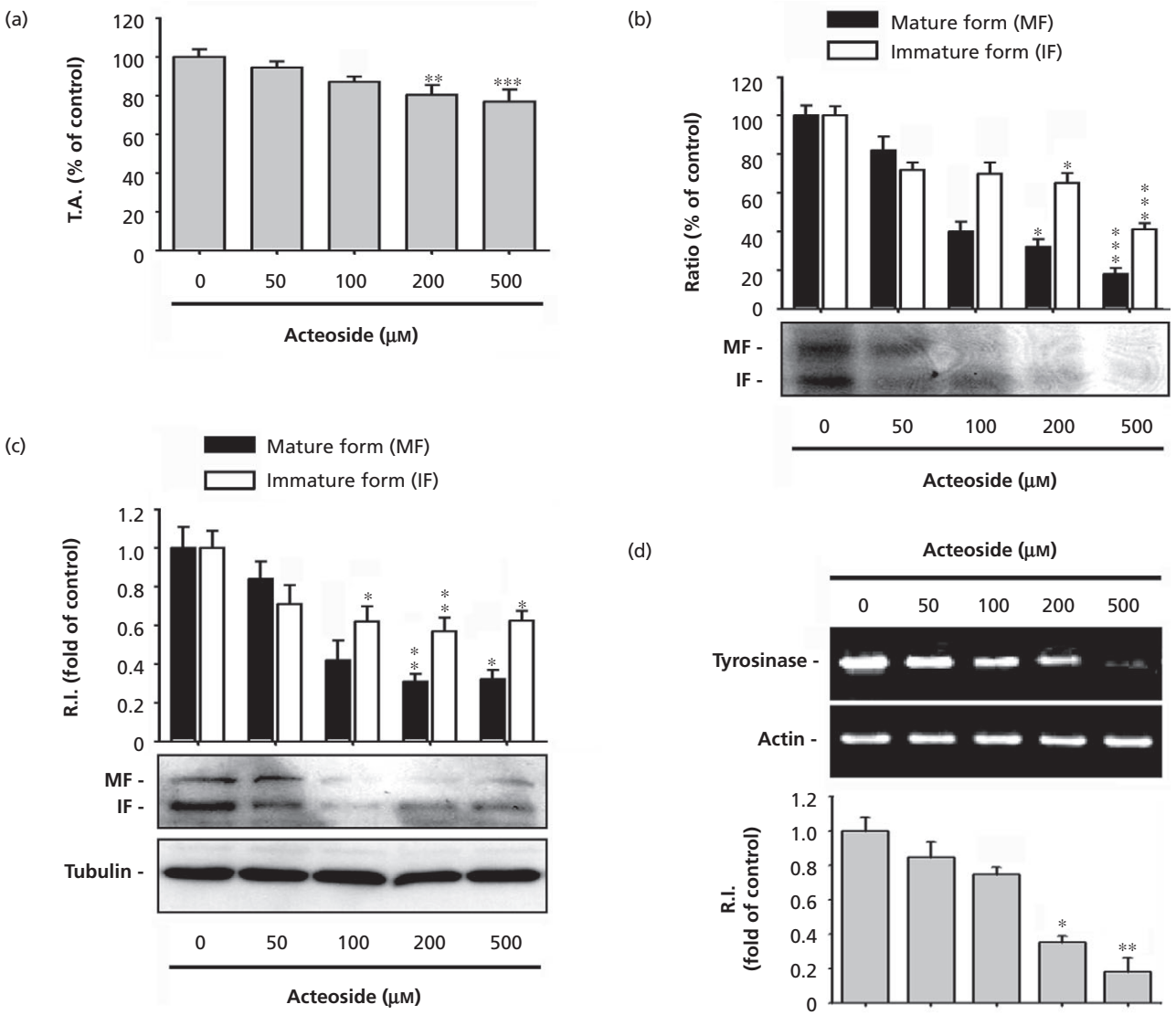

Figure 2 Effect of acteoside on tyrosinase activity and protein expression in B16F10 melanoma cells. Cells were cultured with the indicated concentrations (0–500 μM) of acteoside for two days, and cellular tyrosinase activity was measured using the ELISA plate reader (a) or zymography (b), as described in 'Materials and Methods'. The protein (c) and mRNA levels (d) of tyrosinase were determined by Western blotting and RT-PCR. Tubulin antibody and actin primers were used to control for equal loading of proteins and target cDNA, respectively. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs the untreated control values. T.A., tyrosinase activity; R.I., relative intensity.

between two and three days of treatment, whereas there was no significant difference after five days of treatment.

Regulatory effects of acteoside on TRPs, ERK, and MITF in B16F10 melanoma cells

In addition to tyrosinase, the tyrosinase-related proteins, or TRPs, regulate melanin biosynthesis through induction of enzymes that convert DOPAchrome to DHICA and oxidize DHICA in melanogenic pathways. Dependent on acteoside concentration, the level of TRP1 protein decreased significantly in B16F10 cells, whereas TRP2 expression did not change (Figure 4a).

We also tested the effects of acteoside on MITF expression and ERK phosphorylation in B16F10 melanoma cells. This is because MITF and ERK-mediated signalling regulate tyrosinase expression and the TRPs in contradictory ways.[13] Western-blot analysis revealed that MITF protein levels decreased in proportion to acteoside concentration (Figure 4b). In contrast, p-ERK increased after acteoside treatment, which suggested the involvement of ERK signalling in the MITF down-regulation.

ERK inhibition stimulates melanogenesis in acteoside-treated cells through the induction of MITF, tyrosinase and TRP-1

We evaluated changes in ERK signalling and effects on its downstream melanogenic factors in acteoside-treated B16F10 cells. Pretreatment of cells with the ERK inhibitor PD98059

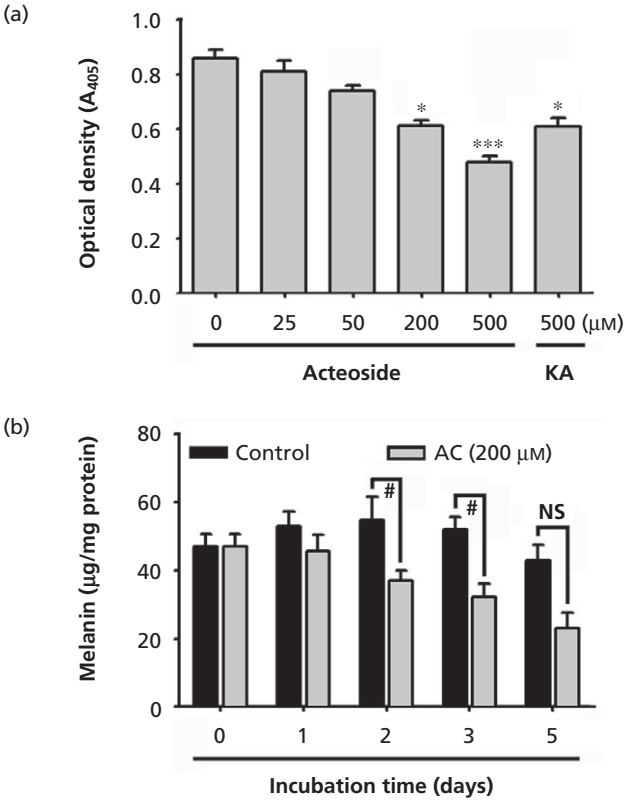

Figure 3 Effect of acteoside on melanin synthesis in B16F10 melanoma cells. (a) Cells were cultured with increasing doses (0–500 μM) of acteoside or 500 μM kojic acid for two days. Cell pellets were then resuspended in NaOH solution and absorbance of the supernatants was measured at 405 nm. (b) Cells were also exposed to 200 μM acteoside for the indicated times (0–5 days). The melanin content was determined by comparison with the standard curves for synthetic melanin. $^{*}P < 0.05$, $^{***}P < 0.001$ vs the untreated control values. $^{\#}P < 0.05$ indicates significant difference between the experiments. KA, kojic acid. NS, not significant.

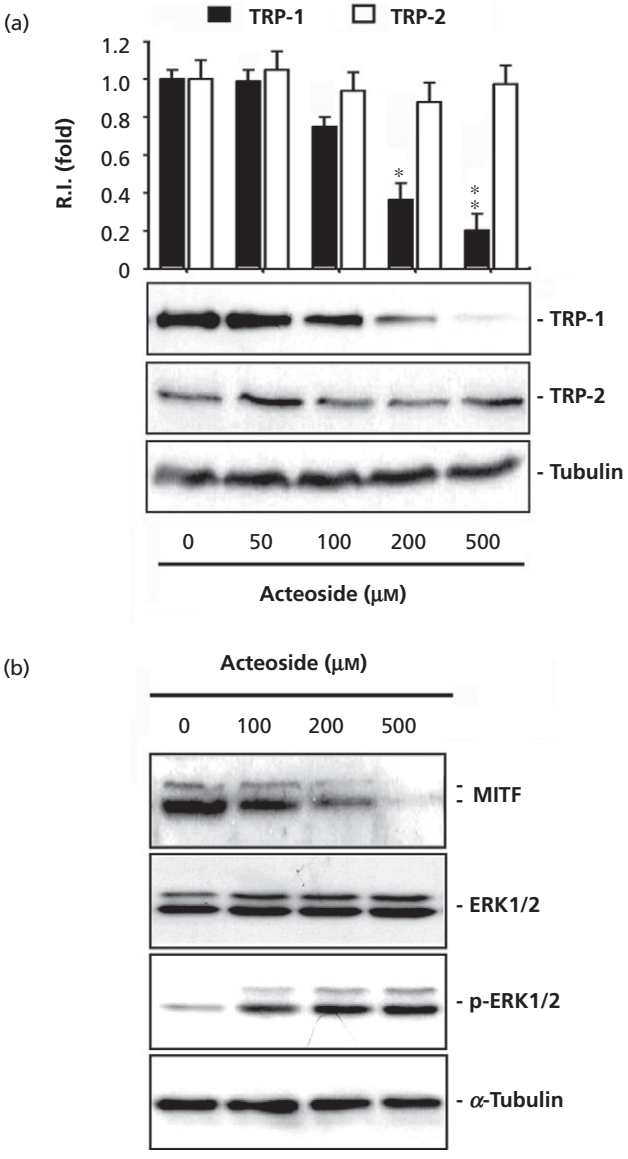

Figure 4 Acteoside decreases levels of TRP-1 and MITF proteins, but increases ERK phosphorylation in B16F10 cells. (a) Cells were treated with acteoside for two days as indicated, and expression of TRP proteins was determined by immunoblotting. $^{*}P < 0.05$, $^{**}P < 0.01$ vs the untreated control values. (b) Cells were exposed to increasing doses (0–500 μM) of acteoside for 12 h, and cell lysates were analysed on Western blots with antibodies against MITF and p-ERK. Equal protein loading was checked by reaction with tubulin and phosphorylation-independent ERK antibodies.

(20 μM) abolished the acteoside-induced ERK phosphorylation (Figure 5a) and attenuated the transient acteoside-induced decrease in MITF protein (Figure 5b). Pretreatment with ERK inhibitor also restored levels of tyrosinase and TRP-1 proteins, which were down-regulated in acteoside-treated cells. We interpreted these results to mean that acteoside-mediated reduction in MITF protein levels is correlated with reduced tyrosinase and TRP-1 levels, and that ERK signalling regulates this pathway. To further explore the role of ERK in melanin regulation, we determined the tyrosinase activity and melanin contents in cells exposed to acteoside in the presence and absence of PD98059. As expected, acteoside significantly reduced cellular tyrosinase activity and melanin levels, but the ERK inhibitor blocked this reduction (Figure 5c and 5d). Especially, treatment with 20 μM PD98059 alone appeared to increase tyrosinase activity and melanin synthesis, compared with the untreated control cells (data not shown). Thus we concluded that the inhibition of ERK signalling augments melanogenesis, whereas acteoside prevents melanin pigmentation by activating ERK, which reduces MITF, tyrosinase and TRP-1 protein levels in melanoma cells.

Acteoside prevents α-MSH-stimulated melanogenesis in B16F10 melanoma cells

Since cAMP is a key intermediary in α-MSH-induced melanin pigmentation,[12] we tested the effect of acteoside on intracellular cAMP and melanogenesis in B16F10 cells stimulated with α-MSH. Photographs of cell pellets in Figure 6a show the visual results of α-MSH-stimulated melanin synthesis and inhibition of this melanogenesis by acteoside. We confirmed these results by enzymatic assay (Figure 6b) and zymographic analysis (Figure 6c) of tyrosinase. In addition,

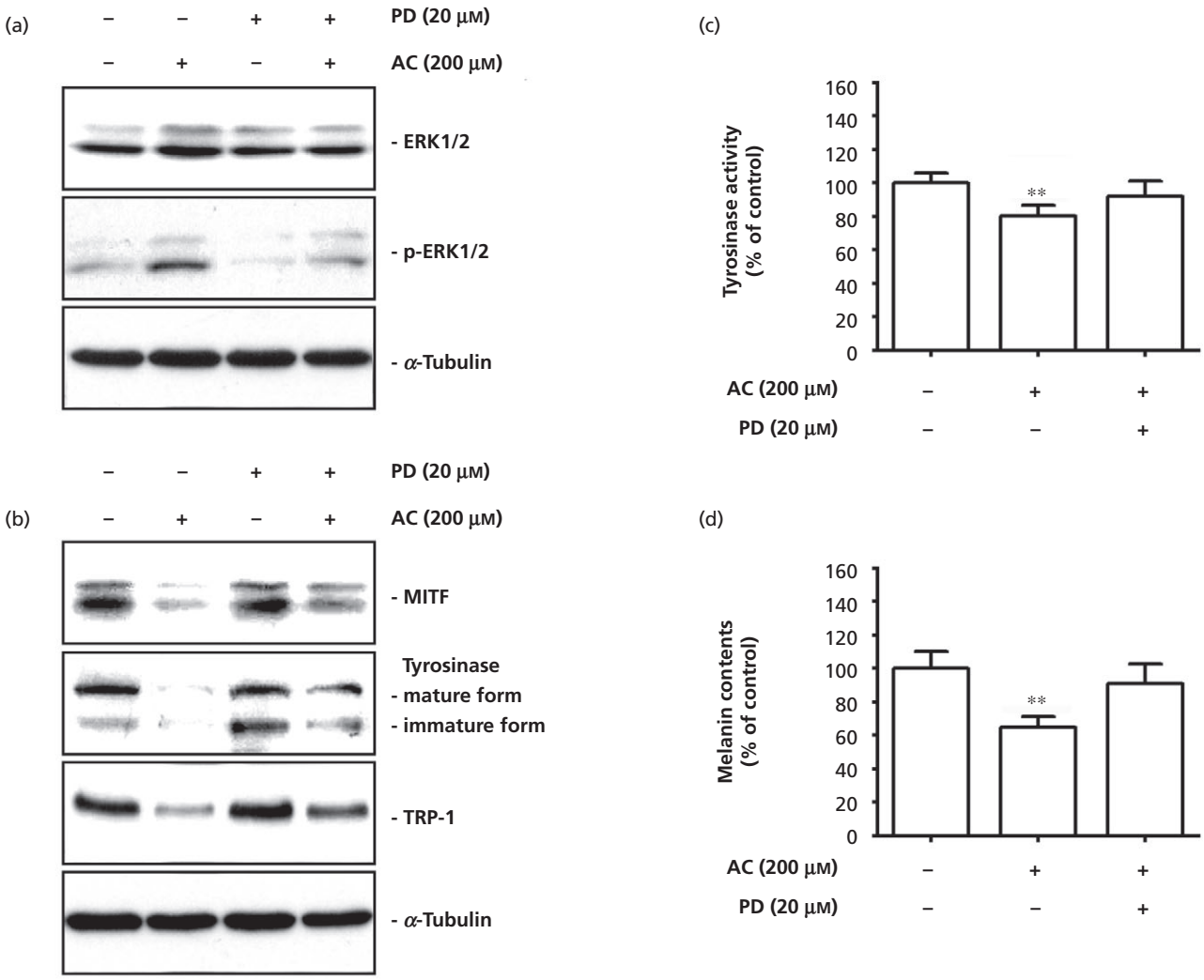

Figure 5 ERK inhibitor abolishes acteoside-mediated reductions in MITF, tyrosinase and TRP-1 proteins with the subsequent increase of tyrosinase activity and melanin content in melanoma cells. (a) Cells were exposed to 200 μM acteoside in the presence and absence of a specific ERK inhibitor, PD98059 (20 μM), for 12 h, and cell lysates were analysed for intracellular ERK and p-ERK. (b) The same cell lysates were also analysed on Western blots using antibodies against MITF, tyrosinase and TRP-1, and tubulin antibody as a loading control. In addition, B16F10 cells were pretreated with 20 μM PD98059, then treated with 200 μM acteoside and further incubated for two days. Tyrosinase activity (c) and melanin content (d) were measured as described in 'Materials and Methods'. $^{**}P < 0.01$ vs the untreated control values. AC, acteoside.

we found that the α-MSH-stimulated melanin synthesis and its inhibition by acteoside occur when more than 100 μM of the compound was added (Figure 6d). We proceeded to determine the levels of cAMP in α-MSH-treated B16F10 cells with and without acteoside. We observed that α-MSH increases intracellular cAMP, but that acteoside at 200 and 500 μM significantly attenuated this increase (Figure 6e). Acteoside at the same concentrations also inhibited α-MSH-stimulated MITF expression in the cells (Figure 6f). These results suggest that acteoside inhibits α-MSH-induced melanogenesis in B16F10 melanoma cells.

Acteoside absorbs UV-A and UV-B

We determined the UV-blocking capacity of acteoside by measuring the absorption spectra in the regions of UV-A (350–370 nm) and UV-B (270–290 nm). Acteoside (250 μM) showed absorption maxima at 283 nm and 335 nm, which correspond to UV-B and UV-A, respectively (Figure 7). Acteoside, at a concentration greater than 1 mM, showed a prominent absorption band extending from about 200 to 400 nm (data not shown). Acteoside thus displays excellent UV-blocking capacity, more specifically for UV-B than for UV-A.

Discussion

The biological activity of acteoside attracts interest from diverse perspectives. Although we found little information on the effects of acteoside on melanin pigmentation, it is reported to prevent hyperpigmentation. We previously found that acteoside inhibits cellular MMP activity, which indicates a potential cosmetic value.[15] This was supported by the report

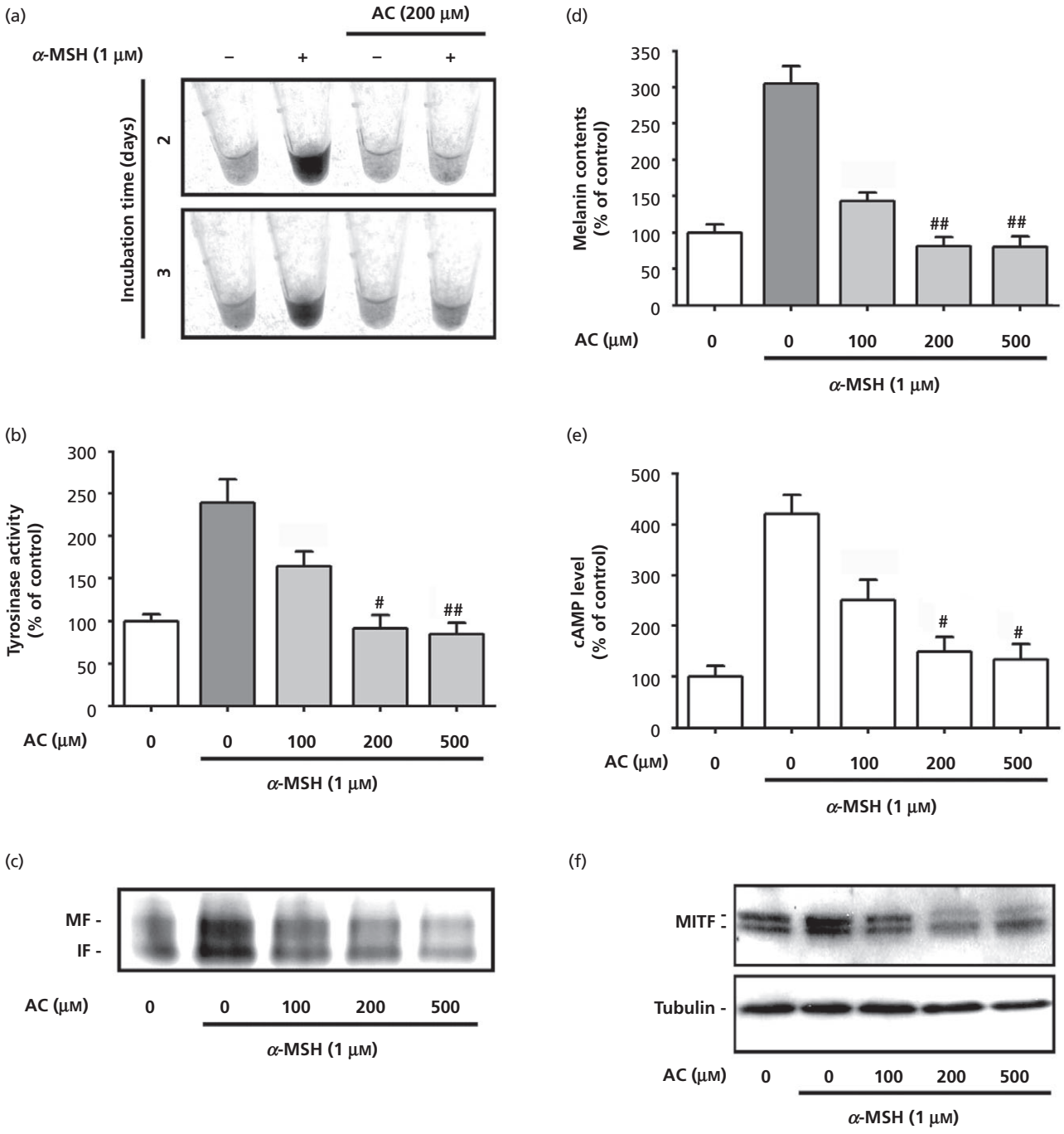

Figure 6 Acteoside significantly attenuates α-MSH-stimulated melanogenesis in B16F10 melanoma cells. Cells were stimulated with 1 μM α-MSH in the presence of the indicated concentrations of acteoside and analysed at the indicated times for melanogenesis. (a) Photographs show cell pellets collected at two and three days after the stimulation. Cell lysates were also prepared after two days of treatment for tyrosinase enzymatic assay (b), tyrosinase zymography (c) and melanin analysis (d). (e) Cellular cAMP level was determined 1 h after treatment with the indicated doses of acteoside and/or α-MSH. (f) MITF protein was measured by Western blot analysis in cells exposed to acteoside (from 0 to 500 μM) in the presence and absence of 1 μM α-MSH for 6 h. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs the α-MSH treatment alone.

that acteoside inhibits α-MSH-stimulated melanogenesis in a murine melanoma cell line.[20] These findings led us to study in greater detail the mechanisms whereby acteoside inhibits melanin biosynthesis. In this study, we demonstrated the inhibitory effects of acteoside in B16F10 melanoma cells and can now suggest plausible mechanisms for these effects. First of all, the potential of acteoside to inhibit melanogenesis in both the presence and absence of α-MSH via the regulation of ERK-mediated or cAMP-mediated signalling pathways is the major significant finding in this study.

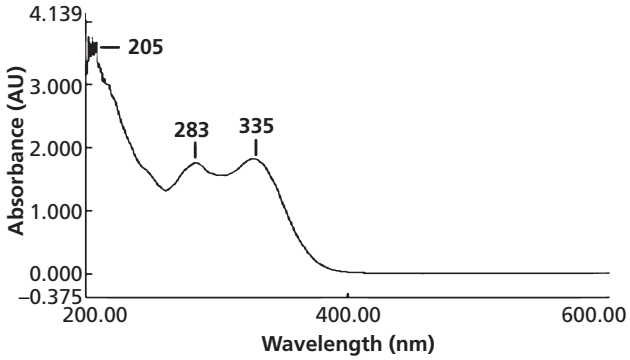

Figure 7 Absorption of UV-A and UV-B by acteoside.

Tyrosinase is the rate-limiting enzyme in melanin biosynthesis, and thus melanin content in cells correlates with the tyrosinase protein level and catalytic activity.[5] We first investigated the ability of acteoside to directly inhibit tyrosinase activity and melanin synthesis in cell-free assay systems. Acteoside showed greater potency as an inhibitor of melanin synthesis than of monophenolase or diphenolase activity, similar in potency to the positive controls arbutin and kojic acid, in the same concentration range. This result was similar to that when melanoma cells were exposed to acteoside, where acteoside alone (200 μM) decreased tyrosinase activity and melanin synthesis to 80.3 and 65%, respectively, compared with the untreated control cells. This is believed to be derived from the capacity of acteoside to diminish TRP-1 and MITF levels in addition to a direct inhibition of tyrosinase activity. The mature form of cellular tyrosinase also showed greater sensitivity to acteoside than the immature form did. Furthermore, acteoside inhibited melanin pigmentation in B16F10 melanoma cells, in proportion to the decrease in tyrosinase activity and protein expression. Consequently, these findings suggested that acteoside inhibits melanogenesis by directly and/or indirectly suppressing tyrosinase activity and several signalling molecules involved in melanin synthesis.

In addition to tyrosinase, TRP-1 and TRP-2 are involved in melanogenesis regulation. Tyrosinase-related protein-2, or DOPA chrome tautomerase, produces DHICA from DOPA chrome, which is derived from DOPA quinone in the absence of thiols; and TRP-1 catalyses the oxidation of DHICA.[6,25] Huh *et al*.[26] found that the sodium channel blocker propafenone inhibits the expression of TRP-1 and TRP-2 proteins in human epidermal melanocytes. In this study, we found that acteoside significantly reduced TRP-1 protein expression, but not TRP-2, in B16F10 cells. Consequently, reduced TRP-1 expression may be related more directly to the decrease in melanin content in acteoside-treated cells.[26,27]

To better understand the molecular mechanisms involved in the effects of acteoside on melanogenesis, we evaluated targets of ERK-mediated signalling in acteoside-treated cells. Current investigations support a regulatory role for ERK signalling in melanogenesis. As an example, UV radiation-induced melanin biosynthesis in human melanocytes is related to ERK activation.[28] Kim *et al*.[13] reported that sphingosine-1-phosphate (S1P) decreases melanin pigmentation through sustained ERK activation. A specific ERK inhibitor abolished this S1P-mediated decrease in melanin, in correlation with increases in tyrosinase activity and expression of tyrosinase, TRP-1 and MITF proteins. In this study, we found that acteoside increased p-ERK, and thus inhibited melanogenesis, in a dose-dependent manner. In contrast, pretreatment of cells with the ERK inhibitor, PD98059, preserved the melanin content in acteoside-treated cells with corresponding increases in tyrosinase activity and protein expression. Acteoside may therefore block melanin synthesis in B16F10 melanoma cells through ERK activation and subsequent down-regulation of tyrosinase, MITF and TRP-1. In contrast, it has been reported that acteoside showed anti-inflammatory activity in vascular endothelium through the suppression of ERK and JNK phosphorylation.[19] Although we do not explain an exact mechanism involved in the opposite function of acteoside on ERK activation, it is suggested that cellular responses to acteoside might differ according to the origins of cells examined and the experimental conditions.

MITF is a transcription factor that directly regulates melanogenesis by stimulating tyrosinase and TRPs.[29–31] The roles of MITF in melanogenesis are also closely linked to ERK signalling pathways.[13] Thus MITF presents a major investigational target in developing new whitening agents. Here we showed that the cellular level of MITF protein declines as the acteoside concentration increases in culture medium. The ERK inhibitor abolished the acteoside-induced decrease in MITF protein, which was consistent with changes in levels of tyrosinase and TRP-1 proteins. Our results thus suggest that the inhibition of melanin synthesis by acteoside correlates with the increased of ERK phosphorylation and suppression of MITF, followed by decreases in tyrosinase and TRP-1 expression. These results are also quite similar to the observation that baicalein inhibited melanin synthesis by inducing MITF down-regulation and subsequent tyrosinase inhibition through ERK activation.[32] Baicalein is an active compound in some herbal medicines and has been shown to exert various beneficial effects.[33] The bioactivity of this compound is due to its antioxidant property. It is important to note that acteoside contains a strong free radical scavenging potential with a redox cycling activity. This may suggest that the anti-melanogenesis effect of such compounds are associated with their antioxidant properties as well as with the activation of ERK. More detailed experiments using various types of melanocytes will be needed to further elucidate the signalling pathways involved in acteoside-mediated anti-melanogenesis.

We also studied the effect of acteoside on α-MSH-stimulated pigmentation in B16F10 melanoma cells. α-MSH stimulates tyrosinase and melanin biosynthesis by activation of adenylyl cyclase, which increases intracellular cAMP.[12,34] Acteoside significantly inhibited α-MSH-stimulated tyrosinase activity in B16 melanoma cells and this coincides with a reduction in intracellular cAMP.[20] This study supports previous findings in demonstrating that α-MSH mediated an increase in melanin content and acteoside inhibited this increase in B16F10 cells. We observed that this inhibition by acteoside occurred through abolition of the increase in tyrosinase activity and cellular cAMP that α-MSH had induced. We found that acteoside induced a concentration-dependent decrease in MITF protein in α-MSH-stimulated cells. Previous findings show that ERK signalling is activated during

cAMP-induced melanogenesis in B16 melanoma cells.[35] Our present findings confirm that acteoside down-regulates α-MSH-stimulated melanogenesis through inhibition of adenylyl cyclase activation and regulation of the ERK-MITF cascade. However, in the absence of α-MSH, acteoside inhibits melanin synthesis via the down-regulation of MITF and tyrosinase by mainly inducing ERK activation.

Conclusions

In this study, we discovered that acteoside inhibits melanogenesis by decreasing tyrosinase protein expression and activity, and that this involves ERK activation, which reduces expression of MITF and tyrosinase downstream in the ERK-signalling cascade. We also demonstrated the potential of acteoside to inhibit α-MSH-stimulated melanin synthesis through regulation of intracellular cAMP and MITF levels. We observed the UV spectra of acteoside, which indicate that it may serve as a sunscreen against UV-induced DNA damage and mutagenesis in the skin. We want to emphasize the potential value of acteoside as a cosmetic agent for skin whitening and protection, although this potential awaits further investigation.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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