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Research Paper

Acetoside inhibits α-MSH-induced melanin production in B16 melanoma cells by inactivation of adenyl cyclase

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Abstract

Objectives The aim of the study was to determine the mechanism of the whitening effect of acteoside.

Methods We used tyrosinase activity and melanin production stimulated in B16 melanoma cells by α-melanocyte stimulating hormone (α-MSH) or forskolin to measure the whitening effect of acteoside.

Key findings Acteoside did not directly inhibit mushroom tyrosinase activity, but dose-dependently inhibited tyrosinase activity and melanin production in B16 melanoma cells stimulated by 1 μmol/l α-MSH. Acteoside also reduced cyclic AMP levels in cells stimulated by 1 μmol/l α-MSH, suggesting direct inhibition of adenyl cyclase. Acteoside also inhibited production of both melanin and cyclic AMP in cells stimulated by 1 μmol/l forskolin, an adenyl cyclase activator. Acteoside showed antioxidant activity in a cell-free DPPH (1-diphenyl-2-picrylhydroazyl) assay and inhibited generation of intracellular reactive oxygen species.

Conclusions These results suggest that the whitening activity of acteoside results from inhibition of adenyl cyclase and α-MSH signalling.

Keywords acteoside; anti-oxidant; cAMP; melanin; tyrosinase

Introduction

Melanin pigmentation in mammals occurs in melanocytes. Melanin synthesis is regulated by a cascade of enzymatic reactions controlled at the level of tyrosinase, which converts tyrosine into dopaquinone and controls the rate-limiting step of melanin production. Melanocytes can be stimulated by various effectors, including 1-oleyl-2-acetyl-glycerol,[1] ultraviolet B radiation[2] and α-melanocyte stimulating hormone (α-MSH).[3] Both 1-oleyl-2-acetyl-glycerol and ultraviolet B radiation induce melanogenesis via protein kinase C activation,[4,5] whereas α-MSH stimulates adenyl cyclase activation. The cyclic AMP (cAMP) pathway plays a key role in the regulation of melanogenesis.[3,6]

Abnormal hyperpigmentations, such as melanoma, freckles and senile lentigines, can be treated with depigmenting agents. Arbutin, an active ingredient from Uvae ursi folium, (*Arctostaphylos uva-ursi* (L.) Spreng., folium) is widely used as a whitening agent.[7] Acteoside and other phenylpropanoid glycosides are contained in plants used in traditional herbal medicine. Some of these phenylpropanoid glycosides appear to have various biological activities, such as antibacterial, analgesic, anti-inflammatory and antioxidant activities.[8–10] However, there are few data on the depigmenting mechanism of acteoside. We therefore measured the inhibitory effect of acteoside on tyrosinase activity and melanin production in B16 melanoma cells.

Materials and Methods

Materials

Acteoside, a phenylpropanoid glycoside, was isolated from *Clerodendron trichotomum Thunberg* in our laboratory. The ELISA kit for the determination of cAMP was from Amersham Pharmacia (Piscataway, NJ, USA). Mushroom tyrosinase, 1-diphenyl-2-picrylhydroazyl (DPPH), MTT, ascorbic acid and arbutin were purchased from Sigma Chemical Co. (St Louis, MO, USA).

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Cell culture

B16 melanoma cells obtained from the Korean Cell Line Bank (Seoul, Korea) were grown in Dulbecco's modified Eagle minimum essential medium supplemented with 10% fetal bovine serum (Gibco) in 5% CO_2. The cells were used for measurements of melanin, cAMP and production of reactive oxygen species (ROS).

Cell viability

Cell viability was determined using the MTT-based colorimetric assay.[11] Cells in 96-well plates (8×10^5 cells/well) were exposed to acteoside at 37°C for 72 h. Then 20 μl of MTT solution (5 mg/ml in phosphate-buffered saline) was added and incubated for 3 h. After aspirating the supernatant from the wells, 100 μl DMSO was added to dissolve the formazan crystals. The absorbance of each well was then read at 520 nm.

DPPH scavenging activity

To determine antioxidant activity of acteoside in a cell-free system, a DPPH radical scavenging assay was used, with slight modifications.[12] Briefly, DPPH was dissolved in ethanol to make a 100 μmol/l solution. Then 20 μl of the test compound in ethanol (or ethanol as control) was added to 180 μl DPPH solution and incubated in the dark for 20 min. The decrease in DPPH absorption was measured at 517 nm.

Measurement of reactive oxygen species generation

To determine antioxidant activity of acteoside in a cellular system, the level of intracellular ROS was quantified by 2′,7′-dichlorofluorescein diacetate (DCF-DA) fluorescence. The B16 melanoma cells were suspended in 20 ml Krebs buffer and incubated with 20 μmol/l DCF-DA for 1 h at 37°C. The cells were washed twice with Krebs buffer and then suspended in Krebs buffer at a density of 10^6 cells/ml. The cells were incubated with acteoside for 10 min, and then ROS generation was induced by 1 mg/ml silica for 30 min at 37°C. Fluorescence intensity was measured using excitation and emission wavelengths of 485 and 535 nm, respectively, using a spectrophotometer.[13]

Tyrosinase assay

The activity of tyrosinase was determined spectrophotometrically by measuring the production of 3,4-dihydroxyphenylalanine (DOPA)chrome from L-tyrosine or L-DOPA at 475 nm. The reaction mixture (450 μl) containing 10 mmol/l sodium phosphate buffer (pH 6.8), 10 mmol/l L-DOPA (or 1.7 mM L-tyrosine) and test compound was incubated by adding 50 μl tyrosinase (50 U/ml for L-DOPA; 250 U/ml for L-tyrosine) for 30 min at 37°C.

Melanin assay

B16 melanoma cells treated with test compound and α-MSH or forskolin (both 1 μmol/l) in culture dishes (60 mm) were incubated for 72 h at 37°C in a CO_2 chamber. The cells were suspended in 400 μl 10 mmol/l sodium phosphate buffer (pH 6.8) containing 1% Triton X-100. After shaking to lyse the cells, the lysates were clarified by centrifugation at 10 000 rpm for 10 min. Tyrosinase activity of the supernatant was then determined.

For the measurement of melanin, the pellet was dissolved in 100 μl 1 M NaOH and 200 μl distilled water. The samples were incubated at 90°C for 1 h. Absorbance at 405 nm was compared with a standard curve of fungal melanin.

cyclic AMP assay

B16 melanoma cells pretreated with acteoside were stimulated with α-MSH or forskolin (both 1 μmol/l) for 60 min at 37°C. The total amount of cellular cAMP was determined using an enzyme immunoassay protocol provided by Amersham Pharmacia Biotech and expressed as fmole/mg protein. Values were corrected for protein content, determined using the bincinchoninic acid method.[14]

Statistical analysis

Results are represented as means ± SD and were analysed statistically with analysis of variance. Differences between groups were determined using Newman–Keul's test. The level of significance was set at less than 5%.

Results

Antioxidant activity of acteoside

The antioxidant activity of acteoside was determined using the DPPH radical scavenging assay in a cell-free system and measurement of intracellular ROS in B16 melanoma cells. In the DPPH radical scavenging assay, acteoside dose dependently removed DPPH free radicals (Figure 1). Ascorbic acid, a well-known antioxidant, removed DPPH free radicals by 78% at 100 μmol/l; acteoside reduced levels by 67% at the same concentration. In B16 melanoma cells, silica (1 mg/ml) increased intracellular ROS generation by 260% compared with control, which was dose-dependently inhibited by acteoside (Figure 2).

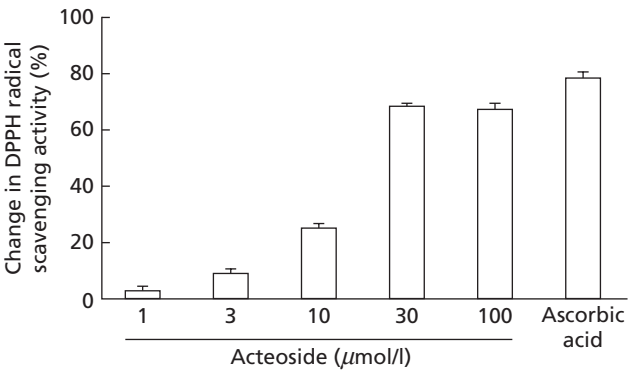

Figure 1 Anti-oxidant activity of acteoside in the 1-diphenyl-2-picrylhydroazyl (DPPH) radical scavenging assay. Results are means ± SD from four separate experiments and are obtained from the equation: ([absorbance of control – absorbance of acteoside])/[absorbance of control] × 100.

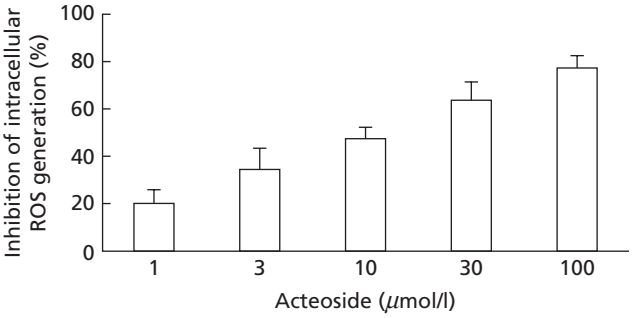

Figure 2 Effect of acteoside on silica-induced intracellular generation of reactive oxygen species in B16 melanoma cells, quantified by DCF-DA fluorescence. Silica increased intracellular reactive oxygen species (ROS) generation by 260% over control, which was dose-dependently inhibited by acteoside. Results are means ± SD from six separate experiments and are obtained from the equation: ([fluorescence of silica – fluorescence of acteoside]/[fluorescence of silica]) × 100.

Effect of acteoside on tyrosinase activity and melanin production

ROS scavengers are closely related to anti-ageing and whitening activity. To investigate the effect of acteoside on whitening activity, B16 melanoma cells were exposed to acteoside for 72 h. Acteoside did not show any cytotoxic effects at a concentration of 100 μmol/l, as determined using the MTT assay (data not shown).

Tyrosinase is the rate-limiting step in melanin biosynthesis. Tyrosinase activity is measured using two substrates: L-tyrosine and L-DOPA. Arbutin is a competitive inhibitor against L-tyrosine and a non-competitive inhibitor against L-DOPA. Arbutin significantly inhibited mushroom tyrosinase using L-tyrosine and L-DOPA as substrates by 85% and 37%, respectively, whereas acteoside did not affect mushroom tyrosinase activity (Figure 3). However, in α-MSH-stimulated B16 melanoma cells, acteoside dose-dependently inhibited both tyrosinase activity and melanin production (Figures 4 and 5). Acteoside at 100 μmol/l inhibited α-MSH-stimulated tyrosinase activity and melanin production by 94.2% and 84.0%, respectively, and forskolin-stimulated tyrosinase activity and melanin production by 71.4% and 69.8%, respectively (Figures 4 and 5).

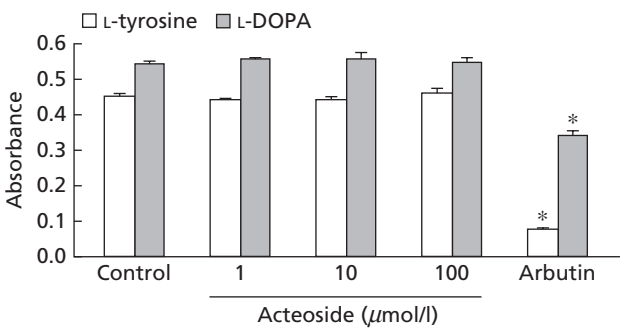

Figure 3 Effect of acteoside on purified mushroom tyrosinase. Results are means ± SD from four separate experiments. *$P < 0.05$ vs control.

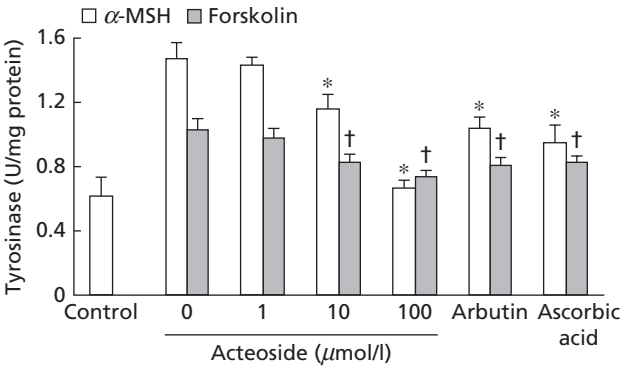

Figure 4 Effect of acteoside on tyrosinase in B16 melanoma cells stimulated by α-melanocyte-stimulating hormone or forskolin (both 1 μmol/l). Results are means ± SD from six separate experiments. *$P < 0.05$ vs α-melanocyte stimulating hormone (α-MSH); †$P < 0.05$ vs forskolin.

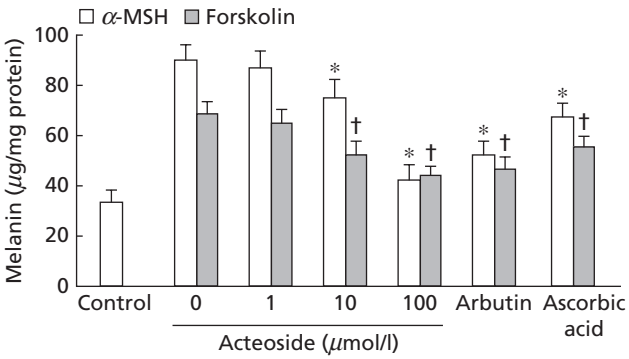

Figure 5 Effect of acteoside on melanin synthesis in B16 melanoma cells stimulated by α-melanocyte-stimulating hormone or forskolin (both 1 μmol/l). Results are means ± SD from six separate experiments. *$P < 0.05$ vs α-melanocyte stimulating hormone (α-MSH); †$P < 0.05$ vs forskolin.

Effect of acteoside on cyclic AMP production

We next measured intracellular cAMP levels in B16 melanoma cells stimulated by α-MSH and forskolin, an adenyl cyclase activator (both 1 μmol/l). Acteoside significantly inhibited cAMP production in B16 melanoma cells stimulated by α-MSH or forskolin, whereas arbutin and ascorbic acid did not affect cAMP formation (Figure 6).

Discussion

The antioxidant activity of acteoside was determined using a DPPH radical scavenging assay in a cell-free system and intracellular ROS generation in a cellular system. Ascorbic acid removed DPPH free radicals by 78% at 100 μmol/l and acteoside reduced levels by 67% at the same concentration. Silica dose-dependently induces intracellular ROS generation,[15] and here increased intracellular ROS generation in B16 melanoma cells by 260% over controls, which was dose-dependently inhibited by acteoside (Figure 2). Acteoside can also decrease lipid peroxidation induced by free-radical-induced oxidative

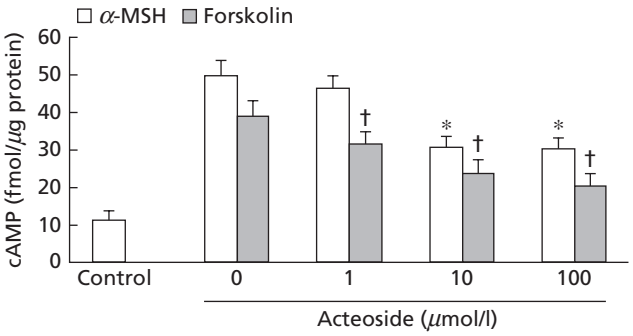

Figure 6 Effect of acteoside on cyclic AMP levels in B16 melanoma cells stimulated by α-melanocyte-stimulating hormone or forskolin (both 1 μmol/l). Results are means ± SD from four separate experiments. $^{*}P < 0.05$ vs α-melanocyte stimulating hormone (α-MSH); $^{\dagger}P < 0.05$ vs forskolin.

stress in endothelial cells.[16] Phenolic hydroxyl groups have potent radical-scavenging effects,[17] and acteoside contains these groups.

Antioxidant activity is closely related to anti-ageing and whitening activity. In the skin, tyrosinase helps eliminate ROS[18] and can use superoxide to produce melanin.[18,19] Tyrosinase is an important enzyme in the process of melanin biosynthesis, converting tyrosine to DOPA quinone. As this process is the rate-limiting step in melanin biosynthesis, tyrosinase inhibitors may be whitening agents. Tyrosinase activity is measured using two substrates, L-tyrosine and L-DOPA. Arbutin (a competitive inhibitor against L-tyrosine and a non-competitive inhibitor against L-DOPA[7]) significantly inhibited mushroom tyrosinase, measured using L-tyrosine and L-DOPA, by 85% and 37%, respectively. Acteoside did not affect mushroom tyrosinase activity using L-tyrosine and L-DOPA as substrates, suggesting that antioxidant activity alone does not directly act on tyrosinase.

Acteoside dose-dependently inhibited both tyrosinase activity and melanin production after α-MSH and forskolin stimulation, but was not cytotoxic. Direct inhibition of mushroom tyrosinase activity did not correlate with the inhibition of cellular tyrosinase or melanin production in cultured melanocytes.[7] Ascorbic acid also inhibited both tyrosinase activity and melanin production in α-MSH-stimulated B16 melanoma cells. Ascorbic acid also inhibits melanin synthesis *in vivo*, and is useful as a whitening agent in cosmetics. Matsuda and colleagues have suggested that the influence of ascorbic acid on the monopherase activity of tyrosinase results from its ability to reduce enzymatically generated *O*-quinones.[20]

In B16 melanoma cells, α-MSH activates tyrosinase and melanogenesis via adenyl cyclase activation.[3] Acteoside significantly inhibited intracellular cAMP levels stimulated by 1 μmol/l α-MSH or forskolin (an adenyl cyclase activator).[21] We previously reported that acteoside inhibited α-MSH-induced tyrosinase expression in B16 melanoma cells,[22] potentially via blocking cAMP formation by adenyl cyclase. Therefore, the whitening activity of acteoside may be due to the inhibition of adenyl cyclase downstream of α-MSH.

Conclusions

To determine the whitening effect of acteoside, we measured tyrosinase activity and melanin production in B16 melanoma cells stimulated by α-MSH or forskolin. Although acteoside did not directly inhibit mushroom tyrosinase activity, it dose-dependently inhibited tyrosinase activity and melanin production stimulated by α-MSH or forskolin, an adenyl cyclase activator. Acteoside also dose-dependently inhibited cAMP levels. Therefore, acteoside may show whitening activity by inhibiting adenyl cyclase downstream of α-MSH stimulation.

Declarations

Conflict of interest

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

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