Effects of Sesquiterpene Lactones on Melanogenesis in Mouse B16 Melanoma Cells

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In this study, we examined the effect of sesquiterpene lactones isolated from Calea urticifolia and Tanacetum parthenium (feverfew) on melanogenesis in mouse B16 melanoma cells. In response to 3-isobutyl-1-methylxanthin (IBMX), B16 melanoma cells underwent differentiation characterized by increased melanin biosynthesis. Treatment of sesquiterpene lactones at lower concentration (1 μM) significantly blocked IBMX-induced melanogenesis, but did not induce the inhibitory activity of cell growth. Among them, 2,3-epoxyjuanislamin exhibited a potent inhibitory effect on melanogenesis. Treatment of B16 cells with 2,3-epoxyjuanislamin elicited significant decreases in tyrosinase protein and mRNA levels. These results demonstrated that the inhibitory effects of sesquiterpene lactones on melanin biosynthesis may be due to the suppression of tyrosinase expression.

Key words melanin; melanogenesis; tyrosinase; sesquiterpene lactone

Melanin production is principally responsible for skin color and plays an important role in prevention of sun-induced skin injury.1–3 Melanin is produced by melanocytes in the basal layer of epidermis.1–3) Synthesis of melanin starts from the conversion of the amino acid L-tyrosine to dopaquinone by tyrosinase, the enzyme catalyzing the rate-limiting step for the melanin biosynthesis.4) This tyrosinase process is involved in abnormal accumulation of melanin pigments (hyperpigmentation). Therefore, many reports have described pharmacologic and cosmetic agents that inhibit tyrosinase activity or that block melanogenic pathways, leading to skin lightening.5,6)

Calea urticifolia belongs to a family of Compositae has been used as a traditional medicinal plant for a variety of diseases such as gastritis, infection and cancer in El Salvador. Calea urticifolia is also known to contain abundant sesquiterpene lactones with a germacrane skeleton. Recently, various interesting biological activities such as apoptosis induction,7) anti-oxidant effect8) and suppressive activity against adipocyte differentiation9) were found in sesquiterpene lactones from Calea urticifolia. On the other hand, Tanacetum parthenium (feverfew) is also a member of Compositae family and is distributed among Europe, North America and Australia. Parthenolide is a sesquiterpene lactone that has been isolated from feverfew, and it has several biological activities including the induction of apoptosis and inhibition of nuclear factor (NF)-κB.10) Although a wide range of pharmacological effects exhibited by sesquiterpene lactones have been reported, the melanogenesis induced by sesquiterpene lactones has not been studied. In the present study, we examined the inhibitory effect of sesquiterpene lactones isolated from Calea urticifolia on melanogenesis in mouse B16 melanoma cells.

MATERIALS AND METHODS

Materials Five sesquiterpene lactones (calealactone B, arucanolide, 2,3-epoxyjuanislamin, 2,3-epoxycalealactone A, and calealactone A) extracted from leaves of Calea urticifolia (Compositae) and parthenolide, a sesquiterpene lactone extracted from Tanacetum parthenium (feverfew) were dissolved in dimethyl sulfoxide (DMSO) and added to the cell cultures. 3-Isobutyl-1-methylxanthin (IBMX) and the antibody to β-actin were from Sigma. Protease inhibitor mixture (Complete™) was from Roche. The antibody to tyrosinase was from Santa Cruz Biotechnology. Anti-rabbit and -mouse antibodies conjugated with horseradish peroxidase and the chemiluminescence kit (ECL Plus Western blotting detection reagents) were obtained from GE healthcare. Other reagents were of the highest quality available.

Cell Culture Mouse B16 melanoma cells (4A5) were obtained from Riken Cell Bank (Tsukuba, Japan). The cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, at 37 °C in a humidified, CO₂-controlled (5%) incubator.

Determination of Melanin Content The B16 cells were washed with phosphate buffer saline (PBS) and dissolved in 2 N NaOH for 1 h at 60 °C. The absorbance at 470 nm was measured and melanin content was measured using the authentic standard of synthetic melanin.

Measurement of Cell Viability Cell viability was determined by trypan blue dye exclusion assay.

Measurement of Tyrosinase Activity The B16 cells were lysed by incubating at 4 °C for 30 min in lysis buffer (10 mM Tris–HCl, pH 7.5, 1% NP-40, 0.1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 1 mM EDTA) containing protease inhibitors (Complete™ protease inhibitor mixture). The lysates were centrifuged at 15000×g for 30 min to obtain the supernatant as source of tyrosinase. The reaction mixture contained 50 mM phosphate buffer, pH 6.8, 0.05% L-dopa and the supernatant. After incubation at 37 °C for 20 min, dopachrome formation was monitored by measuring absorbance at wavelength 470 nm.

Western Blot Analysis Cells were lysed by incubating at 4 °C for 30 min in lysis buffer containing protease inhibitors and centrifuged at 15000×g for 30 min. The resultant supernatant (solvulized proteins) was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)
on 7.5% polyacrylamide gel. Proteins were transferred electrophoretically onto a PVDF membrane. Blocking was performed in Tris–buffered saline containing 5% skim milk powder and 0.05% Tween-20. Blots were incubated with the appropriate primary antibodies, and then further incubated with horseradish peroxidase-conjugated secondary antibody. Proteins were visualized using an ECL detection system.

RNA Isolation and Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Total RNA was extracted from B16 melanoma cells using TRIZOL-Reagent according to manufacturer’s protocol. RNA samples (2 µg/reaction) were reverse-transcribed with Superscript III in the presence of oligo-dT, and RT reaction was used for amplification with Taq polymerase. The resulting cDNA was amplified using specific primers. The primers used were as follows; for tyrosinase 5’-GGCCAGCTTTCAGGACAGGT-3’ and 5’-TGGTGCTTTCATGGGCAAAATC-3’. Specific primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Clontech) were added as a control for the same reverse transcriptase product. Amplification conditions were 94 °C (30 s), 60 °C (30 s), 72 °C (40 s) for 21 cycles (tyrosinase) or 18 cycles (GAPDH). The PCR product were electrophoresed on 1.3% agarose gel containing ethidium bromide.

RESULTS AND DISCUSSION

Figure 1 shows the major sesquiterpene lactones isolated from leaves of *Calea urticifolia* and feverfew. The isolation procedures of these compounds were described in the previous papers.11) Five sesquiterpene lactones (calealactone B, arucanolide, 2,3-epoxyjuanislamin, 2,3-epoxycalealactone A, and calealactone A) extracted from leaves of *Calea urticifolia* and parthenolide, which has been reported to be a typical and simple germacranolide extracted from feverfew. Melanogenesis in B16 melanoma cells was initiated by the addition of IBMX and was assessed by determination of intracellular melanin content. As shown in Fig. 2A, all compounds tested showed inhibitory effects on melanogenesis in B16 melanoma cells which were treated with IBMX. Since it has been previously reported that arucanolide, one of sesquiterpene lactones isolated from *C. urticifolia*, induces apoptosis in cancer cell lines,7) we examined the number of viable cells grown in the presence of various sesquiterpene lactones. Cell viability was not significantly changed at 1 µM sesquiterpene lactones (Fig. 2B).

Among six sesquiterpene lactones, we studied further the most potent melanogenesis inhibitor, 2,3-epoxyjuanislamin in subsequent experiments. As shown in Fig. 3A, the levels of melanin content were measured in the presence of 2,3-epoxyjuanislamin. The cell viability was determined by trypan blue exclusion test. Melanin content and cell number were expressed as the percentage of the values obtained in the control cells. Data represent the mean±S.D. of two different experiments each carried out in duplicate.
of melanin content were reduced in a concentration-dependent manner with the maximal level at 1 μM. Viable cell number was not significantly changed at 1 μM 2,3-epoxyjuanislamin but slightly decreased at 2 μM (Fig. 3B).

To prove the mechanism by which 2,3-epoxyjuanislamin reduces melanin contents in B16 melanoma cells, we examined the effect of 2,3-epoxyjuanislamin on activity and expression of the melanogenic enzymes. Tyrosinase, the enzyme catalyzing the rate-limiting step for melanin biosynthesis is known as a well characterized marker of differentiation in melanocytes and melanoma cells. Although the activity of tyrosinase was greatly enhanced by the treatment with IBMX, its level was not increased but rather reduced in the presence of 2,3-epoxyjuanislamin (Fig. 4A). Furthermore, the inhibition of tyrosinase activity by 2,3-epoxyjuanislamin was well correlated with the decrease in expression levels of tyrosinase (Fig. 4B).

To examine whether the decreased protein level of tyrosinase was responsible for the decrease in the mRNA expression in 2,3-epoxyjuanislamin-treated B16 cells, we examined the levels of tyrosinase mRNA by RT-PCR. As shown in Fig. 4C, 2,3-epoxyjuanislamin exhibited a significant decrease in the level of tyrosinase mRNA. These results suggested that the down-regulation of tyrosinase expression by 2,3-epoxyjuanislamin occurred at the transcriptional level.

In summary, the present study has demonstrated that sesquiterpene lactones, including parthenolide exhibited antimelanotic activity in B16 melanoma cells. Especially, 2,3-epoxyjuanislamin exerted a potent inhibitory effect on melanogenesis by modulating the transcriptional machinery of tyrosinase mRNA. Although we have obtained useful fundamental information regarding to melanogenic signaling in B16 melanoma cells in the present study, it cannot be directly extrapolated to human pigmentation. Sesquiterpene lactones are considered to be useful resources for cosmetic agents, but further studies are required to define the detailed mechanisms at the molecular level underlying the effect of sesquiterpene lactones on melanogenesis in normal human epidermal melanocytes and are under current progress in our laboratory.

REFERENCES