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Mechanism of the melanogenesis stimulation activity of (−)-cubebin in murine B16 melanoma cells

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Abstract—(−)-Cubebin showed a melanogenesis stimulation activity in a concentration-dependent manner in murine B16 melanoma cells without any significant effects on cell proliferation. Tyrosinase activity was increased at 24–72 h after addition of cubebin to B16 cells, and then intracellular melanin amount was increased at 48–96 h after the treatment. The expression levels of tyrosinase were time-dependently enhanced after the treatment with cubebin. At the same time, the expression levels of tyrosinase mRNA were also increased after addition of cubebin. Furthermore Western blot analysis revealed that cubebin elevated the level of phosphorylation of p38 mitogen-activated protein kinase (MAPK). SB203580, a selective inhibitor of p38 MAPK, completely blocked cubebin-induced expression of tyrosinase mRNA in B16 cells. These results suggested that cubebin increased melanogenesis in B16 cells through the enhancement of tyrosinase expression mediated by activation of p38 MAPK.

1. Introduction

Melanogenesis is a multistage process involving melanin synthesis, melanin transport, and melanosome release. Melanin synthesis is stimulated by various effectors, including α-melanocyte-stimulating hormone (α-MSH), theophylline, cyclic AMP (cAMP)-elevating agents (forskolin, isobutylmethylxanthine,[1] and glycyrrhizin[2]), and ultraviolet light. During the course of our screening program for the development of gray hair prevention agents from the natural resources, (−)-cubebin (cubebin) has been isolated from leaves of *Piper nigrum* L. (Piperaceae) as an active lignan which showed a potent melanogenesis stimulation activity in cultured murine B16 melanoma cells (B16 cells) without any significant effects on cell proliferation.[3–5] A lignan, cubebin is a new entry as a melanogenesis stimulating substance. However, the mechanism of melanogenesis stimulation by cubebin is unknown hitherto.

In this paper, we examined the effects of cubebin in B16 cells on intracellular melanin amount, tyrosinase activity, and the signaling mechanism involved in the expression of tyrosinase.

2. Results and discussion

2.1. Effect of cubebin on melanin amount and cell proliferation in B16 cells

The cells were treated with various concentrations of cubebin for 72 h. Melanogenesis was assessed by determination of intracellular melanin amount/protein amount which is shown in percentage value. Cell proliferation was also expressed as the percentage value. Each percentage value in the treated cells was calculated with respect to that in the control cells. The effect of cubebin on melanin amount and cell proliferation in B16 cells is shown in Figure 1. Cubebin showed a melanogenesis stimulation activity in a concentration-dependent manner (Fig. 1a) without any significant effects on cell proliferation at a concentration of 2–10 μM, although 20 μM cubebin slightly decreased the cell proliferation (Fig. 1b). As reported in the previous paper,[3] the activity

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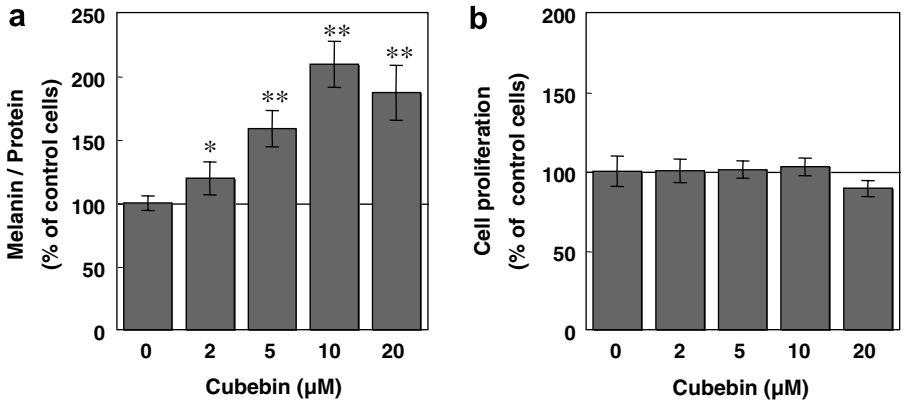

Figure 1. Effect of cubebin on melanin amount and cell proliferation in B16 cells. The cells were treated with various concentrations of cubebin for 72 h. (a) The melanin amount was determined. (b) The cell viability was determined by the trypan blue exclusion test. Melanin amount and cell proliferation were expressed as percentage values. Each percentage value in the treated cells was calculated with respect to that in the control cells. Data represent means ± SD of two different experiments each carried out in duplicate. *Significantly different from the control cells ($^{*}p < 0.05$, $^{**}p < 0.01$).

of cubebin was about 1.2-fold of that of theophylline, a reference compound in the melanogenesis stimulation assay in B16 cells. It has been reported that theophylline increased both gamma-glutamyl transpeptidase- and tyrosinase-reactive cells, as a result, theophylline enhanced pigmentation in B16 cells.[6]

2.2. Effect of cubebin on tyrosinase activity in B16 cells

To elucidate the mechanism of melanogenesis stimulation by cubebin, B16 cells were treated with cubebin at a concentration of 10 μM, because this concentration was found to be the most effective on the basis of the above results (Fig. 1). Melanogenesis is regulated by the activity of tyrosinase, a rate limiting enzyme in melanin biosynthesis.[7] Since melanin is derived from the precursor dopaquinone that is formed by tyrosinase oxidation of L-tyrosine, tyrosinase plays an important role in melanin synthesis. Thus we examined the effect of cubebin on tyrosinase activity. Each percentage value of tyrosinase activity in the treated cells was calculated with respect to that in the pre-treated cells. After the treatment of B16 cells with cubebin, melanin amount was significantly increased at 48 h and reached the maximal levels at 72 and 96 h (Fig. 2a). As shown in Figure 2b, tyrosinase activity was enhanced during 24–96 h after the addition of 10 μM cubebin.

2.3. Effect of cubebin on tyrosinase and tyrosinase mRNA expressions in B16 cells

In order to clarify the further mechanism of tyrosinase activation by cubebin, the levels of tyrosinase expression in B16 cells were examined by Western blot analysis. As shown in Figure 3a, the tyrosinase expression was enhanced in 6.2-fold of increase compared with that of the pre-treated cells at 48 h after the treatment of 10 μM cubebin.

Therefore, we next examined the effect of cubebin on the level of tyrosinase mRNA expression in B16 cells by using RT-PCR. As shown in Figure 3b, the level of tyrosinase mRNA expression increased 1.8-fold compared with the pre-treated cells at 12 h after the addition of 10 μM cubebin. Based on the above-mentioned results, it is suggested that the increment of tyrosinase mRNA expression at 12 h caused the tyrosinase expression at 24–48 h (Fig. 3a) followed by tyrosinase activation at 48–72 h (Fig. 2b). Although there are some discrepancies between tyrosinase protein expression, mRNA expression, and tyrosinase activity, these differences may be attributable to poor association between protein and mRNA expression for tyrosinase as often observed in other enzymes. Additionally, activity of tyrosinase depends not only on amount of protein but also on activation process in Golgi apparatus and Golgi-associated endoplasmic reticulum.

2.4. Effect of cubebin on phosphorylation of p38 MAPK in B16 cells

Microphthalmia-associated transcription factor (MITF) is involved in the pigmentation, proliferation, and survival of melanocytes.[8,9] The activation of MITF, a transcription factor that regulates tyrosinase gene expression, is known to be a critical event during melanogenesis.[10] During the process of melanogenesis in melanocytes, it has been known that p38 mitogen-activated protein kinase (MAPK) cascade activates MITF, whereas S6 kinase cascade and extracellular signal-regulated kinase (ERK) cascade[11] depress MITF. Recently, the p38 MAPK cascade was demonstrated to be involved in the melanogenesis in B16 melanoma cells induced by α-MSH.[12] More recently negative regulation of melanogenesis by phospholipase D1 through mammalian target of rapamycin (mTOR)/p70 S6 kinase 1 (S6K1) signaling in B16 cells has been reported.[13]

The activation of p38 MAPK, ERK 1/2, and p70 S6K1 in B16 cells by treatment with 10 μM cubebin was examined by Western blot analysis with using the mouse anti-tyrosinase antibody for phosphorylated forms of p38 MAPK, ERK 1/2, and p70 S6K1. As shown in Figure 4, a time-course analysis revealed that the phosphoryla-

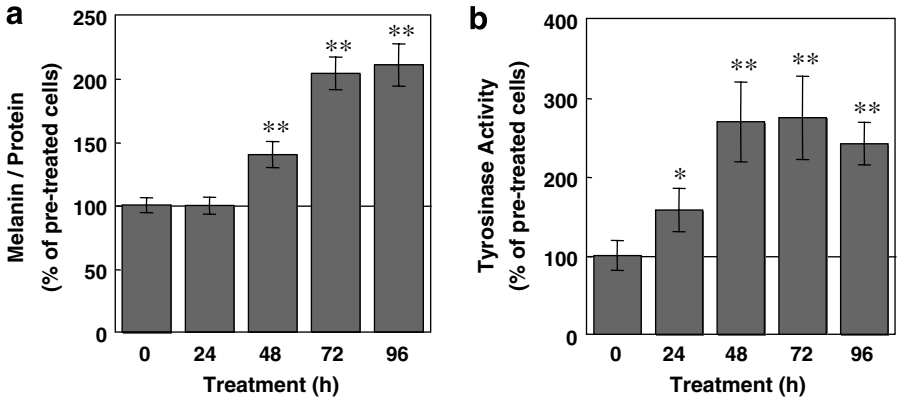

Figure 2. Effect of cubebin on tyrosinase activity in B16 cells. The cells were treated with 10 μM cubebin for the indicated times. (a) Melanin amount was determined. (b) Tyrosinase activity was determined by measuring the formation of dopachrome. Melanin amount and tyrosinase activity were expressed as percentage values. Each percentage value of tyrosinase activity in the treated cells was calculated with respect to that in the pre-treated cells. Data represent means ± SD of two different experiments each carried out in duplicate. *Significantly different from that in the pre-treated cells ($^{*}p < 0.05$, $^{**}p < 0.01$).

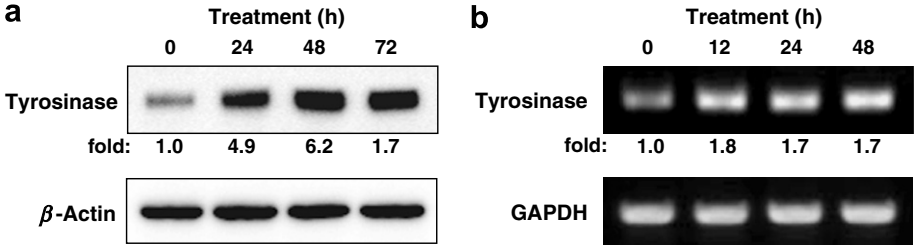

Figure 3. Effects of cubebin on tyrosinase and tyrosinase mRNA expressions in B16 cells. The cells were treated with 10 μM cubebin for the indicated times. (a) The levels of tyrosinase expression were determined by Western blot analysis using the mouse anti-tyrosinase antibody. (b) The levels of tyrosinase mRNA were determined by RT-PCR analysis.

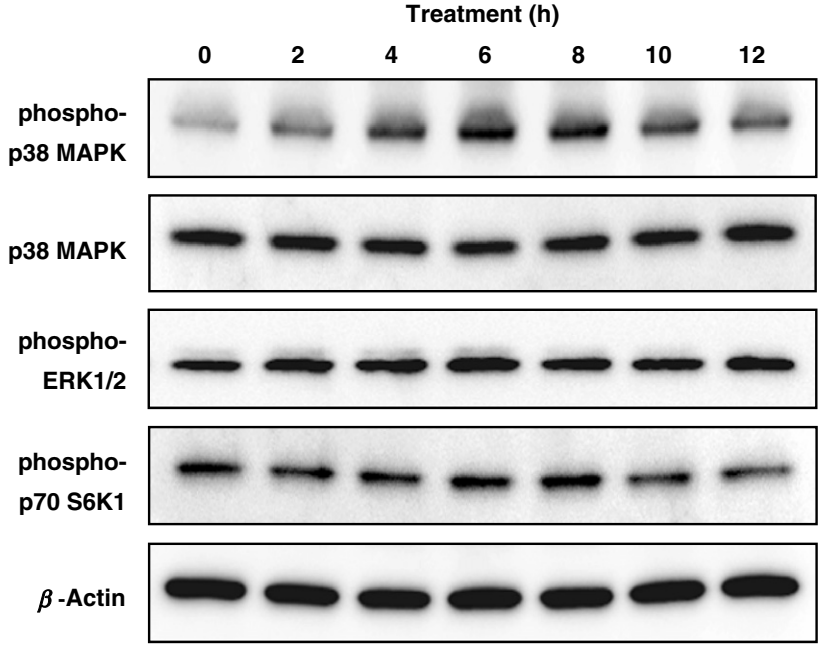

Figure 4. Effect of cubebin on phosphorylation of p38 MAPK in B16 cells. The cells were treated with 10 μM cubebin for the indicated times. Phosphorylation of p38 MAPK, ERK 1/2, and p70 S6K1 was assessed by Western blot analysis with using the mouse anti-tyrosinase antibody for phosphorylated forms of p38 MAPK, ERK 1/2, and p70 S6K1.

tion of p38 MAPK was significantly enhanced at 4–8 h after the treatment with cubebin, whereas no effect was observed in the levels of phosphorylation of ERK 1/2 and p70 S6K1. By using SB203580, a selective inhibitor

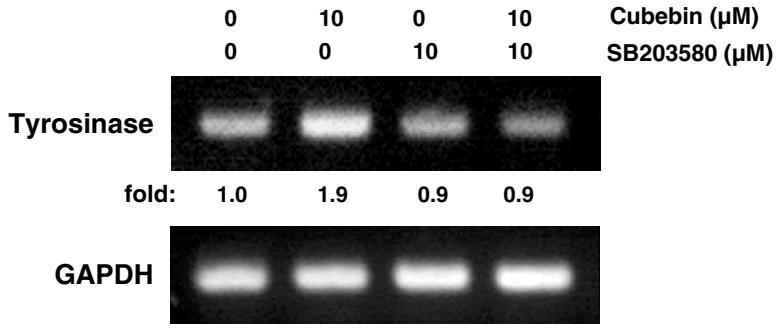

Figure 5. Effect of cubebin with SB203580, a selective inhibitor of p38 MAPK, on tyrosinase mRNA expression in B16 cells. The cells were treated with 10 μM cubebin for 12 h with or without 10 μM SB203580. The level of tyrosinase mRNA was determined by RT-PCR analysis.

of p38 MAPK, we examined whether p38 MAPK plays an important role in the cubebin-induced activation of tyrosinase. As shown in Figure 5, the level of tyrosinase mRNA expression by 10 μM cubebin was shown to increase 1.9-fold compared with that of the pre-treated cells. On the other hand, 10 μM SB203580 completely blocked cubebin-induced expression of tyrosinase mRNA. These results indicated that cubebin may enhance the phosphorylation of p38 MAPK leading to the melanogenesis in B16 cells, but may not enhance the phosphorylations of both ERK 1/2 and p70 S6K1 which exert a negative regulatory role in the melanogenesis.

3. Conclusion

Cubebin showed a melanogenesis stimulation activity in a concentration-dependent manner in B16 cells. After the treatment with 10 μM cubebin, tyrosinase activities were increased at 24–96 h, and then intracellular melanin amount was increased at 48–96 h. The expression levels of tyrosinase and of tyrosinase mRNA were enhanced by the treatment with cubebin. Cubebin elevated the level of phosphorylation of p38 MAPK, whereas SB203580 completely blocked cubebin-induced expression of tyrosinase mRNA. Thus, it was suggested that one of the mechanisms for cubebin-induced melanogenesis in B16 cells is attributable to the increase of tyrosinase gene expression through positive regulator, MITF, initiated by cubebin-induced activation of p38 MAPK as shown in Figure 6.

4. Experimental

4.1. Materials

Natural (−)-cubebin was isolated from *Piper nigrum* leaf in our previous report.[3] 3,4-Dihydroxyphenylalanine (L-dopa) and synthetic melanin were purchased from Sigma (St. Louis, MO). SB203580 was from Promega. Protease inhibitor mixture (Complete™) was from Roche. The antibody to tyrosinase was from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against phosphor-specific p38 MAPK, phosphor-specific ERK 1/2, phosphor-specific p70 S6K1, and p38 were from Cell Signaling Technology (Beverly, MA). Anti-rabbit and

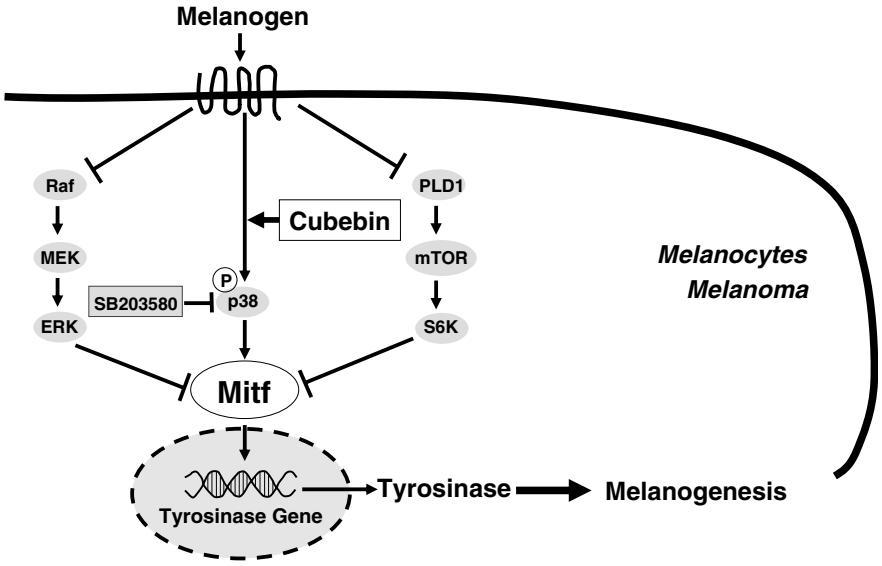

Figure 6. A proposed scheme showing the activation mechanism of cubebin on melanogenesis.

mouse antibodies conjugated with horseradish peroxidase and the chemiluminescence (ECL) kit were obtained from Amersharm Pharmacia (Piscataway, NJ). Other reagents were of the highest quality available.

4.2. Cell culture

Murine B16 melanoma cells were purchased from Riken Cell Bank (Tsukuba, Japan). The cell were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 100 U/ml of penicillin, and 100 μg/ml of streptomycin, at 37 °C in a humidified, CO_2-controlled (5%) incubator.

4.3. Melanogenic assays

Cells (4×10^4cells) were seeded on 24-well plates, and treatment with cubebin began 24 h after seeding. Cubebin dissolved in DMSO was added to the cell cultures with a final DMSO concentration of 0.1% v/v.

For determination of melanin amount according to the previous method,[13] the B16 cells were washed with phosphate-buffered saline (PBS) and dissolved in 2 N NaOH for 1 h at 60 °C. The absorbance at 470 nm of each sample was measured, and melanin amount was determined by using the authentic standard of synthetic melanin, then protein concentration of each sample was assayed using the DC protein assay reagent (Bio-Rad, Hercules, CA) with BSA as a standard. Intracellular melanin amount/protein amount was shown in percentage values. Each percentage value in the treated cells was calculated with respect to that in the pre-treated cells.

The cell proliferation was determined by the trypan blue exclusion test. Cell proliferation was shown in percentage values. Each percentage value in the treated cells was calculated with respect to that in the control cells.

4.4. Tyrosinase activity

For measurement of tyrosinase activity according to the previous method,[13] the cells were washed with ice-cold PBS and then lysed by incubating at 4 °C for 30 min in RIPA buffer (10 mM Tris–HCl, pH 7.5, 1% NP-40, 0.1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, and 1 mM EDTA) containing protease inhibitors (Complete™ protease inhibitor mixture). The lysates were centrifuged at 15,000*g* for 30 min to obtain a supernatant as source of tyrosinase. The reaction mixture in which contained 50 mM phosphate buffer, pH 6.8, 0.05% L-dopa and the supernatant (tyrosinase) was incubated at 37 °C for 20 min. After incubation, dopachrome formation was assayed by measuring absorbance at 475 nm. Tyrosinase activity was shown in percentage values. Each percentage value in the treated cells was calculated with respect to that in the pre-treated cells.

4.5. Electrophoresis and Western blot analysis

According to the previous method,[13] B16 cells were lysed in RIPA buffer containing protease inhibitors and phosphatase inhibitor cocktail and centrifuged at 15,000*g* for 30 min. The resultant supernatant (solubilized 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 ECL detection system.

4.6. RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from B16 cells using TRIZOL-reagent according to manufacturer's protocol. RNA samples (2 μg/reaction) were reverse-transcribed with Superscript 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′-GGCCAGCTTTCAGGCAGAGGT and 5′-TGGTGCTTCATGGGCAAAATC. Specific primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Clontech, CA) 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 products were electrophoresed on 1.3% agarose gel containing ethidium bromide.

4.7. Statistical analysis

In statistical analysis, we performed one-way ANOVA using a StatView software (SAS Institute Inc., Cary, NC).

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