SPANDIDOS PUBLICATIONS

Curcumin suppresses α-melanocyte stimulating hormone-stimulated melanogenesis in B16F10 cells

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Received February 3, 2010; Accepted March 30, 2010

DOI: 10.3892/ijmm_00000440

Abstract. The present study was designed to assess the potential inhibitory activity of curcumin on the α-melanocyte stimulating hormone (α-MSH)-stimulated melanogenesis signal pathway in B16F10 melanoma cells. The molecular mechanism of curcumin-induced inhibitory activity on the α-MSH-stimulated melanogenesis signal pathway, including expression of melanogenesis-related proteins and activation of melanogenesis-regulating proteins, was examined in B16F10 cells. Curcumin suppressed the cellular melanin contents and the tyrosinase activity in α-MSH-stimulated B16F10 cells. In addition, the expression of melanogenesis-related proteins such as microphthalmia-associated transcription factor (MITF), tyrosinase, and tyrosinase-related protein 1 and 2 was suppressed by curcumin in the α-MSH-stimulated B16F10 cells. Notably, a melanogenesis-regulating signal such as mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) or phosphatidylinositol 3-kinase (PI3K)/Akt was activated by curcumin in the B16F10 cells treated with or without α-MSH. The suppressive activity of curcumin on α-MSH-induced melanogenesis was down-regulated by PD98059 and by LY294002. Our results suggest that the suppressive activity of curcumin on α-MSH-stimulated melanogenesis may involve the down-regulation of MITF and its downstream signal pathway through the activation of MEK/ERK or PI3K/Akt.

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Key words: curcumin, melanogenesis, tyrosinase, microphthalmia-associated transcription factor, extracellular signal-regulated kinase, Akt

Introduction

Melanogenesis occurs in melanocytes through an enzymatic process which is catalyzed by tyrosinase, a key enzyme of melanogenesis that converts tyrosine to melanin pigments (1,2). The melanotropic hormone, α-melanocyte-stimulating hormone (α-MSH), binds to the melanocortin 1 receptor that leads to the activation of adenylate cyclase (AC), increased intracellular cyclic adenosine monophosphate (cAMP) levels and the activation of protein kinase A (PKA). PKA is reported to phosphorylate cAMP response element-binding protein (CREB), known to be an activator of microphthalmia-associated transcription factor (MITF) gene expression (3). MITF plays an important role in melanogenesis as the major transcriptional activator of tyrosinase expression (1-3).

Recently, phosphatidylinositol 3-kinase (PI3K)/Akt has been suggested to be involved in the negative regulation of melanogenesis (4,5). LY294002, a specific inhibitor of PI3K, was reported to stimulate tyrosinase expression, which leads to an increase in melanin production (6). In addition, the inhibition of extracellular signal-regulated kinase (ERK) signaling was reported to induce hyperpigmentation by increasing tyrosinase activity, thus suggesting that the activation of ERK signaling down-regulates melanogenesis by inhibiting tyrosinase activity and expression (7-9). These studies demonstrated that improved methods of melanogenesis inhibition do not suppress the activity of tyrosinase as much as they control the tyrosinase upstream signaling pathway associated with its activation and expression.

The natural compound curcumin (diferuloylmethane), a polyphenol, is the active ingredient in turmeric (*Curcuma longa* Linn.), a herbal remedy and dietary spice (10,11). Recent scientific research has confirmed that curcumin possesses anti-oxidant, anti-inflammatory, anti-bacterial, anti-amyloid and anti-tumor properties (12,13). In addition, curcumin has been reported to regulate intracellular signaling mechanisms involving protein kinases, including mitogen-activated protein kinases (MAPK), ERK, c-Jun N-terminal kinase (JNK), PKA, protein kinase C (PKC) and PI3K/Akt, as well as transcription factors such as nuclear factor κB (NF-κB), signal transducer

and activator of transcription (STAT) and CREB (13). Notably, melanogenesis-regulating proteins, which include PKA, PKC, PI3K/Akt, ERK and CREB, are also modulated by curcumin. We previously reported that bioassay-guided fractionation from *Curcuma longa* reduced melanin synthesis (14). Therefore, we hypothesized that curcumin may also affect the melanogenesis signaling pathway.

In the present study, we examined the molecular mechanism of curcumin-induced inhibitory activity on the α-MSH-stimulated melanogenesis signal pathway, including the expression of melanogenesis-related proteins and the activation of melanogenesis-regulating proteins. To our knowledge, this study is the first to show the suppressive activity of curcumin itself on the melanogenesis signaling pathways, including the reduced expression of MITF and tyrosinase, and the activation of mitogen-activated protein kinase kinase (MEK)/ERK and PI3K/Akt.

Materials and methods

Chemicals and antibodies. α-MSH, arbutin, L-DOPA, methylthiazolyldiphenyl-tetrazolium bromide (MTT), PD98059 and LY294002 were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). The antibodies recognizing phospho-MEK (#9121) and total MEK (#9122) were obtained from Cell Signaling Technology (Danvers, MA, USA). Phospho-Akt (SC-7985-R), Akt (C-20), and phospho-ERK1/2 (E-4), ERK1/2 (K-23), tyrosinase (H-109), tyrosinase-related protein 1 (TRP-1; H-90) and 2 (TRP-2; H-150), MITF (H-50), and actin (H-300) antibodies were supplied by Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA).

Cell culture. B16F10 mouse melanoma cells (CRL 6323) were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM), 10% fetal bovine serum and 1% penicillin/streptomycin (10,000 U and 100 μg/ml, respectively) in a humidified atmosphere containing 5% CO_2 in air at 37˚C.

Cell viability assay. The general viability of the cultured cells was determined through the reduction of 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide to formazan. After treating the curcumin, the cells were incubated for 48 h at 37˚C in an atmosphere containing 5% CO_2. MTT (1 mg/ml in PBS) was then added to each well at 1/10 volume of media. The cells were incubated at 37˚C for 3 h, and dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals. The absorbance was measured at 570 nm using a spectrophotometer.

Measurement of cellular melanin contents. The melanin content was measured using a slight modification of a method reported previously (15). In brief, the cells were treated with the test substances in DMEM for 5 days. The cell pellets were then dissolved in 500 μl solution of 1 N NaOH in 10% DMSO at 80˚C for 1 h. The relative melanin content was determined by measuring the absorbance at 475 nm in an ELISA reader. A standard synthetic melanin curve (0 to 500 μg/ml) was prepared in triplicate for each experiment.

Tyrosinase activity assay. The tyrosinase activity was estimated by measuring the level of L-DOPA (16). Cells that had been grown in 6-well dishes were treated as indicated for 3 days in DMEM. The cells were then washed in ice-cold phosphate-buffered saline and lysed in 500 μl of phosphate buffer (0.1 M), pH 6.8, containing 1% (w/v) Triton X-100. The cellular extract was clarified by centrifugation at 13,000 x g for 25 min. The tyrosinase substrate, L-DOPA (2 mg/ml), was prepared in the same lysing phosphate buffer. Two hundred microliters of each extract was placed in a 96-well plate, and the enzymatic assay was begun by adding 2 μl of an L-DOPA solution at 37˚C. The control wells contained 200 μl of the lysis buffer. The absorbance at 405 nm was read every 10 min for at least 1 h at 37˚C using an ELISA reader.

Western blot analysis. The B16F10 melanoma cells were cultured in 60-mm dishes with or without different effectors for the times indicated in the figure legends. The cells were then lysed in buffer A containing 50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM 4-(2-aminoethyl) benzene-sulfonyl fluoride hydrochloride, 100 units/ml aprotinin, 10 mM NaF and 1 mM Na_3VO_4. The samples (30 μg) were resolved by 10% SDS-PAGE, transferred to polyvinylidene difluoride membranes, and then exposed to the appropriate antibodies. The proteins were visualized using an ECL system purchased from Amersham Biosciences Inc. (Piscataway, NJ, USA) using horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies.

Data analysis. Data are expressed as the mean ± SEM. The means and standard errors were calculated and the Student's t-test was implemented using SigmaPlot (San Jose, CA, USA). A P-value <0.05 was considered statistically significant.

Results

Curcumin suppresses the melanin synthesis of B16F10 melanoma cells through the inhibition of tyrosinase activity. To determine the effect of curcumin on cell viability, B16F10 melanoma cells were treated with 0-100 μM curcumin, incubated for 48 h and assayed with MTT. The results showed that curcumin had no significant cytotoxic effect on the cells at concentrations ranging from 1-10 μM (Fig. 1). To further determine the effect of curcumin on melanin synthesis, the cells were then exposed to α-MSH (200 nM) in the presence of curcumin (10 μM) or arbutin (50 μg/ml), a typical tyrosinase inhibitor. The cellular melanin contents were then measured. Fig. 2a shows that the melanin contents of the cells treated with curcumin were reduced to the amounts in the control. Fig. 2b also shows that the tyrosinase activity of the curcumin-treated cells was significantly reduced to the level of the untreated control cells. This result suggests that the suppressive activity of curcumin on melanogenesis is due to the down-regulation of tyrosinase activity.

Curcumin suppresses the expression of tyrosinase protein by down-regulating MITF expression. In order to determine if the suppressive activity of curcumin is linked to the expression levels of melanogenesis-related proteins such as tyrosinase,

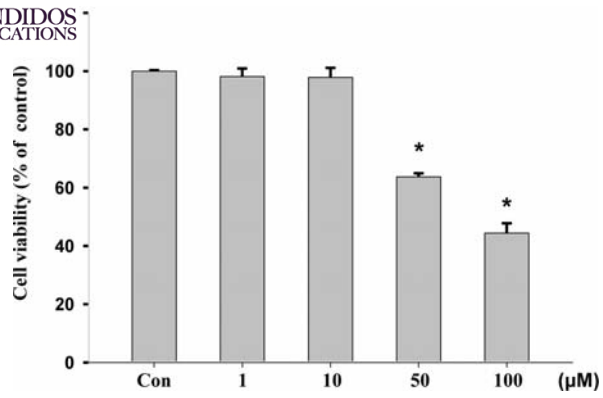


Figure 1. Effect of curcumin on cell viability in B16F10 cells. The cells were treated with various concentrations of curcumin for 48 h. Cell viability was determined by MTT assay. Each percentage value in the treated cells was calculated with respect to that in the control cells. Data are expressed as the mean ± SEM of three independent experiments carried out in triplicate. *P<0.05 compared with the control.

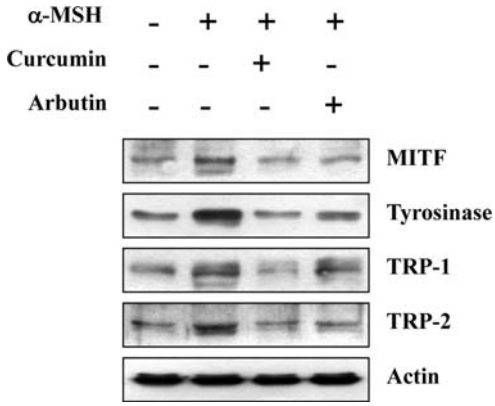


Figure 3. Effect of curcumin on the expression of melanogenesis-related proteins in B16F10 cells. The cells were exposed to α-MSH (200 nM) in the presence of curcumin (10 μM) or arbutin (50 μg/ml) for 48 h. The expression levels of the tyrosinase, TRP-1, TRP-2 and MITF proteins were examined by Western blot analysis as described in Materials and methods. Equal protein loading was confirmed by actin expression.

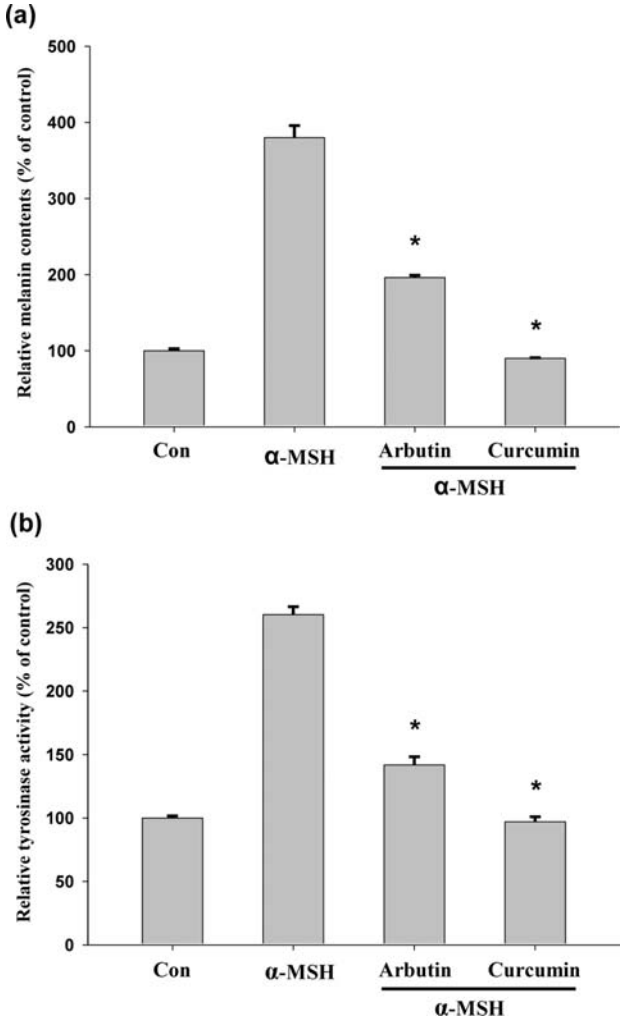


Figure 2. Effects of curcumin on cellular melanin synthesis and tyrosinase activity in B16F10 cells. The cells were exposed to α-MSH (200 nM) in the presence of curcumin (10 μM) or arbutin (50 μg/ml). The cellular melanin contents (a) and the cellular tyrosinase activity (b) were determined and reported as percentages. Each percentage in the treated cells is reported relative to that in the control cells. Arbutin was used as a positive control for tyrosinase inhibition. Data are expressed as the mean ± SEM of three independent experiments carried out in triplicate. *P<0.05 compared with α-MSH treated cells.

tyrosinase-related proteins (TRPs) and MITF, the cells were exposed to α-MSH in the presence of curcumin or arbutin for 48 h, and the protein extracts were then subjected to SDS-PAGE and Western blot analysis. Fig. 3 shows that α-MSH-induced expression of MITF, tyrosinase, TRP-1 and TRP-2 proteins was suppressed by curcumin treatment. This result indicates that the suppressive activity of curcumin on melanogenesis is linked to the down-regulation of MITF-tyrosinase expression signaling pathways.

The suppressive mechanism of curcumin on melanogenesis is involved in the activation of the PI3K/Akt or ERK signaling pathways. The inhibition of PI3K/Akt by LY294002 was previously reported to enhance melanogenesis by increasing intracellular cAMP levels (6), while the inhibition of the ERK pathway was found to induce hyperpigmentation (8). Thus, we examined whether curcumin affects the activation of PI3K/Akt using antibodies against phospho-ERK1/2, phospho-Akt and phospho-MEK (an upstream activator of ERK). B16F10 melanoma cells were exposed to α-MSH in the presence of curcumin for the indicated amounts of time, and the protein extracts were then subjected to SDS-PAGE and Western blot analysis. As shown in Fig. 4a, the phosphorylated forms of MEK, ERK1/2 and Akt were reduced at early time points, 15 min to 6 h after exposure. However, the phosphorylated proteins were increased at later time points, from 6 to 48 h.

To further confirm the result shown in Fig. 4a, we examined the effect of curcumin on the expression of phosphorylated proteins in serum-starved B16F10 melanoma cells. Fig. 4b shows that curcumin induced the phosphorylation of MEK, ERK1/2 and Akt at 6 and 9 h after treatment, which suggests that the suppressive activity of curcumin on melanogenesis is related to the up-regulation of the phosphorylation of MEK, ERK and Akt at late time points.

The suppressive mechanism of curcumin on melanogenesis is regulated by the specific inhibitors of PI3K/Akt or ERK. Since curcumin activates the phosphorylation of MEK, ERK and Akt, we hypothesized that PD98059, a selective inhibitor

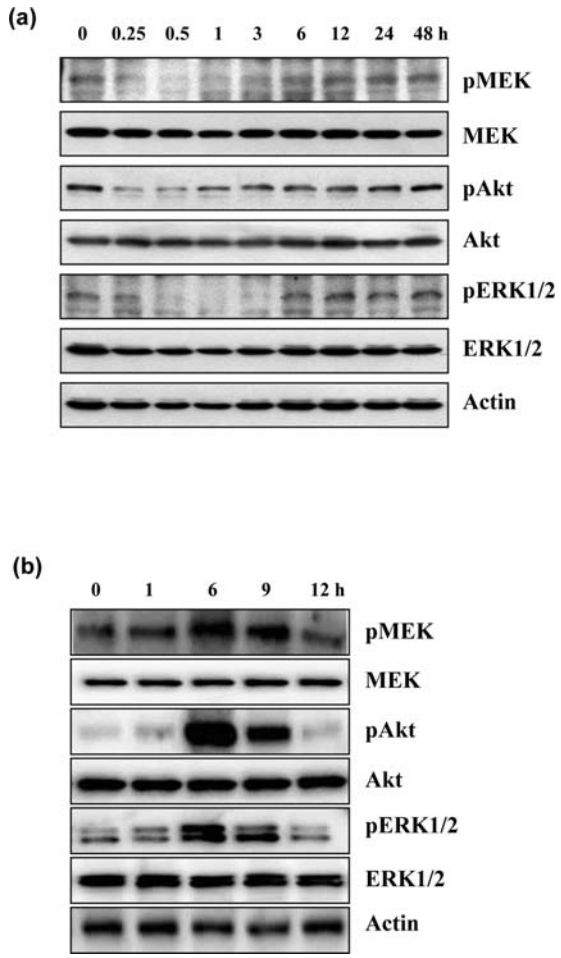


Figure 4. Effect of curcumin on the phosphorylation of MEK, ERK1/2 and Akt in B16F10 cells. The cells were exposed to curcumin (10 μM) for the indicated lengths of time following treatment with 200 nM of α-MSH (a) or serum starvation (b). The expression levels of the phospho-MEK, MEK, phospho-ERK1/2, ERK1/2, phospho-Akt and Akt proteins were examined by Western blot analysis as described in Materials and methods. Equal protein loading was confirmed by actin expression.

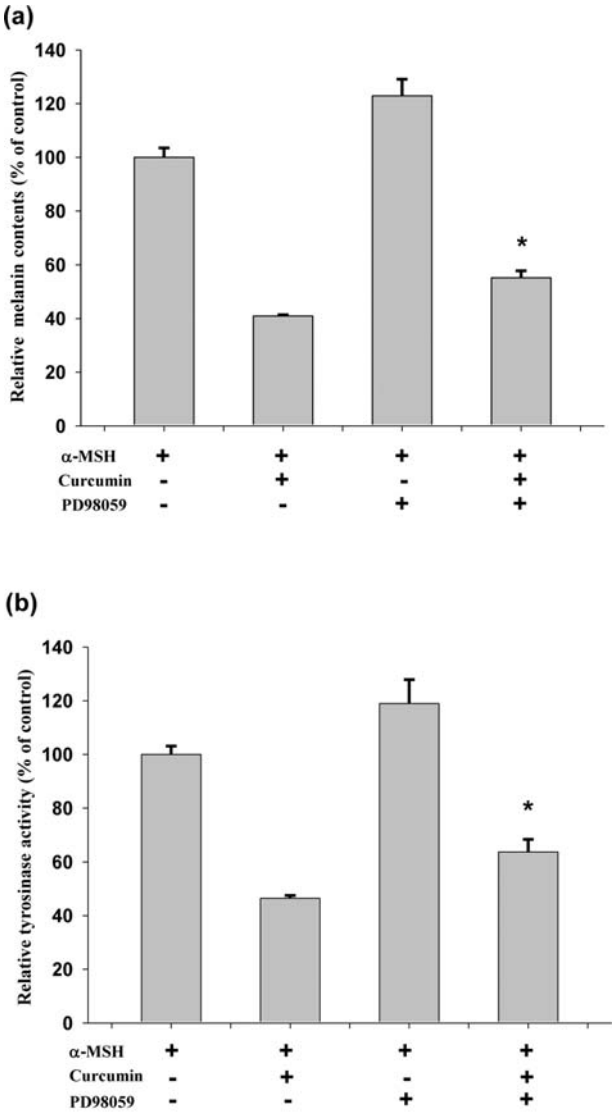


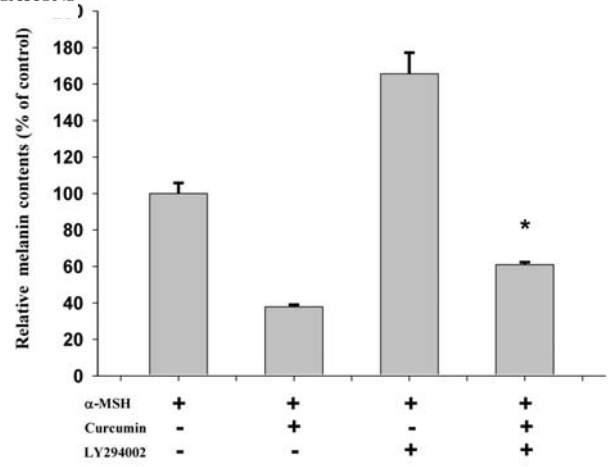
Figure 5. Effect of PD98059 on cellular melanin synthesis (a) and tyrosinase activity (b) in B16F10 cells treated with curcumin. The cells were exposed to α-MSH (200 nM) in the presence of curcumin (10 μM) and PD98059 (20 μM). The cellular melanin contents and tyrosinase activity were determined and expressed as percentages. Each percentage in the treated cells is reported relative to that in the control cells. Data are reported as the mean ± SEM of three independent experiments carried out in triplicate. *P<0.05 compared with the cells co-treated with α-MSH and curcumin.

of MEK and ERK, and LY294002, a selective inhibitor of PI3K, inhibit the suppressive activity of curcumin on melanogenesis. The cells were exposed to α-MSH (200 nM) in the presence of curcumin (10 μM) and PD98059 (20 μM) or LY294002 (20 μM), and the cellular melanin contents and tyrosinase activity were subsequently measured. Fig. 5 shows that the melanin contents and the tyrosinase activities of the cells co-treated with curcumin and α-MSH increased from 40.9 and 46.5% to 55.2 and 63.7%, respectively, following treatment with PD98059. The melanin contents and the tyrosinase activities of the cells co-treated with curcumin and α-MSH increased from 37.9 and 41.3% to 61 and 80.2%, respectively, following treatment with LY294002 (Fig. 6).

Since inhibition of PI3K/Akt or MEK/ERK was shown to inhibit the suppressive activity of curcumin on melanogenesis, we examined the effect of PD98059 and LY294002 on the expression level of melanogenesis-related proteins in B16F10 melanoma cells co-treated with curcumin and α-MSH. Fig. 7 shows that the curcumin-induced suppression of α-MSH-induced MITF, tyrosinase, TRP-1 and TRP-2 expression was inhibited by PD98059 or LY294002. These results suggest that the suppressive activity of curcumin on melanogenesis is regulated by the specific inhibitors of anti-melanogenic signal pathways involving the phosphorylation of MEK, ERK and Akt.

Discussion

Turmeric powder, the powdered dry rhizome of *C. longa* Lynn., gives specific flavor and color to curry, and has been traditionally used as a folk remedy to treat inflammatory disorders (17). The active component of turmeric powder, curcumin, was discovered to be an anti-oxidant and an angiogenesis inhibitor (18). Moreover, a variety of biological activities, including the inhibition of several signal transduction pathways involving PKA, PKC, PI3K/Akt, ERK and CREB, have been reported (10,13). In particular, PKA is known as a



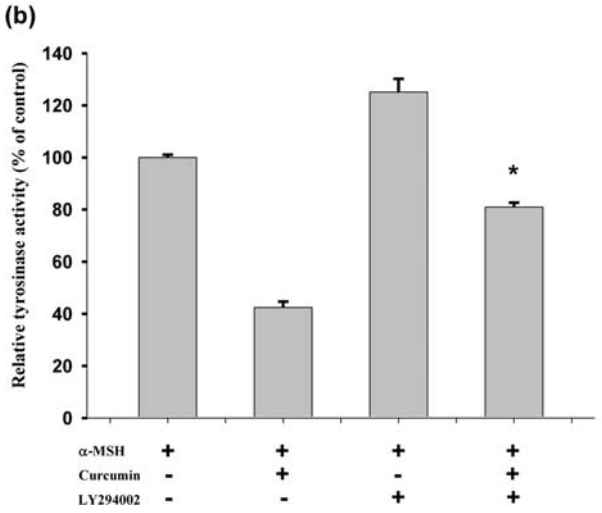


Figure 6. Effect of LY294002 on cellular melanin synthesis (a) and tyrosinase activity (b) in B16F10 cells treated with curcumin. The cells were exposed to α-MSH (200 nM) in the presence of curcumin (10 μM) and 20 μM of LY294002. The cellular melanin contents and tyrosinase activity were determined and expressed as percentages. Each percentage in the treated cells is reported relative to that in the control cells. Data are reported as the mean ± SEM of three independent experiments carried out in triplicate. *P<0.05 compared with the the cells co-treated with α-MSH and curcumin.

key mediator of α-MSH-induced melanogenesis, and PI3K/Akt or MEK/ERK signaling is known to regulate α-MSH-induced melanogenesis (6-9).

Accordingly, it was hypothesized that curcumin might inhibit the melanogenesis-related signaling pathways, including the inhibition of MITF-tyrosinase signaling and/or activation of ERK-Akt signaling. However, in spite of all of the progress in curcumin research, no study has appropriately addressed its suppressive activity on melanogenesis (14). In the present study, we demonstrated that curcumin suppressed α-MSH-induced melanogenesis involving the inhibition of tyrosinase activity and the down-regulation of melanogenesis-related proteins, particularly tyrosinase, in B16F10 melanoma cells (Figs. 2 and 3).

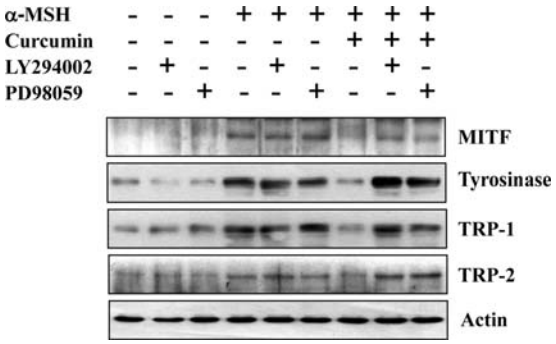


Figure 7. Effect of PD98059 and LY294002 on the expression of the melanogenesis-related proteins in B16F10 cells treated with curcumin. The cells were exposed to α-MSH (200 nM) in the presence of curcumin (10 μM) and 20 μM of PD98059, or curcumin and 20 μM of LY294002 for 48 h. The expression levels of the tyrosinase, TRP-1, TRP-2 and MITF proteins were examined by Western blot analysis as described in Materials and methods. Equal protein loading was confirmed by actin expression.

Transcriptional regulation of tyrosinase expression is mainly dependent on the expression of MITF, which is up-regulated by PKA and down-regulated by ERK or PI3K/Akt (6-9). While activation of ERK is reported to induce phosphorylation and degradation of MITF, inhibition of ERK is known to induce an increase in cAMP, a key intermediator of α-MSH-induced melanogenesis in melanocytes (7,8). On the other hand, activation of PI3K/Akt is reported to inhibit AC-dependent production of cAMP and, consequently, its activation results in the inhibition of melanogenesis (4). In the present study, curcumin induced the phosphorylation of MEK, ERK and Akt at 6 and 9 h after treatment (Fig. 4b). However, the reduction of phospho-ERK, phospho-Akt and phospho-MEK occurred at early time points, while the increase in the phosphorylated proteins was at late time points (Fig. 4a). The reduction in the phosphorylated proteins at early time points appears to be associated with hormonal melanogenesis inducers such as α-MSH. In addition, our results (Fig. 4b) corroborate the finding that an increase in phosphorylated proteins at late time points is caused by curcumin.

The results shown in Fig. 4 contrast with those noted in various curcumin-treated carcinoma cell lines, all of which showed decreased ERK and Akt activity upon exposure to relatively high doses of curcumin (19-22). Relatively high doses of curcumin (20 μM and greater) result in the induction of apoptosis and cell cycle arrest, which is accompanied by a switch-off survival signal pathway involving the inhibition of MEK/ERK or PI3K/Akt, as described in previous reports (19-22). It was previously reported that curcumin induces the activation of PI3K/Akt at a relatively low dose (10 μM) in a human melanoma cell line (23). Therefore, it is possible that the suppression of melanogenesis in B16F10 melanoma cells may require a relatively low dose (10 μM) of curcumin without side effects such as growth arrest and apoptosis.

The signaling pathway of α-MSH-induced melanogenesis involves the activation of AC, which leads to an increase in cAMP levels. Previous studies have demonstrated that curcumin inhibits the downstream molecular targets of cAMP, which include PKA and CREB (20,24). In the present study,

the melanin contents and tyrosinase activity of the cells co-treated with curcumin and α-MSH were slightly increased by treatment with the selective inhibitors of PI3K, MEK or ERK (Figs. 5 and 6). The curcumin-induced suppression of α-MSH-induced MITF, tyrosinase, TRP-1 and TRP-2 expression was inhibited by the selective inhibitors (Fig. 7). These results suggest that curcumin-induced activation of MEK/ERK or PI3K/Akt partly accounts for the anti-melanogenic effect of the compound. Namely, the suppressive activity of curcumin on melanogenesis may not only be related to the down-regulation of AC-dependent production of cAMP through the activation of MEK/ERK or PI3K/Akt, but may also be associated with the inhibition of cAMP-downstream molecules such as PKA and CREB.

In conclusion, curcumin suppresses the cellular melanin contents and the tyrosinase activity in α-MSH-stimulated B16F10 cells, suppressing the expression of MITF, tyrosinase, and TRPs, and phosphorylating MEK/ERK and PI3K/Akt. Overall, these results suggest that the suppressive activity of curcumin on melanogenesis involves the down-regulation of AC-dependent production of cAMP through the activation of MEK/ERK or PI3K/Akt, and also involves the inhibition of cAMP-downstream molecules such as PKA and CREB.

Acknowledgements

This study was supported by Blue-Bio Industry Regional Innovation Center (RIC08-06-07) of Dongeui University as a RIC program under the Ministry of Knowledge Economy and Busan City.

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