Berberine regulates melanin synthesis by activating PI3K/AKT, ERK and GSK3β in B16F10 melanoma cells

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Abstract. Berberine, an isoquinoline alkaloid, has a wide range of beneficial properties, including anti-bacterial, anti-inflammatory, anti-cancer, and cholesterol-lowering effects. Recently findings suggest that berberine improves glucose and lipid metabolism disorders. In the present study, we examined the mechanism underlying the inhibitory effect of berberine on α-melanocyte-stimulating hormone (α-MSH)-stimulated B16F10 melanoma cells. The results showed that berberine attenuated α-MSH induction of the microphthalmia-associated transcription factor (MITF) and tyrosinase in a dose-dependent manner. To elucidate the mechanism underlying the inhibitory effect of berberine, we examined the effect of α-MSH-stimulated phosphorylation of PI3K/AKT, ERK, and GSK3β. The results showed that treatment with berberine resulted in a reduction in the phosphorylation of PI3K/AKT, ERK, and GSK3β. Taken together, the results suggested that berberine inhibits melanin synthesis and tyrosinase activity by downregulating the expression of MITF and tyrosinase. Thus, these findings may contribute to the potential application of berberine in the prevention and treatment of skin pigmentation disorders.

Introduction

Human skin color ranges from white to brown to black due to the presence of the pigment melanin, which is crucial in protecting the skin against ultraviolet (UV) light and other environmental challenges. Melanogenesis, a highly regulated process, is modified by transcriptional, translational, and post-translational mechanisms. Marked cellular and molecular connections among cell populations have been identified in the skin; however, the key players are fibroblasts, keratinocytes, and melanocytes (1). The excessive accumulation of melanin can lead to hyperpigmentation or even skin cancer (2,3). Many inhibitors of melanin synthesis have been isolated from natural sources, including flavonoids, kojic acid, arbutin, and hydroquinone (4-9). Previous findings have shown that arbutin increases the pigmentation of cultured human melanocytes, while kojic acid has toxic and mutagenic effects when used for prolonged periods of time (10-12). Therefore, the safety and effectiveness of certain natural herbal extracts for skin pigmentation that have other properties including anti-cancer, anti-inflammatory, and anti-bacterial activity have been investigated. These well-known materials are polyphenol, curcumin, and rosemary (13-15).

Berberine, an isoquinoline alkaloid, is a common component of a variety of plant species, particularly in the Berberidaceae family. Although berberine has a wide range of pharmacological effects, including anti-bacterial, anti-inflammatory, anti-cancer and cholesterol-lowering effects (16-19), the precise mechanisms involved remain to be determined. In this study, we examined the inhibitory effect of berberine on α-melanocyte stimulating hormone (α-MSH)-induced melanogenesis in B16F10 melanoma cells in addition to the underlying mechanism of action.

Materials and methods

Materials. Berberine chloride hydrate, mushroom tyrosinase, 3,4-dihydroxy-L-phenylalanine (L-DOPA), PD 98059 (ERK inhibitor), BIO (GSK3β) and 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). LY 294002 (PI3K inhibitor) was purchased from Calbiochem (Darmstadt, Germany). Dulbecco's modified Eagle's medium (DMEM), fetal calf serum, trypsin EDTA, phosphate-buffered saline (PBS), and penicillin/streptomycin were purchased from Welgene, Inc. (Daegu, Korea). Antibodies specific to tyrosinase and β-actin were purchased from Santa

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Abbreviations: MITF, microphthalmia-associated transcription factor; α-MSH, α-melanocyte-stimulating hormone

Key words: B16F10 melanoma cells, melanin synthesis, tyrosinase, microphthalmia-associated transcription factor, phosphotidyl inositol 3-kinase/AKT, extracellular signal-regulated kinases, glycogen synthase kinase 3β

Cruz Biotechnology, Inc. (USA). Antibodies specific to phospho-ERK1/2 (Thr202/Tyr204), phospho-AKT, GSK3β and β-catenin were from Cell Signaling Technology (Beverly, MA, USA).

Cell cultures. The B16F10 melanoma cells were purchased from ATCC (American Type Culture Collection; Manassas, VA, USA). The cells were maintained at 37˚C in a humidified atmosphere of 95% air and 5% CO_2 in DMEM supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 100 U/ml penicillin and 10 μg/ml streptomycin.

Cell viability assay. A cell viability assay was determined using an MTT assay. Briefly, the cells were seeded at a desity of $2x10^5$ cells/well in 6-well plates. Prior to treatment, the cells were cultured for 24 h in serum-free medium. Following treatment with the indicated concentrations of 0, 0.01, 0.1, 1 and 10 μg/ml berberine in each well, the cells were incubated for 24 h. The supernatant was removed and MTT solution was added to each well prior to incubation for 4 h. Subsequently, 1.5 ml of dimethylsulfoxide (DMSO) was added to each well to solubilize any deposited formazon. Folowing incubation of 10 min at room temperature, optical density (OD) was determined at 540 nm on an ELISA plate reader (ThermoMax Microplate Reader; Molecular Devices, Sunnyvale, CA, USA). The percentage of viable cells in each well was calculated with respect to the OD value of living cells of the control group (100%).

Measurement of melanin content. Cells ($2x10^5$ cells) were incubated in 6-well plates overnight. After 24 h incubation, α-MSH (1 μM) was added per well and treated with increasing concentrations of berberine (0.01-10 μg/ml) in phenol-red free DMEM for 3 days. Then, 200 μl of medium were transferred in 96-well plates and the OD value was measured using an ELISA plate reader (VersaMax ELISA Microplate Reader; Molecular Devices) at 400 nm. Cell numbers were then counted using a haemocytometer. Melanin productions were expressed as percentages of those of untreated controls.

Tyrosinase activity. Tyrosinase activity was assayed as DOPA oxidase activity. In order to measure tyrosinase activity in cells, B16F10 melanoma cells were seeded at a density of $1x10^5$ cells in 6-well plates, and incubated in DMEM with berberine. After 3 days, the cells were washed with PBS and lysed with lysis buffer [0.1 M phosphate buffer (pH 6.8) containing 1% Triton X-100]. The cells were then disrupted by freeze-thawing, and lysates were clarified by centrifugation at 21,000 x g for 20 min. After quantifyingthe protein content using a protein assay kit (Bio-Rad, Hercules, CA, USA), the cell lysates were adjusted to the same amount of protein with a lysis buffer. Reaction mixtures consisting of protein, 10 mM L-DOPA and 0.1 M phosphate buffer were assayed in 96-well plates at 37˚C. Absorbance was measured every 10 min for at least 1 h at 475 nm using an ELISA reader followed by incubation at 37˚C. A cell-free assay system was used to investigate the direct tyrosinase effect of berberine. A reaction mixture consisting of 10 μg/ml mushroom tyrosinase and 10 mM L-DOPA was assayed in 96-well plates at 37˚C. After 30 min, absorbance was measured as described above at 475 nm.

Western blot analysis. Treated whole cell extracts were lysed in RIPA buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 0.5% Triton X-100, 0.1% sodium dodecyl sulfate (SDS) (Sigma-Aldrich Co.) and a protease inhibitor cocktail tablet (Roche Diagnostics, Indianapolis, IN, USA) for preparation of the cell extracts. The protein concentration of the extracts was estimated with Bradford reagent (Bio-Rad) using bovine serum albumin (BSA) as the standard.

For western blot analysis, cell lysates containing 20 μg/ml of proteins were resolved on a 10% polyacrylamide gel. The separated proteins were then transferred onto a PVDF membrane (Immobilon; Millipore, Bedford, MA, USA). The membrane was saturated with 5% dried milk in tris-buffered saline containing 0.5% Tween-20. A western blot analysis was performed by first incubating the membrane in primary antibodies, such as MITF, Tyrosinase, p-AKT, t-AKT, p-ERK, t-ERK, p-GSK3β, t-GSK3β and β-antin overnight at 4˚C, followed by further incubation with horseradish peroxidase-conjugated secondary antibody (Vector Laboratories, Inc., Burlingame, CA, USA). The membrane developed in the enhanced chemiluminescence (ECL) Western detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Quantitative RT-PCR. Total RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions after treatment and quantified by an ND1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) at an absorbance of 260 nm. cDNA was synthesized with 2 μg of denatured total RNA in a final volume of 20 μl of buffer containing $MgCl_2$, KCl, dNTPs, and oligo-dT reverse transcriptase by incubation at 42˚C for 60 min. The cDNA obtained was amplified with the following primers: microphthalmia-associated transcription factor (MITF) forward, 5'-CGCCTGATCTGGTGAATCG-3' and reverse, 5'-CCTGGCTGCAGTTCTCAAGAA-3'; tyrosinase forward, 5'-TTGCCACTTCATGTCATCATAGAATATT-3' and reverse, 5'-TTTATCAAAGGTGACTGCTATACAAAT-3'; and GAPDH forward, 5'-CGTCCCGTAGACAAAATGGT-3' and reverse, 5'-TTGATGGCAACAATCTCCAC-3'. Quantitative PCR was performed with a C1000 Touch™ ThermalCycler (Bio-Rad) using SYBR-Green (Takara, Shiga, Japan). Reactive mixtures were incubated for 40 cycles at 95˚C for 15 sec; 58˚C for 45 sec and 72˚C for 20 sec. Gene expression was normalized to those of the housekeeping gene encoding GAPDH.

Immunocytochemistry. B16F10 melanoma cells were seeded in two-chamber slides. After incubation for 24 h, immunocytochemistry (ICC) was performed. The slides were fixed with 4% paraformaldehyde for 30 min at room temperature. After washing with DPBS, the cells were permeabilized with 0.05-0.1% Triton X-100 in PBS or TBST for 10-15 min at room temperature and then blocked with 5% BSA in 0.05% Triton X-100 for 30 min at room temperature. The slides were incubated with tyrosinase and MITF antibodies at 4˚C for overnight. After washing, the slides were incubated with fluorescein isothiocyanate (FITC)-conjugated secondary antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). These slides were mounted using fluorescent mounting medium with 4',6-diamidino-2-phenylindole (DAPI) (Golden Bridge

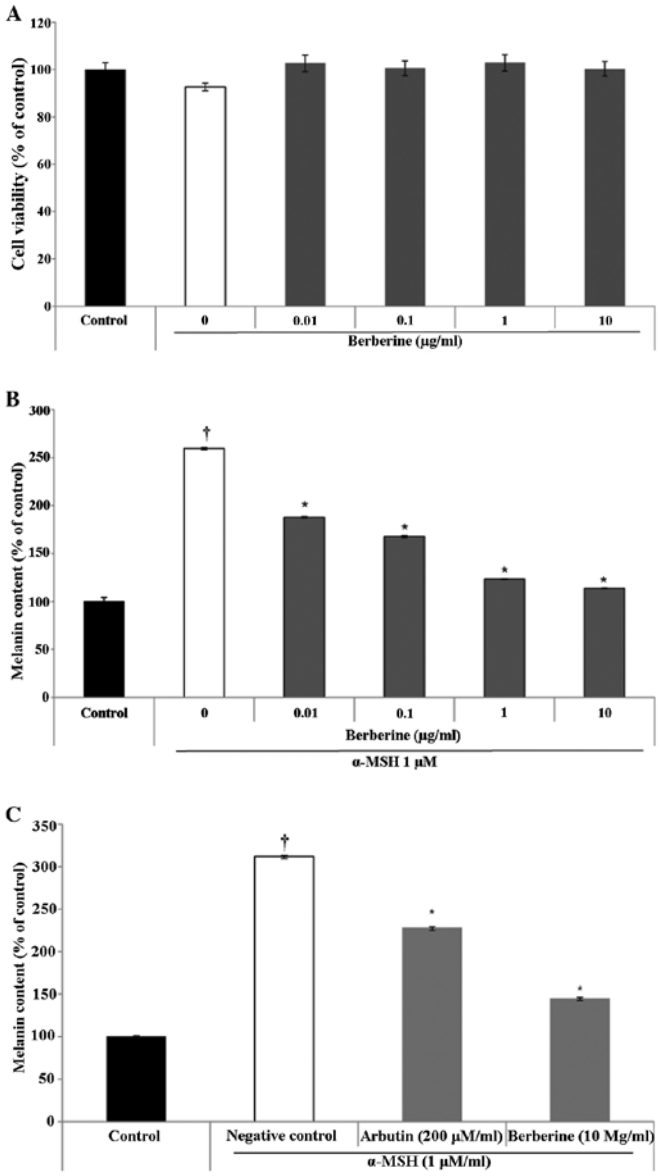

Figure 1. Effect of viability and melanin contents by berberine in B16F10 melanoma cells. (A) After B16F10 melanoma cells were incubated for 24 h in 96-well plates, the cells were treated with various concentrations of berberine (0, 0.01, 0.1, 1 and 10 μg/ml) for 24 h. Cell viability was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. (B and C) After cells were incubated for 24 h in 6-well plates, the cells were co-treated with various concentrations of berberine and α-melanocyte-stimulating hormone (α-MSH) (1 μM/ml) for 72 h. Arbutin (200 μM/ml) was used as a positive control. Melanin contents by berberine were largely decreased in a dose-dependent manner in the MSH-stimulated B16F10 melanoma cells. Results are expressed as a percentage of viability and melanin contents compared to the control. Data are expressed as the mean ± SD (n=3). †P<0.05 vs. control, *p<0.05 vs. α-MSH-stimulated.

International, Inc., Mukilteo, WA, USA) and observed by fluorescence microscopy (Olympus IX71; Olympus, Tokyo, Japan).

Statistical analysis. Statistical analyses were performed using SPSS version 18.0 for Windows (SPSS Inc., Chicago, IL, USA). Results are expressed as the means ± SD. Data were analyzed by one-way ANOVA followed by a Duncan's test for multiple comparisons. A two-tailed value of p<0.05 was considered statistically significant.

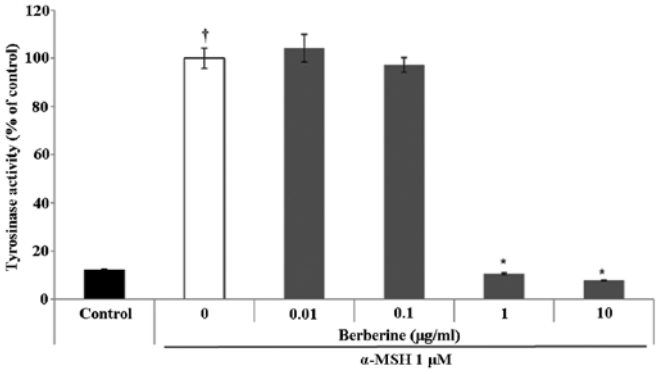

Figure 2. Effect of tyrosinase activity by berberine in B16F10 melanoma cells. After cells were incubated for 24 h in 6-well plates, the cells were co-treated with various concentrations of berberine (0, 0.01, 0.1, 1 and 10 μg/ml) and α-melanocyte-stimulating hormone (α-MSH) (1 μM) for 72 h. The tyrosinase activity of the berberine-treated cells was measured. Each determination was made in triplicate, and the data shown are the mean ± SD. †P<0.05 vs. control, *p<0.05 vs. α-MSH-stimulated.

Results

Effect of viability and melanin contents by berberine in B16F10 melanoma cells. After examining the effects of berberine on cell viability, we examined the effects of berberine on the melanin content. As the berberine did not decrease the viability of cells up to a concentration of 10 μg/ml (Fig. 1A), this concentration was used in subsequent experiments. We quantified the melanin content and observed that even 10 μg/ml of berberine inhibited melanin production significantly. At concentrations of 10 μg/ml, cells were still viable, and the cellular melanin content decreased to 2.3-fold (Fig. 1B). In addition, the inhibitory effect for melanin contents by berberine (10 μg/ml) was more effective compared to that of arbutin (200 μM/ml), which is known to inhibit tyrosinase activity (Fig. 1C).

Effect of tyrosinase activity by berberine in B16F10 melanoma cells. Many inhibitors of melanin synthesis induce inhibition of tyrosinase directly. Thus, to investigate the direct effects of berberine on tyrosinase, we measured the tyrosinase activity of mushroom tyrosinase in a cell system and a cell-free system. In the cell system, tyrosinase activity was significantly decreased by berberine at concentrations ranging from 1 to 10 μg/ml (Fig. 2), whereas berberine was found to have no direct inhibitory effect in the cell-free system (data not shown).

Effect of berberine MITF and tyrosinase levels on B16F10 melanoma cells. To elucidate the mechanism of melanogenesis, including the expression of tyrosinase and MITF, underlying the effect of berberine, B16F10 cells were treated with the extract prior to stimulation with α-MSH for 24, 48 and 72 h. qPCR and western blot analysis were conducted to analyze the resulting cell lysates. Fig. 3A shows that the α-MSH-stimulated expression of MITF and tyrosinase mRNA was suppressed by berberine (10 μg/ml) treatment. Furthermore, as shown in Fig. 3B, 10 μg/ml of berberine decreased the α-MSH-stimulated protein expression of tyrosinase and MITF. The decreased protein level of tyrosinase was confirmed by ICC (Fig. 3C).

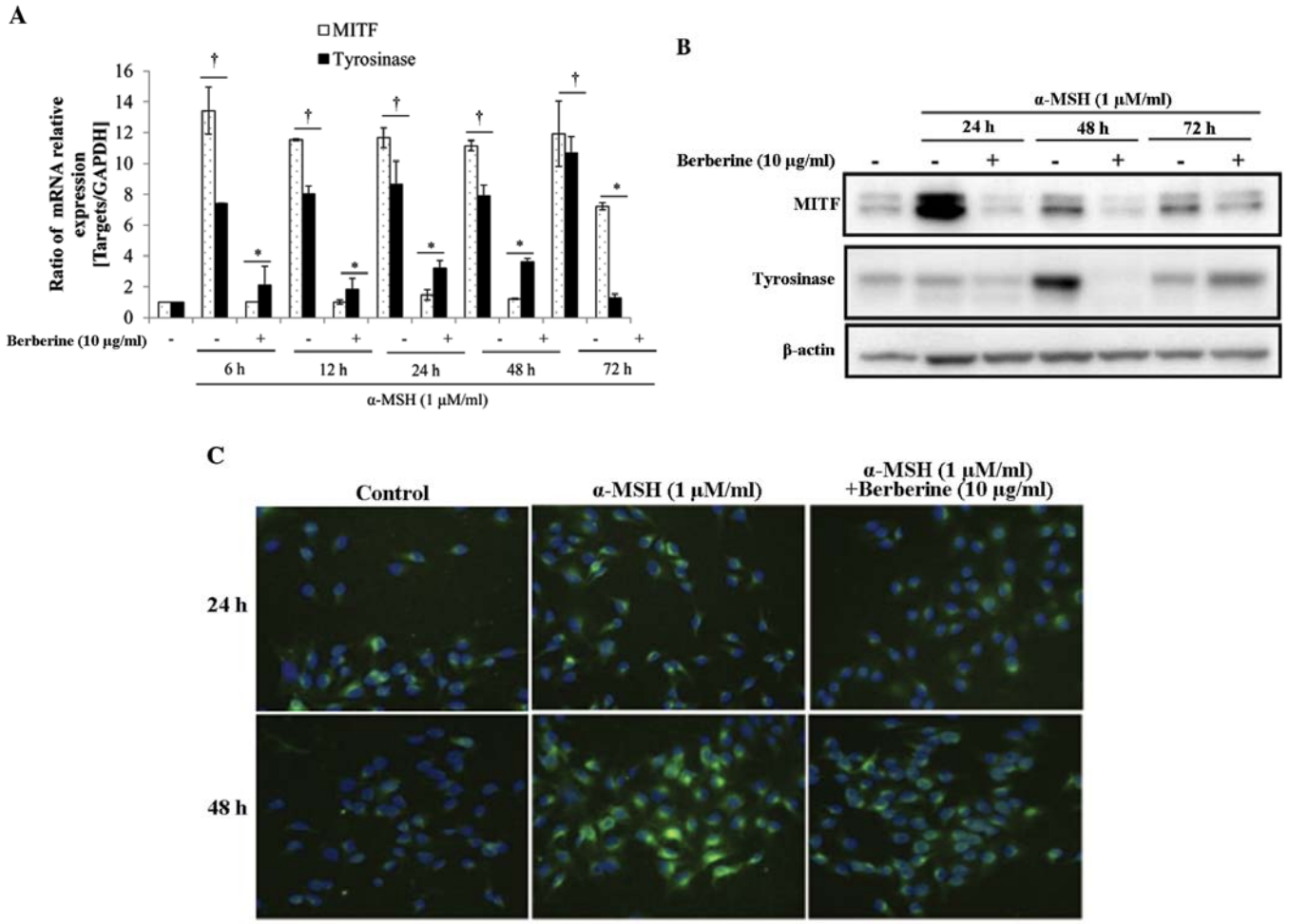

Figure 3. Effect of berberine microphthalmia-associated transcription factor (MITF) and tyrosinase levels of B16F10 melanoma cells. (A and B) B16F10 melanoma cells were co-treated with various concentrations of berberine (0, 0.01, 0.1, 1 and 10 μg/ml) and α-melanocyte-stimulating hormone (α-MSH) (1 μM) for the indicated time points. The expression levels of MITF and tyrosinase were examined by (A) qPCR and (B) western blot analysis. Equal mRNA and protein was confirmed using GAPDH. (C) The expression of tyrosinase was confirmed by immunocytochemical (ICC) staining using specific anti-tyrosinase antibodies (green). Corresponding 4',6-diamidino-2-phenylindole (DAPI) nuclear staining (blue) and the merged images are shown. Each determination was made in triplicate, and the data shown are the mean ± SD. †P<0.05 vs. control, *p<0.05 vs. α-MSH-stimulated (magnification, x400).

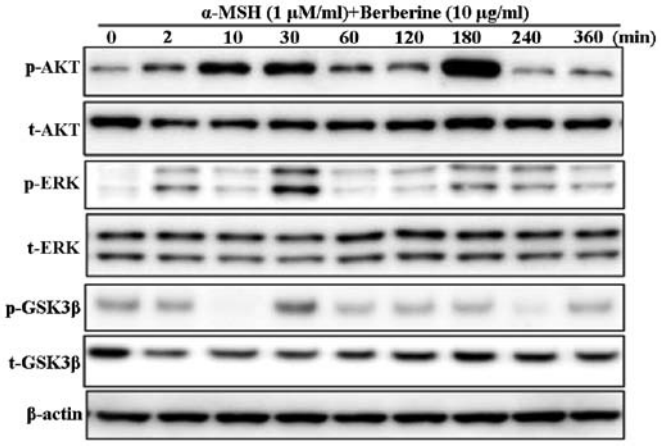

Figure 4. Effects of berberine on the melanogenesis-related signaling pathway in B16F10 melanoma cells. B16F10 melanoma cells were co-treated with berberine (10 μg/ml) and α-melanocyte-stimulating hormone (α-MSH) (1 μM) for the indicated time points. The expression levels of the melanogenesis-related signaling pathway, which involves the phosphorylation of PI3K/AKT, ERK and GSK3β, were examined by western blot analysis. Equal protein loadings were confirmed by AKT, ERK, GSK3β and β-actin expression.

Effects of berberine on the melanogenesis-related signaling pathway in B16F10 melanoma cells. The PI3K/AKT, ERK and GSK3β signaling pathways regulate melanogenesis. To elucidate the mechanism underlying the melanogenesis effect of berberine, B16F10 melanoma cells were exposed to α-MSH (1 μM/ml) in the presence of berberine (10 μg/ml) for the indicated time points, and the protein extracts were then analyzed by western blot analysis. As shown in Fig. 4, berberine induced the phosphorylation of PI3K/AKT, ERK and GSK3β at early time points, i.e., 30 min to 3 h after exposure.

Effects of berberine on the melanogenesis-related signaling pathways by specific inhibitors in B16F10 melanoma cells. Since berberine activates phosphorylation of the PI3K/AKT, ERK, and GSK3β signaling pathways, we hypothesized that LY 294002, a selective inhibitor of PI3K; PD 98059, a selective inhibitor of ERK; and BIO, a selective inhibitor of GSK3β, inhibits the suppressive effect of berberine on melanogenesis. B16F10 melanoma cells were treated with berberine (10 μg/ml) in the presence or absence of LY 294002 (20 μM/ml) or PD 98059 (10 μM/ml) or BIO (1 μM/ml) for 3 days, and the extracellular

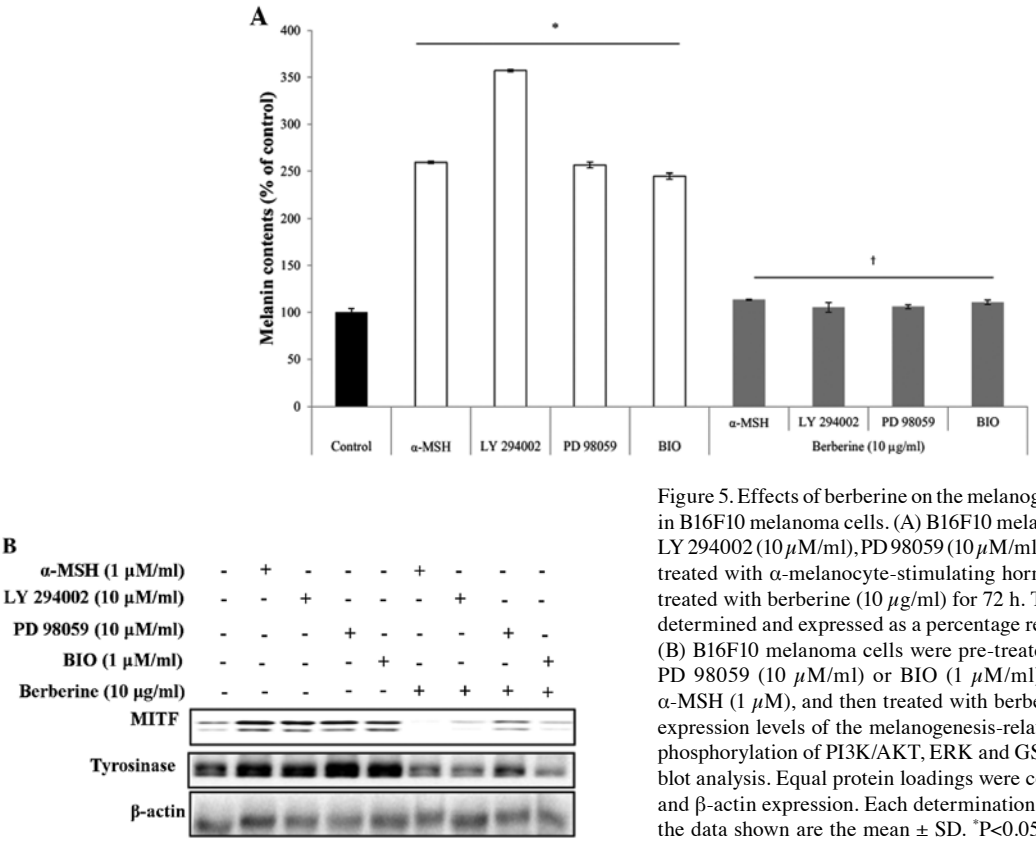

Figure 5. Effects of berberine on the melanogenesis-related signaling pathway in B16F10 melanoma cells. (A) B16F10 melanoma cells were pre-treated with LY 294002 (10 μM/ml), PD 98059 (10 μM/ml) or BIO (1 μM/ml) for 2 h, or were treated with α-melanocyte-stimulating hormone (α-MSH) (1 μM), and then treated with berberine (10 μg/ml) for 72 h. The cellular melanin content was determined and expressed as a percentage relative to that of the control cells. (B) B16F10 melanoma cells were pre-treated with LY 294002 (10 μM/ml), PD 98059 (10 μM/ml) or BIO (1 μM/ml) for 2 h, or were treated with α-MSH (1 μM), and then treated with berberine (10 μg/ml) for 30 min. The expression levels of the melanogenesis-related signaling pathway, involving phosphorylation of PI3K/AKT, ERK and GSK3β, were examined by western blot analysis. Equal protein loadings were confirmed by AKT, ERK, GSK3β and β-actin expression. Each determination was performed in triplicate, and the data shown are the mean ± SD. *P<0.05 vs. control, †p<0.05 vs. α-MSH or inhibitors-stimulated.

melanin release was measured. As shown in Fig. 5A, addition of the inhibitors increased the amount of melanin in the media. Berberine treatment inhibited this melanin release (Fig. 5A).

To further confirm the results shown in Fig. 5A, we examined the effect of the inhibitors on the expression level of melanogenesis-related proteins in B16F10 melanoma cells co-treated with berberine, α-MSH or inhibitors (Fig. 5B). The data are consistent with Fig. 5A, showing that the phosphorylation of the PI3K/AKT, ERK, and GSK3β signaling pathway is associated with the reduction of melanogenesis by berberine. Thus, these results suggested that the reduction of melanogenesis by berberine is regulated by the specific inhibitors of anti-melanogenic signaling pathways, which involved the phosphorylation of the PI3K/AKT, ERK, and GSK3β signaling pathways.

Discussion

Berberine, an isoquinoline alkaloid, has a wide range of beneficial properties, including anti-bacterial, anti-inflammatory, anti-cancer and cholesterol-lowering effects (16-19). However, the exact mechanisms underlying these effects remain to be identified. Recently, studies on the effects of berberine found that it improved glucose and lipid metabolism disorders by increasing insulin sensitivity through the adjustment of adipokine secretions in preadipocytes in primary culture and in metabolic syndrome patients (20,21). The anti-angiogenic activity of berberine via the downregulation of hypoxia-inducible factor-1, VEGF, and proinflammatory mediators was confirmed (22).

In this study, we examined the expression of melanogenesis-related proteins including MITF and tyrosinase, and melanogenesis-regulating molecules including PI3K/AKT, ERK and GSK3β in order to elucidate the potential mechanisms by which berberine inhibits melanogenesis in B16F10 melanoma cells.

In particular, the PI3K/AKT and ERK signaling pathways are known as key mediators of α-MSH-induced melanogenesis (23-25). The GSK3β signaling pathway, which has been widely induced in the regulation of cell homeostasis due to its ability to phosphorylate a broad range of substrates, including glycogen synthase, the microtubule-associated protein τ, and β-catenin, is also known to decrease the binding of MITF to the tyrosinase promoter (26).

This study has demonstrated that berberine decreases melanin synthesis and tyrosinase activity. Furthermore, we studied the effect of berberine on MITF and tyrosinase expression in B16F10 melanoma cells using RT-PCR and western blot analysis, and their expression was confirmed by ICC. Our data showed that expression of MITF and tyrosinase was attenuated by berberine (Fig. 3A-C).

Berberine inhibits the signaling pathways involved in α-MSH-induced melanogenesis. Since activation of the PI3K/AKT, ERK, and GSK3β signaling pathways is associated with the inhibition of hyperpigmentation by decreasing MITF and tyrosinase activity (23-26), berberine may inhibit melanin synthesis by inducing the phosphorylation of PI3K/AKT and ERK. Using the specific inhibitors, LY 294002, PD 98059 and BIO, we found that berberine inhibited induced-melanin content, MITF and tyrosinase.

In conclusion, berberine significantly inhibits melanogenesis in B16F10 melanoma cells. The berberine-induced phosphorylation of PI3K/AKT, ERK, and GSK3β may contribute to anti-melanogenic effects. The results that the beneficial effects of berberine on skin diseases caused by stress including UV light, are secondary to its inhibition of melanin synthesis. Further studies are required to assess the safety and efficacy of berberine for clinical use.

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