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# Lucidone, a Novel Melanin Inhibitor from the Fruit of *Lindera erythrocarpa* Makino

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**The effects of lucidone on tyrosinase and antimelanogenic activity were investigated. Initially, we found that lucidone strongly inhibits the activity of mushroom tyrosinase. The effects of lucidone on tyrosinase were further examined in α-MSH-induced B16 melanoma cells. Lucidone significantly inhibits tyrosinase activity and leads to decreased melanin content in cultured B16 melanoma cells. Lucidone also attenuates the expression of tyrosinase and MITF (Microphthalmia-associated Transcription Factor) protein in a concentration-dependent manner, as shown by western blot. Quantitative real-time reverse-transcription polymerase chain reaction (RT-PCR) confirmed that lucidone inhibits the expression of tyrosinase mRNA. Accordingly, the effects of lucidone on the ERK signaling pathway were also investigated, but lucidone was not found to play major role in the induction of ERK activation. Our data indicate that the antimelanogenic activity of lucidone is probably due to its inhibition of tyrosinase activity and the suppression of tyrosinase and MITF expression.** 

*Keywords*: lucidone; antimelanogenic; tyrosinase inhibition; melanin; MITF.

## INTRODUCTION

The color of mammalian skin and hair is determined by a number of factors, the most important of which is the degree and distribution of melanin pigmentation. Melanin is one of the most widely distributed pigments and is found in bacteria, fungi, plants and animals. In animals, it is secreted by melanocyte cells distributed in the basal layer of the dermis (Kim and Uyamma, 2005). It is a heterogeneous, polyphenol-like biopolymer with a complex structure and color varying from yellow to black (Choi *et al*., 2006). The control of melanogenesis is an important strategy in the treatment of abnormal skin pigmentation for cosmetic purposes (Im *et al*., 2002).

Melanin synthesis is mainly controlled by tyrosinase, a copper-containing enzyme that catalyzes two distinct reactions in melanin synthesis, *viz* the hydroxylation of tyrosine by monophenolase action and the oxidation of 3,4-dihydroxy-L-phenylalanine (L-DOPA) to *o*-dopaquinone by diphenolase action (Song *et al*., 2006). *o*-Dopaquinone is unstable in aqueous solution and rapidly suffers a non-enzymatic cyclization to leukodopachrome, which is further oxidized non-enzymatically by another molecule of *o*-dopaquinone to yield dopachrome and one molecule of regenerated L-DOPA (Cooksey *et al*., 1997). The modulators of melanogenesis can act directly on tyrosinase activity, which is responsible for the conversion of melanosomes to keratinocytes (Kim *et al*., 2006b). It has been reported that transcription factors such as lymphoid-enhancing factor-1 are involved in the expression of tyrosinase-related proteins such as TRP-1 and TRP-2 (Sato and Toriyama, 2009).

Recently, another transcription factor, micropthalmia associated transcription factor (MITF), was shown to play a key role in melanocyte survival, development and differentiation (Kim *et al*., 2006a). However, tyrosinase plays the critical regulatory role in melanin biosynthesis. Therefore, many tyrosinase inhibitors that suppress melanogenesis have been extensively studied with the aim of developing preparations for the treatment of hyperpigmentation (Masamoto *et al*., 2003).

Lucidone is a naturally occurring cyclopentenedione, which was initially isolated from the fruit of *Lindera lucida* (Lauraceae) (Lee, 1968; Leong *et al*., 1997) and subsequently from other species such as *Lindera erythrocarpa* (Oh *et al*., 2005; Wang *et al*., 2008). Our recent *in vitro* studies suggest that lucidone exerts inhibition of LPS-induced inflammation in murine macrophage cells (Wang *et al*., 2008; Senthil Kumar and Wang, 2009). However, the information regarding antimelanogenic and tyrosinase activity of lucidone is lacked. On other hand, the antimelanogenic effect of naturally occurring phytochemicals has been studied (Kim and Uyama, 2005; Parvez *et al*., 2007). So far, it remains unknown that the naturally occurring phyto-compound, lucidone, could inhibit melanagenesis or tyrosinase activity in cultured melanoma cells. The major purpose of this study was to investigate the antimelanogenic and tyrosinase activity of lucidone in cultured melanoma cells. These results will elucidate the possible action modes from lucidone against melanogenesis and tyrosinase enzyme activity.

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## MATERIALS AND METHODS

**Chemicals and reagents.** Lucidone was prepared according to the protocol described previously (Wang *et al*., 2008). The purity of lucidone was above 99% according to high performance liquid chromatography (HPLC) and $^1$H-NMR analysis. Roswell Park Memorial Institute (RPMI) medium and other cell culture reagents including fetal bovine serum (FBS) were purchased from GIBCO BRL Life Technologies/Invitrogen (Grand Island, NY, USA). Tyrosinase (EC 1.14.18.1, activity of 6680 units/mg), melanin, α-MSH and ascorbic acid were purchased from Sigma-Aldrich (St Louis, MO, USA), SYBR GreenER qPCR super mix for ABI from Invitrogen (Carlsbad, CA, USA). Anti-rabbit polyclonal tyrosinase and anti-rabbit monoclonal MITF antibodies were obtained from Abcam (Cambridge, UK) and used as received unless otherwise noted.

**Mushroom tyrosinase inhibition assay.** Mushroom tyrosinase inhibition assays were performed in 96-well microplate format using ELISA microplate reader (μ-Quant, Bio-Tek Instruments, Winooski, VT, USA) according to a previously developed method (Hearing and Tsukamoto, 1991; Azhar-ul-Haq *et al*., 2006; Kim *et al*., 2006b). The compound was initially screened for inhibition of the *o*-diphenolase activity of tyrosinase using L-DOPA as substrate, and the concentration of lucidone required for 50% inhibition (the $IC_{50}$) determined. Lucidone was dissolved in 1% of DMSO at concentrations of 1, 5 and 10 μg/mL. Thirty units of mushroom tyrosinase (28 nM) were pre-incubated with the lucidone in 50 mM sodium phosphate buffer (pH 6.8) for 10 min at 25°C. Then the L-DOPA (4 μM) was added and the reaction monitored by measuring the change in absorbance at 475 nm (37°C) due to the formation of dopaquinone for 10 min. The inhibition of the enzyme activity was calculated as follows, Percent inhibition (%) = $[B - S/B] \times 100$, where $B$ and $S$ were the absorbance for the blank and samples, respectively. Ascorbic acid (5 μg/mL) was used as standard tyrosinase inhibitor.

**Cell viability assay.** Cell viabilities were determined using the MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)-based cell viability assay. MTT is a pale yellow substrate that produces a dark blue formazan product when incubated with living cells, due to mitochondrial cleavage of an MTT ring. Briefly, B16 murine melanocytes ($1 \times 10^6$) were seeded on 96-well plates and treated with lucidone (1, 5 and 10 μg/mL) for 2 h and then incubated with or without α-MSH (100 nM/mL) for 24 h. After 24 h incubation medium were removed and cells were incubated in fresh medium with MTT (1 mg/mL) for 1 h at 37°C and then the absorbance was recorded at 570 nm using an ELISA reader. Survival of melanocytes after treatment with or without lucidone and α-MSH was calculated using the following formula, viable cell number (%) = $OD_{570}$ (treated cells)/$OD_{570}$ (control cells) × 100.

**Inhibition of tyrosinase activity in murine melanoma cells.** B16 melanoma cells were cultured in RPMI medium in a humidified atmosphere containing 5% $CO_2$ in air at 37°C. Cells were incubated in the presence or absence of different concentrations of lucidone and then treated for 24 h with or without 100 nM α-MSH. The cells were lysed in 100 μL of 50 mM sodium phosphate buffer (pH 6.8) containing 1% Triton X-100 and 0.1 mM phenylmethylsulfonyl fluoride, and centrifuged at 20,000 × *g* for 30 min at 4°C. The supernatant were placed in a 96-well plate, and the absorbance at 492 nm was read using an ELISA plate reader.

**Inhibition of melanin synthesis in murine melanoma cells.** B16 cells ($1 \times 10^6$) were incubated in the presence or absence of lucidone, and then incubated for 24 h with or without 100 nM α-MSH. After they were washed twice with phosphate buffered saline, samples were dissolved in 100 μL of 1 N NaOH. Total melanin content was estimated by absorbance at 405 nm and comparison with a melanin standard curve.

**Preparation of whole cell, cytosolic and nuclear extract.** The preparation of whole cell extract was previously described (Wang *et al*., 2008). B16 cells ($1 \times 10^6$) were seeded on 6 cm dishes and were treated with lucidone (1, 5 and 10 μg/mL), stimulated with α-MSH (100 nM/mL) and incubated at 37°C, 5% $CO_2$ for 24 h. Ascorbic acid (5 μg/mL) was used as a reference compound. After 24 h, cells were lysed in Mammalian Protein Extraction Reagent (Cayman Chemicals, Ann Arbor, MI, USA) and the lysates were centrifuged (15,000 × *g*) at 4°C for 10 min to remove debris and the supernatant were stored at –80°C until use. The cytosolic and nuclear extracts were prepared following protocol #78833 of the Nuclear and Cytoplasmic Extraction Reagents (NE-PER) kit (Pierce Biotechnology, Rockford, IL, USA). The total protein content was measured by absorbance at 595 nm by the Bradford method (Pierce Biotechnology, Rockford, IL, USA).

**RNA extraction and real time RT-PCR analysis.** Total RNA was isolated from cultured melanocytes using Trizol Reagent according to the manufacturer's instructions (Invitrogen, Grand Island, NY, USA). Quantitative real-time reverse-transcription polymerase chain reaction (RT-PCR) analysis for tyrosinase and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) mRNA were performed using Applied Biosystems (Foster City, CA, USA) detection instrument and software. Quantitative PCR was carried out in 48-well plates with 10 μM forward and reverse primers, and the working solution SYBR green, using a custom PCR master mix, with the following conditions, 95°C for 10 min, followed by 40 cycles at 95°C for 15 s, 55°C for 10 s, 95°C for 15 s for tyrosinase and 95°C for 10 min, followed by 40 cycles at 95°C for 15 s, 60°C for 1 min, 95°C for 15 s for G3PDH. G3PDH, a housekeeping gene, was chosen as an internal standard control for variability in amplification because of differences in starting mRNA concentrations. The sequences of the PCR primers were as follows: Tyrosinase, Forward 5′-TAT TGA GCC TTA CTT GGA AC-3′, reverse 5′-AAA TAG GTC GAG TGA GGT AA-3′ (Maeda *et al*., 2007); G3PDH, forward 5′-TCA ACG GCA CAG TCA AGG-3′, reverse 5′-ACT CCA CGA CAT ACT CAG C-3′. The copy number of each transcript was calculated as the relative copy number normalized to G3PDH copy number.

**Western blot analysis.** Whole cell, cytosolic and nuclear protein extracts from treated and untreated B16 cells (20 μg per lane) were separated by 12% SDS polyacrylamide gel electrophoresis (Immobilon, Millipore Corporation, Bedford, MA, USA) and then transferred to PVDC membrane. The membrane was blocked with 10% skim milk in TBST overnight at 4°C before staining with appropriate antibodies using the Enhanced Chemi-Luminescence (ECL) Western Blotting Reagent (Millipore, Billerica, MA, USA) and image were captured using a VL Chemi-Smart 3000 imager (Viogene Biotek, Taipei, Taiwan).

**Statistical analysis.** Data are expressed as means ± SD. The significance of differences between group means were tested using Student's *t*-test for single comparisons. *P* values less than 0.05 were considered to represent significant differences from α-MSH alone.

## RESULTS AND DISCUSSIONS

The inhibition of tyrosinase has been the subject of numerous studies (Njoo *et al*., 1999; Kim and Uyamma,

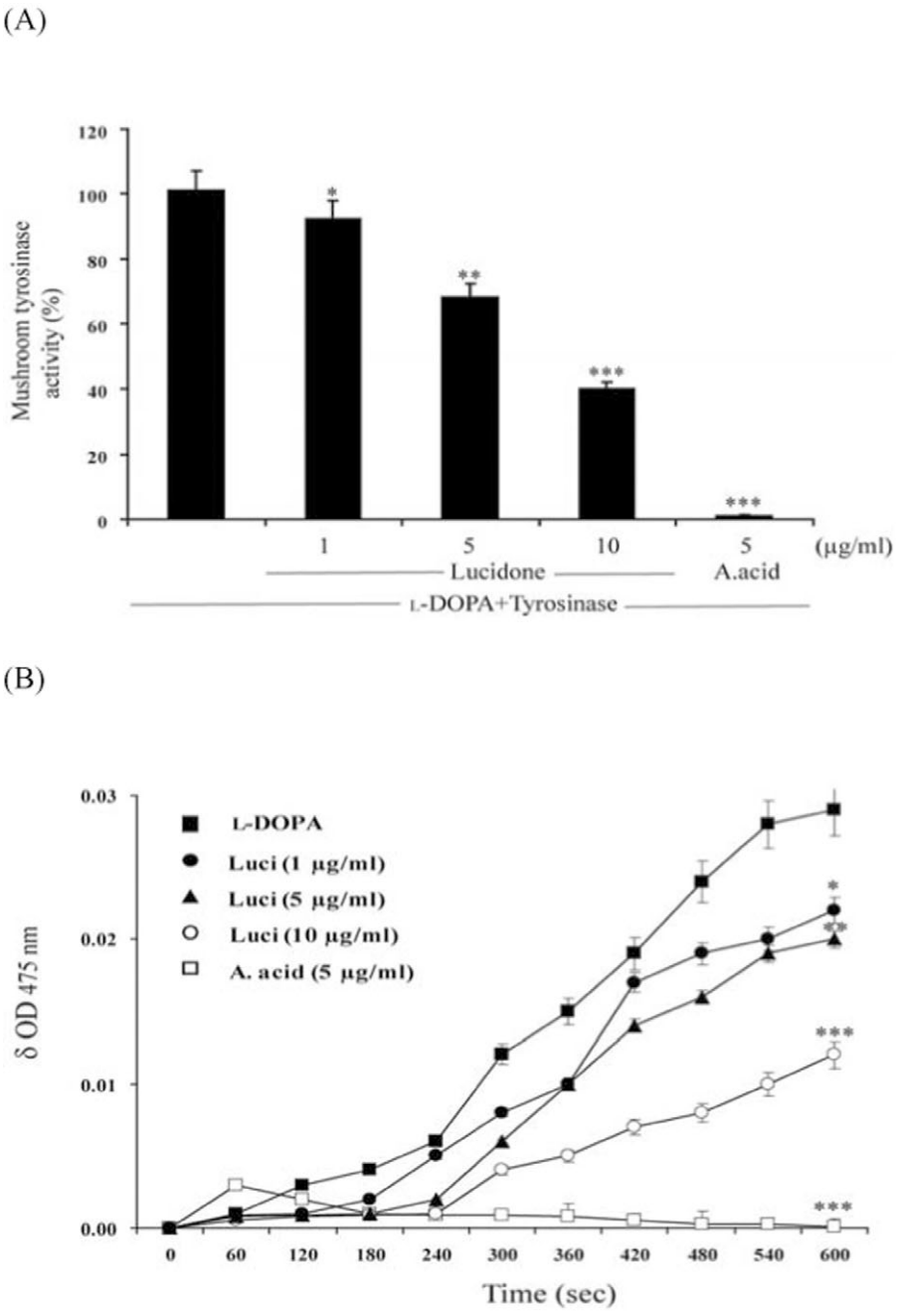


**Figure 1.** Effect of lucidone on mushroom tyrosinase activity for the oxidation of L-DOPA. Course of substrate reaction in the presence of different concentrations of lucidone. Assay conditions: 200 μl system containing 50 mM sodium phosphate buffer pH 6.8, 4 μM L-DOPA as substrate, 28 nM mushroom tyrosinase and indicated concentration of lucidone, at 37°C. (A) The relative activity of mushroom tyrosinase on L-DOPA in the presence of different concentrations of lucidone, compared to the absence of lucidone (100%). (B) The time course of the substrate oxidation reaction. Concentrations of lucidone for curves were 0 (■), 1 (●), 5 (▲) and 10 μg/ml (○); 5 μg/ml of ascorbic acid (□) was used as a positive control. Values represent the mean ± SD of three experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with the α-MSH treated group.

 

2005), and several inhibitors are used as cosmetic additives and medicinal products in the treatment of hyperpigmentation (Kim *et al*., 2006b). In recent tyrosinase inhibitors has been used as a whitening agent or antihyperpigmention agent because of its ability to suppress dermal-melanin production (Piao *et al*., 2002). Many scientists are working to isolate tyrosinase inhibitors from natural products. The present work was designed to investigate the mode of inhibitory action of lucidone on mushroom tyrosinase activity and on cellular tyrosinase activity and melanin content in cultured B16 melanoma cells. Further, to gain molecular insight into the inhibition of melanogenesis by lucidone, we investigated its effect on tyrosinase, extracellular signal-regulated kinase (ERK) 1/2 and MITF molecules. Lucidone is known as an anti-inflammatory and farnacyl protein inhibitory activity (Oh *et al*., 2005; Wang *et al*., 2008; Senthil Kumar and Wang, 2009) but its inhibitory effect on melanogenesis and the modulation of MITF is first reported in this present study.

As Fig. 1A indicates, lucidone exhibits a concentration-dependent inhibition of mushroom tyrosinase activity, with an $IC_{50}$ value of 7.3 μg/mL. Additionally, in time course experiments, lucidone increased the lag time and slowed the increase in absorbance in a concentration-dependent manner, which was directly proportional to its inhibition of dopachrome formation (Fig. 1B). To establish the relative efficacy of lucidone, its

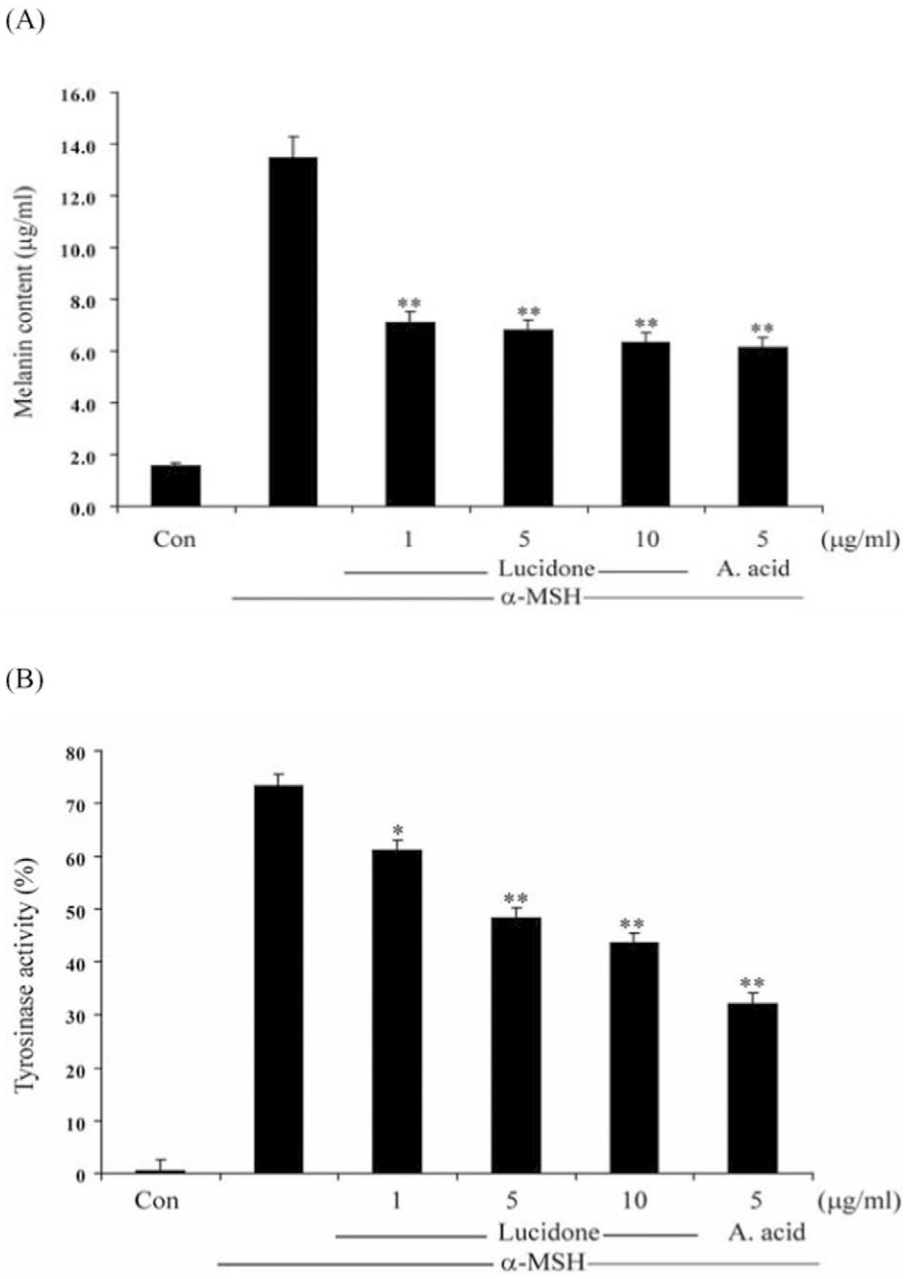


**Figure 2.** Inhibitory effect of lucidone on melanin content and tyrosinase enzyme activity in α-MSH-induced B16 melanoma cells. (A) Melanoma B16 cells were treated with indicated concentrations of lucidone and stimulated with α-MSH. Melanin accumulation was assessed by spectrometry. Cellular melanin was solubilized with 1 N NaOH and measured by absorbance at 405 nm. Cellular melanin content was calculated by comparison with a melanin standard curve. (B) Melanoma cells were treated with indicated concentrations of lucidone and stimulated with α-MSH. The lysates of the whole cells were used as enzyme sources. Effect of lucidone on L-DOPA oxidation velocity was measured at 492 nm. Melanin content and tyrosinase activity were expressed as percentage value. Data represent the mean ± SD of three experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with the α-MSH treated group.

 

inhibitory effects were compared with a well known tyrosinase inhibitor, ascorbic acid. The inhibitory effect of lucidone was significantly less then to the ascorbic acid.

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, and glycyrrhizin), and ultraviolet light (Hirata *et al*., 2007). Initially we investigated whether lucidone has cytotoxic effects; we treated B16 melanoma cells at the various concentrations of lucidone, and cell viability was determined by using MTT assay. However, Lucidone showed no effect on cell viability at the concentrations up to 10 μg/mL, indicating that they are not cytotoxic to B16 cells (data not shown). α-MSH is an activator of melanin synthesis in melanocytes (Kim *et al*., 2006a). In our present study, we subjected α-MSH as a melanogeneic inducer. Melanoma B16 cells were treated with 100 nM/mL of α-MSH for 24 h with or without various concentrations of lucidone. Melanoma B16 cells in resting state released 1.83 μg/mL of melanin during incubation for

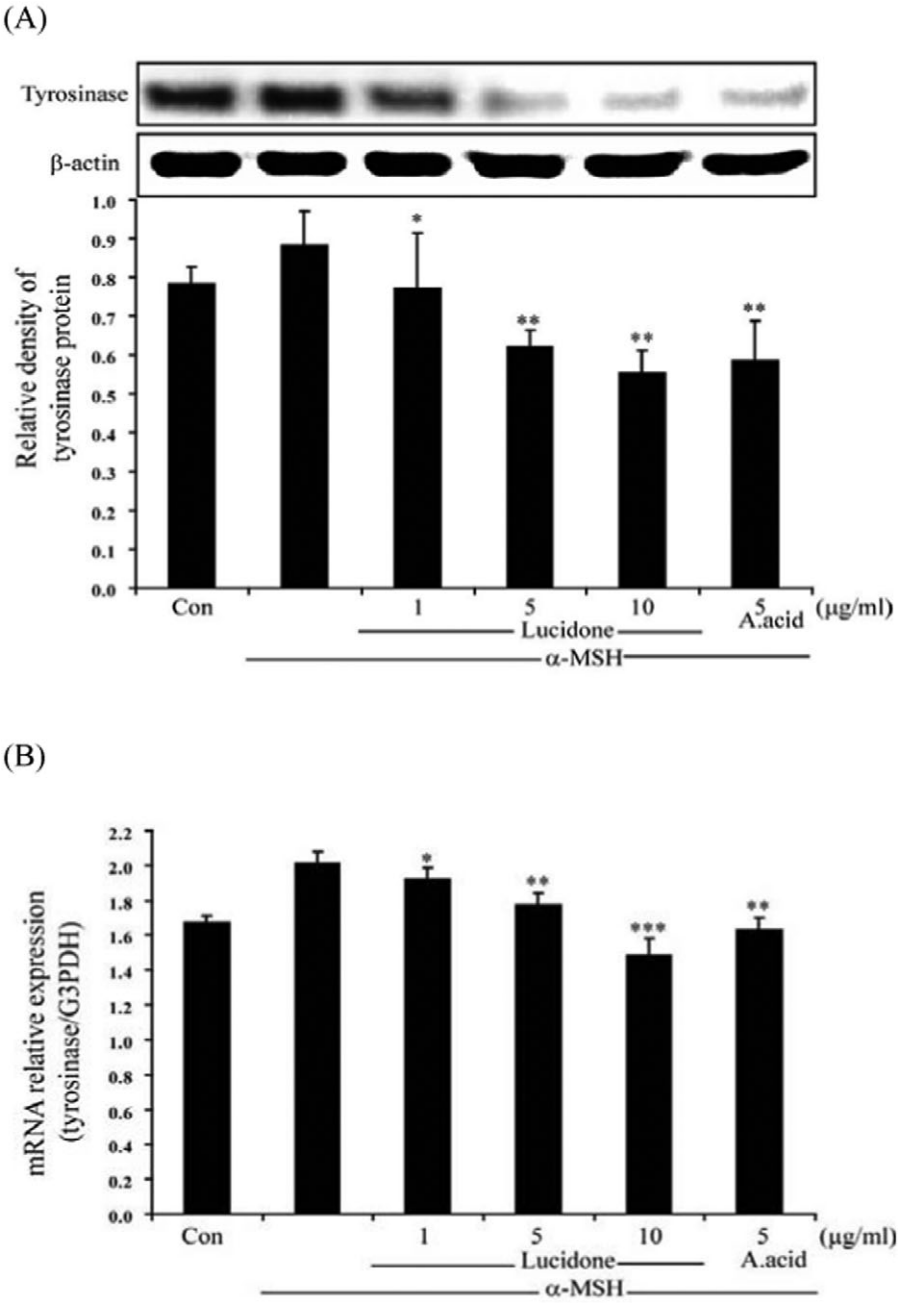


**Figure 3.** Inhibitory effect of lucidone on tyrosinase protein and mRNA expression levels in cultured B16 melanoma cells. (A) Melanoma B16 cells were treated with indicated concentrations of lucidone for 2 h and stimulated with α-MSH for 24 h. Cell lysates were analyzed by western blot with anti-tyrosinase antibody. Relative density of one representative experiment is shown, where tyrosinase signal was normalized to β-actin signal. (B) Relative expression of tyrosinase mRNA in B16 melanoma cells. Total RNA was extracted from cells treated with indicated concentrations of lucidone for 2 h and stimulated with α-MSH for 12 h. The transcription levels of tyrosinase were quantified by RT-PCR. A representative experiment is shown. Tyrosinase mRNA signal was normalized to G3PDH mRNA signal. Values represent the mean ± SD of three experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with the α-MSH treated group.

 

24 h, but markedly increased melanin production, up to 13.6 μg/mL, upon treatment with α-MSH alone for the same period (Fig. 2A). Lucidone showed dose-dependent inhibitory effects of melanin production in α-MSH-induced B16 melanoma cells (Fig. 2A). As a drug control, ascorbic acid also showed inhibition of melanin production in cultured B16 melanoma cells.

Melanogenesis is started by the oxidation of tyrosine, which is oxidized to dopaquinone catalyzed by tyrosinase. Tyrosinase is believed to be the key enzyme in the melanogenesis of animal skin (Choi *et al*., 2006). We hypothesized that the reduction of melanin production in cultured B16 melanoma cells is due to the inhibition of tyrosinase activity. Next we determined whether luci-

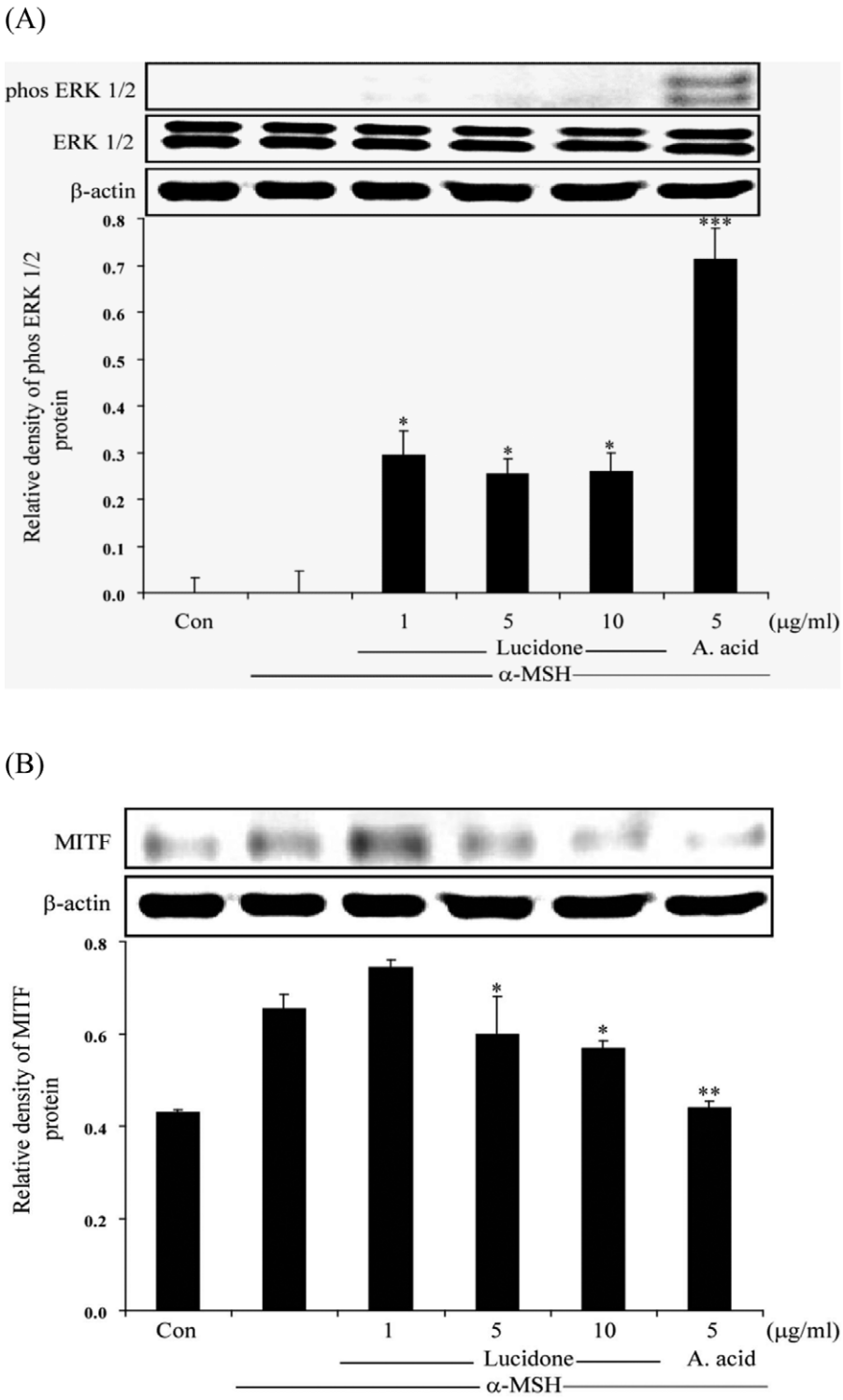


**Figure 4.** Effect of lucidone on activation of ERK and MITF protein stability. (A) Melanoma B16 cells were treated with various concentrations of lucidone for 2 h and stimulated with α-MSH for 24 h. Cytoplasmic fraction of the cells were analyzed by western blot with anti-phospho and total ERK1/2 antibodies. Relative density of one representative experiment is shown, where phosphorylated ERK 1/2 signal was normalized to total ERK 1/2 signal. (B) The nuclear fraction was analyzed by western blot with anti-MITF antibody. Relative density of one representative experiment is shown, where MITF signal was normalized to β-actin signal. Values represent the mean ± SD of three experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with the α-MSH treated group.

 

done can affect catalytic activity of cell-free tyrosinase. Lysates of melanoma B16 cells were stimulated with α-MSH alone were used as enzyme source of the cell-free tyrosinase, and L-DOPA oxidation velocity was measured as the catalytic activity. Lucidone shows significant inhibitory effects on L-DOPA oxidation activity of the cell-free tyrosinase in a dose-dependent manner (Fig. 2B). However, ascorbic acid as a drug control also inhibits catalytic activity of the cell-free tyrosinase (Fig. 2B).

Lucidone reduces melanin production and tyrosinase activity in cultured melanoma cells; to emphasize its mechanism, we determined protein and mRNA levels of tyrosinase. Western blot analysis was carried out with lysates of α-MSH-induced B16 melanoma cells. The B16 cells produced basal levels of tyrosinase (Fig. 3A). Upon treatment with α-MSH alone, melanoma cells markedly increased expression level of tyrosinase protein, lucidone attenuated α-MSH-induced tyrosinase protein level in a dose-dependent manner (Fig. 3A). To further investigate whether inhibitory effect of lucidone on tyrosinase, expression was influenced at the transcriptional level. Figure 3B show lucidone significantly inhibit tyrosinase mRNA level in cultured B16 cells which was confirmed by quantitative RT-PCR analysis. As a drug control, ascorbic acid also showed significant inhibition of protein and mRNA levels of tyrosinase.

MITF is involved in the pigmentation, proliferation, and survival of melanocytes (Hodgekinson *et al*., 1993). The activation of MITF, a transcription factor that regulates tyrosinase gene expression, is a critical event during melanogenesis (Kim *et al*., 2003; Hirata *et al*., 2007). During the melanogenesis in melanocytes, the ERK cascade depresses MITF protein stability in the nucleus (Hodgekinson *et al*., 1993; Kim *et al*., 2003; Xu *et al*., 2000; Kim *et al*., 2004; Hirata *et al*., 2007). The activation of ERK 1/2 in α-MSH-induced B16 melanoma cells by lucidone was examined here by western blot analysis with antibodies against phosphorylated or non-phosphorylated forms of mouse ERK 1/2. As shown in Fig. 4A, activated ERK 1/2 levels were significantly enhanced after the treatment with ascorbic acid as a drug control, whereas very little effect was observed with various concentrations of lucidone. This suggests that lucidone does not play an important role in the ERK pathway in α-MSH-induced B16 melanoma cells. Next we examined whether lucidone affects the α-MSH-induced activation of MITF. Western blots were carried out with nuclear fraction of α-MSH-stimulated melanoma B16 cells. The B16 cells produced basal levels of MITF and markedly increased MITF protein expression upon exposure to α-MSH alone (Fig. 4B). Lucidone decreased α-MSH-induced MITF production in a dose-dependent manner (Fig. 4B), which suggested that suppression of MITF expression by lucidone may be unrelated to the ERK pathway. Further studies are required to elucidate the mechanisms of the downregulation of MITF by lucidone.

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