Baicalein inhibits melanogenesis through activation of the ERK signaling pathway

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Abstract. Baicalein is one of the major flavonoids in *Scutellaria baicalensis*. However, the effects of baicalein on melanogenesis are unknown. The objective of this study was to evaluate the depigmenting capacity of baicalein and to elucidate its mechanism of action. B16F10 mouse melanoma cells were used to examine the effect of baicalein on melanogenesis by measurement of melanin content and tyrosinase activity after treatment. To ascertain the baicalein activity, the effect on two protein kinases, ERK and Akt and downstream microphthalmia-associated transcription factor (MITF) were examined by Western blotting and RT-PCR. Baicalein significantly inhibited melanin synthesis in a concentration-dependent manner without cytotoxicity. Tyrosinase activity was also reduced. Baicalein decreased MITF and tyrosinase levels but did not decrease MITF mRNA. Western blotting showed that baicalein induced ERK activation. Using the specific ERK phosphorylation inhibitor, PD98059, we blocked the hypopigmentation effect, and also abrogated the baicalein-mediated activation of ERK. However, baicalein did not induce Akt activation. These results suggest that the ERK pathway is involved in the melanogenic signaling cascade, and that ERK activation by baicalein reduces melanin synthesis via MITF downregulation and is subsequent to the inhibition of tyrosinase synthesis.

Introduction

Skin pigmentation, which results from the production and distribution of melanin in the epidermis, is the major physiologic defense against solar irradiation. However, increased levels of melanin synthesis can darken the skin and induce a number of hyperpigmentary skin conditions, such as freckles, chloasma, and solar lentigo (1). Tyrosinase catalyzes two rate-limiting steps in the synthesis of melanin which are the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (DOPA) and the oxidation of DOPA to dopaquinone, and inhibitors of tyrosinase have been used in cosmetics as skin-whitening agents (2). Two of these inhibitors, arbutin and kojic acid, are widely used cosmetic agents in Northeast Asia. However, these substances are only weak inhibitors of tyrosinase and can cause skin irritation. In addition, a previous study has reported that kojic acid can result in serious side effects such as cytotoxicity, skin cancer, and dermatitis, and this agent has been banned for cosmetic use in some countries (3).

Therefore, there is a need to develop safer and more effective skin whitening agents. A number of efforts have been made to develop new therapeutic agents against pigmentation abnormalities using novel biologically active compounds from plants and other natural sources (4).

Traditional Chinese medicine has a long history of use for the treatment of chloasma. Although these traditional medicines have been reported to be clinically effective in China, their mechanisms of action and active components have not been studied yet. The search for depigmentation medicine from traditional Chinese herbs is currently focused on agents that inhibit the tyrosinase enzyme itself. However, the biosynthesis of melanin is a complicated process involving many factors. Gene expression, protein degradation, glycosylation, melanosome transfer, and cellular signaling cascades are some of the factors involved (1,5,6). Another way to decrease tyrosinase activity might be to do so indirectly, by blocking one of the upstream processes that regulate it.

Baicalein (5,6,7-trihydroxyflavone) is one of the major flavonoids in *Scutellaria baicalensis*, a plant that has been extensively used in Chinese herbal medicine for a long time. Several biological effects of baicalein such as antiviral, anti-inflammatory, anti-hepatotoxic, and anti-tumor actions have been reported (7-9). An inhibitory effect of baicalein on tyrosinase (10) has also been reported. However, the exact mechanisms by which baicalein decreases tyrosinase activity are unclear.

Therefore, we have conducted an investigation using B16F10 mouse melanoma cells, to ascertain whether baicalein decreases melanogenesis by inhibiting tyrosinase through an upstream action. To do so, we examined its effect on two

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protein kinases, ERK and Akt, that are participants in two different upstream signaling systems regulating an intermediary, micropthalmia-associated transcription factor (MITF), that increases tyrosinase gene expression.

To our knowledge, the study reported here is the first to show direct evidence that baicalein inhibits melanogenesis, and that the mechanism underlying its action may be activation of the ERK signaling pathway.

Materials and methods

Materials. For cell culture, Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco-BRL (Grand Island, NY, USA). The inhibitor PD98059 (no. 9900), chemiluminescent horseradish peroxidase Western blot detection system (LumiGlo Reagent and peroxide no. 7003), and antibodies recognizing phospho-specific Akt (Ser473, no. 9271S), phospho-specific ERK1/2 (Thr202/Tyr204, no. 9101S) were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA). Primary antibodies to tyrosinase (C-19, sc-7833), MITF (no. sc-52938), and actin (I-19, sc-1616) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). L-DOPA, MTT, horseradish peroxidase-conjugated secondary antibodies, baicalein, and kojic acid were obtained from Sigma (St. Louis, MO, USA). All other chemicals used were of the highest purity available. Baicalein and PD98059 were dissolved in dimethylsulphoxide (DMSO). The DMSO final concentration was not greater than 1‰ (v/v) in all experiments.

Cell culture. The B16F10 murine melanoma cell line was obtained from the Tumor Researching Department of The Third Affiliated Hospital of Harbin Medical University, Harbin, China. Cells were cultured in DMEM containing 10% FBS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 0.25 μg/ml amphotericin B at 37˚C in a humidified 95% air/5% CO_2 atmosphere as described previously (11). Drug treatment began 24 h after seeding, and cells were harvested after indicated concertations and times of incubation. For inhibition experiments, B16F10 melanoma cells were pretreated with PD98059 for 1 h and then cultured with baicalein for indicated concertations and times.

Cell growth assay. The effects of baicalein on the growth of B16F10 murine melanoma cells were measured using the MTT assay (12) with some modifications. Briefly, cells ($2.5x10^3$ cells/well) seeded into 96-well plates and incubated with test substances at 37˚C in 5% CO_2 for 48 h. After the addition of 10 μl of MTT solution (dissolved in PBS, 5 mg/ml) per well, plates were incubated for a further 4 h. Supernatants were removed and the formazan crystals of these cells were solubilized in 150 μl DMSO by gentle shaking for 10 min. The amount of formazan was quantified in an ELISA reader (Thermo Fisher Scientific Inc., USA) at 492 nm.

Tyrosinase activity. Tyrosinase activity was determined by the method described by Tomita *et al* (13) with slight modification. B16F10 murine melanoma cells were plated at a density of $2.5x10^3$ cells/well in 96-well plates. After incubation with test substances for 48 h, cells were washed with ice-cold PBS and lysed with phosphate buffer (pH 6.8) containing 1% Triton-X/PBS (90 μl/well) and then frozen at -80˚C for 30 min. After thawing and mixing, 10 μl of 1% L-DOPA was added to each well. Following incubation at 37˚C for 2 h, the absorbance was measured at 492 nm using an ELISA reader (Thermo Fisher Scientific Inc.).

Measurement of melanin content. Melanin contents were measured according to the method of Tsuboi *et al* (14) with slight modifications. Briefly, cells were treated with test substances for 2 days, and cell pellets containing a known number of cells (~$5x10^5$) were dissolved in 0.5 ml of 1 N NaOH at 100˚C for 30 min and centrifuged for 20 min at 16,000 x g. Optical densities (OD) of supernatants were measured at 410 nm using an ELISA reader (Thermo Fisher Scientific Inc.).

Western blot analysis. Cells were lysed in cell lysis buffer [62.5 mM Tris-HCl, pH 6.8, 2% SDS, 5% ß-mercaptoethanol, 2 mM phenylmethylsulfonyl fluoride, protease inhibitors (Complete, Roche, Mannheim, Germany), 1 mM Na_3VO_4, 50 mM NaF, and 10 mM EDTA]. Protein (50 μg per lane) was then separated by SDS-polyacrylamide gel electrophoresis and blotted onto PVDF membranes, which were saturated with 5% dried milk in Tris-buffered saline containing 0.4% Tween-20. Blots were incubated with the appropriate primary antibodies at a dilution of 1:1,000, and then further incubated with horseradish peroxidase-conjugated secondary antibody. Bound antibodies were detected using an enhanced chemiluminescence plus kit (Amersham International, Little Chalfont, UK). Results were analyzed using a Bio-Rad GS-700 imaging densitometer (Hercules, CA, USA).

Reverse transcription-polymerase chain reaction (RT-PCR). To determine the amount of mRNA expressed, RT-PCR analysis was performed by techniques described earlier (15) with slight modifications. Briefly, total RNA was isolated from cells using RNeasy Mini Kit (Qiagen, Valencia, CA, USA). RT-PCR was carried out with 3 μg isolated total RNA sequentially in the same tube using Qiagen one step RT-PCR kit (Qiagen). All procedures were conducted following the manufacturer's instructions. Oligonucleotide primers used for PCR were as follows: MITF upstream 5'-GTATGAACACGCACTCTC TCGA-3' and downstream 5'-CTTCTGCGCTCATACTG CTC-3'. The reaction was cycled 25 times for 60 sec at 94˚C, 60 sec at 56˚C, and 60 sec at 72˚C. Reaction mixture (50%) was analyzed by electrophoresis on 1% agarose gels and stained with ethidium bromide. In order to check the reproducibility of the results, each experiment was carried out more than three times. Specific primers for GAPDH were used as a control.

Statistical analysis. All data are expressed as mean ± standard deviation (SD). Comparisons were made either among various doses for each medicine or between baicalein and kojic acid at 1 and 10 μM. The former comparison was implemented using one-way analysis of variance (ANOVA) test and the latter was examined by an independent t-test. Dunnett T3 *post-hoc* test was then performed if ANOVA test revealed a significant result. Also, the difference in melanin content among PD98059,

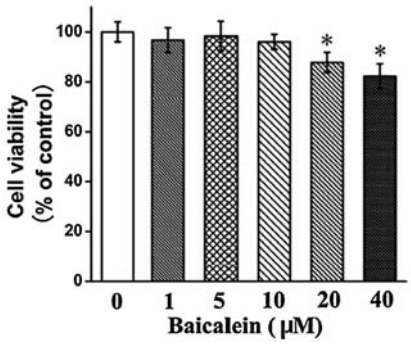

Figure 1. Cell viability of B16F10 melanoma cells after 48 h treatment with baicalein (n=9/per dose). Mean ±standard deviation (SD) is shown. *Significant difference from control.

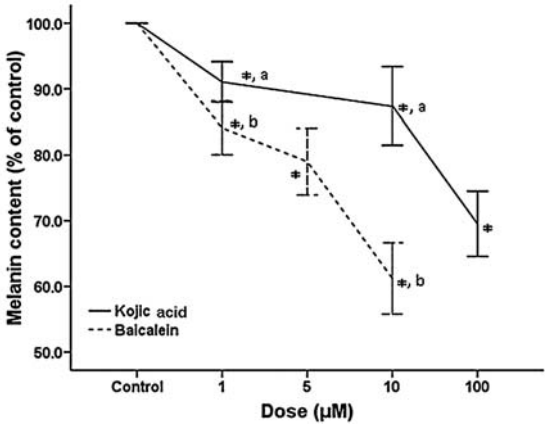

Figure 2. Melanin content in cells treated with baicalein or kojic acid for 48 h (n= 9/ per dose). Mean ±standard deviation (SD) is shown. One-way ANOVA followed by Dunnett's T3 test was performed to test the difference among varied doses. The difference between baiclein and kojic acid was compared with an independent t-test. *Significant difference from control. [a,b]Significant difference between baicalein and kojic acid.

baicalein and the mixed group (PD98059 + baicalein) was tested by ANOVA and Dunnett T3 test. Furthermore, the dose-response trend was confirmed by using linear regression analysis. All statistics were performed with SPSS 15.0 software (SPSS Inc, Chicago, IL). Two-sided $p<0.05$ was considered as a statistical difference.

Results

Inhibitory effect of baicalein on melanin synthesis in B16F10 melanoma cells without cytotoxicity. To determine whether baicalein had a cytotoxic effect on B16F10 melanoma cells, we incubated these cells for 48 h with various concentrations of baicalein, and measured cell viability using the MTT assay. Baicalein showed no cytotoxicity at concentrations from 1 to 10 μM (Fig. 1).

The effect of baicalein on melanogenesis was examined next. Fig. 2 shows the comparison of the effects of baicalein and the tyrosinase inhibitor kojic acid on melanogenesis in B16F10 cells. Cells treated with baicalein (1-10 μM) showed a significant and concentration-dependent decrease in melanin. This decrease was significantly greater than the decrease achieved with kojic acid, which at a concentration of

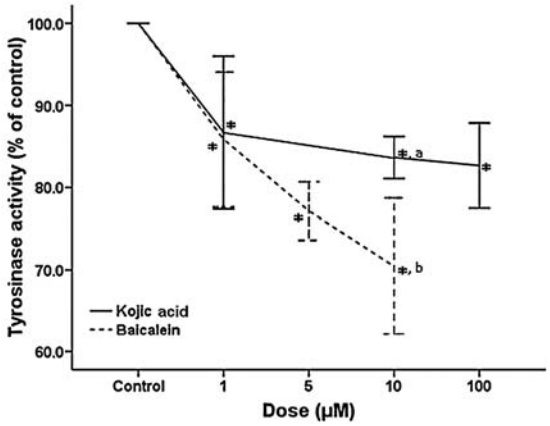

Figure 3. Tyrosinase activity in cells treated with baicalein or kojic acid for 48 h (n=9/per dose). Mean ± standard deviation (SD) is shown. One-way ANOVA followed by Dunnett's T3 test was performed to test the difference among varied doses. The difference between baiclein and kojic acid was compared with independent t-test. *Significant difference from control. [a,b]Significant difference between baicalein and kojic acid.

100 μM produced less inhibition than that produced by 10 μM baicalein. The combined results show that baicalein (1-10 μM) has a relatively strong anti-melanogenesis effect on B16F10 melanoma cells without accompanying cytotoxicity.

Downregulation effects of baicalein and kojic acid on tyrosinase activity in B16F10 melanoma cells. Concentrations of 1-10 μM baicalein, in addition to suppressing melanogenesis, inhibited the activity of tyrosinase, the rate-limiting enzyme in melanogenesis (Fig. 3). Moreover, the inhibition effect of kojic acid was weaker at concentrations 1-100 μM than baicalein at concentrations 1-10 μM. These results show that baicalein decreases tyrosinase activity, and by this action decreases melanin synthesis.

Baicalein decreases tyrosinase and MITF protein levels but does not decrease MITF mRNA. MITF is an upstream intermediary that increases melanin synthesis by increasing transcription of the tyrosinase gene (16). To test whether baicalein decreases tyrosinase activity by acting on MITF, B16F10 melanoma cells were treated with different concentrations of baicalein for 48 h, and MITF and tyrosinase protein levels were then assayed by Western blotting (Fig. 4A). Baicalein treatment (1-10 μM) reduced both MITF and tyrosinase protein levels in a dose-dependent manner (Fig. 4A).

Because decreased MITF gene expression might be the mechanism responsible for diminished levels of MITF protein seen as a result of baicalein treatment, we examined whether baicalein (1-10 μM) decreased MITF gene transcription. At these concentrations, no significant change was seen in the levels of MITF mRNA in baicalein-treated cells (Fig. 4B).

Baicalein induces phosphorylation of ERK but not Akt. Baicalein decreased MITF protein levels, but not by decreasing the transcription of its gene. Phosphorylation of the upstream kinase, ERK, has been reported to increase MITF degradation by phosphorylating this protein (17,18). Therefore, we investigated whether baicalein induced ERK phosphorylation. Incubation with 10 μM baicalein (Fig. 5) caused an increase

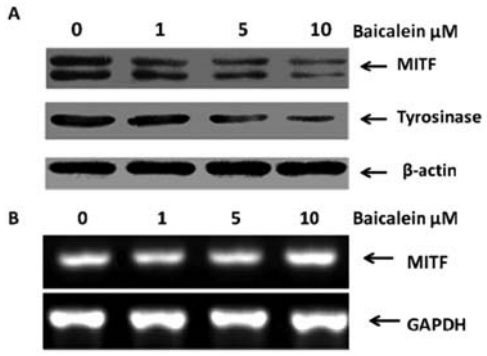

Figure 4. Baicalein decreases MITF and tyrosinase expression in B16 melanoma cells. B16 melanoma cells were treated with the indicated concentrations of baicalein for 48 h. (A) Dose-dependent decrease in MITF and tyrosinase protein levels. (B) No effects of baicalein on MITF mRNA levels are seen.

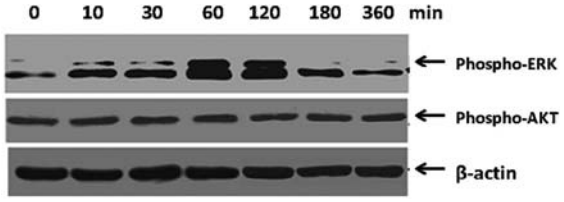

Figure 5. Baicalein induces ERK activation. B16 melanoma cells were treated with 10 μM baicalein for the indicated times. Whole cell lysates were then subjected to Western blot analysis using antibodies against phospho-specific ERK and Akt. Equal protein loadings were confirmed using anti-actin antibody.

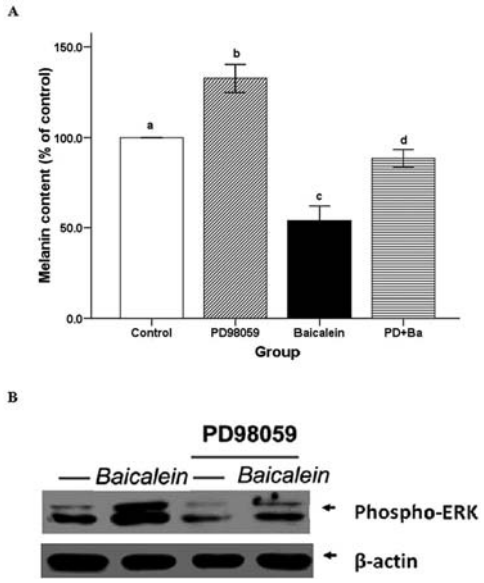

Figure 6. (A) Melanin content in cells treated with 20 μM PD98059, 10 μM baicalein or both for 48 h (n=9/per group). Mean ± standard deviation (SD) is shown. One-way ANOVA followed by Dunnett T3 test was performed. Different letters indicate significant differences between the treatments. (B) PD98059 blocks the baicalein-induced increase in phospho-ERK. After treatment for 1 h, whole cell lysates were subjected to Western blot analysis with antibodies against phospho-specific ERK. Equal protein loadings were confirmed using anti-ß-actin antibody.

in ERK kinase phosphorylation that reached high levels at 60-120 min of incubation. Phosphorylation of this enzyme had completely disappeared by 180 min.

The Akt pathway is another kinase pathway known to be involved in melanogenesis (19,20). Baicalein caused no change in the phosphorylation levels of this kinase (Fig. 5). These results show that baicalein induces phosphorylation of ERK but not of Akt, and reduces MITF protein, but not MITF mRNA, in B16F10 melanoma cells.

Effect of PD98059 on melanin synthesis in baicalein-treated B16F10 melanoma cells. Because baicalein appeared to decrease melanin synthesis by increasing the phosphorylation and activation of ERK, we investigated whether its effect on melanin synthesis is prevented by the addition of PD98059, a selective inhibitor of MEK (MAPK/ERK), the upstream activator of ERK. PD98059 decreased both baicalein-induced phosphorylation of ERK and baicalein-induced inhibition of melanogenesis (Fig. 6A and B). Thus, these results imply that baicalein-induced inhibition of melanogenesis occurs through phosphorylation of ERK.

Discussion

We have shown in this study that in B16F10 mouse melanoma cells, baicalein inhibits tyrosinase and melanogenesis indirectly in the following manner. It causes the phosphorylation of ERK and MITF, decreases MITF protein but not MITF mRNA, and decreases both tyrosinase activity and melanin levels. We did not examine whether baicalein had an additional direct inhibitory effect on the tyrosinase itself.

Recent studies have shown that baicalein exhibits anticancer (21), and antiproliferative activities (22). The ERK pathway, initiated through membrane receptor-induced increases in cAMP, regulates cell proliferation and differentiation in many types of cells (23,24) and this pathway is involved in the regulation of melanin synthesis (25). The Akt/PKB pathway, initiated through receptor binding and the production of inositol phospholipids, plays an important role in cell growth regulation and apoptosis inhibition (26,27). Activation of the Akt signaling pathway is also implicated in the regulation of melanogenesis (20). Baicalein acts through the ERK pathway and not through the Akt pathway. The relative roles and importance of the two pathways in regulating melanogenesis remain to be determined.

Several other melanogenesis inhibitors from natural sources are either in clinical use or are under investigation. Two direct inhibitors of tyrosinase, arbutin and kojic acid, are in clinical use. Terrein, a fungal metabolite, does not inhibit tyrosinase directly, but like baicalein, phosporylates ERK, but not Akt. However, terrein is active at the range of 10-100 μM and baicalein is active at 1-10 μM, a 10-fold lower concentration range (28).

Four substances, cimicifuga extract, haganin A, ceramide, and partially purified *Curcuma longa* (29-33) phosphorylate both ERK and Akt. Haganin A, *in vitro* is also a non-competitive inhibitor of tyrosinase. For all substances, both ERK and Akt inhibition decreased their effect on melanogenesis.

Most preceding studies of melanogenesis-inhibiting substances have been preformed *in vitro*, in cell culture. The compounds investigated offer a variety of methods to decrease

melanogenesis, competitive and non-competitive inhibition of the rate-limiting enzyme, inhibition of the ERK or Akt regulatory pathway or combinations of these mechanisms. The relative effectiveness and tolerability *in vivo* of chronic topical use for each of these compounds remains to be determined.

In summary, we investigated the hypopigmentary effect of baicalein and its underlying mechanism. In this study, we found that baicalein has a considerable ability to inhibit melanin synthesis but no accompanying cytotoxic activity. Our results suggest that baicalein inhibits melanogenesis in B16F10 melanoma cells by downregulating MITF through ERK activation leading to the inhibition of tyrosinase production. Therefore, to further delineate baicalein as a potential skin-whitening agent, we intend to examine the skin-whitening effects of baicalein in an animal model.

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