

Partially purified *Asiasari radix* inhibits melanogenesis through extracellular signal-regulated kinase signaling in B16F10 cells

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Abstract. The active fraction of the extract of *Asiasari radix*, the 60% methanol chromatographic fraction from the ethyl acetate layer (PPAR), was used to investigate the melanogenesis signal pathway in B16F10 melanoma cells. PPAR led to significantly decreased melanin synthesis and tyrosinase activity in a dose-dependent manner. PPAR also reduced the expression of melanogenesis-related proteins including microphthalmia-associated transcription factor (MITF), tyrosinase, and tyrosinase-related protein (TRP)s while down-regulating the expression of mRNA of MITF and tyrosinase. Melanogenesis-regulating signals, such as mitogen-activated protein kinase (MEK)/extracellular signal-regulated kinase (ERK), phosphatidylinositol 3-kinase (PI3K)/Akt and p38 mitogen-activated protein kinase (MAPK) were evaluated, and ERK was activated by PPAR. The selective inhibitor of MEK/ERK, PD98059, abrogated all suppressive activities of PPAR on melanin biosynthesis, tyrosinase activation and expression of melanogenesis-related proteins. These results suggest that ERK activation by PPAR contributes to reduced melanin synthesis via ERK signal pathway-mediated suppression of MITF and its downstream signal pathway.

Introduction

Melanogenesis is a multistage process that involves a cascade of enzymatic reactions regulated by tyrosinase and tyrosinase-related protein (TRP)s (1,2). Tyrosinase is a key enzyme that catalyses the hydroxylation of L-tyrosine to 3,4-dihydroxy-phenylalanine (DOPA) and the subsequent oxidation of DOPA into dopaquinone (3). The intracellular concentration of cAMP plays a critical role in melanogenesis through activation of protein kinase A (PKA) and cAMP response element binding protein (CREB) transcription factor and promotes an increase in the expression of microphthalmia-associated transcription factor (MITF). MITF plays an important role as a transcriptional activator of genes that encode melanogenesis-related proteins such as tyrosinase and TRPs (4,5).

It has been reported that activation of the extracellular signal regulated kinase (ERK) and phosphatidylinositol 3-kinase (PI3K)/Akt pathways induces hypopigmentation, suggesting that these signaling pathways regulate melanogenesis via MITF degradation or inhibition (6-8). Conversely, p38 mitogen-activated protein kinase (MAPK) has been implicated in melanogenesis via up-regulation of tyrosinase expression in B16F10 cells (9). Recent studies have focused on the specific mechanisms involved in melanogenesis to develop new therapeutic agents for skin pigmentation. The results of these studies have shown that inhibition of melanogenesis may control tyrosinase upstream signaling pathways such as a ERK, PI3K/Akt and p38 MAPK.

Asiasari radix has long been used in traditional Korean medicine to treat headaches, neuralgia, chronic bronchitis, asthma and allergies, as well as for its anti-bacterial effects. However, the extract of *A. radix* was found to significantly suppress melanin biosynthesis in our preliminary examinations, which indicated that some of its active ingredients influenced the signaling pathways involved in melanogenesis. In this study, we investigated the effects of partial purification from *A. radix* (PPAR) on the inhibition of melanin synthesis, including melanogenesis-related signaling pathways, in B16F10 cells.

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Materials and methods

Preparation of PPAR. Dry powdered roots of *Asiasarum sieboldii* (300 g) were immersed in 8 l of distilled water and boiled under reflux for 150 min. The resulting extract was then centrifuged (2,000 x g for 20 min at 4°C) and filtered through a 0.2 µm filter. The filtrate was then evaporated to dryness under reduced pressure. Next, the dried extract (102.3 g) was dissolved in water and further partitioned in succession with ethyl acetate (2 l x 5), n-butanol, and distilled water, which produced fractions of 21.6, 41.2, and 37.1 g, respectively. The ethyl acetate fraction was concentrated by rotary vacuum evaporation (Eyela, Tokyo, Japan). The 2.0 g residue was then subjected to octadesylsilane (ODS) open column chromatography using a 50x183 mm YMC-GEL (Kyoto, Japan) and eluted with the following, incrementally increasing solvent system: 100% distilled water, 20, 40, 60, 80, 100% methanol (MeOH), 100% dichloromethane. Seven fractions were obtained, concentrated under reduced pressure, and freeze-dried. The active 60% MeOH fraction (109.2 mg) was used as the PPAR and a voucher specimen (WC34-2-4) and deposited at the School of Korean Medicine, Pusan National University.

Materials. 3,4-dihydroxyphenylalanine (L-DOPA), Melanin, PD98059 and β-actin antibody were purchased from Sigma (St. Louis MO, USA). A monoclonal antibody to MITF was purchased from Millipore (Bedford, MA, USA). Tyrosinase (C-19), TRP1 (G-17), dopachrome tautomerase (Dct, D-18), phospho-MEK (pMEK, Ser218/Ser222), MEK (12-B), phospho-ERK1/2 (pERK1/2, E-4), ERK1/2 (K-23), phospho-Akt (pAkt, Ser473), Akt (C-20), phospho-p38 (pp38, D-8) and p38 (H-147) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies recognizing phospho-CREB (pCREB, Ser133, 1B6) and CREB (48H2) were obtained from Cell Signaling Technology (Danver, MA, USA). All other reagents used in this study were of the highest quality available.

Cell culture. B16F10 mouse melanoma cells (CRL 6323) were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco Life Technologies, France) with 10% fetal calf serum and 1% penicillin/streptomycin in a 5% CO₂ humidified incubator at 37°C.

MTT assay. The general viability of the cultured cells was determined through the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) to formazan as described previously (10). After treating the PPAR, cells were incubated for 48 h at 37°C in an atmosphere containing 5% CO₂. MTT (1 mg/ml in PBS) was then added to each well at 1/10 volume of media. Cells were incubated at 37°C for 3 h, and DMSO was to dissolve the formazan crystals. The absorbance at 570 nm was measured using a spectrophotometer (Spectra MAX 190, Molecular Devices, Sunnyvale, CA, USA).

Determination of the melanin content. The melanin content was measured using a slight modification of the method described by Tsuboi *et al* (11). Briefly, cells were washed with PBS and then dissolved in 1 N NaOH in 10% DMSO at 80°C for 1 h. The absorbance was then measured at 475 nm,

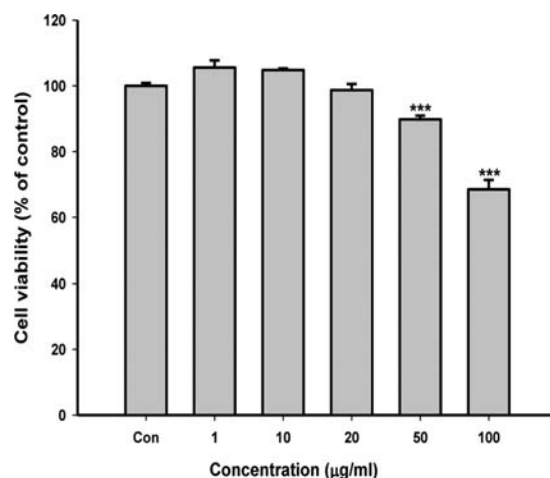


Figure 1. Effect of PPAR on B16F10 cell viability. Cells were treated with various concentrations of PPAR for 48 h. Cell viability was determined by an MTT assay. Each percentage value in treated cells was calculated with respect to that in control cells. Data expressed as the means ±SEM of three independent experiments carried out in triplicate. *P<0.05, **P<0.01, ***P<0.001 vs control.

with standard melanin being used to quantify the melanin content and expressed as a percentage of the control.

Measurement of tyrosinase activity. The tyrosinase activity was measured based on its dopa oxidase activity using a slight modification of the method reported by Tomita *et al* (12). Briefly, cells were lysed by incubation at 37°C for 30 min in RIPA buffer (0.1 M sodium phosphate, pH 7.0, 1% Triton X-100, 0.1 mM PMSF, 1 mM NaF). Lysates were then centrifuged at 10,000 x g for 20 min to obtain the supernatant as the crude tyrosinase extract for the activity assay. The reaction mixture contained 0.1 M sodium phosphate (pH 7.0), 0.05% L-DOPA and the supernatant (tyrosinase source). After incubation at 37°C for 1 h, the dopachrome was monitored by measuring the absorbance at 405 nm in a microplate reader.

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was prepared from B16F10 melanoma cells using Trizol reagent™ (Invitrogen, Paisley, UK) according to the manufacturer's protocols. cDNA was synthesized using 2 µg of total RNA and oligo_{dT}(18) primer with taq polymerase (Promega) in a 20-µl reaction system. Reverse transcription was conducted by incubating the mixture at 37°C for 45 min and then termination at 95°C for 5 min. The following primers were used, 5'-CCCGTCTCTGGAACTTGATCG-3' (forward), and 5'-CTGTACTCTGAGCAGCAGGTG-3' (reverse) for MITF; 5'-GAGAAGCGAGTCTTGATTAG-3' (forward) and 5'-TGG TGCTTCATGCGCAAATC-3 (reverse) for tyrosinase; 5'-ATGAGAAGGAGATCACTGC-3' (forward) and 5'-CTG CGCAAGTTAGGTTTTGT-3' (reverse) for β-actin. PCR was conducted by subjecting the reaction mixture to 30 cycles of 30 sec at 94°C, 30 sec at 55°C, and 60 sec at 72°C. The PCR products were then electrophoresed on 1.5% agarose gels and stained with ethidium bromide. All data were normalized to β-actin primers.

Western blot analysis. Cells were cultured and then harvested using RIPA buffer A containing 20 mM Tris-HCl, pH 8.0,

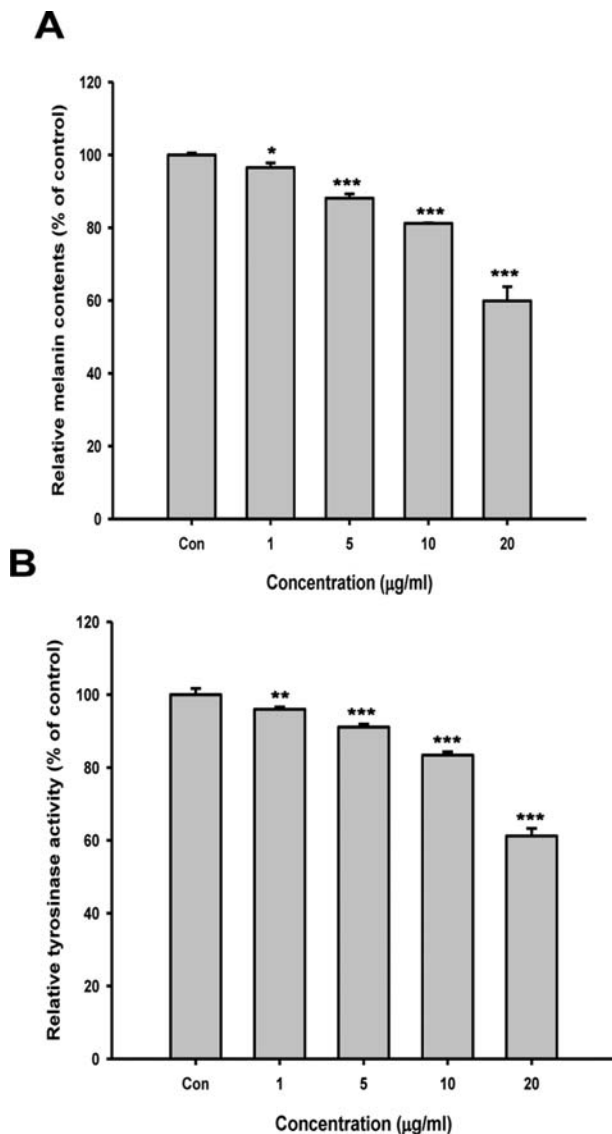


Figure 2. Effect of PPAR on cellular melanin synthesis (A) and tyrosinase activity (B) in B16F10 cells. Cells were treated with 1-20 µg/ml of PPAR. Each percentage value for treated cells was reported relative to that of control cells. The results shown are the means \pm SEM and representative of three independent experiments. *P<0.05, **P<0.01, ***P<0.001 versus control.

150 mM NaCl, 1% NP-40, 2 mM EDTA, 1 mM PMSF, 10 mM NaF, 1 mM Na₃VO₄ and protease inhibitors (Sigma). The protein concentrations were then determined by a Bradford assay (BioRad, Richmond, CA, USA), after which 30 µg of protein were separated by electrophoresis on a 10% SDS-polyacrylamide gel and then transferred to a nitrocellulose membrane (Whatman, Germany). The membrane was blocked with 5% skim milk and incubated with MITF (1:200), tyrosinase (1:250), TRP1 (1:250), Dct (1:250), anti-pMEK (1:1,000), anti-MEK (1:1,000), anti-pERK1/2 (1:1,000), anti-ERK1/2 (1:1,000), anti-pAkt (1:1,000), anti-Akt (1:1,000), anti-p38MAPK (1:1,000), anti-p38 MAPK (1:1,000), anti-pCREB (1:1,000) or anti-CREB (1:1,000). All bands were visualized using horseradish peroxidase-conjugated secondary antibodies (1:2,000, Santa Cruz Biotech, CA, USA) using an enhanced chemiluminescence system (Pierce Biotech, Rockford, IL, USA). The results of the Western blotting assays reported here are representative of at least three experiments.

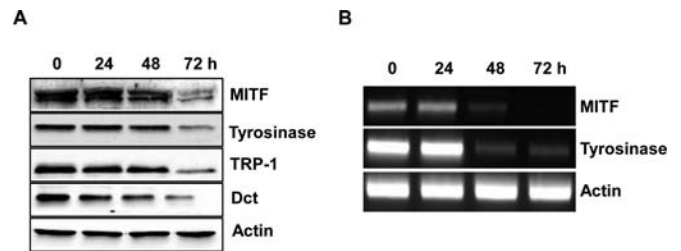


Figure 3. Time-course effect of PPAR on the expression of melanogenesis-related proteins in B16F10 cells. Cells were treated with 20 µg/ml of PPAR for the indicated times. (A) Western blotting assays were conducted to evaluate the expression levels of MITF, tyrosinase, TRP1, Dct and β -actin on the same blot. (B) Representative RT-PCR results of MITF, tyrosinase mRNA expression. Average mRNA expression as normalized with β -actin.

Statistical analysis. All data were expressed as the mean \pm SEM. The means, standard errors, and Student's t-test were determined using SigmaPlot (San Jose, CA, USA). A P-value <0.05 was considered to indicate statistical significance.

Results

Effects of PPAR on melanin biosynthesis and tyrosinase activity. Initially, we investigated the cytotoxic effects of PPAR in cells treated with 1-100 µg/ml of the extract. PPAR showed no cytotoxic effect on B16F10 cells at a concentration of 0-20 µg/ml (Fig. 1). To determine the effects of PPAR on melanin synthesis and tyrosinase activity, cells were exposed to concentrations ranging from 0-20 µg/ml. As shown in Fig. 2, the cellular melanin content and tyrosinase activity decreased significantly in response to treatment with PPAR and this decrease occurred in a dose-dependent fashion. These findings suggest that PPAR regulates tyrosinase activity and subsequently inhibits melanin synthesis in B16F10 cells.

Effect of PPAR on the expression of melanogenic proteins and mRNA. To investigate the effect of PPAR on melanogenesis-related proteins, B16F10 cells were treated with 20 µg/ml of PPAR for 24, 48 and 72 h. As shown in Fig. 3A, the expression of melanogenic proteins showed time-dependent down-regulation in response to treatment with PPAR. The effects of PPAR on the level of MITF and tyrosinase mRNA expression were also evaluated. The results revealed that the mRNA levels of MITF and tyrosinase decreased significantly in a time-dependent manner in response to PPAR (Fig. 3B). These findings indicate that the inhibitory activity of PPAR does not directly suppress the activity of tyrosinase as much as it regulates the tyrosinase upstream signaling pathway related to its activation and expression.

Effect of PPAR on melanogenesis-related signaling pathways. Activation of the ERK, PI3K/Akt and p38 MAPK pathways was involved in the regulation of melanogenesis. Therefore, we examined PPAR to determine if it influenced the MEK/ERK, PI3K/Akt and p38 MAPK signaling pathways with transcription factor CREB. As shown in Fig. 4, PPAR significantly up-regulated activation of ERK1/2 from early time points in B16F10 cells. However, PPAR did not activate PI3K/Akt or p38. These results suggest that the suppressive mechanism of PPAR on

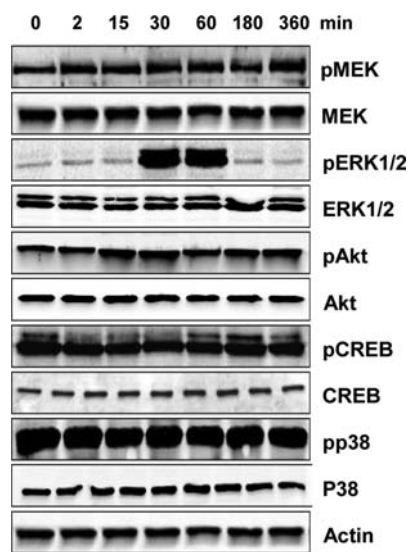


Figure 4. Effect of PPAR on the expression of melanogenesis-related proteins in B16F10 cells. After 24 h of starvation, B16F10 cells were treated with 20 μ g/ml PPAR for the indicated times. Western blotting assays were used to examine the phosphorylation of MEK, ERK1/2, Akt, CREB and p38 MAPK. Equal protein loading was confirmed by β -actin expression.

melanogenesis is involved in activation of the ERK signaling pathway.

Effect of PD98059 on the suppressive mechanism of PPAR. Because PPAR activates the phosphorylation of ERK, we examined PD98059, a selective inhibitor of MEK/ERK, to determine if it inhibited the suppressive activity of PPAR on melanogenesis. As shown in Fig. 5A and B, the melanin synthesis and tyrosinase activity in cells co-treated with PPAR and PD98059 was higher than in cells treated with PPAR alone. PD98059 abrogated PPAR-induced MITF down-expression and partially recovered tyrosinase, TRP1 and Dct expression (Fig. 5C). These results suggest that ERK activation mediated by PPAR contributes to reduced melanin synthesis via MITF and its downstream signal pathway.

Discussion

A. radix, which primarily originates from *Asiasarum sieboldii* or *Asiasarum heterotropoides*, has long been used for the treatment of various pains and inflammatory disorders of the

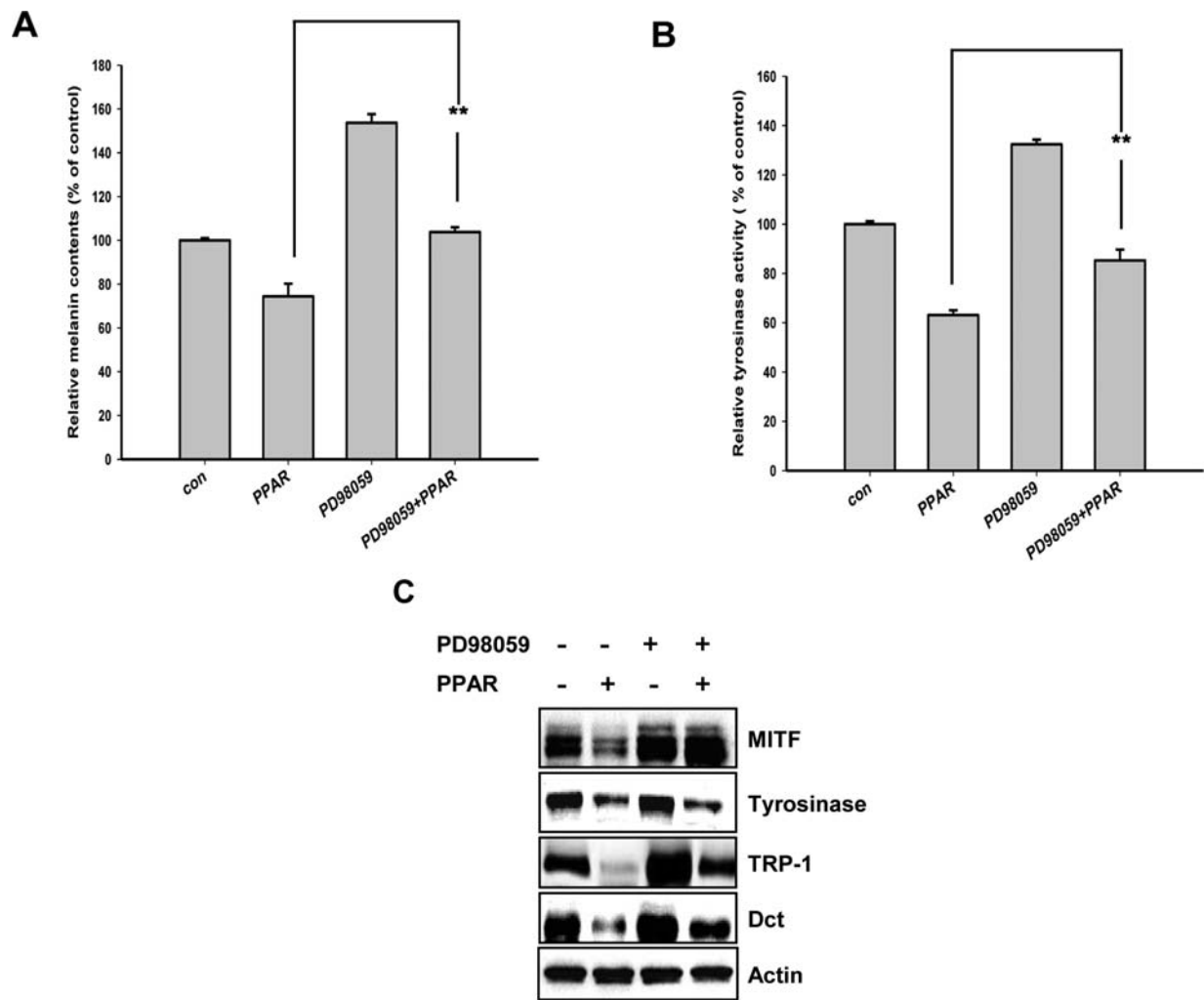


Figure 5. Effect of PD98059 pretreatment on cellular melanin synthesis (A) and tyrosinase activity (B) in B16F10 cells. B16F10 cells were pretreated in the absence (-) or presence (+) of 20 μ M of PD98059 for 1 h followed by incubation with 20 μ g/ml of PPAR. The results shown are the means \pm SEM and representative of three independent experiments. ** P <0.01 versus control. (C) B16F10 cells were pretreated in the absence (-) or presence (+) of 20 μ M PD98059 for 1 h, after which the cells were treated with or without 20 μ g/ml of PPAR. Proteins were extracted and subjected to Western blotting assays and the expression levels of MITF, tyrosinase, TRP1 and Dct and β -actin were determined on the same blot.

respiratory tract in traditional Korean medicine. Accordingly, considerable effort has been directed towards evaluating the various biological activities of *A. radix*, particularly its antinociception and memory enhancing effects (13,14). The extract of *A. radix* enhances memory via activation of ERK1/2 in the rat hippocampus (14), which suggests that *A. Radix* contains active ingredients that would be useful for regulation of melanogenesis-related signal pathways, including inhibition of MITF-tyrosinase signaling and/or activation of MEK/ERK, PI3K/Akt and p38 MAPK signaling.

Therefore, in this study, bioassay-guided purification was conducted by determining the inhibitory effect of *A. radix* on melanin synthesis and tyrosinase activation in B16F10 cells. The ethyl acetate layer was concentrated and the residual aqueous suspension and subjected to ODS open column chromatography with aqueous MeOH followed by dichloromethane. The results revealed that the melanogenesis-inhibitory activity was concentrated in the 60% MeOH fraction (fraction four).

Partial purification was then used to investigate the effects of this extract on melanogenesis in B16F10 cells. We found that decreased melanin synthesis and tyrosinase activity was accompanied by reduced MITF, tyrosinase and TRPs expression (Figs. 2 and 3). Therefore, we evaluated MITF and tyrosinase mRNA expression to determine if PPAR had an effect on the MITF transcription level. The results revealed that MITF and tyrosinase mRNA decreased in a time-dependent fashion in PPAR-treated cells (Fig. 3B).

These results indicate that the suppressive activity of PPAR on melanogenesis is not due to the direct down-regulation of tyrosinase activity. The suppressive activity of PPAR on melanogenesis is linked to down-regulation of MITF-tyrosinase expression signaling pathways. Transcriptional regulation of melanogenic proteins is primarily dependent on the expression of MITF, which is a master transcriptional regulator of key melanogenic enzymes.

Recently, several reports have indicated that the activation of ERK and PI3K/Akt signaling reduced melanin synthesis through MITF degradation and inhibition, respectively. Activation of ERK by c-kit stimulation phosphorylates MITF at serine 73, and the phosphorylated MITF is followed by MITF ubiquitination and degradation (15-17). Activation of the PI3K/Akt signaling pathway has also been implicated in the inhibition of melanogenesis (18,19). The inhibition of PI3K/Akt signaling plays a key role in the phosphorylation of MITF on serine 298, which leads to stimulation of tyrosinase expression (8).

Conversely, it is well known that the p38 MAPK cascade is involved in melanogenesis in B16 melanoma cells. The activation of p38 MAPK up-regulates MITF expression and promotes the transcription of tyrosinase (20). CREB is a MITF transcription factor that is involved in the melanogenic signaling pathway through phosphorylation. Phosphorylated CREB interacts with CREB binding protein (CBP) to activate the expression of MITF, which results in the indirect activation of tyrosinase promoter by MITF (21).

Therefore, we investigated PPAR to determine whether it activates the MEK/ERK, PI3K/Akt and p38 MAPK pathways with CREB in B16 melanoma cells. As shown Fig. 4, we found that PPAR only induced activation of the ERK pathway from

the early time points, and it did not activate the Akt or p38 MAPK pathways. To confirm the involvement of ERK signaling, we evaluated the effects of PD98059 as a selective MEK/ERK inhibitor on PPAR-induced down-regulated melanogenesis. The PPAR-reduced melanin synthesis was recovered by PD98059 via abrogation of PPAR-mediated MITF, tyrosinase and TRPs expression (Fig. 5).

Generally, our results show that ERK signaling was activated by PPAR and that the MITF and tyrosinase mRNA and protein levels were attenuated. Furthermore, treatment with a selective MEK/ERK inhibitor abrogated the PPAR-mediated activity of melanogenesis. These results suggest that ERK plays an important role in PPAR-mediated hypopigmentation through MITF and its downstream signal pathway, including tyrosinase and TRPs. The ERK activation induces MITF phosphorylation and degradation, which results in reduced tyrosinase expression and melanin synthesis (6). In the present study, phosphorylated CREB was slightly decreased at early time points and the transcriptional level of MITF was also reduced by PPAR. We propose that PPAR inhibits pigmentation by indirectly regulating tyrosinase via ERK activation and subsequent MITF down-regulation. However, there is a possibility that CREB activation by PPAR is partially controlled at the MITF transcriptional level with ERK-mediated MITF degradation.

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