Okadaic Acid Suppresses Melanogenesis via Proteasomal Degradation of Tyrosinase

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Okadaic acid is a C 38 fatty acid derivative that is known to specifically inhibit the activity of protein phosphatase 2A (PP2A). Previously, we reported that inhibition of PP2A by okadaic acid elicited extracellular signal-regulated kinase (ERK) activation, and that PP2A may be involved in melanogenesis. However, the effects of okadaic acid on melanogenesis have not been completely evaluated. In the present study, we investigated the molecular mechanisms involved in okadaic acid modulation of melanin synthesis in a spontaneously immortalized mouse melanocyte cell line, Mel-Ab. Treatment with okadaic acid inhibited melanin production in a dose-dependent manner. Moreover, okadaic acid led to a decrease in tyrosinase protein levels without altering mRNA expression. Therefore, we investigated whether the decreased level of tyrosinase by okadaic acid was related to proteasomal degradation of tyrosinase. We found that MG132, a proteasome inhibitor, almost completely abolished both the downregulation of tyrosinase levels and the inhibition of melanin synthesis by okadaic acid. Taken together, our data indicate that okadaic acid inhibits melanin synthesis via proteasomal degradation of tyrosinase.

Key words melanocyte; melanogenesis; tyrosinase; okadaic acid

The color of human skin is mainly due to the pigment melanin, which is produced within melanosomes, a specialized intracellular organelle of melanocytes. Melanogenesis is promoted by various stimulators such as UV irradiation, cytokines, growth factors, and hormones. Tyrosinase is the critical enzyme involved in melanogenesis as it catalyses the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (DOPA) and the oxidation of DOPA to dopaquinone. Thus, melanin synthesis is regulated predominately by the expression and activity of tyrosinase.

Microphthalmia-associated transcription factor (MITF) is known to regulate the expression of genes related to diverse biological processes such as proliferation, survival, and pigmentation. MITF is a key transcription regulator that binds to the M box of the tyrosinase promoter and controls the expression of tyrosinase. Moreover, MITF expression has been reported to be involved in the Wnt pathway. Activation of the Wnt signaling pathway induces glycogen synthase kinase 3β (GSK3β) inactivation (phosphorylation) and subsequent β-catenin accumulation. Accumulated β-catenin then binds the lymphoid-enhancing factor/T-cell factor (LEF/TCF) in the nucleus, thereby ultimately enhancing MITF expression. In contrast, GSK3β is known to phosphorylate MITF at Ser298 and augment the affinity of MITF for the tyrosinase promoter, resulting in increased tyrosinase mRNA expression.

Okadaic acid is a C 38 fatty acid (C 38H 68O 13) derivative that specifically inhibits the activity of protein phosphatase 2A (PP2A), a serine/threonine phosphatase. PP2A has been reported to regulate the activity of extracellular signal-regulated kinase (ERK) family kinases. We previously reported that inhibition of PP2A by okadaic acid elicits ERK activation and that there may be an association between melanogenesis and PP2A regulation. However, the effects of okadaic acid on melanogenesis have not been completely investigated. In the present study, we examined the molecular mechanisms involved in okadaic acid regulation of melanin synthesis in Mel-Ab cells.

MATERIALS AND METHODS

Reagents and Antibodies Okadaic acid, 12-O-tetradecanoylphorbol-13-acetate (TPA), cholera toxin (CT), Triton X-100, Tris, β-mercaptoethanol, phenylmethylsulfonyl fluoride, synthetic melanin, α-melanocyte-stimulating hormone (α-MSH), and α-DOPA were all purchased from Sigma (St. Louis, MO, U.S.A.). MG132 was obtained from Calbiochem (San Diego, CA, U.S.A.). Okadaic acid and MG132 were dissolved in dimethyl sulfoxide (DMSO), and α-MSH in distilled water. Fetal bovine serum (FBS) was purchased from HyClone (Logan, UT, U.S.A.) and the protease inhibitor cocktail Complete was obtained from Roche (Mannheim, Germany). Antibodies used in this study included phosho-specific ERK1/2 (Thr202/Tyr204, no. 9101), phosho-specific Akt (Ser473, no. 9271), phosho-specific GSK3β (no. 9336), total GSK3β (no. 9315), and β-catenin (no. 9581), which were from Cell Signaling Technology (Beverly, MA, U.S.A.). Okadaic acid, MG132, and α-MSH were purchased from NeoMarkers (Fremont, CA, U.S.A.). Microphthalmia Ab-1 (CS, MS-771-P0) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.).

Cell Culture For many experiments in this study we used Mel-Ab cells, a spontaneously immortalized mouse melanocyte cell line that synthesizes large quantities of melanin. Mel-Ab cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS, 1 ng CT, 100 ng TPA, 100 units/mL penicillin, and 100 µg/mL penicillin.

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streptomycin at 37°C in 5% CO₂. B16F10 murine melanoma cells were cultured in DMEM supplemented with 10% FBS, 100 units/mL penicillin, and 100 µg/mL streptomycin at 37°C in 5% CO₂.

**Cell Viability Assay** Cell viability was estimated using a crystal violet assay. After treating Mel-Ab or B16F10 cells with okadaic acid for 4 d or 3 d, respectively, cells were stained with 0.1% crystal violet in 10% ethanol for 5 min at room temperature and then rinsed four times with distilled water. The crystal violet retained by adherent cells was extracted with 95% ethanol and cell viability was analyzed by measuring absorbance at 590 nm with an enzyme-linked immunosorbent assay (ELISA) reader (VERSAMax; Molecular Devices, Sunnyvale, CA, U.S.A.).

**Measurement of Melanin Content** Mel-Ab cells were incubated with okadaic acid for 3 or 4 d, observed under a phase-contrast microscope (Olympus Optical Co., Tokyo, Japan), and photographed using a DCM300 digital camera (Scopetek, Inc., Hangzhou, China) supported by ScopePhoto software (Scopetek, Inc.). Melanin content was analyzed as described previously. Briefly, cells were treated with okadaic acid and then harvested. The resulting cell pellets were dissolved in 1 mL of 1 N NaOH at 100°C for 30 min and centrifuged for 20 min at 16000×g. The optical densities (ODs) of the supernatants were assessed at 400 nm using an ELISA reader. Standard curves of synthetic melanin (0–300 µg/mL) were prepared in triplicate for each experiment. B16F10 cells were incubated in six-well plates at a density of 5×10⁴ cells/well for 24 h. The cells were treated with okadaic acid and incubated for 3 d in the presence of α-MSH (1 µM). The supernatant was analyzed at 400 nm and the number of cells was then enumerated using a hemocytometer.

**Western Blot Analysis** Cells were lysed in cell lysis buffer containing 62.5 mM Tris–HCl pH 6.8, 2% sodium dodecyl sulfate (SDS), 5% β-mercaptoethanol, 2 mM phenylmethylsulfonyl fluoride, protease inhibitor cocktail, 1 mM Na₃VO₄, 50 mM NaF, and 10 mM ethylenediaminetetraacetic acid (EDTA). Aliquots of cell lysates were separated by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride (PVDF) membranes, which were then blocked with 5% skim milk in Tris-buffered saline containing 0.5% Tween20. Blots were incubated with the appropriate primary antibodies at a dilution of 1:1000, and then further incubated with horseradish peroxidase-conjugated secondary antibody. All blots were developed using an enhanced chemiluminescence detection system (Thermo Fisher Scientific Inc., Rockford, IL, U.S.A.).

**Reverse Transcription-Polymerase Chain Reaction (RT-PCR)** To characterize mRNA expression, total RNA was isolated from cells using an RNeasy Mini kit (Qiagen, Valencia, CA, U.S.A.). Then, 1 µg of RNA was reverse transcribed using the iScript cDNA Synthesis System (Bio-Rad Laboratories, Hercules, CA, U.S.A.). The obtained cDNA was amplified with specific primers for tyrosinase (forward, 5′-GGCCGAGCTTTCGAGGAGGATG-3′ and reverse, 5′-TTGCTGCTTCTATGGGCAAAATC-3′). The PCR conditions for tyrosinase were 30 cycles of the following: 1 min at 95°C, 1 min at 59°C, and 1 min at 72°C. The resulting PCR products were visualized by electrophoretic separation on 1.5% agarose gels with Safe-Pink® DNA gel stain (Gendepot, Barker, TX, U.S.A.). Specific primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as a control.

**Statistical Analysis** Statistical significance between groups was determined by Student’s t-test and a significance level of p<0.05 was used.

**RESULTS**

**Effect of Okadaic Acid on Cell Viability** The effect of okadaic acid on cell viability was estimated using a crystal violet assay. Mel-Ab and B16F10 cells were incubated with okadaic acid for 4 d and 3 d, respectively, at concentrations of 0–200 nM. Treatment with okadaic acid showed no effects on the viability of Mel-Ab cells or B16F10 cells over a concentration range of 0–100 nM, indicating that okadaic acid is not cytotoxic to the cells at concentrations of 0–100 nM (Figs. 1A, B). However, okadaic acid at 200 µM was found to be cytotoxic in Mel-Ab cells and B16F10 cells.

**Effects of Okadaic Acid on Melanin Synthesis** To investigate the effect of okadaic acid on melanin production, Mel-Ab cells were incubated with okadaic acid at concentrations of 0–100 nM. Okadaic acid treatment significantly decreased the melanin content of Mel-Ab cells (Fig. 1C), indicating that okadaic acid induces obvious hypopigmentation in these cells. To confirm the effects of okadaic acid on melanogenesis, we performed the same experiments with the B16F10 cell line. B16F10 cells were treated with okadaic acid (0–100 nM) for 3 d in the presence of α-MSH (1 µM). Under these conditions, okadaic acid exhibited obvious inhibitory effects on melanin synthesis in B16F10 cells (Fig. 1D). Furthermore, Mel-Ab cells were observed under a phase contrast microscope and, as shown in Fig. 1E, were found to have reduced melanin pigmentation following okadaic acid treatment, in a dose-dependent manner.

**Effects of Okadaic Acid on the Signaling Pathways Involved in Melanogenesis** It has been suggested that the ERK and Akt signaling pathways are involved in the regulation of melanin synthesis. Moreover, okadaic acid has been reported to induce ERK phosphorylation. Thus, we investigated whether okadaic acid treatment affected other signaling pathways, in addition to ERK, in melanocytes. We found that while okadaic acid induced ERK activation shortly after being introduced to cells, Akt activation was not altered (Fig. 2A). In addition, okadaic acid led to GSK3β phosphorylation, though levels of β-catenin did not change in okadaic acid-treated cells (Fig. 2A).

We next examined changes in MITF and tyrosinase protein levels following okadaic acid treatment in long-term cultures (Fig. 2B). Despite having hypopigmentary effects, okadaic acid treatment did not change MITF protein levels. In contrast, okadaic acid decreased tyrosinase protein levels after 24–48 h of incubation. The protein levels of tyrosinase were recovered at 72 h post-okadaic acid treatment. Moreover, GSK3β phosphorylation (inactivation) was induced in a time-dependent manner by okadaic acid (Fig. 2C). It has been reported that activated (dephosphorylated) GSK3β phosphorylates MITF at Ser298 and ultimately enhances melanin mRNA expression in melanocytes. Our results suggested that okadaic acid could reduce the mRNA level of tyrosinase and thus, we investigated the transcription level of tyrosinase mRNA by RT-PCR. However, as shown in Fig. 2D, okadaic acid did not decrease tyrosinase mRNA levels in a manner...
that was comparable to that of tyrosinase protein.

Okadaic Acid Regulates Levels of Tyrosinase via Proteasomal Degradation  We next determined if the decrease in tyrosinase level was due to proteasome-mediated proteolytic degradation by okadaic acid. Melanocytes were incubated with okadaic acid for 3 d in the absence or presence of proteasome inhibitor MG132, after which time melanin content was determined. MG132 abrogated the inhibition of melanin synthesis by okadaic acid (Fig. 3B). Taken together, these results indicate that okadaic acid may inhibit melanin synthesis through the proteasomal degradation of tyrosinase.

DISCUSSION

The literature indicates that there may be a relationship between PP2A activity and melanin synthesis.\(^{14,15}\) Previously, we reported that heat treatment decreases pigmentation through PP2A inactivation.\(^ {14}\) Moreover, PP2A is involved in
melanososome aggregation\textsuperscript{21}) and may also participate in melanogenesis\textsuperscript{17}). In the present study, we investigated the effects of okadaic acid, a PP2A inhibitor, on melanin synthesis. We found that okadaic acid treatment decreased melanin content in Mel-Ab cells and B16F10 cells. These data indicate that okadaic acid has a hypopigmentary effect.

It has been previously reported that ERK activation decreased melanin synthesis via MITF downregulation\textsuperscript{18,20}). Although okadaic acid (100 nM) slightly induced ERK activation, MITF protein levels and tyrosinase mRNA levels were not changed (Figs. 2B, D). Thus, okadaic acid-induced ERK activation may not be involved in hypopigmentation.

Active GSK3\textbeta\ is known to phosphorylate MITF at Ser298 and enhance MITF binding to the tyrosinase promoter, ultimately augmenting tyrosinase mRNA expression\textsuperscript{10}). Okadaic acid treatment is also known to induce GSK3\textbeta phosphorylation (inactivation) in a model of neurodegeneration\textsuperscript{22}). We observed that okadaic acid treatment elicited GSK3\textbeta phosphorylation (inactivation) in Mel-Ab cells (Fig. 2C). However, we found that okadaic acid did not affect tyrosinase mRNA levels, unlike its effect on tyrosinase protein levels (Fig. 2D). Therefore, we ruled out the involvement of GSK3\textbeta phosphorylation in okadaic acid-induced hypopigmentation.

As okadaic acid treatment resulted in decreased tyrosinase protein levels without changing mRNA expression, it was likely that tyrosinase protein levels were being regulated by protein degradation. Thus, we investigated whether the downregulation of tyrosinase by okadaic acid was a result of proteasome-mediated proteolytic degradation. As expected, MG132, a proteasome inhibitor, almost completely abrogated the downregulation of tyrosinase levels by okadaic acid (Fig. 3B). Furthermore, MG132 treatment eliminated the decrease of melanin content by okadaic acid (Fig. 3A).

Recently, the p38 mitogen-activated protein kinase (MAPK) pathway has been reported to be involved in tyrosinase degradation\textsuperscript{23}). Thus, we determined whether okadaic acid induces the phosphorylation of p38 MAPK in Mel-Ab cells. Although p38 MAPK was phosphorylated in okadaic acid-treated cells, SB203580, a p38 MAPK inhibitor, did not abrogate the inhibition of melanin synthesis by okadaic acid, indicating that p38 MAPK may not be the primary signaling pathway involved in okadaic acid-induced hypopigmentation (data not shown).

Interestingly, fatty acids have been shown to regulate melanogenesis via tyrosinase degradation. Specifically, linoleic acid (an unsaturated fatty acid) expedited the proteasomal degradation of tyrosinase, whereas palmitic acid (a saturated fatty acid) delayed this process\textsuperscript{24,25}). Given that okadaic acid is a fatty acid derivative, our results indicate that okadaic acid may regulate the tyrosinase level via the ubiquitin-proteasome pathway.
In summary, the present study evaluated the effects of okadaic acid on melanogenesis. Our results suggest that okadaic acid decreases melanin synthesis. Moreover, we found that okadaic acid downregulated the tyrosinase level and the results are presented below each lane.

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