Possible involvement of proteolytic degradation of tyrosinase in the regulatory effect of fatty acids on melanogenesis

Hideya Ando,[1,*,†,§] Yoko Funasaka,[*] Masahiro Oka,[*] Akiko Ohashi,[*] Minao Furumura,[†] Jun Matsunaga,[†] Naoko Matsunaga,[†] Vincent J. Hearing,[†] and Masamitsu Ichihashi[*]

Department of Dermatology,* Kobe University School of Medicine, Kobe, Japan; Laboratory of Cell Biology,† National Cancer Institute, National Institutes of Health, Bethesda, MD; Central Research Institute,§ SUNSTAR Inc., Takatsuki, Osaka, Japan

Abstract **The purpose of this study was to investigate the mechanism of fatty acid-induced regulation of melanogenesis. An apparent regulatory effect on melanogenesis was observed when cultured B16F10 melanoma cells were incubated with fatty acids, i.e., linoleic acid (unsaturated, C18:2) decreased melanin synthesis while palmitic acid (saturated, C16:0) increased it. However, mRNA levels of the melanogenic enzymes, tyrosinase, tyrosinase-related protein 1 (TRP1), and tyrosinase-related protein 2 (TRP2), were not altered. Regarding protein levels of these enzymes, the amount of tyrosinase was decreased by linoleic acid and increased by palmitic acid, whereas the amounts of TRP1 and TRP2 did not change after incubation with fatty acids. Pulse-chase assay by [^{35}S]methionine metabolic labeling revealed that neither linoleic acid nor palmitic acid altered the synthesis of tyrosinase. Further, it was shown that linoleic acid accelerated, while palmitic acid decelerated, the proteolytic degradation of tyrosinase. These results suggest that modification of proteolytic degradation of tyrosinase is involved in regulatory effects of fatty acids on melanogenesis in cultured melanoma cells.**—Ando, H., Y. Funasaka, M. Oka, A. Ohashi, M. Furumura, J. Matsunaga, N. Matsunaga, V. J. Hearing, and M. Ichihashi. **Possible involvement of proteolytic degradation of tyrosinase in the regulatory effect of fatty acids on melanogenesis.** ***J. Lipid Res.*** **1999.** 40: **1312–1316.**

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Free fatty acids have been shown to have remarkable regulatory effects on melanogenesis in cultured B16F10 murine melanoma cells. Unsaturated fatty acids, such as oleic acid (C18:1), linoleic acid (C18:2), or α-linolenic acid (C18:3), decreased melanin synthesis and tyrosinase activity, while saturated fatty acids, such as palmitic acid (C16:0) or stearic acid (C18:0), increased it (1–3).

Melanin synthesis is regulated by melanogenic enzymes, e.g., tyrosinase, tyrosinase-related protein 1 (TRP1), and tyrosinase-related protein 2 (TRP2). Tyrosinase is a bifunctional enzyme which plays a pivotal role in the modulation of melanin production (4), first by catalyzing the hydroxylation of tyrosinase to DOPA and, in a second enzymatic step, by catalyzing the oxidation of DOPA to DOPAquinone. TRP2, which functions as DOPAchrome tautomerase, catalyzes the rearrangement of DOPAchrome to 5,6-dihydroxyindole-2-carboxylic acid (DHICA), and TRP1 oxidizes DHICA to a carboxylated indole-quinone, both of them working at downstream points in the melanin biosynthetic pathway (5).

A previous study demonstrated that linoleic acid or palmitic acid had little effect on tyrosinase mRNA levels although these fatty acids regulated tyrosinase activity dramatically (2). Those data suggested that fatty acid-induced regulation of tyrosinase activity is due to post-transcriptional events. However, there have been no reports as to how fatty acids act in the post-transcriptional regulation of tyrosinase activity. Moreover, effects on melanogenic enzymes other than tyrosinase, i.e., TRP1 and TRP2, by fatty acids have not yet been evaluated. The purpose of this study was to investigate the mechanism of fatty acid-induced regulation of melanogenesis, especially for the fatty acid-induced post-transcriptional control of tyrosinase, and to examine the effect of fatty acids on TRP1 and TRP2.

MATERIALS AND METHODS

Cell culture

B16F10 murine melanoma cells were cultured in Eagle's minimal essential medium, supplemented with 10% heat-inactivated (56°C, 30 min) free fatty acid-free fetal bovine serum and 2 mm l-glutamine, at 37°C in a humidified atmosphere containing 5%

Abbreviations: BSA, bovine serum albumin; DHICA, 5,6-dihydroxyindole-2-carboxylic acid; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; TRP, tyrosinase-related protein.

[1] To whom correspondence should be addressed.

CO_2. Free fatty acid-free fetal bovine serum was prepared using three extractions with equal volumes of diethyl ether in a separation funnel to remove free fatty acids from fetal bovine serum (2). The residual ether in the fetal bovine serum was then removed using an evaporator. Twenty-five μm linoleic acid or palmitic acid, or an equal volume of solvent (ethanol) alone, was added to the culture medium approximately 3 h after cell seeding. The cells were incubated with the fatty acids for 24, 48, or 72 h, and cell numbers (determined by counting in a hemocytometer chamber), melanin contents, and tyrosinase activities were determined in triplicate for each treatment, as detailed below.

Melanin measurement

Melanin content was measured using a modification of a previously reported method (6, 7). Approximately 10^7 cells were pelleted by centrifugation at 1,000 *g* for 5 min and then washed twice with phosphate-buffered saline. After further centrifugation, the supernatant was decanted, the precipitated cells were resuspended in 200 μl of distilled water, and 1 ml of ethanol–ether 1:1 (vol/vol) was added to remove opaque substances other than melanin. Melanin is insoluble in ethanol–ether (6). This mixture was stored and suspended at room temperature for 15 min. After further centrifugation (3,000 *g* for 5 min), the precipitate was solubilized by treatment with 1 ml of 1 N NaOH/10% dimethyl sulfoxide at 80°C for 30 min in a capped test-tube. The absorbance was measured at 470 nm, and the melanin content per cell was calculated and expressed as a percentage of control (=100%).

Tyrosinase assay

Tyrosinase activity was assayed as DOPA oxidase activity using a modified version of a previously reported method (8). Approximately 10^7 cells were pelleted and then washed twice with phosphate-buffered saline. After centrifugation, the supernatant was decanted. The cell pellet was dissolved in 1.0 ml of 0.5% sodium deoxycholate (Sigma) in distilled water and allowed to stand at 0°C for 15 min. Tyrosinase activity was analyzed spectrophotometrically by following the oxidation of DOPA to DOPAchrome at 475 nm. The reaction mixture, consisting of 3 ml of 0.1% l-DOPA (Sigma) in 0.1 m phosphate buffer (pH 6.8), was mixed with the cell lysate (the reaction mixture was freshly prepared every 2 h). Assays were performed at 37°C in a spectrophotometer. The rate was measured during the first 10 min of the reaction while it was linear. Corrections for auto-oxidation of l-DOPA in controls were made. Specific activity was defined as the amount of DOPAchrome formed (absorbance at 475 nm) per 10 min per cell, and is expressed as a percentage of control (=100%).

Northern blot analysis

To identify the expression of melanogenic enzymes at the mRNA level, Northern blot analysis was performed. After incubation with linoleic acid or palmitic acid for 2/3, 3, 6, 18, 24, or 48 h, total cellular RNAs from the treated cells were isolated using Isogen (Nippon Gene Corp., Toyama, Japan). Total RNAs (50 μg) were size-fractionated on 1.0% agarose/formaldehyde gels and blotted onto a cellulose nitrate membrane (Schleicher and Schuell, Dassel). The blots were prehybridized in 40% formamide/10% Denhardt's solution/4 × SSC/7 mm Tris-HCl, pH 7.4/20 μg/ml sheared salmon sperm DNA for 1 h at 42°C. They were then hybridized at 42°C overnight with murine tyrosinase cDNA, human TRP1 or TRP2 cDNA which had been random prime-labeled with [α-^{32}P]dCTP (Amersham). The membranes were washed twice in 2 × SSC, 0.1% sodium dodecyl sulfate (SDS) for 15 min, twice at room temperature, and twice in 0.1 × SSC, 0.1% SDS for 30 min at 65°C, and then exposed to a phosphorimaging plate for 1 h. The total radioactivity of each hybridized band was quantified using a Fujix Bio-Image analyzer BAS2000 (Fuji Photo Film Co. Ltd., Kanagawa, Japan). As an internal loading control, the membranes were rehybridized with human β-actin cDNA (Clontech Laboratories, CA) which had been random prime-labeled with [α-^{32}P]dCTP. The mRNA levels were adjusted to the relative amounts of β-actin mRNA.

DOPA staining of electrophoresed gels

To identify the amount of DOPA-positive tyrosinase and the effect of glycosylation of tyrosinase, DOPA staining of electrophoresed gels was performed as follows. After incubation with linoleic acid or palmitic acid for 24, 48, or 72 h, treated cells were solubilized in 0.1 m sodium phosphate buffer (pH 6.8) containing 1% Triton X-100, 1 mm phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml aprotinin, and 10 μg/ml leupeptin, without mercaptoethanol or heating. Twenty μg of total protein from each cell extract was resolved by SDS-polyacrylamide gel electrophoresis (PAGE), along with molecular weight markers (Amersham), on 7.5% acrylamide gels. Protein content was measured using the Bio-Rad protein Assay Kit (Bio-Rad, Richmond, CA) with bovine serum albumin (BSA) as a standard. Gels containing tyrosinase activity were placed in a flat-bottom container with 200 ml of rinse buffer (0.1 m NaH_2PO_4, pH 6.8) and equilibrated for 30 min at room temperature with gentle shaking. The rinse buffer was then drained from the gels and the gels were transferred into 200 ml staining solution, which contained the rinse buffer supplemented with 5 mm l-DOPA, and incubated in the dark for 3 h at 37°C. Tyrosinase activity was visualized in the gels as dark bands containing DOPA-melanin (9).

Western blotting

To identify the amount of tyrosinase, Western blotting was performed. After incubation with linoleic acid or palmitic acid for 24, 48, or 72 h, the treated cells were solubilized in Nonidet P-40/SDS (1% Nonidet P-40, 0.01% SDS, 0.1 m Tris-HCl, pH 7.2, 100 μm PMSF, and 1 μg/ml aprotinin). Ten μg of total protein from each cell extract was resolved by SDS-PAGE on 7.5% acrylamide gels. Protein contents were measured using the Bio-Rad protein Assay Kit (Bio-Rad) with BSA as a standard. Proteins in the gel were transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore Corp., Bedford, MA). The membranes were blocked with 5% BSA and incubated with specific peptide antisera (1:1000 dilution), anti-PEP7, anti-PEP1, and anti-PEP8 antibodies, which recognize the COOH termini of tyrosinase, TRP1, and TRP2, respectively (10,11). Normal rabbit serum was used as a control. Subsequent visualization of antibody binding was carried out with Enhanced Chemiluminescence (Amersham Corporation) according to the manufacturer's instructions. Films were processed for densitometric analysis using an imaging densitometer (FDU-3, Shimadzu).

Metabolic labeling and immunoprecipitation

These techniques were performed as previously detailed (11, 12). To identify effects on translation of tyrosinase cells were grown to semiconfluence, pretreated with linoleic acid or palmitic acid for 48 h, and then pulsed for 30 min with [^{35}S]methionine (Amersham, Buckinghamshire, England) in methionine/cysteine-free medium in the presence or absence of linoleic acid or palmitic acid. To identify alterations in the degradation of tyrosinase or TRP1, cells grown to semiconfluence were pretreated with linoleic acid or palmitic acid for 48 h, and were then pulsed for 4 h, or were pulsed for 4 h and then chased for 4 h in complete medium containing unlabeled methionine and cysteine in the presence or absence of linoleic acid or palmitic acid. Radiolabeled cells were then harvested with trypsin and solubilized at 4°C for 60 min in 1% Nonidet-P40, 0.01% SDS, 0.1 m Tris-HCl, pH 7.2, 1 μg/ml aprotinin and 100 μm PMSF. Routinely,

5×10^6 dpm of ^{35}S-labeled extracts were incubated with antibodies (αPEP7 for tyrosinase, αPEP1 for TRP1), complexed with protein G-agarose, and washed, and immune complexes were resolved by SDS-PAGE using 7.5% acrylamide gels and then characterized by autoradiography. Autoradiograms were developed by exposing X-ray film to membranes at −75°C for 1 to 3 days. Films were processed for densitometric analysis using an imaging densitometer (FDU-3, Shimadzu).

RESULTS

Linoleic acid decreases and palmitic acid increases melanogenesis

Visible changes in melanin content were observed in cell pellets incubated with fatty acids; linoleic acid decreased melanogenesis and palmitic acid increased it visibly in a time-dependent manner (**Fig. 1a**). The growth rate of cultured B16F10 murine melanoma cells was not significantly altered in the presence of linoleic acid or palmitic acid during the 72-h incubation period (Fig. 1b), indicating that the fatty acid-induced regulatory effects on melanogenesis of melanoma cells occurred without affecting cell proliferation. After incubation with fatty acids, melanin content was regulated in a time-dependent manner; linoleic acid decreased melanin content to 30% and palmitic acid increased it to 150% the level of solvent-treated control cells (Fig. 1c). Further, tyrosinase activity was modulated by fatty acid treatments in a manner correlating well with the regulatory effects on melanin production, i.e., after 72 h incubation linoleic acid suppressed tyrosinase activity to 50% and palmitic acid raised the activity to 190%, compared with controls (Fig. 1d).

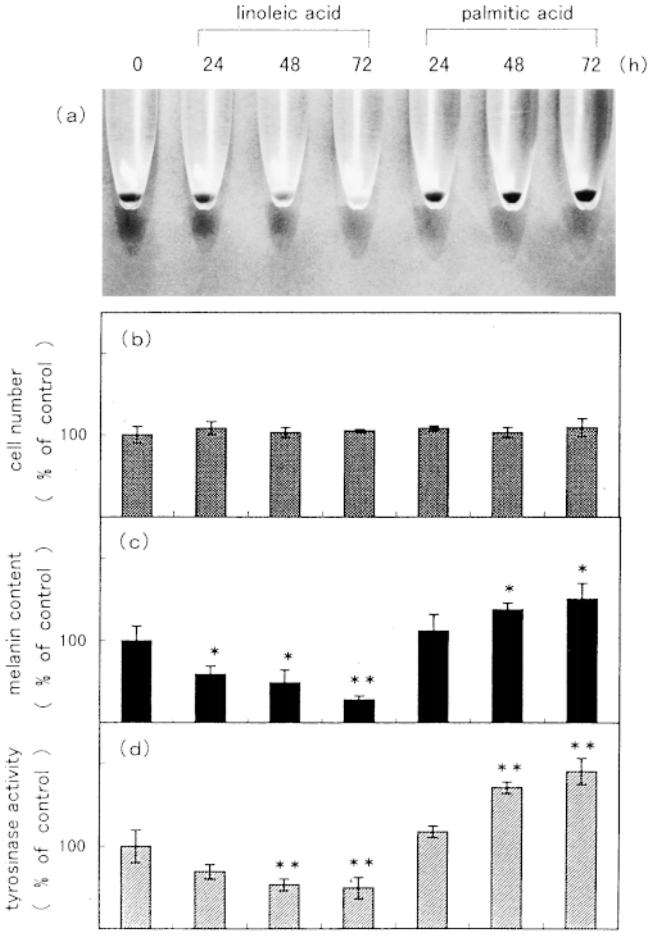

Fig. 1. The pellets (a), growth rates (b), melanin contents (c), and tyrosinase activities (d) of cultured B16F10 murine melanoma cells after treatment with linoleic acid (25 μm) or palmitic acid (25 μm), compared with control (solvent-treated) cells. Linoleic acid decreased both melanin content and tyrosinase activity while palmitic acid increased them, without affecting cell proliferation, in a time-dependent manner. The left lane (0) indicates the control, and treatment periods of 24, 48, and 72 h are as labeled above. Data are expressed as a percentage of control and are mean values of triplicate determinations ± SD. A Student's *t*-test was used for statistical analysis of the data. (* $P < 0.05$; ** $P < 0.01$, versus control)

No alteration in melanogenic enzyme mRNA levels was observed after treatment with linoleic acid or palmitic acid

As previously reported (2), linoleic acid or palmitic acid had little or no effect on tyrosinase mRNA levels after 2/3–48 h incubation. Similarly, mRNA levels of tyrosinase-related proteins, e.g., TRP1 and TRP2, were also not altered by the fatty acid-treatment (**Fig. 2**), indicating that

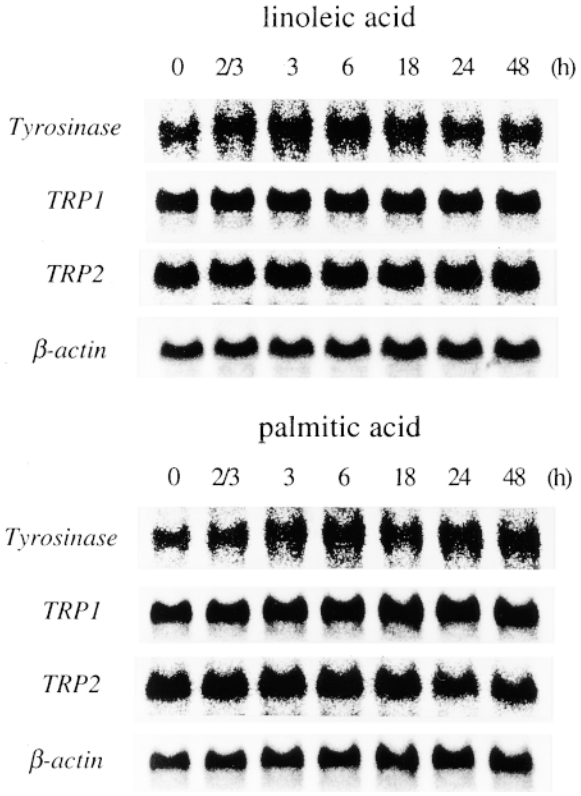

Fig. 2. Northern blot analysis of tyrosinase, TRP1, and TRP2 mRNAs from cultured B16F10 murine melanoma cells after treatment with linoleic acid (25 μm) or palmitic acid (25 μm). Linoleic acid or palmitic acid had little effect on the mRNA expression level of melanogenesis-related enzymes. The left lane (0) indicates the control, and treatment periods of 2/3, 3, 6, 18, 24, and 48 h are as labeled above. β-Actin mRNA is shown as an internal loading control.

the fatty acid-induced regulation of melanogenesis is due to post-transcriptional events of melanogenic enzymes.

Tyrosinase protein (but not TRP1 or TRP2) was decreased by linoleic acid and increased by palmitic acid

Using DOPA staining of electrophoresed gels, it was clear that DOPA reactivity of tyrosinase was decreased by linoleic acid but increased by palmitic acid, in a time-dependent manner. Linoleic acid decreased the DOPA reaction to 50% and palmitic acid increased it to 200% after 72 h incubation, compared with solvent-treated controls. In addition, the molecular weight of glycosylated tyrosinase was not altered by incubation with linoleic acid or palmitic acid, indicating that fatty acids did not alter the glycosylation of tyrosinase (**Fig. 3a**).

Western blotting revealed that tyrosinase protein levels were decreased by linoleic acid and increased by palmitic acid during the 72-h incubation period in a manner that correlated well with the DOPA reactivity. Linoleic acid decreased tyrosinase protein amount to 30%, and palmitic acid increased it to 130% after 72 h incubation, compared with solvent-treated controls. In contrast, the amount of TRP1 and TRP2 proteins showed little change after incubation with fatty acids up to 72 h (Fig. 3b).

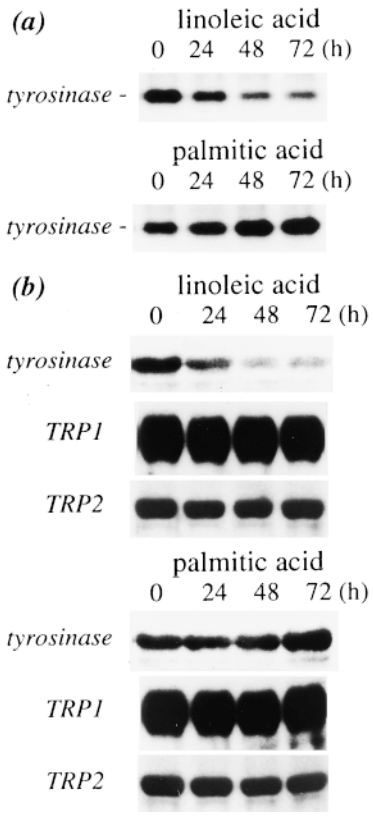

Fig. 3. DOPA staining of tyrosinase (*a*) and Western blot analysis of tyrosinase, TRP1 and TRP2 (*b*) from cultured B16F10 murine melanoma cells after treatment with linoleic acid (25 μm) or palmitic acid (25 μm). Linoleic acid decreased DOPA-reactive tyrosinase and protein levels of tyrosinase, while palmitic acid increased them. Protein levels of TRP1 and TRP2 were not significantly altered. The left lane (0) indicates the control, and treatment periods of 24, 48, and 72 h are as labeled above.

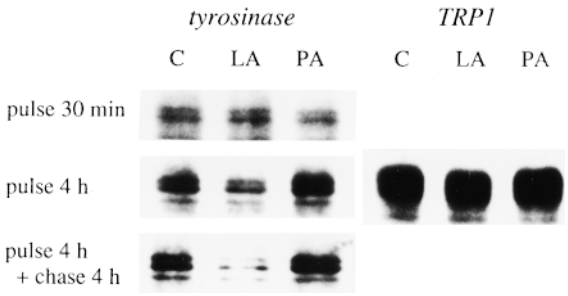

Fig. 4. Metabolic labeling and immunoprecipitation analysis of tyrosinase synthesis and tyrosinase/TRP1 degradation in cultured B16F10 murine melanoma cells after treatment with linoleic acid (25 μm) or palmitic acid (25 μm). Linoleic acid (LA) and palmitic acid (PA) had little effect on the synthesis of tyrosinase (at 30 min), while linoleic acid accelerated, and palmitic acid decelerated, proteolytic degradation of tyrosinase, but not of TRP1. Upper lane: 30-min pulse for tyrosinase synthesis; middle and lower lanes: 4-h pulse, or 4-h pulse and then 4-h chase for tyrosinase/TRP1 degradation; C, control.

Linoleic acid and palmitic acid do not alter the synthesis but affect the proteolytic degradation of tyrosinase

Immunoprecipitation assays of cells metabolically radiolabeled for 30 min revealed that fatty acids did not alter the rate of tyrosinase synthesis (**Fig. 4**, upper lane), indicating that the linoleic acid-induced decrease or the palmitic acid-induced increase of tyrosinase protein was due to post-translational effects. On the other hand, after cells were metabolically radiolabeled for 4 h, and ^{35}S-labeled tyrosinase was chased in the presence of fatty acids for another 4 h, it was clear that linoleic acid accelerated and palmitic acid decelerated the proteolytic degradation of tyrosinase in a dramatic manner. Linoleic acid decreased [^{35}S]tyrosinase levels to 40%, and palmitic acid increased it to 140%, after a 4-h pulse, and linoleic acid decreased [^{35}S]tyrosinase to 10% and palmitic acid increased it to 150% after a 4-h pulse and then a 4-h chase, compared with solvent-treated control (=100%) (Fig. 4, middle and lower lanes). In the case of TRP1, cells incubated with linoleic acid or palmitic acid and then ^{35}S-labeled for 4 h showed strong reactivity with TRP1 protein, indicating that proteolytic degradation by fatty acids is rather specific to tyrosinase (Fig. 4, middle lane).

DISCUSSION

A positive relationship between tyrosinase activity and tyrosinase mRNA levels in murine melanoma cells has been reported (13, 14), although no correlation between them was observed in human melanocytes or in human/murine melanoma cells (2, 15). Using melanogenic regulatory agents, 12-*O*-tetradecanoylphorbol 13-acetate was shown to decrease melanogenesis and to down-regulate the abundance of tyrosinase mRNA (2, 14), while cyclic adenosine monophosphate was shown to increase melanogenesis and to up-regulate tyrosinase mRNA levels (2, 13). Contrary to these tyrosinase transcriptional control-

ling agents, linoleic acid and palmitic acid were shown to regulate melanogenesis by post-transcriptional modification of tyrosinase (2). The data in this study demonstrate that linoleic acid and palmitic acid do not alter the abundance of TRP1 or TRP2 mRNAs, indicating that fatty acid-induced regulation of melanogenesis was due to post-transcriptional control of melanogenic enzymes.

To date, it is not known how fatty acids affect the protein levels of melanogenic enzymes. Interestingly, this study showed that the tyrosinase protein level was altered specifically, by incubation of linoleic acid or palmitic acid in cultured murine melanoma cells but that TRP1 and TRP2 were not. These results suggest that the amount of each melanogenic enzyme, e.g., tyrosinase, TRP1 and TRP2, is not always consistent with regulation of melanogenesis. Tyrosinase plays a critical regulatory role in melanin biosynthesis and it is suggested that tyrosinase activity is pivotal, especially for the fatty acid-induced regulation of melanogenesis. Further, an experiment using the DOPA reaction to reveal abnormal tyrosinase glycosylation, as previous reports showed that glycosylation inhibitors, such as glucosamine and tunicamycin, produced aberrant isoforms of tyrosinase (16), demonstrated clearly that such changes did not occur after treatment with fatty acids. In addition, the reactivity of DOPA staining was consistent with protein levels of tyrosinase seen by Western blot. Thus these results strongly suggest that alteration of melanogenesis by fatty acids, with little or no change in mRNA levels for melanogenic enzymes, is due to the modification of tyrosinase protein.

In order to investigate the mechanism underlying the linoleic acid and palmitic acid regulation of tyrosinase protein, immunoprecipitation assays of metabolically radiolabeled cells were used. Tyrosinase was found to be synthesized within melanoma cells rapidly, as significant quantities of pulse-labeled enzyme could be detected within 30 min, as previously reported (12). Linoleic acid and palmitic acid did not alter the synthesis of tyrosinase, suggesting that the fatty acid-induced regulation of melanogenesis was due to post-translational modification. A previous study showed that tyrosinase in melanocytes was synthesized rapidly and that active degradation occurred spontaneously (12). Recently, it was also reported that tyrosinase in melanocytes and in melanoma cells is degraded endogenously by proteasomes, and that, especially in melanoma cells, proteolytic degradation of tyrosinase occurs within a few hours (17). Although TRP1 degradation was not affected, linoleic acid and palmitic acid modulated the rates of the proteolytic degradation of tyrosinase after a 4-h pulse, and the effect became more apparent after an additional 4-h chase. These findings suggest that linoleic acid and palmitic acid regulate the proteolysis of tyrosinase, which in turn leads to the alteration of tyrosinase protein. More definitive data are needed to further clarify the mechanism involved in the fatty acid-regulated degradation of tyrosinase. However, our results explain how linoleic acid and palmitic acid regulate melanogenesis without altering mRNA levels of melanogenic enzymes.

In summary, the present study suggests that accelerated or decelerated proteolytic degradation specifically of tyrosinase, but not of TRP1 or TRP2, is involved in the regulation of melanogenesis by linoleic acid and palmitic acid in cultured murine melanoma cells.

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REFERENCES

1. Shono, S., and K. Toda. 1981. Pigment Cell 1981/Phenotypic Expression in Pigment Cells. University of Tokyo Press, Tokyo. 263–268.
2. Ando, H., A. Itoh, Y. Mishima, and M. Ichihashi. 1995. Correlation between the number of melanosomes, tyrosinase mRNA levels, and tyrosinase activity in cultured murine melanoma cells in response to various melanogenesis regulatory agents. *J. Cell. Physiol.* **163:** 608–614.
3. Ando, H., A. Ryu, A. Hashimoto, M. Oka, and M. Ichihashi. 1998. Linoleic acid and α-linolenic acid lightens ultraviolet-induced hyperpigmentation of the skin. *Arch. Dermatol. Res.* **290:** 375–381.
4. Hearing, V. J., and M. Jiménez. 1987. Mammalian tyrosinase: the critical regulatory control point in melanocyte pigmentation. *Int. J. Biochem.* **19:** 1141–1147.
5. Kobayashi, T., K. Urabe, A. Winder, C. Jiménez-Cervantes, G. Imokawa, T. Brewington, F. Solano, J. C. García-Borrón, and V. J. Hearing. 1994. Tyrosinase related protein 1 (TRP1) functions as a DHICA oxidase in melanin biosynthesis. *EMBO J.* **13:** 5818–5825.
6. Oikawa, A., and M. Nakayasu. 1973. Quantitative measurement of melanin as tyrosinase equivalents and as weight of purified melanin. *Yale J. Biol. Med.* **46:** 500–507.
7. Hosoi, J., E. Abe, T. Suda, and T. Kuroki. 1985. Regulation of melanin synthesis of B16 mouse melanoma cells by 1α,25-dihydroxy vitamin D_3 and retinoic acid. *Cancer Res.* **45:** 1474–1478.
8. Wrathal, J. R., C. Oliver, S. Silagi, and E. Essner. 1973 Suppression of pigmentation in mouse melanoma cells by 5-bromodeoxyuridine. *J. Cell. Biol.* **57:** 406–423.
9. Laskin, J. D., and L. A. Piccinini. 1986. Tyrosinase isozyme heterogeneity in differentiating B16/C3 melanoma. *J. Biol. Chem.* **261:** 16626–16635.
10. Jiménez, M., K. Tsukamoto, and V. J. Hearing. 1991. Tyrosinases from two different loci are expressed by normal and by transformed melanocytes. *J. Biol. Chem.* **266:** 1147–1156.
11. Tsukamoto, K., I. J. Jackson, K. Urabe, P. M. Montague, and V. J. Hearing. 1992. A second tyrosinase-related protein, TRP-2, is a melanogenic enzyme termed DOPAchrome tautomerase. *EMBO J.* **11:** 519–526.
12. Jiménez, M., K. Kameyama, W. L. Maloy, Y. Tomita, and V. J. Hearing. 1988. Mammalian tyrosinase: biosynthesis, processing, and modulation by melanocyte-stimulating hormone. *Proc. Natl. Acad. Sci. USA.* **85:** 3830–3834.
13. Hoganson, G. E., F. Ledwitz-Rigby, R. L. Davidson, and B. B. Fuller. 1989. Regulation of tyrosinase mRNA levels in mouse melanoma cell clones by melanocyte-stimulating hormone and cyclic AMP. *Somatic Cell Mol. Genet.* **15:** 255–263.
14. Fuller, B. B., I. Niekrasz, and G. E. Hoganson. 1990. Down-regulation of tyrosinase mRNA levels in melanoma cells by tumor promoters and by insulin. *Mol. Cell. Endocrinol.* **72:** 81–87.
15. Naeyaert, J. M., M. Eller, P. R. Gordon, H-Y. Park, and B. A. Gilchrest. 1991. Pigment content of cultured human melanocytes does not correlate with tyrosinase message level. *Br. J. Dermatol.* **125:** 297–303.
16. Imokawa, G., and Y. Mishima. 1982. Loss of melanogenic properties in tyrosinases induced by glycosylation inhibitors within malignant melanoma cells. *Cancer Res.* **42:** 1994–2002.
17. Halaban, R., E. Cheng, Y. Zhang, G. Moellmann, D. Hanlon, M. Michalak, V. Setaluri, and D. N. Hebert. 1997. Aberrant retention of tyrosinase in the endoplasmic reticulum mediates accelerated degradation of the enzyme and contributes to the dedifferentiated phenotype of amelanotic melanoma cells. *Proc. Natl. Acad. Sci. USA.* **94:** 6210–6215.