Fatty Acids Regulate Pigmentation Via Proteasomal Degradation of Tyrosinase – A New Aspect of Ubiquitin-Proteasome Function

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The abbreviations used are: DMSO, dimethyl sulfoxide; ER, endoplasmic reticulum; LLnL, N-Acetyl-Leu-Leu-norleucinal; MG132, benzyloxy carbonyl-Leu-Leu-leucinal; NF-κB, nuclear factor-kappaB; PBS, phosphate-buffered saline; PKC, protein kinase C; SDS, sodium dodecyl sulfate
ABSTRACT

Fatty acids are common components of biological membranes that are known to play important roles in intracellular signaling. We report here a novel mechanism by which fatty acids regulate the degradation of tyrosinase, a critical enzyme associated with melanin biosynthesis in melanocytes and in melanoma cells. Linoleic acid (unsaturated fatty acid, C18:2) accelerated the spontaneous degradation of tyrosinase while palmitic acid (saturated fatty acid, C16:0) retarded the proteolysis. The linoleic acid-induced acceleration of tyrosinase degradation could be abrogated by inhibitors of proteasomes, the multicatalytic proteinase complexes that selectively degrade intracellular ubiquitinated proteins. Linoleic acid increased the ubiquitination of many cellular proteins, while palmitic acid decreased that compared to untreated controls, when a proteasome inhibitor was used to stabilize ubiquitinated proteins. Immunoprecipitation analysis also revealed that treatment with fatty acids modulated the ubiquitination of tyrosinase, i.e. linoleic acid increased the amount of ubiquitinated tyrosinase while in contrast palmitic acid decreased it. Furthermore, confocal immunomicroscopy showed that the colocalization of ubiquitin and tyrosinase was facilitated by linoleic acid and was diminished by palmitic acid. Taken together, these data support the view that fatty acids regulate the ubiquitination of tyrosinase and are responsible for modulating the proteasomal degradation of tyrosinase. In broader terms, the function of the ubiquitin-proteasome pathway might be regulated physiologically, at least in part, by fatty acids within cellular membranes.

Key words: linoleic acid / lipid / melanin / melanoma / palmitic acid / pigment
INTRODUCTION

Although the dynamic roles of lipids, including fatty acids, as participants controlling many cellular activities such as mediators in the intracellular signaling network and as precursors for ligands that bind to nuclear receptors has been well studied (1;2), the role of fatty acids in regulating intracellular protein turnover in mammalian cells is poorly understood. Among known pathways of intracellular protein breakdown that are functional in eukaryotic cells, the ubiquitin-proteasome pathway has been in the limelight recently because of its importance in the selective elimination of abnormal proteins that may arise by mutations, by neurotoxicity or by intracellular denaturation (3-6) as well as the proteolysis of short-lived proteins that need to be rapidly removed for physiological regulatory purposes (7;8).

Tyrosinase (monophenol, L-dopa:oxygen oxidoreductase, EC 1.14.18.1) is a type I membrane glycoprotein which is the critical rate-limiting enzyme involved in melanin biosynthesis (9;10). Some pigmentary disorders such as oculocutaneous albinism are caused by the dysfunction of tyrosinase and related melanogenic enzymes, and the aberrant retention of those proteins in the endoplasmic reticulum (ER) results in their degradation by proteasomes which leads to the hypopigmented phenotype (11;12). Previous studies have shown that tyrosinase is synthesized in the ER and is processed rapidly through the Golgi apparatus in/after which active degradation of tyrosinase occurs spontaneously (13;14). It has also been reported that tyrosinase can be degraded endogenously by proteasomes (11;15). These studies support the notion that an ideal balance between tyrosinase synthesis and degradation is necessary for regulating pigmentation in mammalian skin, hair and eyes.

Free fatty acids have been shown to have remarkable regulatory effects on melanogenesis, i.e. unsaturated fatty acids such as oleic acid (C18:1), linoleic acid (C18:2) and α-linolenic acid (C18:3) decrease melanin synthesis and tyrosinase activity, while saturated fatty acids such as palmitic acid (C16:0) and stearic acid (C18:0) increase it (16;17). In fact, linoleic acid has proven to be useful as a topical agent to prevent hyperpigmentary disorders such as melasma that are caused by tyrosinase dysfunction (17;18). Among the various fatty acids, linoleic acid and palmitic acid are abundant components of cell membranes in the epidermis (19), and we have been investigating the mechanisms by which these fatty acids regulate tyrosinase metabolism, e.g. synthesis, processing and/or degradation. In those studies, we have found that tyrosinase mRNA levels were not altered following incubation with linoleic acid or palmitic acid,
indicating that these fatty acids regulate tyrosinase activity via post-transcriptional events (20). In addition, metabolic labeling and immunoprecipitation analyses revealed that linoleic acid accelerated but palmitic acid decelerated the proteolysis of 35S-labeled tyrosinase (21). However, the mechanism involved in the fatty acid-induced regulation of tyrosinase degradation is poorly understood. In this study, we evaluated the effects of fatty acids on the ubiquitin-proteasome pathway, where membrane proteins are selectively degraded, and we further explored the possibility whether this pathway could be involved in the fatty acid-induced regulation of tyrosinase degradation.

**MATERIALS AND METHODS**

*Antibodies and Reagents.* Antibodies used were αPEP7; a rabbit polyclonal antibody generated against a synthetic peptide [KLH-CDKDDYHSLYQSHL-COOH] which corresponds to the carboxyl terminus of mouse tyrosinase (22), Ub(P4D1); a mouse monoclonal IgG1 antibody which corresponds to amino acids 1-76 representing the full length of bovine ubiquitin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-20S proteasome; a rabbit polyclonal antibody (Calbiochem, San Diego, CA, USA) which recognizes the α-subunit, and anti-PA700; a rabbit polyclonal antibody (Calbiochem) which recognizes ATPase/non-ATPase subunits. MG132 (benzyloxy carbonyl-Leu-Leu-leucinal, also known as Cbz-LLL) was from Calbiochem, while LLnL (N-Acetyl-Leu-Leu-norleucinal, also known as ALLN), cycloheximide, the saturated free fatty acid palmitic acid (hexadecanoic acid) and the unsaturated free fatty acid linoleic acid (cis-9, 12-octadecadienoic acid) were all from Sigma (St Louis, MO, USA).

*Cell Cultures and Treatments.* B16F10 mouse melanoma cell lines, which stably express tyrosinase activity and produce melanin, were grown in Eagle’s minimal essential medium (Sigma) containing 10% heat-inactivated (56°C, 30 min) fetal bovine serum (HyClone, Logan, UT, USA) at 37°C in a humidified atmosphere with 5% CO2. Free fatty acids, proteasome inhibitors and cycloheximide were dissolved in dimethyl sulfoxide (DMSO). Final DMSO concentrations were 0.1% or less, which did not influence cell viability or tyrosinase function. For evaluating alterations of tyrosinase content and ubiquitin-proteasome pathway related proteins, cells were treated with linoleic acid (25 µM) or palmitic acid (25 µM) for 24, 48 and 72 h. We previously showed that 25 µM of each fatty acid had no effect on the proliferation of B16 cells (21). To measure tyrosinase degradation, cells were pretreated with linoleic acid (25
µM) or palmitic acid (25 µM) for 72 h and then were treated with cycloheximide (1 µg/ml) for an additional 4 or 8 h in the presence of linoleic acid or palmitic acid, with or without incubation in the presence of proteasome inhibitors (MG132 at 120 nM or LLnL at 3 µM). To detect ubiquitinated proteins and ubiquitinated tyrosinase, cells were treated with linoleic acid (25 µM) or palmitic acid (25 µM) for 24 h in the presence or absence of MG132 (120 nM). In these experiments, cells were grown to confluence at which time they were harvested to detect ubiquitinated proteins. For confocal microscopy, cells were treated with linoleic acid (25 µM) or palmitic acid (25 µM) for 72 h in the presence or absence of MG132 (120 nM) for the last 24 h before fixation.

**Western Blot Analysis.** Treated cells were solubilized in lysis buffer consisting of 0.1 M Tris-HCl, pH 7.4, containing 1% Nonidet P-40, 0.01% sodium dodecyl sulfate (SDS), 2.4 µM MG132, and complete protease inhibitor cocktail (Roche, Mannheim, Germany) for 30 min at 4°C. After centrifugation (15,000 g for 10 min at 4°C), the supernatants were used as cell extracts. Protein concentrations were measured using the BCA protein assay kit (Pierce, Rockford, IL, USA). The cell extracts were mixed with Tris-glycine SDS sample buffer (2X) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% 2-mercaptoethanol and were boiled at 95°C for 5 min. Five or 10 µg total protein from each extract was separated by SDS-polyacrylamide gel electrophoresis on 8-16% gradient Tris-glycine gels (Invitrogen), and were transferred electrophoretically to Immobilon-P membranes (Millipore, Bedford, MA, USA). Those membranes were blocked in 10% non-fat dry milk in TBS-T (20 mM Tris-HCl, pH 7.4, 137 mM NaCl, 0.1% Tween 20) overnight at 4°C and were then incubated with primary antibodies diluted (as noted in the Figure legends) in 2% non-fat dry milk in TBS-T for 2 h at 23°C. Prior to the probing with the anti-ubiquitin antibody, the blotting membranes were denatured in 20 mM Tris-HCl, pH 7.4, containing 6 M guanidine-HCl and 5 mM 2-mercaptoethanol for 30 min at room temperature (23), washed 5 times (5 min each) with TBS-T, and then blocked with 10% non-fat dry milk. After 5 washes with TBS-T, the blotting membranes were incubated in horseradish peroxidase-linked anti-rabbit or anti-mouse whole antibodies (1:10,000) (Amersham, Piscataway, NJ, USA) in TBS-T for 2 h at 23°C. After 5 washes with TBS-T, the immunoreactivities of the blots were detected using an ECL-plus Western Blotting Detection System (Amersham), according to the manufacturer’s instructions.
The BenchMark prestained protein ladder (Invitrogen) was used to establish the molecular weight curve for the Western blotting. For reprobing, a Re-Blot Western blot recycling kit (Chemicon International, Temecula, CA, USA) was used to strip the antibodies and the blots were restarted from the blocking step with 10% non-fat dry milk in TBS-T, and were then recycled for another antibody. The expression levels of tyrosinase and other proteins were quantified by measuring the optical densities of specific bands using an image analysis system with NIH Image software, version 1.62.

**Immunoprecipitation.** Cell extracts (200 µg total protein in 400 µl of lysis buffer) were incubated with 20 µl normal rabbit serum (Sigma) with continuous mixing for 2 h at 4°C. Protein-G Sepharose 4 Fast Flow (150 µl, Amersham) pre-equilibrated in lysis buffer (1:1 volume) was added and the cell extracts were subjected to further mixing for 2 h at 4°C. After centrifugation (2,000 g for 1 min at 4°C), the supernatants were used as pre-cleared cell extracts. The pre-cleared cell extracts were incubated with 10 µl αPEP7 antibody or with 10 µl normal rabbit serum as a control. After continuous mixing for 2 h at 4°C, 20 µl Protein-G Sepharose suspended in lysis buffer was added and was further mixed for 2 h at 4°C. After the antigen-antibody complexes were precipitated by brief centrifugation, the pellets were washed 6 times with 800 µl lysis buffer. Finally, absorbed proteins were eluted with 50 µl Tris-glycine SDS sample buffer with 2-mercaptoethanol at 95°C for 5 min. Each supernatant (20 µl) was separated on 8-16% Tris-glycine SDS gels, and was then transferred to Immobilon-P membranes. Ub(P4D1) was used to detect ubiquitinated tyrosinase precipitated with αPEP7. The detection method was the same as indicated in the protocol for Western blot analysis.

**Immunofluorescence Microscopy.** Confocal microscopy was performed using double indirect immunofluorescence as previously described (24;25). Cells were seeded in 2-well Lab-Tek chamber slides (Nalge Nunc Intl, Naperville, IL, USA) and incubated with linoleic acid or palmitic acid for 72 h at 37°C in Eagle’s minimum essential medium containing 10% heat-inactivated fetal bovine serum. Twenty four h before fixation (48 h after cell seeding), 120 nM MG132 was added to some plates to stabilize the ubiquitinated proteins. After 3 washes in phosphate-buffered saline (PBS) at 37°C, the cells were fixed in 4% paraformaldehyde for 15 min at 4°C. After 3 further washes in PBS in room temperature, the cells were permeabilized with 100% methanol for 15 min at 4°C and were then blocked with 5% normal goat serum and 5% normal horse serum in PBS for 1 h at room temperature. The cells were then incubated with a
mixture of the polyclonal and the monoclonal antibody (at the dilution noted in the Figure legends) in PBS containing 2% normal goat serum and 2% normal horse serum overnight at 4°C. After 5 washes in PBS at room temperature, the polyclonal antibody was reacted with goat anti-rabbit IgG labeled with Texas red (1:100) and the monoclonal antibody was reacted with horse anti-mouse IgG labeled with fluorescein (1:100) (Vector Laboratories, Burlingame, CA, USA), followed by nuclear counterstaining with 4’,6-diamidino-2-phenylindole (Vector). Reactivity was classified into three categories, according to whether they showed red, green, or yellow fluorescence. The yellow fluorescence was indicative of colocalization of the red and green fluorescence signals. All preparations were examined with a confocal microscope (LSM 510; Zeiss), equipped with HeNe, argon and krypton laser sources.

RESULTS

Involvement of the ubiquitin-proteasome pathway in the fatty acid-induced regulation of tyrosinase degradation. The amount of tyrosinase and of the ubiquitin-proteasome pathway-related proteins, i.e. ubiquitin (free mono-ubiquitin), 20S proteasome (a core catalytic protease complex of the 26S proteasome) and PA700 (also known as the 19S proteasome, a regulatory complex that binds to both ends of the 20S proteasome) (26;27) were determined by Western blotting after incubation with various concentrations of linoleic acid or palmitic acid for 72 h (Fig. 1). In agreement with previous results (21), the amount of tyrosinase was decreased by linoleic acid and was increased by palmitic acid in dose-dependent manners, and this was consistent with the colors of the cell pellets. In contrast, the amount of the ubiquitin-proteasome pathway-related proteins did not change appreciably after incubation with either fatty acid, i.e. the effects of fatty acids on the ubiquitin-proteasome pathway did not alter those proteins.

We previously reported preliminary evidence that linoleic acid accelerates, while palmitic acid decelerates, the proteolytic degradation of tyrosinase, as revealed by pulse-chase analysis (21). In the present study, we assessed the fate of tyrosinase to confirm the rate of its proteolysis using cycloheximide, a protein synthesis inhibitor, after prolonged treatment with fatty acids. The concentration of cycloheximide used was 1 µg/ml, a level previously shown to inhibit protein synthesis in these cells by more than 80% (28). The degradation of tyrosinase occurred in a time-dependent manner after 4 or 8 h of treatment with cycloheximide (Fig. 2A, lanes 2 and 3 vs lane 1). Further, accelerated degradation of tyrosinase was observed (again in a
time-dependent manner) in cells pretreated with linoleic acid for 72 h (lanes 4-6). In contrast, cells pretreated with palmitic acid for 72 h had increased levels of tyrosinase, and showed a decelerated rate of tyrosinase degradation (lanes 7-9). The rate of tyrosinase degradation was increased or decreased by treatment with linoleic acid or palmitic acid, respectively (Fig. 2B).

We next examined whether the linoleic acid-induced decrease of tyrosinase was due to its proteolytic degradation by proteasomes. We used the proteasome inhibitors MG132, a membrane permeable proteasome inhibitor, which has been used to study the role of proteasomes in the breakdown of membrane proteins within the ER (28), and LLnL, which is a neutral cysteine protease inhibitor that also blocks proteasome-mediated proteolysis (5). The concentration of each proteasome inhibitor used was determined by its cytotoxicity of less than 40% growth inhibition, i.e. the cell number after 72 h incubation with 120 nM MG132 was 73.4% and with 3 μM LLnL was 64.4%, compared to the control. Linoleic acid decreased the level of tyrosinase in a time-dependent manner, and this was most significant after pretreatment of the cells for 72 h (Fig. 3A, lanes 1-4). The decrease of tyrosinase induced by linoleic acid could be blocked by proteasome inhibitors (MG132 or LLnL) after co-incubation for 72 h (Fig. 3A, lanes 5 and 6). To further confirm the involvement of proteasomes in the linoleic acid-induced acceleration of tyrosinase degradation, we used cycloheximide to block protein synthesis. The decrease in tyrosinase protein in cells pretreated with linoleic acid for 72 h in the presence of cycloheximide could be abrogated by co-incubation with MG132 or LLnL (Fig. 3B). In contrast, treatment with trans-epoxysuccinyl-L-leucylamido(4-guanidino)-butane (E-64), an inhibitor of cysteine proteases that does not block proteasome activity (5;29), or with (2S,3S)-trans-epoxysuccinyl-L-leucylamido-3-methylbutane ethyl ester (EST; also known as E-64d), which also inhibits lysosomal thiol proteases, had no effect on the accumulation of tyrosinase, even at concentrations as high as 30 μM (data not shown).

**Regulatory effect of fatty acids on cellular ubiquitination and on ubiquitinated tyrosinase.** To elucidate the mechanism by which the ubiquitin-proteasome pathway is involved in the fatty acid-induced regulation of tyrosinase degradation, we evaluated the effects of fatty acids on cellular ubiquitination in general. To do that, it was necessary to stabilize ubiquitinated proteins which would normally be immediately degraded by proteasomes, and we used MG132 at 120 nM to accomplish that. We determined an incubation time of 24 h to estimate the proper alteration of ubiquitination to compare the relative amounts of tyrosinase. Very low band
intensities of ubiquitinated proteins were observed in the absence of MG132 (Fig. 4A, lanes 1-3 on the left), while increased band intensities of ubiquitinated proteins were observed in the presence of MG132 and in the presence or absence of fatty acids (Fig. 4A, lanes 4-6 on the left). Interestingly, treatment with linoleic acid in the presence of MG132 showed a 1.6-fold increase in ubiquitinated proteins (lane 5) while treatment with palmitic acid in the presence of MG132 showed a 0.6-fold decrease (lane 6) compared to the MG132-treated control (lane 4). The mean values (from 3 independent experiments) of -fold increase or decrease compared to MG132-treated controls is shown at the top of each lane. The image on the right side of Fig. 4A shows the reprobing for tyrosinase using the same membrane after stripping the ubiquitin antibody. The degradation of tyrosinase was prevented by MG132 and similar band intensities of tyrosinase were obtained (Fig. 4A, lanes 4-6 on the right). Comparison of these tyrosinase bands clearly show that fatty acids regulate the ubiquitination of cellular proteins (compare lanes 4-6 in both panels of Fig. 4A).

We then performed immunoprecipitation analysis to more critically evaluate the effects of fatty acids on the ubiquitination of tyrosinase. Since we wanted to clarify whether fatty acids regulated the ubiquitination of tyrosinase, equal amounts of tyrosinase after incubation with fatty acids needed to be immunoprecipitated. Since the amount of tyrosinase could be standardized by incubation with MG132 for 24 h (Fig. 4A, right), we employed that same protocol. Western blotting (Fig. 4B) with a ubiquitin antibody using a membrane on which tyrosinase immunoprecipitated by the tyrosinase antibody had been loaded, showed a band of ubiquitinated tyrosinase in which protein masses were shifted to ~90 kDa. Quantitation of these bands revealed that treatment with linoleic acid in the presence of MG132 increased the amount of ubiquitinated tyrosinase by 1.8-fold, while treatment with palmitic acid in the presence of MG132 decreased it to 0.7-fold, compared to the control. The serum control did not show a band of ubiquitinated tyrosinase (lane 4 in Fig. 4B). The mean value of -fold increase or decrease compared to the MG132-treated control (in 3 independent experiments) is shown for each lane in the enlarged view (Fig. 4B, right). These results demonstrate that linoleic acid enhances while palmitic acid diminishes the ubiquitination of tyrosinase.

**Intracellular association of tyrosinase and ubiquitin after incubation with fatty acids.** To evaluate the intracellular association of tyrosinase and ubiquitin after incubation with fatty acids, we used confocal immunohistochemistry with αPEP7 (tyrosinase) and Ub(P4D1)
(ubiquitin). Tyrosinase staining is detected by red fluorescence and ubiquitin staining is detected by green fluorescence, while in the merged images, yellow fluorescence indicates the colocalization of the two signals. In untreated controls (Fig. 5, left), little colocalization of tyrosinase and ubiquitin are seen, but treatment with MG132 increases that considerably. After incubation with linoleic acid for 72 h in the absence of MG132, the intensity of tyrosinase (red) was decreased and it was localized in the perinuclear area, while the subcellular localization of ubiquitin in fatty acid-treated or control cells was ubiquitous, with some concentration in the nuclei. In contrast, linoleic acid-treated cells in the presence of MG132 showed similar intensities of tyrosinase to the controls (because of the abrogation of tyrosinase degradation) and similar levels of aggregated ubiquitin were observed in the cytoplasm. In the merged images of MG132-untreated cells, little association of tyrosinase and ubiquitin was observed in the fatty acid-treated or control cells, although a slight yellow fluorescence was seen in the palmitic acid-treated cells. In contrast, in the merged images of MG132-treated cells, yellow fluorescence indicating the association of tyrosinase and ubiquitin was observed in the fatty acid-treated and control cells. It is apparent that a large amount of ubiquitin colocalized with tyrosinase in the linoleic acid-treated cells, i.e. almost all of the small granular globules of ubiquitin were yellow. In contrast, the association of tyrosinase and ubiquitin in the palmitic acid-treated cells was greatly reduced in the presence of MG132. These results show that linoleic acid enhances and palmitic acid diminishes the association of tyrosinase and ubiquitin compared with the control, which is consistent with the immunoprecipitation analyses above which showed increased or decreased amounts of ubiquitinated tyrosinase following fatty acid treatment.

**DISCUSSION**

Fatty acids are ubiquitous components of cell membranes and are an important source of biological energy. Recent studies have revealed that fatty acids also play an important role in various cell functions such as intracellular signaling, but the comprehensive and nonspecific functions of fatty acids seem to veil their mechanisms. One example of the role of fatty acids in intracellular signal transduction is protein kinase C (PKC) which functions as a critical signaling pathway for cells that recognize and respond to a variety of extracellular stresses (30-32). Following the finding that PKC is activated by unsaturated fatty acids (33), it has been shown that various cis-unsaturated fatty acids, such as oleic, linoleic, linolenic, arachidonic (C20:4) and
docosahexaenoic (C22:6) acids greatly enhance the diacylglycerol-dependent activation of PKC, notably at basal levels of Ca\(^{2+}\) concentration (34-37). In contrast, saturated fatty acids and trans-unsaturated fatty acids do not have similar effects (38). As for their roles in the regulation of transcription, linoleic acid has been shown to induce DNA synthesis, c-fos, c-jun and c-myc mRNA expression and mitogen-activated protein kinase activation in rat vascular smooth muscle cells (39). Recent studies have also revealed that linoleic acid activates nuclear factor-kappaB (NFκB) in vascular endothelial cells (40-42). Further, linoleic acid and its metabolites can modulate tyrosine phosphorylation and the activities of key signal transduction proteins in a growth factor mitogenic pathway (43). Taken together, those studies have shed light on the close relationship between fatty acids and the networks of various signaling pathways.

Our study demonstrates a very interesting function of fatty acids by showing that physiologically relevant fatty acids can elicit diverse effects on ubiquitin-proteasome function. To date, various physiological and nonphysiological treatments that regulate the ubiquitin-proteasome pathway have been reported. For example, dramatic activation of proteasomes can be induced by various treatments in vitro, including incubation with basic polypeptides, SDS, guanidine HCl, heating at 55°C or fatty acids, while glycerol helps maintain proteasome activity in the latent form (29;44-46). Regarding the fatty acid-induced regulation, the proteolytic activity of 20S proteasomes is increased by physiological concentrations of fatty acids such as oleic acid and linoleic acid in rat skeletal muscle (47) or spinach leaves (48). More recently, it was reported that 15(S)-hydroxyeicosatetraenoic acid increased expression of the regulatory components of the ubiquitin-proteasome pathway, e.g. the proteasome subunit and E2 ubiquitin-conjugating enzymes, possibly through the intervention of NF-κB and this process could be inhibited by eicosapentaenoic acid (C20:5) (49). Thus, a number of reports have shown that various treatments regulate proteasome activity, however, there have been no reports to date on the contrasting regulation of the ubiquitin-proteasome pathway, especially on the ubiquitin system, induced by fatty acids or lipids or any other physiologically relevant factors.

In this study, we have demonstrated that linoleic acid and palmitic acid regulate the proteasomal degradation of tyrosinase in contrasting manners by way of relative increases or decreases in the ubiquitination of tyrosinase. Ubiquitin-protein ligation requires the sequential action of three enzymes, e.g. an E1 activating enzyme, E2 ubiquitin-conjugating enzymes (ubiquitin-carrier proteins), and E3 ubiquitin-protein ligases (50). Since the selectivity of protein
degradation is determined mainly at the stage of ubiquitin ligation, one possibility is that fatty acids may be able to activate or inhibit an as-yet unidentified E3 ubiquitin ligase for tyrosinase. Although in this study, the amount of ubiquitinated tyrosinase was altered by incubation with fatty acids, the total amount of ubiquitinated proteins in the cells was also changed, suggesting that tyrosinase could be among the ubiquitinated proteins regulated by the fatty acid-modulated ubiquitin system. We still know very little about the mode of action of fatty acids on the ubiquitin system, but we would like to propose a model in which fatty acids regulate the ubiquitination of cellular proteins, including tyrosinase, which then becomes targeted for proteasomal degradation. Further study will be needed to clarify the precise mechanism(s) involved in this process, and why tyrosinase, but not the tyrosinase-related proteins Tyrp1 and Dct, is regulated at this level. This is the first report of fatty acid-induced contrasting effects on the ubiquitination of a membrane glycoprotein.

In summary, this study focused on the diverse contributions of unsaturated and saturated fatty acids in the ubiquitin-proteasome pathway-mediated degradation of tyrosinase. Our results suggest that fatty acids act as intrinsic factors that modulate the proteasomal degradation of membrane glycoproteins, such as tyrosinase, and presumably other membrane proteins. Taken together, these results imply a novel mechanism for the involvement of fatty acids in regulating the selective degradation of a melanogenic enzyme via the ubiquitin-proteasome pathway, and possibly in the modulation of the functions of other proteins. The investigative system presented here is an excellent model to further elucidate the roles of fatty acids and other lipids on cell functions mediated by the ubiquitin-proteasome pathway-dependent membrane protein degradation, which in turn should provide insights to better understand the nature of the ubiquitin-proteasome pathway.

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FIGURE LEGENDS

**Fig. 1.** Effects of fatty acids on melanin production, tyrosinase and ubiquitin-proteasome pathway-related proteins. Cells were cultured for 72 h in medium containing 6.25, 12.5 or 25 µM linoleic acid or palmitic acid, or DMSO only (control). Western blotting (5 µg protein/lane) with primary antibodies recognizing tyrosinase (αPEP7 at 1:5,000), mono-ubiquitin (Ub(P4D1) at 1:5,000), 20S proteasome (at 1:2,000) or PA700 (at 1:2,000). Numbers at the left indicate protein masses in kDa.

**Fig. 2.** Tyrosinase degradation after incubation with fatty acids. (A) Kinetics of tyrosinase degradation in cells pretreated with DMSO only (control), with linoleic acid (25 µM) or with palmitic acid (25 µM) for 72 h. Cells were then treated with cycloheximide (1 µg/ml) to inhibit protein synthesis for 4 or 8 h where noted. During exposure to cycloheximide, linoleic acid or palmitic acid was added continuously. Western blotting of tyrosinase probed with αPEP7 in whole cell lysates (10 µg protein/lane) is shown. Data are expressed as a percentage of control and are mean values of triplicate determinations±SD. (B) Graphical representation of the relative band intensities of tyrosinase; the initial tyrosinase levels in lanes 1, 4 and 7, which are the bands before treatment with cycloheximide, were recalculated to 100% and the respective rates of tyrosinase degradation are shown.

**Fig. 3.** Involvement of proteasomes in the linoleic acid-induced acceleration of tyrosinase degradation. Western blotting of tyrosinase probed with αPEP7 in whole cell lysates (10 µg protein/lane) is shown. Data are expressed as a percentage of control and are mean values of triplicate determinations±SD. (A) The amount of tyrosinase in cells after treatment with linoleic acid (25 µM) for 24, 48 or 72 h in the presence or absence of proteasome inhibitors (120 nM MG132 or µM LLnL). (B) Abrogation of the linoleic acid-induced acceleration of tyrosinase degradation by proteasome inhibitors. Cells were pretreated with linoleic acid (25 µM) for 72 h and then cycloheximide (1 µg/ml) was added to the medium in the presence or absence of proteasome inhibitors (120 nM MG132 or 3 µM LLnL) for 8 h.

**Fig. 4.** Regulatory effects of fatty acids on the ubiquitination of general cellular proteins and of
tyrosinase. Numbers at the left indicate protein masses in kDa. (A) Cells were treated with linoleic acid (25 µM), palmitic acid (25 µM) or DMSO only (control) for 24 h in the presence or absence of MG132 (120 nM). Left, Western blotting (WB) of ubiquitin probed with Ub(P4D1) in whole cell lysate (5 µg protein/lane). Right, after stripping, the same membrane was reprobed with the tyrosinase antibody (αPEP7). Band intensities in lanes 4-6 of the left panel were measured at 70 kDa and the upper range enclosed by the bracket and were recalculated to X-fold increase or decrease as compared to the control. (B) Cells were treated with linoleic acid (25 µM), palmitic acid (25 µM) or DMSO only (control) for 24 h in the presence of MG132 (120 nM). Cells were then immunoprecipitated (IP) with the tyrosinase antibody (αPEP7) or with normal rabbit serum as a control (serum ctrl) and Western blotting of ubiquitin probed with Ub(P4D1) was performed. Arrowheads indicate the bands of heavy or light chains of IgG. Band intensities were recalculated to X-fold increase or decrease compared to the control after subtraction of the background intensity of the serum control.

**Fig. 5.** Immunofluorescence confocal microscopy showing the intracellular distribution of tyrosinase (Tyr, red) and ubiquitin (Ubi, green) after incubation with fatty acids. Cells were treated with linoleic acid (25 µM), palmitic acid (25 µM) or DMSO only (control) for 72 h in the presence (lower) or absence (upper) of MG132 (120 nM) for the last 24 h of treatment before fixation. Cells were stained with antibody (αPEP7 at 1:20 or Ub(P4D1) at 1:5), followed by nuclear counterstaining with 4’,6-diamidino-2-phenylindole (blue). The small panels show tyrosinase (Tyr) and ubiquitin (Ubi) while the large panels represent the merged images indicating colocalization of Tyr and Ubi as yellow fluorescence.
Figure 1

|        | linoleic acid | palmitic acid |
|--------|---------------|---------------|
| control| 6.25 µM       | 6.25 µM       |
|        | 12.5 µM       | 12.5 µM       |
|        | 25 µM         | 25 µM         |

- **tyrosinase**: 80
- **mono-ubiquitin**: 8
- **20S proteasome**: 37
- **PA700**: 50

Linoleic acid and palmitic acid tested at different concentrations (6.25 µM, 12.5 µM, 25 µM) for tyrosinase, mono-ubiquitin, 20S proteasome, and PA700.
Figure 2

A

|                  | control | linoleic acid | palmitic acid |
|------------------|---------|---------------|---------------|
| cycloheximide:   | -       | +             | +             |
| h                | -       | 4             | 8             |

B

Graph showing the relative amount of tyrosinase (% initial intensities) over time (0, 4, 8).
Figure 3

A
linoleic acid: - 24 h 48 h 72 h 72 h 72 h
MG132: - - - - 72 h -
LLnL: - - - - - 72 h

B
linoleic acid: 72 h 72 h 72 h 72 h
cycloheximide: - 8 h 8 h 8 h
MG132: - - 8 h -
LLnL: - - - 8 h
Figure 4

**A**

|          | ctrl | LA | PA | ctrl | LA | PA |
|----------|------|----|----|------|----|----|
| MG132    | -    | -  | -  | +    | +  | +  |

Fold: 1.0 1.6 0.6

**B**

|          | ctrl | LA | PA | serum | ctrl |
|----------|------|----|----|-------|------|
| MG132    | +    | +  | +  | +     | +    |

Fold: 1.0 1.8 0.7

**WB:** ubiquitin

**IP:** tyrosinase

**WB:** ubiquitin

**WB:** tyrosinase
### Figure 5

|                | control | linoleic acid | palmitic acid |
|----------------|---------|---------------|---------------|
| **MG132 -**    | ![Image](image1.png) | ![Image](image2.png) | ![Image](image3.png) |
| **MG132 +**    | ![Image](image4.png) | ![Image](image5.png) | ![Image](image6.png) |
Fatty acids regulate pigmentation via proteasomal degradation of tyrosinase - A new aspect of ubiquitin-proteasome function

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