Correlation between the number of melanosomes, tyrosinase mRNA levels, and tyrosinase activity in cultured murine melanoma cells in response to various melanogenesis regulatory agents

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Correlation between the Number of Melanosomes, Tyrosinase mRNA Levels, and Tyrosinase Activity in Cultured Murine Melanoma Cells in Response to Various Melanogenesis Regulatory Agents

培養マウス黒色腫細胞のメラニン色素生成調節物質処理における メラノソーム数、チロシナーゼｍＲＮＡレベル、および チロシナーゼ活性の相関性について

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Key words : dibutyryl cAMP, linoleic acid, melanoma cell, melanosome, palmitic acid, phorbol ester, tyrosinase

ABSTRACT

Tyrosinase is the rate limiting enzyme critically associated with melanin synthesis. The melanosomes are specialized membrane-bound organelles within melanocytic cells in which melanin polymers are ultimately deposited. To determine whether tyrosinase correlates with the number of melanosomes, we examined the relationship between tyrosinase activity, tyrosinase mRNA levels, and the number of melanosomes in B16 murine melanoma cells, using melanogenesis regulatory agents. 12-*O*-Tetradecanoylphorbol 13-acetate (TPA) or linoleic acid decreased tyrosinase activity, while dibutyryl cyclic adenosine monophosphate (dbcAMP) or palmitic acid increased it. The tyrosinase mRNA levels were not always correlated with tyrosinase activity, i.e., TPA down-regulated, dbcAMP up-regulated, while linoleic acid or palmitic acid did not alter the message levels, indicating that fatty acid-regulation of melanogenesis was due to post-transcriptional events. The number of melanosomes changed when agents which modulate the tyrosinase gene expression were added, since TPA decreased, dbcAMP increased, and linoleic acid or palmitic acid did not alter their number. These results suggest that the number of melanosomes changed in relation to tyrosinase mRNA level but not to tyrosinase activity in response to melanogenesis regulatory agents.

INTRODUCTION

The most important characteristic of differentiation in melanocytic cells is the presence of melanin pigment. Melanin synthesis is enzymatically regulated by the rate limiting enzyme termed tyrosinase. Tyrosinase (EC 1.14.18.1) is a bifunctional copper-containing enzyme which controls the modulation of melanin production (Pawelek, 1976; Hearing and Jimenez, 1987), firstly by catalyzing the hydroxylation of tyrosine to dopa and, in a second enzymatic step, catalyzing the oxidation of dopa to dopaquinone. Melanin pigment is a heterogeneous biopolymer formed from various intermediate products derived from dopaquinone and it is synthesized within melanosomes, the specialized membrane-bound organelles observed in melanocytic cells.

To date, a large number of melanogenesis regulatory factors have been reported. Among these, phorbol esters, such as 12-*O*-tetradecanoylphorbol 13-acetate (TPA) and phorbol dibutyrate were shown to have inhibitory effects on melanin synthesis, while intracellular cyclic adenosine monophosphate inducers, such as melanocyte-stimulating hormone and dibutyryl cyclic adenosine monophosphate (dbcAMP) were shown to increase melanogenesis in murine melanoma cells (Mufson et al., 1979; Fuller and Viskochil, 1979; Ludwig and Niles, 1980; Laskin et al., 1983; Jimenez et al., 1988; Niles and Loewy, 1989; Hoganson et al., 1989; Fuller et al., 1990; Park et al., 1992). Further, saturated fatty acids, such as palmitic acid (C16:0) and stearic acid (C18:0), have been shown to activate melanogenesis, while unsaturated fatty acids, such as oleic acid (C18:1) and linoleic acid (C18:2), were shown to inhibit it in murine melanoma cells (Shono and Toda, 1981; Ando et al., 1990). These fatty acid-induced antagonistic effects on melanogenesis are of interest, since these fatty acids have a similar structure except for the number of hydrocarbons and unsaturated bonds.

Recently, a positive relationship between tyrosinase activity and tyrosinase

mRNA level in murine melanoma cells was reported (Hoganson et al., 1989; Fuller et al., 1990), although no correlation between them was observed in human melanocytes and melanoma cells (Naeyaert et al., 1991). On the other hand, tyrosinase gene expression due to the transfection of tyrosinase cDNA either into fibroblasts (Bouchard et al., 1989; Winder, 1991) or amelanotic melanocytes (Whitaker et al., 1989; Larue et al., 1990; Ando et al., 1993a) resulted in the appearance of pigmented membrane-bound cytoplasmic vesicles or large numbers of melanosomes. The relationships between tyrosinase activity, tyrosinase gene expression, and the number of melanosomes were reported with some inconsistent results using chicken/mouse/human melanocytes or melanoma cells, although details of these correlations have not been analyzed directly. Therefore, to understand the relationship between these, we assessed the relationships between tyrosinase activity, tyrosinase mRNA level, and the number of melanosomes using melanogenesis regulatory agents, e.g., TPA, dbcAMP, linoleic acid, and palmitic acid, in cultured murine melanoma cells. We found that tyrosinase activity did not always correlate with tyrosinase mRNA level, and also found that the number of melanosomes changed in relation to tyrosinase mRNA level but not to tyrosinase activity in response to melanogenesis regulatory agents.

MATERIALS AND METHODS

Cell cultures

B16F10 murine melanoma cells were cultured in Eagle's minimal essential medium supplemented with 10% heat inactivated (56℃, 30 min) fetal bovine serum (HyClone Laboratories, Logan, UT) and 2 mM L-glutamine at 37℃ in a humidified atmosphere of 5% CO_2. TPA (25 nM), dbcAMP (250 μM),

linoleic acid (25 μ M), or palmitic acid (25 μ M) (all from Sigma Chemical Co., St. Louis, MO) was added to culture medium approximately 3 h after cell seeding. Three days later, the incubated cells were reseeded with fresh medium and reagents. After 6 days incubation, the cells were harvested and used for melanin measurements, tyrosinase assays, and electron microscopic observations.

Melanin measurements

Melanin content was measured by a modification of the method of Oikawa and Nakayasu (1973) and Hosoi et al. (1985). Approximately 10^7 cells were pelleted by centrifugation at 1,000 X g for 5 min, and then washed twice with phosphate-buffered saline. After centrifugation (1,000 X g for 5 min), the supernatant was decanted. The precipitate was resuspended with 200 μ l of distilled water and then 1 ml of ethanol:ether (1:1 volume) was added in order to remove opaque substances other than melanin. This was stored and suspended at room temperature for 15 min. Melanin cannot be dissolved in ethanol/ether (Oikawa and Nakayasu, 1973). After further centrifugation (3,000 X g for 5 min), the precipitate was solubilized by treatment with 1 ml of 10% dimethyl sulfoxide in 1 N NaOH aqueous solution at 80℃ for 30 min in capped test tubes. The absorbance was measured at 470 nm, and melanin content per cell was calculated and expressed as a percentage of untreated cells.

Tyrosinase assays

Tyrosinase activity was assayed as dopa oxidation activity with a modification of the method used by Wrathal et al. (1973). 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 ℃ for 15 min. Tyrosinase activity was analysed spectrophotometrically by following the oxidation of L-dopa to dopachrome at 475 nm. The reaction mixture consisted of 3.0 ml of 0.1% L-dopa (Sigma) in 0.1 M phosphate buffer (pH 6.8) added to the cell lysate, and suspended. The reaction mixture was freshly made every 2 h. Assays were performed at 37 ℃ with a two-wavelength/double beam Spectrophotometer (U-3210, Hitachi, Japan). The rate was measured during the first ten minutes of the reaction while it was linear. Correction for L-dopa auto-oxidation was made. Specific activity was defined as formation of dopachrome (absorbance at 475 nm) per 10 min per cell, and expressed as a percentage of untreated cells.

RNA preparation and Northern blotting

Total cellular RNA was isolated using the guanidium isothiocynate method of Chirgwin et al. (1979). For Northern blot analysis, 10 μ g of total cellular RNA was electrophoresed in a formaldehyde-1% agarose gel and blotted onto nitrocellulose paper. After baking at 80℃ for 2 h and prehybridization, the blot was hybridized with randomly primed ^{32}P-labeled murine tyrosinase cDNA clone Tyrs-J (Yamamoto et al., 1989) or ^{32}P-labeled β -actin cDNA in a solution containing 4 X SSC (Maniatis et al., 1982), 40% formamide, 10% dextran sulfate, 0.02 M Tris-HCl (pH 7.4), 1 X Denhardt's solution, 250 μ g/ml yeast tRNA, and 20 μ g/ml salmon sperm DNA at 42 ℃ and washed in 0.1 X SSC, 0.5% sodium dodecyl sulfate at 65 ℃. The papers were then dried and autoradiographed. The autoradiograms were scanned using a densitometer (FDU-3, Shimadzu, Japan), and the tyrosinase mRNA levels were adjusted to the relative amount of β -actin mRNA levels, which was used as an internal control.

Electron microscopic observation

Cultured B16 melanoma cells were washed twice with 0.1 M phosphate buffer (pH 7.4) and fixed with 2% glutaraldehyde in the same buffer for 2 h. After being washed twice with the buffer for 15 min, the cells were fixed with 1% osmium tetroxide for 2 h. After fixation, they were dehydrated in graded concentrations of alcohol and propylene oxide, and embedded in Epon 812. Ultrathin sections were stained with 1% uranyl acetate and Reynolds' lead citrate, and examined with an electron microscope (JEM-100SX, JEOL, Japan).

Quantitative analysis of melanosome

Quantitative analysis of melanosomes was carried out by electron microscopy by counting the number and classifying the degree of melanization. The number of melanosomes in individual cells were counted over 100 electron micrographs of different cells. The cytoplasmic area of each cell was also measured individually by an image analyzer (Avio SP-500, OLYMPUS, Japan), and the total number of melanosomes seen in individual cells was recalculated for values per 100 μ m^2. Furthermore, each melanosome was quantitatively analyzed based on the degree of melanization according to the method of Ando and Mishima (1991). For evaluating melanization, melanosomes were quantified by melanization degree according to the percent volume of melanin pigment within a melanosome: I = none, II = up to 30% of melanosome field, III = 31-70%, and IV = 71-100%.

RESULTS

Effect on melanogenesis

The melanogenesis regulatory effects of TPA, dbcAMP, linoelic acid, or palmitic acid observed in cell pellets are shown in figure 1. As previously reported (Mufson et al., 1979; Shono and Toda, 1981; Hoganson et al., 1989; Fuller et al., 1990; Ando et al., 1990), TPA or linoleic acid inhibited melanogenesis, while dbcAMP or palmitic acid apparently increased it. TPA or dbcAMP showed a slight inhibition of the growth rate of B16 melanoma cells, but linoleic acid or palmitic acid did not affect the growth during 6 days incubation (Fig. 2a). Therefore, there was no correlation between melanin synthesis and cell growth under our experimental conditions.

The results of the quantitative measurements of melanin content are shown in figure 2b. These results correlate well with the visible appearance shown in figure 1. After 6 days incubation, TPA or linoleic acid decreased, but dbcAMP or palmitic acid increased melanin content to 19.2%, 28.8%, 191.4%, and 150.8%, respectively, as compared to untreated control cells (=100%).

The activity of tyrosinase is shown in figure 2c. These results correlated with the regulatory effects on melanin content. After 6 days incubation, TPA or linoleic acid decreased, but dbcAMP or palmitic acid increased tyrosinase activity to 0% (not detectable), 35.7%, 463.2% and 351.5%, respectively, as compared to untreated control cells (=100%). The inhibitory effect of TPA on tyrosinase activity was stronger than that of linoleic acid.

Effect on tyrosinase mRNA level

By Northern blot analysis, several sizes of tyrosinase mRNA were detected, and all signals were positive even after washing in 0.1 X SSC at 65℃. This is consistent with the previous report showing that multiple transcripts of the murine tyrosinase gene are generated by alternative splicing (Ruppert et al., 1988; Porter and Mintz, 1991).

The Northern blot analysis of tyrosinase mRNA from B16 melanoma cells

treated with TPA or dbcAMP for 24 h is shown in figure 3. As previously reported (Hoganson et al., 1989; Fuller et al., 1990; Itoh et al., 1991), TPA decreased the tyrosinase mRNA level significantly (8.2% of untreated), but dbcAMP, on the other hand, caused a 1.8-fold increase over untreated control level.

The tyrosinase mRNA levels of B16 melanoma cells treated with linoleic acid or palmitic acid for 3-72 h are shown in figure 4. The left lane indicates the level of untreated control. In contrast to TPA and dbcAMP, fatty acids had little effect on the transcription of tyrosinase mRNA through 3-72 h incubation, indicating that this fatty acid-regulated melanogenesis is due to post-transcriptional events.

Effects on number and melanization of melanosomes

Representative electron microscopic figures of B16 melanoma cells after 6 days incubation with TPA, dbcAMP, linoleic acid, or palmitic acid, compared with untreated control, are shown in figure 5. Various stages of melanosomes were observed in untreated control cells. Cells incubated with dbcAMP or palmitic acid had a high percentage of highly melanized mature melanosomes, while cells incubated with linoleic acid had many unmelanized immature ones. On the other hand, cells incubated with TPA lost virtually all mature and immature melanosomes.

Figure 6 shows both the number and the degree of melanization of melanosomes observed in 100 μ m^2 cytoplasmic field from 100 different cells incubated with TPA, dbcAMP, linoleic acid, or palmitic acid for 6 days, compared with untreated control. The number of melanosomes in the untreated control was 51.5 / 100 μ m^2. TPA significantly decreased the number to 6.8 / 100 μ m^2, and dbcAMP increased it to 83.0 / 100 μ m^2. On the other hand, linoleic acid and palmitic acid scarcely changed the total number, i.e., linoleic

acid was 48.8 / 100 μ m^2 and palmitic acid was 51.0 / 100 μ m^2, respectively. These numerical changes of melanosomes were related to the tyrosinase mRNA levels shown in figure 3 and 4.

The degree of melanosome melanization correlated similarly with the results of melanin content, i.e., TPA and linoleic acid reduced the percentage of melanosomes containing melanin pigment (stage II-IV) to 24.6% and 15.8%, and dbcAMP and palmitic acid increased it to 58.9% and 69.3%, respectively, as compared to untreated control (53.8%).

DISCUSSION

The present study directly demonstrates the relationship between melanosome production and tyrosinase message level in murine melanoma cells. Recently, it was reported that the transfection of human or murine tyrosinase cDNA into murine fibroblast cells (which have no melanosomes) transformed the cells into brown pigmented ones in which pigmented membrane-bound cytoplasmic organelles were observed (Bouchard et al., 1989; Winder, 1991). In the case of albino chicken or murine melanocytes or human amelanotic melanoma cells, the transfection of tyrosinase cDNA resulted in the appearance of pigmentation and large numbers of newly formed melanosomes were observed (Whitaker et al., 1989; Larue et al., 1990; Ando et al., 1993a), suggesting that the expression of tyrosinase gene alone may be sufficient for melanin synthesis and melanosome induction even in non-melanin producing melanocytes. Melanosomes are considered to be related to lysosomes (Novak and Swank, 1979; Novak et al., 1980) or to peroxisomes (Moellman, 1990), hence it might be possible to assume that tyrosinase gene transcription correlates with the differentiation of lysosomes and/or peroxisomes into melanosomes.

Alteration of melanosome number has been reported to be induced by melanogenesis regulatory agents. Cultured human melanocytes grown in the presence of cholera toxin, a potent inducer of cAMP, exhibited large numbers of melanosomes, while cells grown in TPA showed fewer (Eisinger and Marko, 1982). Our present study confirmed quantitatively these relationships using B16 melanoma cells. Regarding the correlation between the number of melanosomes and tyrosinase mRNA level, lactic acid has been shown to reduce both tyrosinase mRNA level and the number of melanosomes in B16 melanoma cells (Ando et al., 1993b). Despite these observations, however, it was unclear whether the alteration of the number of melanosomes correlated with tyrosinase message level or with tyrosinase activity. In this study, we examined quantitatively the relationship between the number of melanosomes, tyrosinase activity, and tyrosinase mRNA level. TPA decreased while dbcAMP increased the number of melanosomes in relation to the alteration both of tyrosinase activity and tyrosinase mRNA level. On the other hand, neither linoleic acid nor palmitic acid altered both the number of melanosomes and the tyrosinase mRNA level although these fatty acids changed tyrosinase activity significantly. These results suggest that the production of melanosomes in B16 melanoma cells correlates positively with tyrosinase gene expression but not always with tyrosinase activity.

There are some reports on the relationship between tyrosinase activity and tyrosinase mRNA level. Recently, direct correlations between tyrosinase activity and tyrosinase mRNA level in murine melanoma cells were demonstrated using TPA and dbcAMP (Hoganson et al., 1989; Fuller et al., 1990). In contrast, the correlation between them has not been demonstrated using human melanocytes and melanoma cells, suggesting that post-transcriptional regulation of tyrosinase and/or other events may play an essential role in the rate of melanin synthesis in humans (Naeyaert et al., 1991). Whether these contradictory results were due to the different cell species or

due to other events, such as the participation of a preexisting pool of inactive tyrosinase as previously reported (Jimenez et al., 1988) has not been determined yet. Although the results of TPA and dbcAMP in this study were in agreement with previous reports (Hoganson et al., 1989; Fuller et al., 1990), our data presented here using linoleic acid and palmitic acid did not show a correlation between tyrosinase activity and tyrosinase mRNA level in murine melanoma cells similarly to the previous finding in human melanocytes and melanoma cells (Naeyaert et al., 1991). Taken together, the correlation between tyrosinase activity and tyrosinase mRNA level may depend on the melanogenesis regulatory agents used and their mechanisms of the modulation of catalytic efficiency of tyrosinase, i.e., transcriptional regulation which leads to increased or decreased synthesis of tyrosinase and/or post-transcriptional regulation, such as glycosylation of tyrosinase required for its maturation (Imokawa and Mishima, 1982). Whether the alteration of tyrosinase mRNA level is due to the regulation of tyrosinase gene activity or due to the modulation of the turnover of tyrosinase mRNA remains to be determined. In this study, however, it is clarified that tyrosinase activity does not always correlate with tyrosinase mRNA level in murine melanoma cells.

Among the melanogenesis regulatory agents used in this study, TPA elicited the down-regulation of tyrosinase mRNA level fairly rapidly, i.e., within 24 h. We do not know, at present, the half-life of tyrosinase mRNA, but both a rapid decrease of tyrosinase gene expression and a short message half-life would be required for the TPA-induced loss of tyrosinase mRNA within this timeframe. TPA is well known to have biphasic effects on protein kinase C (PKC), one of the major signal transduction pathways regulating cell function, i.e., treatment by TPA initially activates PKC but chronic treatment apparently down-regulates PKC, resulting in its depletion (Rodriguez-Pena and Rozengurt, 1984; Blackshear et al., 1985; Ballester and Rosen, 1985; Stabel et al., 1987). In murine melanoma cells, phorbol dibutyrate was shown to down-regulate

PKC activity within 24 h (Park et al., 1992). Furthermore, recent work has shown that the melanogenesis of human melanocytes and melanoma cells increased through activation of the β isoform of PKC (Park et al., 1993). Taking these findings into consideration, it is suggested that TPA inhibits melanogenesis by down-regulating tyrosinase mRNA level together with the depletion of PKC, which leads to inactivation of preexisting tyrosinase.

In summary, our study presented here has shown that 1) the number of melanosomes changes in relation to tyrosinase mRNA level but not to tyrosinase activity; and 2) tyrosinase activity does not always correlate with tyrosinase mRNA level, in murine melanoma cells in response to various melanogenesis regulatory agents.

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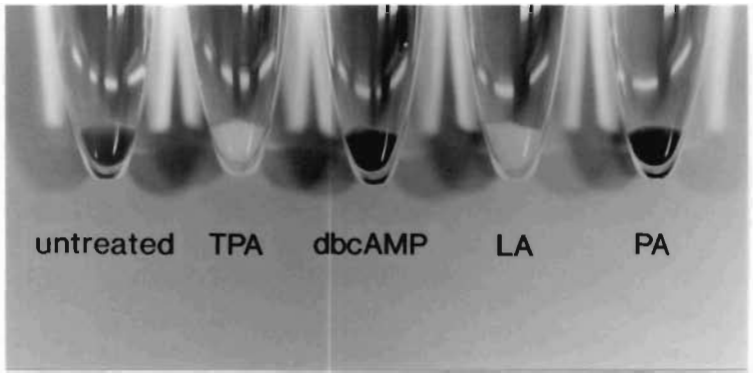

Fig. 1. Pellets of B16 melanoma cells after 6 days incubation with TPA (25 nM), dbcAMP (250 μ M), linoleic acid (LA, 25 μ M), or palmitic acid (PA, 25 μ M), compared to untreated control.

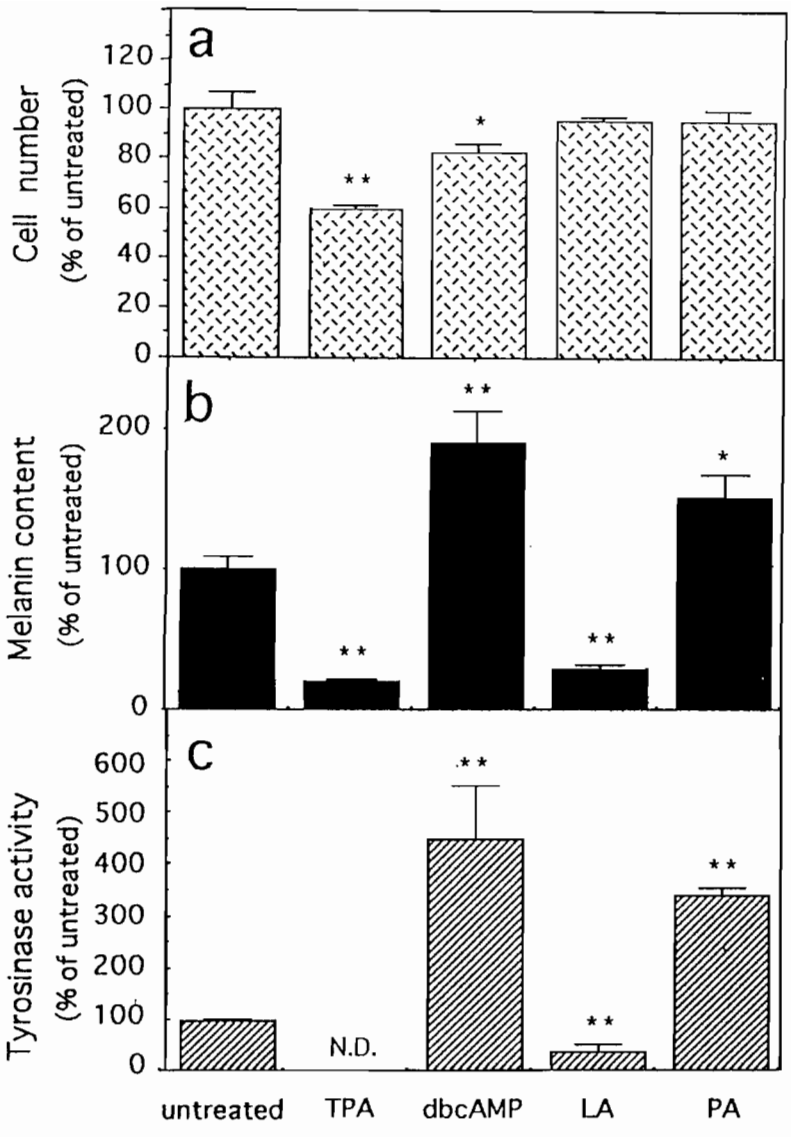

Fig. 2. The growth rate (a), melanin content (b), and tyrosinase activity (c) of B16 melanoma cells after 6 days incubation with TPA (25 nM), dbcAMP (250 μ M), linoleic acid (LA, 25 μ M), or palmitic acid (PA, 25 μ M). Data are expressed as a percentage of control, and are mean values of triplicate determinations $\pm$ SD. A Student's t-test was used for statistical analysis of the data. (* = $p<0.05$; ** = $p<0.01$) N.D. = not detectable.

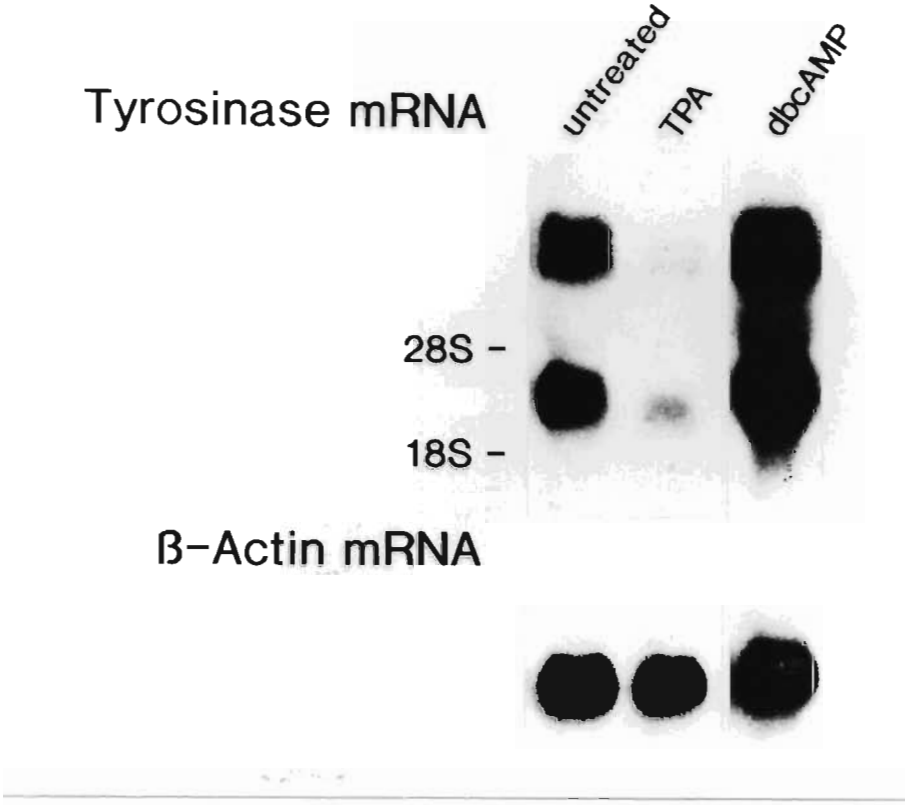

Fig. 3. Northern blot analysis of tyrosinase mRNA from B16 melanoma cells incubated with TPA (25 nM) or dbcAMP (250 μ M) for 24 h, compared to untreated control. The size markers used were 28S and 18S mouse ribosomal RNAs (indicated left).

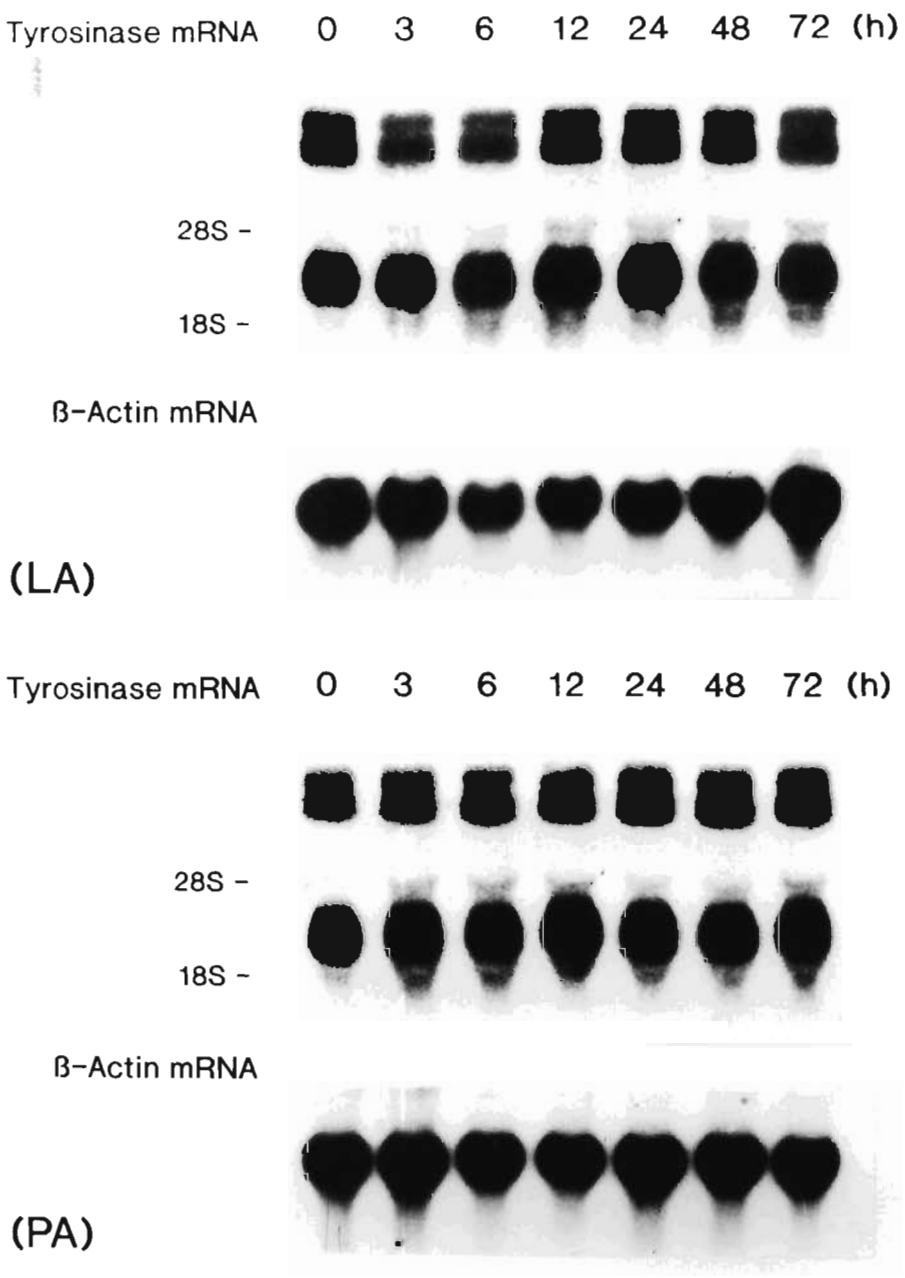

Fig. 4. Northern blot analysis of tyrosinase mRNA from B16 melanoma cells incubated with linoleic acid (LA, upper) or palmitic acid (PA, lower) for 3-72 h. The left lane indicates untreated control, and the treatment periods of 3, 6, 12, 24, 48, and 72 h are labeled above.

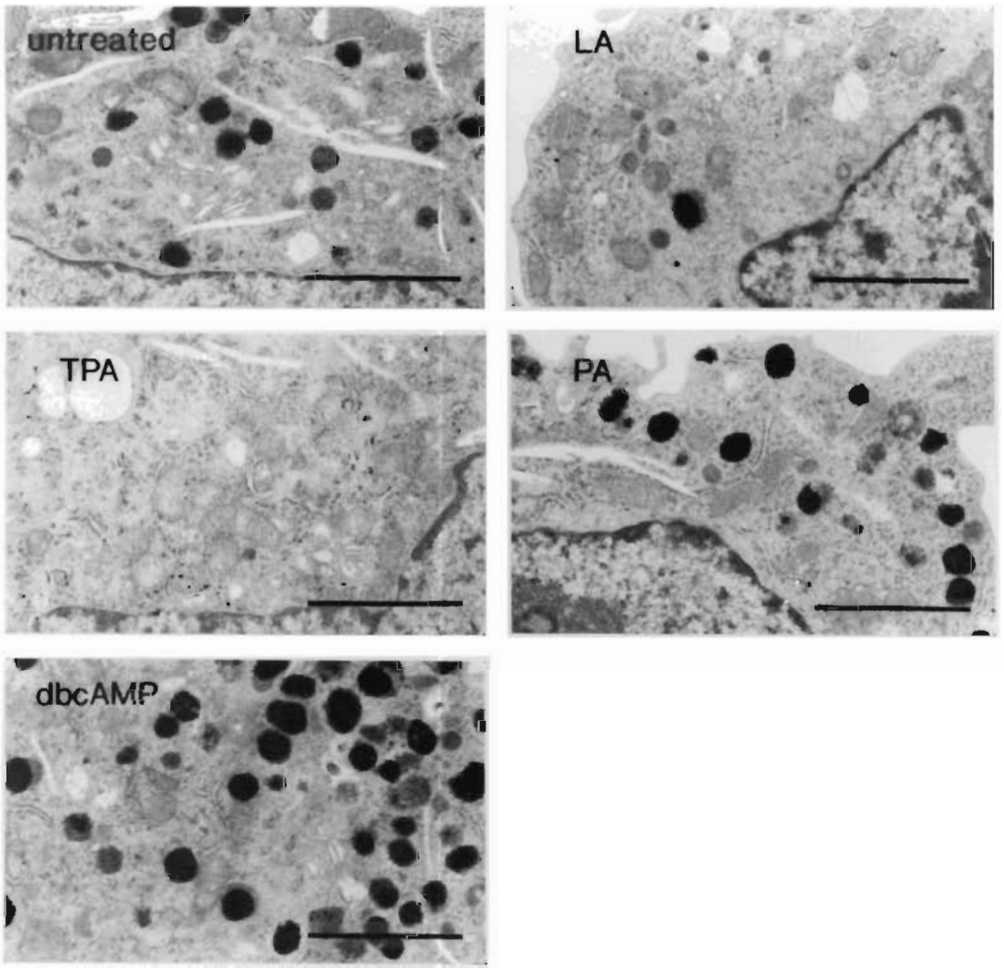

Fig. 5. Representative electron microscopic figures of B16 melanoma cells after 6 days incubation with TPA (25 nM), dbcAMP (250 μ M), linoleic acid (LA, 25 μ M), or palmitic acid (PA, 25 μ M), compared to untreated control. Bar = 2 μ m.

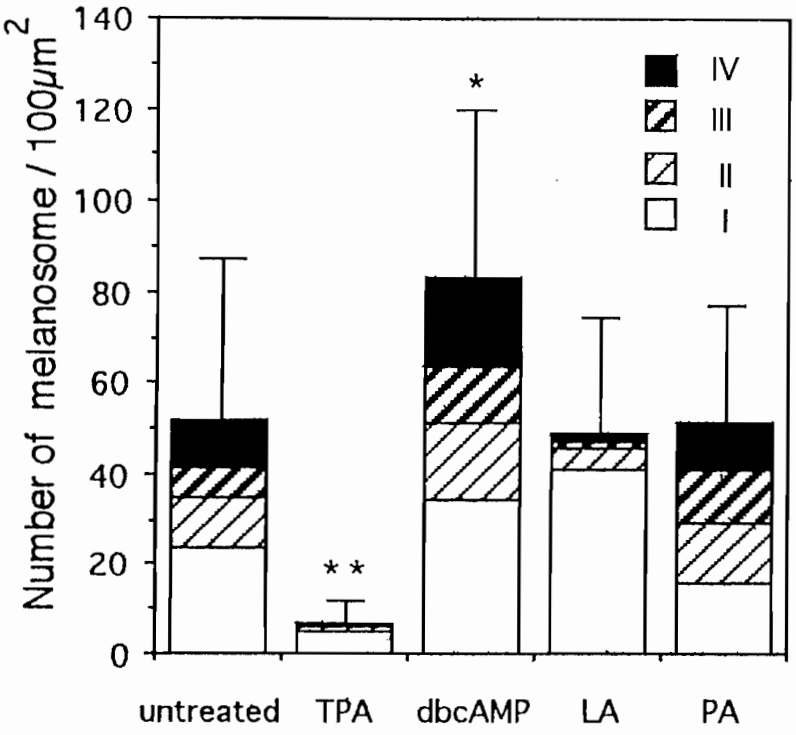

Fig. 6. The number of melanosomes observed in the cytoplasmic field of electron micrographs from 100 different cells each of groups incubated with TPA (25 nM), dbcAMP (250 μ M), linoleic acid (LA, 25 μ M), or palmitic acid (PA, 25 μ M) for 6 days. Data are expressed as the mean number of melanosomes $\pm$ SD per 100 μ m^2. Melanosomes were classified by their degree of melanization according to the extent of the total area occupied by melanin pigment as follows: I = none, II = up to 30% of melanosome field, III = 31-70% and IV = 71-100%. A Student's t-test was used for statistical analysis of the data. (* = $p<0.05$; ** = $p<0.01$)