ABSTRACT

Human promyelocytic leukemia cells (HL-60) undergo differentiation when treated with the tumor promoter, 12-O-tetradecanoylphorbol-13-acetate (TPA). As the primary target for TPA action is membranes, studies were undertaken to determine whether phorbol ester exposure would influence fatty acid metabolism in these cells. In cells incubated with labeled fatty acids for 1 hr, the percentage of distribution of lipid radioactivity is highest in the phospholipid fraction of control cultures, whereas in TPA-supplemented cells, substantially more label is associated with triacylglycerols. The specific activity of phospholipids and triacylglycerols was lower in treated than in control cells; however, the amount of cellular triacylglycerols increased 3.2-fold (lipid per mg protein). The increase in the amount of cellular phospholipids in TPA-treated cells is not as pronounced (approximately 50% above control), and this only occurs at higher concentrations of TPA. At early times after TPA exposure, there is no stimulation of the cellular uptake of labeled fatty acids; however, differentiating cells (24 to 48 hr of TPA), when incubated with label, contained more radioactivity than did control cultures. Cells treated with TPA for 48 hr show a marked decrease in the conversion of [1-14C]stearic acid to monoenoic product (22% of control); this decrease is dose dependent and occurs within 24 to 48 hr of treatment.

Although the phospholipid fatty acid composition of differentiating cells was similar to control cells, acyl groups of triacylglycerols isolated from treated cells showed a marked decrease in the percentage of unsaturates. These data provide evidence which demonstrates that TPA treatment of HL-60 cells has a profound effect on fatty acid metabolism. The lack of an effect of TPA on fatty acid metabolism after short-term exposure to the promoter suggests that the modifications observed may be the result of cellular differentiation rather than a direct effect exerted by the presence of TPA in the culture media.

INTRODUCTION

Berenblum (2), in 1941, discovered that a single application of benzpyrene (initiation) to mouse skin followed by multiple applications of croton oil (promotion) would elicit tumor growth. The chemical properties of croton oil are the 12,13-diesters of the polyfunctional, tetracyclic diterpene alcohol, phorbol (13). TPA,3 the most biologically active tumor-promoting agent of croton oil, has been used widely in the 2-stage mouse skin carcinogenesis system (5) to study the mechanism of tumor promotion. TPA can inhibit differentiation in a number of cell lines [for a review of the effects of tumor promoters on cells in culture, see the paper of Diamond et al. (10)], but TPA can also induce differentiation in HL-60 human promyeloctytic leukemia cells (14, 25). Tumor-promoting phorbol esters alter lipid metabolism in a number of cell systems (8, 15, 19, 23), although the specific mechanisms responsible for these effects have not been delineated. Recently, Paddon and Vance (21) have shown that TPA-stimulated phosphatidylcholine biosynthesis in HeLa cells is due to an increase in the rate of the reaction catalyzed by CTP:phosphocholine cytidylyltransferase.

In a previous paper, we described alterations in phospholipid and triacylglycerol metabolism that occur in TPA-differentiating human leukemia cells (7). In that study, as in other investigations, the effects of TPA on phospholipid metabolism were studied with labeled precursors of the polar head groups. Presently, little is known about the effects of TPA-induced differentiation on fatty acid metabolism. Because fatty acids are precursors of the acyl moieties of phospholipids and triacylglycerols, we undertook this study to determine what effects TPA exerts on the uptake and utilization of fatty acids from the standpoint of the acyl group precursors of the glycerolipids. Membranes are prime targets for phorbol esters (18), and therefore, knowledge of fatty acid metabolism in TPA-treated cells is expected to provide insight about the role of glycerolipids in cellular differentiation.

MATERIALS AND METHODS

Chemicals. Radioisotopes, which were purchased from New England Nuclear, Boston, Mass., included: [1-14C]palmitic acid, 49.2 mCi/mmol; [1-14C]stearic acid, 50.0 mCi/mmol; and [5,6,8,9,11,12,14,15-3H]arachidonic acid, 82.2 Ci/mmol. [9,10-3H]Oleic acid, 5.04 Ci/mmol, was purchased from Amersham/Searle Corp., Arlington Heights, Ill. TPA, purchased from Chemical Carcinogenesis, Eden Prairie, Minn., was dissolved in dimethyl sulfoxide and stored at -20°C under nitrogen. Tripalmitin glycerol, obtained from Applied Science Laboratories, Inc., State College, Pa., was used as a standard for photodensitometric measurements. Solvents used for the analytical procedures were supplied by Burdick & Jackson Laboratories, Inc., Muskegon, Mich.

Cell Culture. Human promyelocytic HL-60 leukemia cells (9) were originally provided by Dr. R. C. Gallo, National Cancer Institute, Bethesda, Md., and had been maintained in the laboratory of Dr. E. Huberman, Biology Division, Oak Ridge National Laboratory. Given the fact that there is some degree of variability among HL-60 cells in various laboratories, morphological and enzymatic differentiation markers had been established for the cells used in these experiments (14) and likewise carried out on the cells used in our previous paper (7). For this reason, differentiation markers were not reassessed during the course of these experiments. The cells were cultured in 75-sq cm Falcon flasks (Falcon Plastics, Oxnard, Calif.) using Roswell Park Memorial Institute Tissue Culture Medium 1640 containing 20% fetal...
calf serum plus penicillin (100 units/ml) and streptomycin (100 µg/ml) from Grand Island Biological Co., Grand Island, N. Y. Cells were seeded at approximately 2 × 10⁶ cells/flask and treated with TPA 24 hr later. Culture medium was used to make serial dilutions of TPA:dimethyl sulfoxide solution and added to cells to provide the desired concentrations of TPA (1 to 8 × 10⁻¹⁰ M). By this method, dimethyl sulfoxide did not exceed 0.0005% in the growth flasks. Radiolabeled fatty acids were added to cell cultures in 20 to 50 µl of absolute ethanol.

**Lipid Analyses.** Termination of experiments and the harvesting of cells have been described (7). Total lipids were extracted by a modified method of Bligh and Dyer (4) in which the methanol contained 2% glacial acetic acid. Total lipid weight was determined using a Cahn 26 electrobalance, and liquid scintillation spectrometry was used for direct analysis of radiolabeled lipid samples.

Total lipids were resolved on Silica Gel G thin layers using a solvent system containing hexane:dioxane:glacial acetic acid (60:40:1, v/v/v). Phospholipids were fractionated on Silica Gel 60 HR layers (Merck, Darmstadt, West Germany) made basic with 1 µmol Na₂CO₃ in a solvent system of chloroform:methanol:glacial acetic acid:water (50: 25:8:4, v/v/v/v). Specific lipid classes were identified by comparison of their Rₜ values with commercial standards, and zonal profile scans (25) were used for analysis of label distribution along the chromatogram.

The fatty acid composition of phospholipids and triacylglycerols isolated from control and TPA-treated cells was determined by gas-liquid chromatographic analysis of the methyl esters (3). The gas chromatograph was equipped with a Hewlett-Packard Model 3390A recorder integrator. Lipid classes were first isolated by preparative thin-layer chromatography. For investigating the desaturation of [1-¹⁴C]stearic acid by HL-60 cells, argentation chromatography (1) was used for separation of saturated and monoenoic methyl esters derived from total cellular lipids. When [1-¹⁴C]palmitic acid was incubated with cells, the metabolic products were analyzed by combined gas-liquid chromatography and collection of ¹⁴CO₂ as described by Blank et al. (23). Cellular triacylglycerols were quantitated by photodensitometry (22) using a protocol described previously (7). Phospholipid phosphorus was determined by the method of Rouser et al. (24), and the method of Lowry et al. (20) was used for determination of cellular protein. Detailed protocols of individual experiments are provided in the legends to charts and tables.

**RESULTS**

The effects of TPA on the cellular utilization of radioactive fatty acids supplemented to growth media would be indicative of alterations in membrane processes associated with cellular differentiation. We therefore used labeled fatty acids to assess glycerolipid acyl group metabolism in human leukemia cells. In initial experiments after a 1-hr incubation with label, the distribution of [1-¹⁴C]stearic acid in total lipids of control and 48-hr TPA-treated cells (8 × 10⁻¹⁰ M) was markedly different; radioactivity was higher in the neutral lipid fraction of treated cells, whereas, in control cells, the highest percentage of ¹⁴C was associated with phospholipids. In both control and TPA-treated cells, the majority of the ¹⁴C was recovered in phospholipids and triacylglycerols (93 to 95%); labeled diacylglycerols and intracellular free stearic acid accounted for the remainder. To evaluate the effects of TPA concentration on the incorporation of [1-¹⁴C]stearic acid into the lipid classes of leukemia cells, the cells were incubated for 48 hr with TPA (1 to 8 × 10⁻¹⁰ M). The data of Chart 1 show that, in cells incubated without TPA, 39 and 61% of the lipid radioactivity are contained within the neutral lipids and phospholipids, respectively. At higher concentrations of TPA, this pattern is reversed, and the ratio of neutral lipid to polar lipid radioactivity increases. At 2 × 10⁻¹⁰ M TPA, the distribution of ¹⁴C in phospholipids and neutral lipids is approximately equal. Analyses of phospholipid radioactivity showed that, of the phosphatidylcholine and phosphatidylethanolamine fractions, the former contained twice the radioactivity; however, these fractions demonstrated a parallel loss of ¹⁴C as the TPA concentration increased. In order to determine the time sequence for the TPA-enhanced incorporation of [1-¹⁴C]stearic acid into neutral lipids, cells were incubated with TPA (8 × 10⁻¹⁰ M) for 2 to 48 hr and labeled for 1 hr. No major differences in the polar and neutral lipid ¹⁴C distribution were noted at 2 hr, although by 4 hr, neutral lipid radioactivity increased. The ratio of phospholipid ¹⁴C to neutral lipid ¹⁴C (dpm/mg cell protein) in untreated cells was 1.6; after 4-, 24-, and 48-hr exposure to TPA, the ratios were 1.3, 0.93, and 0.64, respectively. Thin-layer chromatographic analysis of the neutral lipid fraction showed that, in control cultures, all of the counts were associated with the triacylglycerol fraction, whereas, in the TPA-treated cells, approximately 5% of the neutral glycerolipid radioactivity was in the alkylacylglycerols. To determine the effect of TPA on acyl group metabolism in cells prelabeled with [1-¹⁴C]stearic acid, labeled cells were replated in fresh medium with or without TPA and incubated for 4 hr. In both control and treated cells, there were no differences in total radioactivity of lipids or specific lipid classes.

The distribution of radioactivity between phospholipids and neutral lipids was determined in control and differentiating cells after incubation with [1-¹⁴C]stearic acid at several time periods. The data in Chart 2A shows first that the uptake of labeled fatty acid increased with time, deviating slightly from linearity, in both control and TPA-treated cells. More radioactivity was recovered in treated cells; however, we have found this to vary slightly from experiment to experiment. The time of labeling...
has a profound effect on the relative incorporation of stearic acid into lipids of control (Chart 2B) and TPA-differentiating cells (Chart 2C). At 5 to 30 min, the ratio of phospholipid to neutral lipid radioactivity in treated cells was only slightly modified; whereas, in control cells, phospholipids contained a greater percentage of radioactivity throughout the 60-min time course. By 60 min, the incorporation of radioactivity into phospholipids of TPA-treated cells began to plateau, while assimilation of $^{14}C$ in the neutral lipid fraction continued in a linear manner. In 3 independent experiments, the differences in phospholipid and neutral lipid radioactivity in treated cells at the 60-min labeling period were shown to be significantly different (Student's t test, $p < 0.001$).

In a previous study, we reported that cells treated with TPA contained more triacylglycerol than did control cells. The data of Chart 1 show that, with increasing concentrations of TPA, more stearic acid is directed into triacylglycerols. To ascertain if this increase in triacylglycerol radioactivity is indicative of a mass change, the amount of triacylglycerol was determined in cells exposed to varying concentrations of TPA. The data of Table 1 show that, as the TPA concentration is increased, the amount of cellular triacylglycerols increases dramatically, and with $8 \times 10^{-10} \text{M}$ TPA, this neutral lipid fraction increased 3.2-fold over controls. As shown in Chart 1, an increase in cellular phospholipids occurs only at the highest concentration of TPA. Analysis of sphingomyelin and choline-, serine-, inositol-, and ethanolamine-containing phosphoglycerides showed that the composition percentage of these lipids did not change in control and TPA-treated cells.

Experiments were carried out to compare the metabolism of stearic acid with monoenoic and polyenoic fatty acids in control and treated cells. The data of Table 2 are given as specific activity (dpm/µg lipid) in order to evaluate turnover of the labeled acyl groups within the different lipid classes. In control cells, the highest specific activities occurred with arachidonic and oleic acid in phospholipids and triacylglycerols, respectively. In all instances, the specific activities of the labeled phospholipids and triacylglycerols were lower in the TPA-differentiating cells, and of the 2 lipid classes, the triacylglycerols showed the greatest depression.

The effects of TPA on the uptake of fatty acids by HL-60 cells were assessed in a series of experiments. Cells incubated with TPA ($8 \times 10^{-10} \text{M}$) for 48 hr ($1$-hr $^{14}C$stearic acid labeling period) contained 26% more radioactivity (dpm/µg cell protein); likewise, a higher percentage of $^{3}H$oleic and $^{3}H$arachidonic acids was recovered in treated cells, 18 and 68% above control, respectively. Although there was some degree of variability in the uptake of labeled stearic and oleic acids between control and treated cells, the uptake of labeled arachidonic acid was consistently higher in TPA-treated cells. When $1$-$^{14}C$palmitic acid and TPA were added simultaneously to cultures and incubated for 48 hr, the cells contained approximately twice the radioactivity (dpm/µg protein) compared to cells labeled in the absence of TPA. Qualitative analysis of label in the control and TPA-treated cells showed the latter to contain a 4-fold increase in the amount of triacylglycerol-associated radioactivity. At early times, before the markers of differentiation are expressed, TPA had no influence on the uptake of radiolabeled fatty acids. This was tested by incubating cells with TPA ($8 \times 10^{-10} \text{M}$) for 1, 2, and 4 hr with an
additional 1-hr [^14]C]stearic acid labeling period. Analysis of both control and treated cells showed identical amounts of lipid radioactivity per mg protein.

The possibility that TPA-induced differentiation may alter parameters that govern membrane fluidity was tested by measuring its effect on [1-^14]C]stearic acid desaturation in intact cells (Chart 3). At concentrations of 4 to 8 × 10^{-10} M TPA, the monoenoic product of [1-^14]C]stearic acid was only 22 to 25% of control. The inhibition of desaturation in the HL-60 cells by TPA was found to be time dependent, and by 24 hr, the amount of [1-^14]C]oleic acid recovered from differentiating cells, after [^14]C]stearic acid labeling, was 60% of control; at 48 hr, the inhibition was maximum. Cells incubated with TPA for 4 hr had a conversion rate of stearate to oleate equal to control levels.

The metabolism of palmitic acid was tested to assess the effects of TPA treatment on fatty acid chain elongation and subsequent desaturation (Table 3). Whereas chain elongation (18:0 + 18:1) was not substantially decreased in TPA-differentiating cells, the conversion of saturates to monoenes (16:1 + 18:1) was again lower in cells treated with TPA as compared to controls, 3.6 and 8.9%, respectively. The acyl group composition of lipids from control and TPA-differentiating cells (48-hr treatment, 8 × 10^{-10} M) was analyzed to determine if modification of acyl moieties occurred during TPA-induced differentiation (Table 4). Although there were not appreciable differences in the phospholipid fatty acid profiles between

### Table 3

**Effect of TPA treatment on the distribution of radioactivity from [1-[^14]C]palmitic acid in acyl groups of total glycerolipids from HL-60 cells**

Cells were incubated with TPA (4 × 10^{-10} M) for 47 hr. [1-[^14]C]Palmitic acid (2 μCi) was introduced, and the incubation continued for 1 hr. Cellular lipids were extracted and methylated, and radioactivity distribution was determined as described in "Materials and Methods." Values represent the average ± S.D. of 3 experiments.

<table>
<thead>
<tr>
<th>Acyl group</th>
<th>Control</th>
<th>TPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>16:0</td>
<td>85</td>
<td>90</td>
</tr>
<tr>
<td>16:1</td>
<td>5.6 ± 0.5*a</td>
<td>2.3 ± 0.21</td>
</tr>
<tr>
<td>18:0</td>
<td>5.3</td>
<td>5.5</td>
</tr>
<tr>
<td>18:1</td>
<td>3.3 ± 0.07*b</td>
<td>1.3 ± 0.11</td>
</tr>
</tbody>
</table>

*a p < 0.05 (Student’s t test)

### Table 4

**Acyl group composition of phospholipids and triacylglycerols in control and TPA-differentiated HL-60 cells**

Cells (2.0 × 10^5) were plated and cultured for 24 hr before TPA addition. Treated cells were grown with TPA (8 × 10^{-10} M) for 48 hr. The total time in culture for both control and treated cells was 3 days. Lipids, isolated by preparative thin-layer chromatography, were methylated and analyzed by gas-liquid chromatography as stated in "Materials and Methods."

<table>
<thead>
<tr>
<th>Acyl group</th>
<th>Control</th>
<th>TPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:0</td>
<td>1.34 ± 0.04*b</td>
<td>1.25 ± 0.73</td>
</tr>
<tr>
<td>16:0 DMA</td>
<td>3.53 ± 0.03</td>
<td>2.85 ± 0.87</td>
</tr>
<tr>
<td>16:0</td>
<td>22.12 ± 0.37</td>
<td>21.65 ± 0.77</td>
</tr>
<tr>
<td>18:1</td>
<td>34.33 ± 0.37</td>
<td>3.3 ± 0.11</td>
</tr>
<tr>
<td>18:0 DMA</td>
<td>1.75 ± 0.02</td>
<td>1.79 ± 0.38</td>
</tr>
<tr>
<td>18:0</td>
<td>13.05 ± 0.15*a</td>
<td>14.63 ± 0.43</td>
</tr>
<tr>
<td>18:1</td>
<td>26.11 ± 0.54</td>
<td>25.74 ± 1.23</td>
</tr>
<tr>
<td>18:2</td>
<td>32.39 ± 0.03</td>
<td>30.36 ± 0.83</td>
</tr>
<tr>
<td>18:3</td>
<td>0.99 ± 0.01</td>
<td>1.3 ± 0.17</td>
</tr>
<tr>
<td>20:3 + 22:0</td>
<td>3.1 ± 0.13</td>
<td>3.22 ± 0.31</td>
</tr>
<tr>
<td>20:4 + 22:1</td>
<td>7.67 ± 0.16</td>
<td>7.24 ± 0.64</td>
</tr>
<tr>
<td>22:4 + 24:1</td>
<td>2.37 ± 0.23</td>
<td>2.85 ± 0.64</td>
</tr>
<tr>
<td>22:6</td>
<td>3.36 ± 0.10</td>
<td>2.66 ± 0.33</td>
</tr>
<tr>
<td>Trace quantities</td>
<td>4.24 ± 0.32</td>
<td>4.57 ± 1.35</td>
</tr>
<tr>
<td>% Unsaturated</td>
<td>54.3</td>
<td>52.5</td>
</tr>
<tr>
<td>Unsaturated:saturated</td>
<td>1.2</td>
<td>1.1</td>
</tr>
</tbody>
</table>

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*a p = or < 0.001 (Student’s f test)
b p < 0.05 (Student’s t test)

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control and TPA-treated cells, triacylglycerol analysis revealed a consistent, definite reduction in unsaturates, which was mirrored by a concomitant increase in saturates in differentiating cells.

DISCUSSION

In a previous paper (7), we reported that treatment of human leukemia cells with the tumor promoter, TPA, elicits alterations in phospholipid metabolism during promoter-induced differentiation. Triacylglycerol synthesis was also shown to increase in differentiating cells. Whereas various studies have demonstrated (7, 17, 23) via tracers, such as 32P and labeled precursors of polar head groups, that modifications of lipid metabolism occur in TPA-treated cells and tissues, few investigations of acyl group metabolism have been undertaken (19). In the present study, labeled fatty acids were used to assess the uptake, metabolism, and distribution of acyl groups in polar and neutral lipid constituents of control and TPA-differentiating leukemia cells. Cells treated with TPA and incubated with labeled fatty acids incorporated more radioactivity into neutral lipids, whereas, in untreated cells, a higher percentage of labeled acyl moieties was present in the phospholipids. Exposure of HL-60 cells to TPA causes the cells to attach to the substratum (14), and with lower levels of TPA, a certain population of cells remains suspended. Green and Kehinde (12) reported that sublines of mouse 3T3 cells accumulate triacylglycerols when the cells are in a resting state (monolayers). We tested adherent and floating populations of cells grown with TPA and found that both incorporated more of the labeled fatty acid into triacylglycerols. TPA-treated HL-60 cells have been shown to incorporate more labeled acetate into fatty acids and triacylglycerols than do untreated cells (7). This increase in the amount of cellular triacylglycerols likewise occurs in TPA-exposed cells via direct acylation using fatty acid precursors. It is interesting to note that, whereas a marked stimulation of the incorporation of 32P and choline into phospholipids occurs in TPA-treated cells (7, 17), acyl group metabolism in phospholipids is affected to a much lesser degree. The turnover of the polar portion of the molecule appears to be stimulated more than the hydrophobic moieties of phospholipids.

The specific activity of the intact phospholipid and triacylglycerol fractions isolated from cells labeled with stearic, oleic, or arachidonic acid was lower in the TPA-treated cells; this indicates a slower rate of acyl group turnover in the differentiating cultures. At present, the mechanism responsible for the large increase in the amount of triacylglycerol in differentiating cells is not known; TPA at early times of exposure did not influence the uptake of labeled fatty acids. However, the results from experiments in which TPA and labeled palmitic acid were added simultaneously to cell cultures and incubated for 48 hr indicate that triacylglycerol accumulation may be due to an increased uptake of fatty acids from serum used in the culture medium. More radioactivity was also associated with cellular lipids recovered from 48-hr TPA-treated cells that were exposed to various labeled fatty acids for 1 hr.

When comparing the acyl group composition of lipids from untreated and TPA-treated cells, there were no appreciable differences in the fatty acid profiles of phospholipids, although TPA has been shown to alter membrane fluidity in several lymphoblastoid cells (8) and in rat embryo cells (11). Ip and Cooper (16) found that dimethyl sulfoxide-induced differentiation of HL-60 cells was accompanied by changes in membrane fluidity; however, they observed no modifications in membrane fluidity during TPA exposure. Thus, it appears that HL-60 cells exposed to TPA can maintain membrane fluidity parameters which are crucial to cellular function. The serum required to grow cells is a rich source of fatty acids; this may enable the cells to sustain the required fatty acid composition during TPA treatment. The serum-free culture system for HL-60 cells described recently by Breitman et al. (6) would be useful for determining possible acyl group modification of phospholipids during TPA-induced differentiation. Marked differences were, however, noted in the acyl group composition of triacylglycerols in control and differentiating cells, the latter demonstrating decreased unsaturates and increased saturates. The desaturation of [1-14C]stearic acid was likewise greatly reduced in TPA-treated cells. This was apparent from both the decreased conversion of [1-14C]stearic acid to the corresponding monoene and [1-14C]palmitic acid metabolism via chain elongation and desaturation.

It is well established that TPA elicits profound changes in polar head group metabolism of the glycerophospholipids (7, 17, 23). In the present investigation, labeled fatty acids were used to evaluate acyl group metabolism in differentiating cells. Phorbol esters interact with biomembranes, and thus, they exert direct effects on lipid metabolism. It is not known whether the alterations in fatty acid metabolism noted here are a consequence of TPA-membrane interactions or solely characteristics inherent to the differentiating cell. The lack of an effect of TPA at early time points suggests that the modifications of fatty acid metabolism are the result of cellular differentiation.

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Fatty Acid Metabolism in Phorbol Ester-differentiating Human Leukemia Cells

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