The Differential Susceptibility of A427 and A549 Cell Lines to the Growth-inhibitory Effects of ET-18-OCH₃ Does Not Correlate with the Relative Effects of the Alkyl-lyso phospholipid on the Incorporation of Fatty Acids into Cellular Phospholipids

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ABSTRACT

Proliferation of A427, a lung cancer cell line, was significantly decreased 10 h after incubation with 5 μg/ml 1-O-octadecyl-2-O-methylglycero-3-phosphocholine (ET-18-OCH₃) while the proliferation of A549, another lung cancer cell line, was unaffected until 15 h after incubation with the alkyl-lyso phospholipid. The relative sensitivity of cells to the antiproliferative effect of ET-18-OCH₃ has been postulated to be due to the degree of inhibition of cellular acylation processes. We therefore investigated the effect of 5 μg/ml ET-18-OCH₃ on the incorporation of fatty acids for up to 12 h, into A427 and A549 phospholipids. Significant changes observed in the incorporation of fatty acids into A427 phospholipids by the ALP were a decreased incorporation of oleic acid into PC after 8 h, an increased incorporation of linoleic acid into PE after 12 h, decreased incorporation of arachidonate into PE after 3 h, and increased incorporation into PA after 5 h. Although the above changes affected the distribution of newly esterified fatty acids in the phospholipids, there was no effect on the total quantity of label incorporated in the phospholipid fraction between the experimental and control cells after 12 h. Incubation of A549 cells with ET-18-OCH₃ resulted in decreased esterification of oleic acid into PC, SM, and LPC after 5 h; decreased incorporation of linoleic acid into PE after 12 h; and a decreased incorporation of arachidonate into SM after 1.5 h. After 12 h incubation with ET-18-OCH₃, changes in the distribution of radiolabeled fatty acids were observed in the quantitatively minor phospholipids, SM and LPC. A 20% decrease in the quantity of oleic acid incorporated into the phospholipids was observed in cells incubated with the ALP; however, no differences were observed in the quantity of linoleic or arachidonic acid incorporated into the phospholipids. The lack of common effects of the ALP on the incorporation of fatty acids into A427 and A549 phospholipids, coupled with the absence of changes that were more severe or manifested earlier in the more sensitive A427 cell line, suggests that the effect of ET-18-OCH₃ on the acylation processes depends on the cell type and the fatty acid species and is unlikely to be responsible for the relative sensitivities of the cells to the compound. Radiolabeled ET-18-OCH₃ was used to examine the correlation between the amount of the compound accumulated in A427, A549, MCF7, T84, and LS174T cells and the relative susceptibilities of the cells to the ALP. Our results indicated that a strict correlation did not exist between the quantity of the ALP accumulated and sensitivity to the compound.

INTRODUCTION

In the preceding paper (1) we investigated the hypothesis that the antiproliferative action of ET-18-OCH₃ on cancer cells is mediated through inhibition of LPC:acyl-CoA acyltransferase activity. Using MCF7 and T84 cell lines we examined the effect of ET-18-OCH₃ on the acylation of oleic, linoleic, and arachidonic acids into cellular phospholipids. Our results led to the conclusion that the antiproliferative action of ALP in these cells was unlikely to be mediated through inhibition of the LPC:acyl-CoA acyltransferase or perturbation of any of the acylation processes. Whether these conclusions can be extended to other cancer cell lines remains to be established. Furthermore, because the MCF7 and T84 cell lines used in the previous study were both very sensitive to the growth-inhibitory effects of the ALP, we were unable to provide information on any relationship between perturbation of acylation processes and the relative resistance of cells to the effects of ET-18-OCH₃. It has been postulated that the relative sensitivities of cells to the inhibitory effects of ET-18-OCH₃ is directly related to the degree of interference in the acylation of LPC to PC (2). In the present study, we have investigated the relationship between acylation and the inhibitory action of ET-18-OCH₃ in two lung cancer cell lines under conditions where the growth of one cell line, A427, was inhibited by the ALP whereas growth of the other, A549, was unaffected by the ALP.

MATERIALS AND METHODS

Cell Culture. A549, A427, and LS174T (a colon epithelial cancer cell line) were obtained from the American Type Culture Collection (Rockville, MD) and cultured in media (Gibco) supplemented with 10% fetal bovine serum (Cellect Gold; Flow Laboratories). A549 cells were grown in Ham's F-12 nutrient mixture and A427 cells were cultured in Eagle's minimum essential medium with nonessential amino acids and sodium pyruvate as recommended by the American Type Culture Collection. LS174T cells were grown in Eagle's minimum essential medium with nonessential amino acids and Earle's buffered saline solution. The cells were cultured in T75 flasks (Falcon) at 37°C in 5% CO₂/95% humidified air atmosphere.

Uptake and Incorporation of ET-18-OCH₃ into Cells. Log phase cells growing in 24-well dishes were incubated with 1-O[¹⁴C]octadecyl-2-O-methylglycero-3-phosphocholine (Amersham; 5 μg/ml, 0.5 μCi/well) for selected periods and subsequently washed twice with 0.1% fatty acid free bovine serum albumin (Sigma) in Hanks' buffered saline solution. The cells were trypsinized, transferred to 15-ml polypropylene tubes, and dispered with a 21-gauge needle. Aliquots were taken for the determination of the cell number on a model ZM Coulter Counter and the rest of the cells were pelleted by centrifugation. The supernatant was removed by suction and the pellet was dissolved in 1% sodium dodecyl sulfate in 0.3 M NaOH. The radioactivity associated with the cells was quantified by scintillation counting on a Beckman LS3801. Ecolite (ICN) was used as the scintillant in all experiments.

All other experimental procedures have been described in the preceding paper (1).

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3 The abbreviations used are: ET-18-OCH₃, 1-O-octadecyl-2-O-methylglycero-3-phosphocholine; LPC, lysophosphatidylcholine; PC, phosphatidylinositol; PI, phosphatidylinositol; PE, phosphatidylethanolamine; PA, phosphatic acid; PS, phosphatidylserine; ALP, alkyl-lyso phospholipid.
RESULTS

Effect of ET-18-OCH₃ on the Proliferation of A427 and A549 Cell Lines. In order to select the appropriate concentration of ET-18-OCH₃ for the acylation experiments, the effect of ET-18-OCH₃ on the proliferation of A427 and A549 cells was investigated. Preliminary studies indicated that 5 µg/ml ET-18-OCH₃ was a suitable concentration to use for our studies; therefore, the time-dependent growth inhibition of A427 and A549 cells by 5 µg/ml ET-18-OCH₃ was investigated (Fig. 1). Ten h after incubation with ET-18-OCH₃, a significant difference (P < 0.01) between the control and experimental growth curves was observed for A427 cells which became more pronounced with increasing time. Proliferation of A427 cells ceased between 15 and 24 h in cells incubated with ET-18-OCH₃ (5 µg/ml). Incubation of A549 cells with 5 µg/ml ET-18-OCH₃ did not inhibit proliferation until 15 h (P < 0.005) after incubation with the ALP. The decrease in proliferation increased progressively but even after 24 h proliferation had not ceased. These results clearly indicate that A549 was less susceptible to the antiproliferative effects of ET-18-OCH₃ than A427. After 12 h incubation with 5 µg/ml ET-18-OCH₃ the viability of both cells were unaffected as determined by exclusion of trypan blue (results not shown) and no morphological changes were observed.

Uptake and Incorporation of ET-18-OCH₃ into Cancer Cells. The effect of ET-18-OCH₃ on the proliferation of A427 and A549 cells clearly indicated that these cells were less susceptible to the growth-inhibitory effects of the drug than MCF7 and T84 cells (1). We therefore investigated whether there was a correlation between the quantity of ET-18-OCH₃ incorporated and the sensitivity of the cell to the compound. In addition to A227, A549, MCF7, and T84 cells, LS174T cells were included in the study. All the cells were incubated with 5 µg/ml ET-18-OCH₃ for 12 h. The effects of incubating A427 and A549 cells with 5 µg/ml ET-18-OCH₃ have been described above. Under similar conditions no proliferation of MCF7, T84, and LS174T cells was observed. In fact, between 10 and 12 h, the number of LS174T cells was less than that present at the start of the incubation indicating that the drug had a cytolytic effect on LS174T cells under these conditions. After 12 h, a 24% decrease in number of cells was observed. Thus, the order of increasing susceptibility of the cancer cell lines was as follows: A549 < A427 < MCF7 = T84 < LS174T. The uptake and accumulation (total intracellular content) of ET-18-OCH₃ in A427, A549, MCF7, T84, and LS174T cells were monitored for 12 h (Fig. 2). All the cells accumulated the drug progressively for up to 10 h. After 12 h, MCF7 and LS174T cells accumulated about 5.25 µg/10⁶ cell. This was 1.6-fold greater than the quantities accumulated in T84 and A427 cells and 3-fold greater than that in A549.

Effect of ET-18-OCH₃ on Incorporation of Fatty Acids into A549 Phospholipids. In A549 cells, greater than 75% of total oleic acid incorporated into the phospholipids during the entire incubation period was into PC (Fig. 3A). This was followed by PE and LPC which were labeled to a significantly greater extent than the other phospholipids. Five h after incubation with 5 µg/ml ET-18-OCH₃, the esterification of oleic acid into choline containing phospholipids was significantly decreased. Incorporation into LPC decreased by 50%, SM by 30%, and PC by 20%. No significant changes were observed in the incorporation of oleic acid into the other phospholipids. Experiments with linoleic acid also revealed that the majority (>68%) of the incorporated label in A549 phospholipids was associated with the PC fraction at all times (Fig. 3B). Incubation of A549 cells with 5 µg/ml ET-18-OCH₃ caused a 20% decrease in esterification of linoleic acid into the PE fraction after 12 h. The distribution of arachidonic acid in A549 phospholipids (Fig. 3C) was distinct from that of oleic (Fig. 3A) and linoleic acids (Fig. 3B). Although the initial incorporation was mainly into PC, by the end of the incubation period, the proportion of label in PE (33%) and PC (37%) were similar. A substantial proportion of the arachidonic acid was also associated with the PI fraction while the relative labeling of the PA fraction was less than that observed for linoleic or oleic acids. The only significant effect of ET-18-OCH₃ on the acylation of arachidonic acid into A549 phospholipids was a 30% decrease into the SM fraction beginning after 1.5 h and maintained to the end of the incubation (data not shown). This phospholipid fraction was also the least labeled with arachidonic acid and after 12 h, only about 1.5% of the total label in the phospholipids was associated with SM (Table 1).
Effect of ET-18-OCH₃ on Incorporation of Fatty Acids in A427 Phospholipids. The effect of ET-18-OCH₃ on incorporation of oleic, linoleic, and arachidonic acids into A427 phospholipids was studied in a similar manner to A549. Most of the radiolabeled oleic acid in A427 cells was found in the PC fraction but unlike in A549 cells, the PA fraction rather than the LPC and PE fractions was the next highest labeled fraction after PC (Fig. 4A). ET-18-OCH₃ caused 33 and 20% decreases in the incorporation of labeled oleic acid in A549 LPC by 43% relative to controls (Table 1). Surprisingly no significant changes were observed in the proportion of label in SM (P < 0.05), there were no differences in either the distribution of the newly esterified fatty acids or the total amounts of label incorporated into the phospholipids. With arachidonic acid, the only difference observed was a substantial increase in the proportion of label in PE (Fig. 4B) in contrast to PE in A549 cells (Fig. 3B). In A427 cells incubated with ET-18-OCH₃, a 39% increase in incorporation of linoleic acid into PE was observed after 12 h. The distribution of arachidonic acid in the phospholipids of A427 cells (Fig. 4C) was quite distinct from that in A549 (Fig. 3C). The incorporation of arachidonic acid into PC was substantially higher than incorporation into PE at all times and after 12 h, radioactivity in PC was at least 3-fold higher than in PI and PE. The effect of 5 μg/ml ET-18-OCH₃ on the acylation of arachidonic acid into A427 phospholipids was an 18–28% decrease into PE between 3 and 12 h and an increase of 39 to 47% into PA between 5 and 12 h.

Effect of ET-18-OCH₃ on the Distribution of Newly Incorporated Fatty Acids. Tables 1 and 2 show the distribution of newly incorporated fatty acids in A549 and A427 phospholipids extracted from cells incubated with or without ET-18-OCH₃ for 12 h. ET-18-OCH₃ decreased the proportion of newly esterified oleic acid in A549 LPC by 43% relative to controls (Table 1). Surprisingly no significant changes were observed in the proportion of newly esterified oleic acid in the PC and SM fractions even though significant decreases in the incorporation of labeled oleic acid into these phospholipids were reported (Fig. 3A). A comparison of the quantity of radiolabeled oleic acid in the total phospholipid fraction revealed that A549 cells incubated with ET-18-OCH₃ incorporated 20% less label than control cells. This decrease may explain why differences were not observed in the distribution of newly esterified oleic acid in PC and SM fractions between control and experimental cells. Studies with linoleic acid revealed that, with the exception of a 13% increase in the proportion of label in SM (P < 0.05), there were no differences in either the distribution of the newly esterified fatty acids or the total amounts of label incorporated into the phospholipids. With arachidonic acid, the only difference observed between control cells and cells incubated with the ALP was a

Table 1 Effect of ET-18-OCH₃ on the distribution of newly incorporated fatty acids into A549 cellular phospholipids

<table>
<thead>
<tr>
<th>% of distribution of [³H]oleic acid</th>
<th>% of distribution of [¹⁴C]linoleic acid</th>
<th>% of distribution of [³H]arachidonic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>ET-18-OCH₃</td>
<td>Control</td>
</tr>
<tr>
<td>LPC</td>
<td>6.02 ± 0.23</td>
<td>2.71 ± 0.46</td>
</tr>
<tr>
<td>SM</td>
<td>3.30 ± 0.03</td>
<td>1.50 ± 0.11</td>
</tr>
<tr>
<td>PC</td>
<td>74.61 ± 5.4</td>
<td>67.83 ± 0.50</td>
</tr>
<tr>
<td>LPE</td>
<td>1.26 ± 0.16</td>
<td>1.31 ± 0.21</td>
</tr>
<tr>
<td>PS</td>
<td>1.41 ± 0.26</td>
<td>1.78 ± 0.09</td>
</tr>
<tr>
<td>PI</td>
<td>2.75 ± 0.16</td>
<td>5.43 ± 0.30</td>
</tr>
<tr>
<td>PE</td>
<td>7.42 ± 0.89</td>
<td>10.65 ± 0.73</td>
</tr>
<tr>
<td>PA</td>
<td>3.23 ± 0.48</td>
<td>8.79 ± 0.58</td>
</tr>
<tr>
<td>Radioactivity in phospholipids (dpm/10⁶ cells)</td>
<td>1,008,727 ± 76,764</td>
<td>269,441 ± 8,029</td>
</tr>
</tbody>
</table>

a P < 0.01.
b P < 0.05.
c P < 0.025.
EFFECT OF ET-18-OCH₃ IN A427 AND A549 CELLS

Fig. 4. Effect of ET-18-OCH₃ on incorporation of oleic, linoleic, and arachidonic acids in A427 cellular phospholipids. A427 cells were incubated with [³H]oleic acid (A), [¹⁴C]linoleic acid (B) and [³H]arachidonic acid (C) in the presence (Ο, △, ●, ●) or absence (Ο, △, ●, ●) of 5 µg/ml ET-18-OCH₃. Incorporation of radioactivity into PC (Ο), PE (●), phosphatidylserine (△), PI (△), and PA (●) was determined at different times. Values are the mean of three separate determinations. Asterisks, statistically significant differences between cells incubated with ET-18-OCH₃ and controls of at least P < 0.05.

**Table 2 Effect of ET-18-OCH₃ on the distribution of newly incorporated fatty acids into A427 cellular phospholipids**

A427 cells were incubated with radiolabeled fatty acid in the presence or absence of 5 µg/ml ET-18-OCH₃ for 12 h. The distribution of the radiolabeled fatty acid in the phospholipid classes is expressed as a percentage of the total amount of label in the phospholipid fraction. Each value represents the average of three separate determinations ± SD. Level of significance was compared to the values for controls (Student’s t test).

<table>
<thead>
<tr>
<th>% of distribution of [³H]oleic acid</th>
<th>% of distribution of [¹⁴C]linoleic acid</th>
<th>% of distribution of [³H]arachidonic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>ET-18-OCH₃</td>
</tr>
<tr>
<td>LPC</td>
<td>2.45 ± 0.35</td>
<td>2.35 ± 0.28</td>
</tr>
<tr>
<td>SM</td>
<td>3.07 ± 0.09</td>
<td>4.07 ± 0.74</td>
</tr>
<tr>
<td>PC</td>
<td>65.03 ± 1.34</td>
<td>61.55 ± 2.24*</td>
</tr>
<tr>
<td>LPE</td>
<td>1.73 ± 0.05</td>
<td>1.68 ± 0.17</td>
</tr>
<tr>
<td>PS</td>
<td>2.45 ± 0.17</td>
<td>3.10 ± 0.38*</td>
</tr>
<tr>
<td>PI</td>
<td>6.10 ± 0.53</td>
<td>5.17 ± 0.59</td>
</tr>
<tr>
<td>PE</td>
<td>6.30 ± 0.50</td>
<td>7.17 ± 0.78</td>
</tr>
<tr>
<td>PA</td>
<td>12.16 ± 2.70</td>
<td>14.99 ± 2.34</td>
</tr>
<tr>
<td>Radioactivity in phospholipids (dpm/10⁶ cells)</td>
<td>700,464 ± 40,137</td>
<td>629,549 ± 47,342</td>
</tr>
</tbody>
</table>

* P < 0.05.  
+ P < 0.01.  
& P < 0.025.

20% decrease in the proportion of [³H]arachidonic acid found in the SM fraction, but in light of the small proportion of label found in this phospholipid, it was not surprising that this change did not affect the total amount of label in the phospholipids.

After 12 h incubation of A427 cells with ET-18-OCH₃, the only changes in the distribution of newly esterified oleic acid in A427 phospholipids was a 6% decrease in PC compared to controls (P < 0.05) and a 23% increase in phosphatidylserine (P < 0.05) (Table 2). With linoleic acid, ET-18-OCH₃ caused a 21% increase in proportion of the label in PE compared to controls; while with arachidonic acid, a 14% decrease in PE, a 45% increase in PA, and a 36% increase in SM were observed. No significant differences were observed in the total amount of fatty acid incorporated into the phospholipid fraction in A427 cells incubated with or without ET-18-OCH₃ for all three fatty acids.

DISCUSSION

The results obtained in this and the preceding paper (1) have identified five epithelial cancer cell lines with differential susceptibilities to the antiproliferative effects of ET-18-OCH₃. While the order of increasing susceptibility to 12 h incubation with 5 µg/ml of ET-18-OCH₃ was A549 < A427 < T84 = MCF7 < LS174T, the order of increasing accumulation of the drug was found to be A549 < A427 = T84 < MCF7 = LS174T. The similarity in the extent of uptake and incorporation of ET-18-OCH₃ by T84 and A427 cells in spite of the differences in susceptibility of the cells to the growth-inhibitory effects of ET-18-OCH₃ indicates that there is not a strict correlation between the quantity of the ALP accumulated/cell and the relative susceptibilities of cells to the inhibitory effects of the compound. A similar conclusion has been obtained with Raji and LI210 cells by Fleer et al. (3) and by Chabot et al. (4) in studies with human leukemia cell lines. In contrast, Storch and Munder (5) showed that resistant Meth A cells accumulated much less ET-18-OCH₃ than ALP-sensitive Meth A cells. ET-18-OCH₃ accumulates in cell membranes and affects physical properties of membranes (6–9). Whether such changes are directly responsible for the inhibition of cell growth by the ALP have yet to be established. However, the lack of a direct correlation between the quantity of ET-18-OCH₃ accumulated and the susceptibilities of cells reported in this and previous studies (3, 4, 8) would suggest that other properties of the membrane are critical in determining the susceptibility of cells to the ALP.
The differences in susceptibility of A549 and A427 to the antiproliferative effects of ET-18-OCH₃ permitted a comparison of the effect of the compound on acylation processes in a cell line the proliferation of which was inhibited within the time frame of the experiments, and another cell line the growth of which was unaffected by the ALP under similar conditions. If proliferation was inhibited as a result of perturbation of acylation processes, then the effects of ET-18-OCH₃ on acylation would be expected to occur earlier and/or be more severe in A427 than A549 cells, in order to explain the greater susceptibility of A427 to the growth-inhibitory effects of ET-18-OCH₃. A comparison of the results obtained with A549 and A427 cells indicates that the above expectations were not realized. The only common effect of ET-18-OCH₃ on acylation processes in the two cells was a decrease in the esterification of oleic acid into PC. Surprisingly, this occurred after 5 h of incubation in A549 cells compared to 8 h in A427, the more susceptible cell line. The extent of the decrease was also similar between the two cell lines. The decreased incorporation of oleic acid into LPC and SM observed in A549 was absent in A427 cells which would discount changes in these lipids as being crucial for the growth-inhibitory process. With linoleic acid, opposing effects on the incorporation into PE were observed, a decrease in A549 and an increase in A427. The effect of the ALP on the incorporation of arachidonic acid into A549 and A427 phospholipids were also completely different. Thus even though ALP affected the incorporation of fatty acids into a number of phospholipids, no consistent effect was observed among the fatty acids or between the two cells that would suggest a link between acylation processes and inhibition of proliferation. This supports and extends the conclusions derived from our previous study with MCF7 and T84 cells that disturbances in the acylation processes are unlikely to be a key event in the mechanism of inhibition of proliferation by ET-18-OCH₃ (1).

The above conclusion is at variance with the hypothesis proposing a key role for the inhibition of LPC:acyl-CoA acyltransferase in the antiproliferative effect of ET-18-OCH₃ (2, 10, 11). Although definitive reasons cannot be provided for the differences in our observations and those obtained with Meth A cells which formed the basis of the above hypothesis, the following may be contributing factors. In one study, Meth A cells were incubated for 24 h with concentrations of ET-18-OCH₃ that completely blocked proliferation before the addition of the fatty acids (10). The resultant decrease in incorporation of fatty acids reported could therefore be a consequence of the lack of proliferation rather than the cause. In the study in which both the ALP and fatty acid were added together, the experiments were conducted only with oleic acid (11). We have demonstrated in the present studies that the distribution and effects of ET-18-OCH₃ on the incorporation of fatty acids into phospholipids vary with the fatty acid species. Consequently, it may not be appropriate to draw generalized conclusions from experiments with one fatty acid. The possibility that the mechanism of action of ALP may not be the same for all sensitive cell lines cannot also be discounted.

Because ALP are lipids, it is logical to envisage that the perturbation of lipid metabolism may be one of the mechanisms of the antiproliferative action of these compounds. Although our studies rule out interference in acylation as a universal mechanism, they do not preclude an effect of ALP on de novo lipid synthesis. A reduction in choline incorporation into Meth A and HL-60 cells by ET-18-OCH₃ has been reported (10, 12) suggesting an inhibition of the CDP-choline pathway, the major de novo pathway for the synthesis of PC. A decrease in phospholipid synthesis could severely inhibit the ability of cells to grow and divide. Studies are therefore required to examine the effect of ALP on de novo synthesis of phospholipids as a possible mechanism for the antiproliferative effect of ALP in cancer cells.

REFERENCES


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