Correlation of Ether Lipid Content of Human Leukemia Cell Lines and Their Susceptibility to 1- *O*-Octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine

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Correlation of Ether Lipid Content of Human Leukemia Cell Lines and Their Susceptibility to 1-*O*-Octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine[1]

Marie C. Chabot,[2] Robert L. Wykle, Edward J. Modest, and Larry W. Daniel

Department of Biochemistry, Bowman Gray School of Medicine of Wake Forest University, Winston-Salem, North Carolina 27103

ABSTRACT

A number of synthetic ether-linked phospholipids are selectively cytotoxic to neoplastic cells. However, the mechanisms underlying this selective cytotoxicity are not known. We have investigated the ether-lipid content of HL-60 and K562 human leukemia cells in relation to their sensitivity to 1-*O*-alkyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine (ET-18-OCH_3). HL-60 cells are much more sensitive than K562 cells to the cytotoxic effects of ET-18-OCH_3 and, at the same time, they contain nearly twice as much ether lipid as the more resistant K562 cells. These observations suggested a relation between the cellular ether-lipid content and sensitivity to ET-18-OCH_3. Further evidence linking these properties was obtained when the ether-lipid content of K562 cells was increased by incubating them in medium containing 1-*O*-hexadecyl-*sn*-glycerol. This supplementation not only increased the ether-lipid content of the cells but also increased their sensitivity to ET-18-OCH_3. The 50% inhibitory concentration for ET-18-OCH_3 decreased from 18.4 μM in the control cells to 9.83 μM in the supplemented cells.

INTRODUCTION

ET-18-OCH_3[3] is a synthetic ether-lipid which is selectively cytotoxic to neoplastic cells compared to normal cells (1, 2). The HL-60 human leukemia cell line is highly sensitive while the K562 human leukemia cell line is dramatically less sensitive to the inhibitory activity of ET-18-OCH_3 (3). It was originally proposed that the selective cytotoxicity resulted from the accumulation of the ET-18-OCH_3 in those cells containing low alkyl-cleavage enzyme activity (4–7). However, Hoffman *et al.* (8) found that the specific activity of the alkyl cleavage enzyme in HL-60 cells was similar to that in K562 cells and demonstrated that ET-18-OCH_3 was not a substrate for the alkyl-cleavage enzyme of rat liver (8). Recently Wilcox *et al.* (9) examined the ability of various cell lines to catabolize L-ET-18-OCH_3 and found that L-ET-18-OCH_3 was poorly metabolized by all the cell lines tested including HL-60 and K562. Thus, catabolism of the compound by the alkyl-cleavage enzyme or other mechanisms does not appear to underlie the selective cytotoxicity.

ET-18-OCH_3 is a membrane-active compound which has been shown to partition into the membranes of HL-60 cells (10). As such, it is possible that the membrane lipid environment may contribute to the selective cytotoxicity of ET-18-OCH_3. PC and PE are major components of mammalian cell membranes. Each of these classes may be further divided into three subclasses based on the nature of the linkage at the *sn*-1 position of the glycerol. These are the ester-linked diacyl species and the ether-linked alkylacyl and alk-1-enylacyl species. Several physical properties (*i.e*, dipole moment, phase transition temperature) of ether-lipids differ from those of the corresponding diacyl compounds (11, 12). As a result, it has been speculated that the presence of phospholipid molecules containing alkyl and alk-1-enyl bonds may alter membrane properties. Changes in the physical properties of the membrane have been shown to be of importance in regulating cell growth and the activities of membrane-bound enzymes (13). An alternation in membrane properties may also alter the sensitivity of cells to certain forms of therapy (14). Therefore, we have investigated the relation between the ether-lipid content of cells and their susceptibility to ET-18-OCH_3 using the HL-60 and K562 human leukemia cell lines.

MATERIALS AND METHODS

Cells. HL-60 and K562 human leukemia cell lines were obtained from American Type Culture Collection (Rockville, MD). Both lines were cultured in suspension in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin (100 units/ml), streptomycin (100 μg/ml), 0.22% Na_2HCO_3, and 2 mM L-glutamine (complete RPMI). The cells were maintained at a density of 5.0×10^5 to 1.5×10^6 cells/ml at 37°C in a humidified atmosphere containing 5% CO_2-95% air.

The ether lipid content of K562 cells was modified by incubating the cells in complete RPMI containing 10 μg/ml of 1-*O*-hexadecyl-*sn*-glycerol for 48 h. This lipid was added to cell suspensions in absolute ethanol (1 μl/ml of medium). At the end of the supplementation period, the cells were washed and resuspended in complete RPMI medium for use in subsequent experiments.

Lipid Analysis. Cells were collected by centrifugation at 1200 rpm for 5 min to produce a pellet which was resuspended in deionized H_2O. The cellular lipids were extracted according to the method of Bligh and and Dyer (15); the organic phase was dried under N_2 and then resuspended in $CHCl_3$:CH_3OH (9:1, v/v). The phospholipid classes were separated by thin-layer chromatography on Silica Gel H plates developed in $CHCl_3$:CH_3OH:CH_3COOH:H_2O (50:25:8:3, v/v). The lipids were visualized under ultraviolet light after spraying the layer lightly with primulin solution; the lipids were eluted from the silica gel with $CHCl_3$:CH_3OH:H_2O (1:2.5:0.5, v/v). Phospholipids were quantitated by phosphorus determination according to the method of Rouser (16).

In order to determine the subclass composition of the individual phospholipids, the lipids were dried under N_2 and converted to their diradylglycerobenzoate derivatives by a modification of the method of Blank *et al.* (17). After drying, each phospholipid class was dissolved in 300 μl of ethyl ether and treated with phospholipase C (*Bacillus cereus*) (10 units diluted in 0.5 ml of 0.1 M Tris buffer, pH 7.4, containing 10 mM Ca^{2+}) to remove the phosphobase group. This mixture was stirred vigorously for 2.5 h at room temperature. The resulting diglycerides were extracted from the reaction mixture, dried under N_2, and converted to their benzoate derivatives as follows. The lipids were dissolved in 0.5 ml of dry CH_2Cl_2 containing 10 mg of benzoic anhydride, 4 mg of 4-dimethylaminopyridine, and 12.5 μl of triethylamine and incubated for 2 h at room temperature. Following incubation the reaction mixture was evaporated to dryness, concentrated NH_4OH (1 ml) was added, and the mixture was extracted 3 times with 2 ml of hexane. The benzoate derivatives were dried, dissolved in a small volume of $CHCl_3$, applied to a silicic acid column, eluted from the column with $CHCl_3$ to remove 4-dimethylaminopyridine, and dried under N_2. After dissolving the sample in a small volume of hexane, the alkylacyl-, alk-1-enylacyl-, and diacylglycerobenzoate derivatives were separated using a Perkin-Elmer Series 4 HPLC system equipped with

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[3] The abbreviations used are: ET-18-OCH_3, 1-*O*-alkyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine; L-ET-18-OCH_3, 1-*O*-octadecyl-2-*O*-methyl-*sn*-glycero-3-phosphocholine; PC, choline-containing phosphoglyceride; PE, ethanolamine-containing phosphoglyceride; HPLC, high-pressure liquid chromatography; IC_{50}, 50% inhibitory concentration.

an Econosphere silica column (250 mm x 4.6 mm, 5 μm) (Alltech, Deerfield, IL). The sample was eluted isocratically with cyclohexane:2-propanol (99.85:0.15, v/v) at a flow rate of 1 ml/min. The presence of the chromophoric benzoate group allowed the separated components to be quantitated on-line at 230 nm with a Perkin-Elmer LC-95 UV-visible variable wavelength spectrophotometric detector connected to a Perkin-Elmer LCI-100 laboratory computing integrator for the determination of the peak areas. The retention times of the alkylacyl-, alk-1-enylacyl-, and diacylglycerobenzoates were determined using standards synthesized from lipids of known subclass composition.

Assessment of ET-18-OCH$_3$ Cytotoxicity. The sensitivity of normal and supplemented K562 cells to ET-18-OCH$_3$ was determined by incubating 5×10^5 cells/ml of complete RPMI (no hexadecylglycerol) in 16-mm tissue culture dishes with different concentrations of the drug. After a 48-h incubation, cell viability was evaluated by trypan blue dye exclusion, and the cells were counted with a hemocytometer. The value from each ET-18-OCH$_3$-treated sample was compared to its respective control (supplemented or unsupplemented) and expressed as a percentage of control. By comparing each treated sample to its proper control, the possible effect of any difference in the growth rate of the two groups of cells is eliminated.

Clonogenic Assay. The sensitivity of normal and supplemented K562 cells to ET-18-OCH$_3$ was also determined using semisolid double-layer agar cultures. In these experiments the K562 cells (7.5×10^4 cells/ml) were suspended in RPMI 1640 medium containing 10% fetal bovine serum, penicillin (100 units/ml), streptomycin (100 μg/ml), and 0.3% agarose. Aliquots were overlaid onto pregelled underlayers of the same medium containing 0.5% agarose in 35-mm wells (Linbro plates; Flow Laboratories, Inc., McLean, VA). Cultures were incubated at 37°C in humidified air containing 5% CO_2 with continuous exposure to ET-18-OCH$_3$. The plates were examined for clones with aggregates greater than 40 μm in diameter after 4 days. Colonies were counted by automated image analysis.

Measurement of ET-18-OCH$_3$ Uptake. The amount of L-ET-18-OCH$_3$ taken up by control and supplemented K562 cells was determined by incubating 5×10^5 cells in 1 ml of complete RPMI containing 2 μM L-ET-18-OCH$_3$ (0.77 μCi/ml) for various lengths of time. At the end of the incubation period, an aliquot of the cell suspension was taken for determination of cell number. The remainder of the cells were isolated by centrifugation and extracted according to the method of Bligh and Dyer (15), and their phospholipid phosphorus content was determined using the method of Rouser (16). The amount of radiolabeled L-ET-18-OCH$_3$ incorporated into the cellular lipids was determined using additional cultures. Isolated cells were extracted according to the method of Bligh and Dyer (15), and the cellular lipids were separated by chromatography on Silica Gel G thin-layer plates developed in $CHCl_3$:CH_3OH:CH_3COOH:H_2O (50:25:8:2, v/v). Individual lipid fractions were visualized with iodine vapors and scraped into vials, and the amount of radioactivity in each was determined by scintillation counting. The amount of L-ET-18-OCH$_3$ incorporated into the cells was then calculated and expressed as the nmol percentage of total lipid phosphorus.

Statistical Analysis. The IC_{50} values for ET-18-OCH$_3$ in the control and supplemented K562 cells were compared by a paired *t* test using Number Cruncher Statistical Systems software.

Materials. Cell culture reagents were purchased from Grand Island Biological Co. (Grand Island, NY). All solvents were purchased from Fisher Scientific (Pittsburgh, PA) and were HPLC grade. Thin-layer chromatography plates were from Analtech (Newark, DE), and lipid standards were from Serdary Research Laboratory (London, Ontario, Canada). 1-*O*-Hexadecyl-*sn*-glycerol was from Western Chemical Industries, Ltd. (Vancouver, British Columbia, Canada). Phospholipase C (*B. cereus*, Grad I) was obtained from Boehringer-Mannheim (Indianapolis, IN). Benzoic anhydride, 4-dimethylaminopyridine, and other reagents were purchased from Sigma Chemical Company (St. Louis, MO). The benzoic anhydride was recrystallized from a chloroform:petroleum ether solution prior to use. ET-18-OCH$_3$ was the generous gift of Dr. Wolfgang Berdel, Technical University of Munich (Munich, Federal Republic of Germany). 1-*O*-Octadecyl-2-*O*-methyl-*sn*-glycero-3-phosphocholine was purchased from Novabiochem (Laufelfingen, Switzerland). 1-*O*-[9,10-^{3}H]octadecyl-2-*O*-methyl-*sn*-glycero-3-phosphocholine (56 Ci/mol) was synthesized as described (9).

RESULTS

HL-60 and K562 cells differ dramatically in their sensitivity to ET-18-OCH$_3$ (IC_{50}, 2.0 μM and 18.4 μM, respectively). We now have examined these cells in order to determine whether they differ significantly in their phospholipid composition. K562 cells (31.5 nmol of P_i/10^6 cells) were found to contain approximately twice as much phospholipid as did the HL-60 cells (13.0 nmol of P_i/10^6 cells). Although the total amount of phospholipid varied, little difference in the distribution of the phospholipid classes was observed (Table 1). In both cell types, PC and PE were the major phospholipid classes.

The choline- and ethanolamine-containing phosphoglycerides of many cell types contain diacyl-, alk-1-enylacyl-, and alkylacyl-linked subclasses. Therefore, further analyses were conducted to determine the phospholipid subclass composition of the HL-60 and K562 cells. Phospholipid subclasses were separated and quantitated as their diradylglycerobenzoate derivatives by normal-phase HPLC (see "Materials and Methods"). In K562 cells, only 10.4% of the PC fraction was alkyl linked, whereas in the HL-60 cells, this value was 27.5% (Fig. 1). The percentage of alk-1-enylacyl-PC was also less in the K562 cells. In the PE fraction, the greatest difference between the two cell types was observed in the alk-1-enyl-linked species. Again, K562 cells contained relatively less ether-lipid than did the HL-60 cells (Fig. 1). The lower percentage of ether lipid in the K562 cells was associated with a higher percentage of the diacyl species in PC and PE.

In order to address the possibility that the differential sensitivity of HL-60 and K562 cells to ET-18-OCH$_3$ might be related to the differences in their endogenous ether-lipid content, we increased the ether-lipid content of the K562 cells. Cabot *et al.* (18, 19) have shown that the levels of ether-lipids in cells may be elevated by incubating them in medium supplemented with alkylglycerol. Using a similar protocol, K562 cells were incubated in complete RPMI medium containing 10 μg/ml of 1-*O*-hexadecyl-*sn*-glycerol for 48 h. Cell growth and viability were monitored daily. At all times during the supplementation period, the cells were 100% viable based on trypan blue dye exclusion. However, the growth of the supplemented cells, although not affected after 24 h of incubation, was approximately 75% of control after 48 h.

The phospholipid content and subclass composition of the supplemented cells were then determined. Supplementation of the growth medium with 1-*O*-hexadecylglycerol had minimal effect on the total phospholipid phosphorus content (36.9 nmol of P_i/10^6 cells) or the distribution of the phospholipid classes (Table 1) in the K562 cell line. There was, however, a dramatic

Table 1 *Phospholipid composition of HL-60, K562, and supplemented K562 cells*

HL-60, K562, or K562 cells which had been supplemented with 10 μg/ml of hexadecylglycerol for 48 h were collected, and the lipids were extracted according to the method of Bligh and Dyer (15). Major phospholipid classes were separated by thin-layer chromatography and quantitated according to the method of Rouser (16).

Phospholipid class	% of total phospholipid phosphorus		
	HL-60	K562	K562 sup.
Sph[a]	6.19 ± 0.61[b]	4.48 ± 0.37	3.85 ± 0.44
PC	48.16 ± 0.38	54.96 ± 2.72	55.23 ± 2.10
PS/PI	14.61 ± 1.72	12.35 ± 0.62	13.12 ± 0.80
PE	30.95 ± 1.00	29.36 ± 2.41	27.35 ± 1.62

[a] Sph, sphingomyelin; PS/PI, phosphatidylserine/phosphatidylinositol.
[b] Mean ± SD of at least 4 determinations.

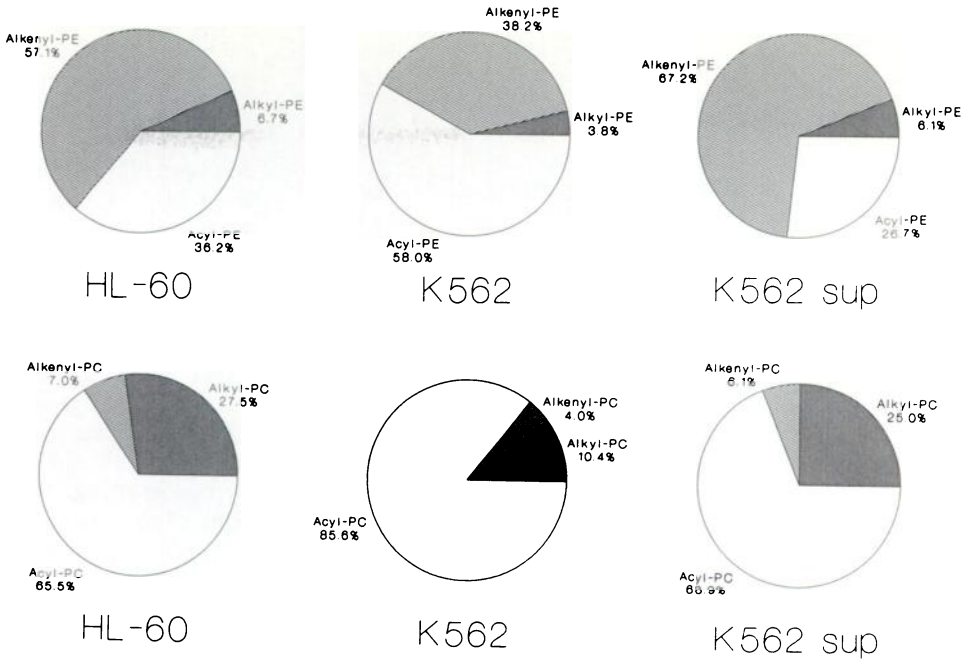

Fig. 1. Phospholipid subclass composition of human leukemia cell lines. HL-60, K562, or K562 cells supplemented with 1-*O*-hexadecyl-*sn*-glycerol (10 μg/ml) for 48 h were collected, and the lipids were extracted according to the method of Bligh and Dyer (15). The PC and PE fractions were isolated by preparative thin-layer chromatography and converted to their diradylglycerobenzoate derivatives. These were separated and quantitated by HPLC as described. Each value represents the mean of 3 or more determinations.

increase in the amount of ether-lipid in the supplemented cells (Fig. 1). Whereas in the control K562 cells only 10% of the PC was alkyl linked, this value increased to 25% after 48 h of supplementation. Similarly, the alk-1-enyl-linked PE increased from 38% in the control cells to 66% in the supplemented cells. In both instances the increase in the ether-lipid content of the cells was accompanied by a loss of the diacyl subclass.

We next investigated whether the hexadecylglycerol-supplemented K562 cells differed in their susceptibility to ET-18-OCH$_3$. In these experiments K562 cells which had or had not been supplemented were washed and resuspended in fresh medium (no hexadecylglycerol) containing various concentrations of ET-18-OCH$_3$ and assayed for cell viability as described in "Materials and Methods" (Fig. 2). Those cells which had previously been incubated in medium containing hexadecylglycerol were significantly more sensitive to ET-18-OCH$_3$. In control cells the IC_{50} for ET-18-OCH$_3$ was approximately 18.4 μM, whereas in the supplemented cells, this value had decreased to 9.83 μM ($P = 0.002$). K562 cells were also supplemented for 24 h with hexadecylglycerol, and their ether-lipid content and susceptibility to ET-18-OCH$_3$ were determined to be intermediate between the cells supplemented for 48 h and control cells (data not shown).

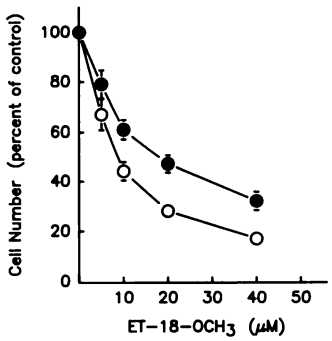

Fig. 2. Dose response of ET-18-OCH$_3$. Normal K562 or K562 cells supplemented with 1-*O*-hexadecyl-*sn*-glycerol (10 μg/ml) for 48 h were washed, resuspended in fresh RPMI medium, and incubated with increasing concentrations of ET-18-OCH$_3$ for 48 h. Cells were counted and assessed for viability by trypan blue dye exclusion. *Points*, mean of 5 determinations from a representative of 4 experiments; *bars*, SD. Data are expressed as a percentage of the cell number. ●, unsupplemented K562 cells; ○, supplemented K562 cells.

In separate experiments, we examined the effect of hexadecylglycerol supplementation on the cytotoxicity of ET-18-OCH$_3$ in K562 cells using a clonogenic assay. In these experiments, the supplemented K562 cells were significantly more sensitive ($P < 0.001$) to the effects of ET-18-OCH$_3$ at low drug concentration (*i.e.*, 5 to 10 μM). For instance, when control K562 cells were treated with 5 μM ET-18-OCH$_3$, the number of colonies was 81.6% of that in untreated samples, whereas this value was 57.9% in the supplemented K562 cell. However, when higher concentrations of ET-18-OCH$_3$ were used in the clonogenic assay, the differences were not statistically significant. Overall, these data suggest that the differential susceptibility of the HL-60 and K562 cell lines may be related in part to the ether-lipid content of these cells.

Having demonstrated that the susceptibility of the leukemia cells to ET-18-OCH$_3$ could be correlated with the ether-lipid content of the cells, we began to investigate possible mechanisms. Storch and Munder (20) have recently reported that uptake of ET-18-OCH$_3$ is decreased in cells which are resistant to its cytotoxicity compared to more sensitive cells. In order to address this possibility, we compared the uptake of L-ET-18-OCH$_3$ in supplemented and control K562 cells (Fig. 3). When control and supplemented K562 cells were incubated with 2 μM L-ET-18-OCH$_3$ (0.77 μCi), no difference in the amount of drug taken up by the cells could be observed. Thus uptake of drug may not be the only factor related to the cytotoxicity of ET-18-OCH$_3$.

DISCUSSION

The HL-60 and K562 human leukemia cell lines are often used in studies of the antineoplastic drug ET-18-OCH$_3$ because of their differential sensitivity to this drug (3). Although various mechanisms have been proposed to account for this difference, none has proven to be satisfactory. Therefore, we have investigated if the ether-lipid content of these cells can influence their sensitivity to ET-18-OCH$_3$. We have found that the HL-60 and K562 cell lines differ not only in their susceptibility to ET-18-

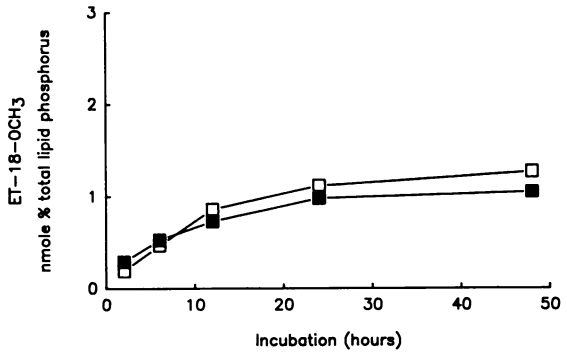

Fig. 3. Uptake of ET-18-OCH$_3$. Normal K562 (□) or K562 cells supplemented with 1-*O*-hexadecyl-*sn*-glycerol (10 μg/ml) for 48 h (■) were washed, resuspended in fresh RPMI medium, and incubated with 2 μM ET-18-OCH$_3$ (0.77 μCi of [^{3}H]ET-18-OCH$_3$/ml) for various lengths of time. At the end of the incubation period, cellular lipid phosphorus and incorporated radiolabeled ET-18-OCH$_3$ were determined as described in "Materials and Methods." The mass of ET-18-OCH$_3$ incorporated into the cells was calculated and is expressed as nmol percentage of total lipid phosphorus. *Points*, mean of two determinations from a representative experiment.

OCH$_3$, but also in their ether-lipid content. This observation suggested that an increase in the ether-lipid content of the cells might be associated with increased sensitivity to the drug. This was supported by our findings that K562 cells supplemented with hexadecylglycerol to increase their ether-lipid content become more sensitive to ET-18-OCH$_3$. Thus in the studies described herein, greater sensitivity to ET-18-OCH$_3$ is correlated with an increase in the ether-containing phospholipids of the cells. This relation may in fact help explain why tumor cells, which in many instances contain higher levels of ether-lipids (21, 22), are more sensitive to ET-18-OCH$_3$ than are normal cells (1, 2).

Although the change in sensitivity of the cells to ET-18-OCH$_3$ may not be directly related to elevated ether-lipids, several mechanisms may be proposed to account for the effect of ether-lipids on cellular function. Changes in the physical properties of the membrane may affect the function of integral membrane proteins which may in turn influence the uptake of nutrients or chemotherapeutic agents. Supplementation of L1210 cells with polyunsaturated fatty acids to alter membrane properties was found to increase their sensitivity to Adriamycin (23, 24). The increased sensitivity to the drug was probably explained by the finding that the accumulation of Adriamycin increased by 30% in the supplemented cells (23). The mechanism underlying the selective cytotoxicity of ET-18-OCH$_3$ may involve the ability of the cells to take up the drug. Storch and Munder (20) recently reported that uptake of ET-18-OCH$_3$ is decreased in cells which are resistant to its cytotoxicity compared to more sensitive cells. Although uptake may be a factor in some cell types, it clearly does not underlie the differential sensitivity of hexadecylglycerol-supplemented and unsupplemented K562 cells. Both groups of cells took up equal amounts of the drug.

A change in the ether-lipid content of the membrane also may be of importance in regulating cell growth and the activities of membrane-bound enzymes (13). Cabot *et al.* (19) demonstrated that supplementation of L-M cells with hexadecylglycerol inhibited phosphatidylcholine metabolism and caused a reduction in the rate of cellular growth. In addition, these investigators found that the alkyl-linked species of phosphatidylcholine was being made at the expense of the diacyl subclass (19). Although we have not investigated the effect of supplementation on the metabolism of PC, our finding of an increase in the ether-linked subclass in K562 cells appears to be consistent with these results.

It is now becoming evident that PC metabolism plays an important role in many cell types. Therefore, it is reasonable to speculate that changes in the metabolism of PC may have a profound effect on cellular function. Agonist-induced hydrolysis of PC by phospholipase C leads to the generation of alkylacyl- and diacylglycerols (25, 26). An increase in the alkyl-linked species of PC at the expense of the diacyl subclass might result in the production of a larger proportion of alkylacylglyceride which has been shown to inhibit protein kinase C (27). Protein kinase C is thought to play an important role in several cellular processes including the control of differentiation and response to growth factors. In addition, ET-18-OCH$_3$ and other alkyl lipids have been shown to inhibit protein kinase C in intact cells and cell-free preparations (28–31). Thus, an increase in endogenous ether-linked lipid combined with treatment of the cells with ET-18-OCH$_3$ may have a profound influence on protein kinase C.

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