Relationship between Arachidonate−Phospholipid Remodeling and Apoptosis[†]

Marc E. Surette,[‡,§] James D. Winkler,[||] Alfred N. Fonteh,[§] and Floyd H. Chilton*[,§]

Section on Pulmonary and Critical Care Medicine and Department of Biochemistry, Bowman Gray School of Medicine, Medical Center Boulevard, Winston-Salem, North Carolina 27157-1054, and Division of Pharmacology and Department of Medicinal Chemistry, SmithKline Beecham Pharmaceuticals, King of Prussia, Pennsylvania 19406

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ABSTRACT: Our previous studies reveal that three structurally distinct inhibitors of the enzyme CoA-independent transacylase, including the antiproliferative alkyllysophospholipid ET-18-O-CH_3, induce programmed cell death (apoptosis) in the promyelocytic cell line HL-60. The objective of the current study was to better elucidate the mechanism responsible for apoptosis. CoA-IT is an enzyme believed to be responsible for the remodeling of long chain polyunsaturated fatty acids like arachidonate between the phospholipids of mammalian cells. The chronic (24−48 h) treatment of HL-60 cells with all three CoA-IT inhibitors resulted in the inhibition of the remodeling of labeled arachidonate from choline- into ethanolamine-containing phospholipid molecular species. GC-MS analysis of the fatty acids in phospholipids revealed that CoA-IT inhibitor treatment induced a marked loss of arachidonate-containing phosphatidylethanolamine and an increase in arachidonate-containing phosphatidylcholine. This redistribution was specific to arachidonate since the mass distribution of linoleic acid in glycerolipids was not affected. In spite of the dramatic redistribution of arachidonate, the total cellular arachidonate content was not altered nor was the relative distribution of total phospholipid classes. The increase of arachidonate in phosphatidylcholine was specifically due to an increase in 1-acyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine species, whereas the loss of arachidonate in PE was from both 1-acyl- and 1-alk-1-enyl-2-arachidonoyl-*sn*-glycero-3-phosphoethanolamine species. The incubation of cells with exogenous arachidonic acid or ethanolamine did not reverse the inhibition of proliferation induced by CoA-IT inhibitor treatment. Incubation with CoA-IT inhibitors also induced the characteristic cytoplasmic and nuclear changes associated with apoptosis as assessed by transmission electron microscopy and DNA fragmentation as determined by flow cytometry. Taken together, these data show that apoptosis in HL-60 cells, induced by blocking arachidonate-phospholipid remodeling, is correlated with a redistribution of arachidonate in membrane phospholipids and suggest that such alterations represent a signal which controls the capacity of cells to proliferate.

Cancer cells have long been known to have altered lipid metabolism when compared to their nonmalignant counterparts. For example, cancer cells generally have an increased phosphatidylcholine/phosphatidylethanolamine ratio and often contain larger pools of 1-ether-linked glycerolipids (Chabot et al., 1990; Gottfried, 1967; Klock & Pieprzyk, 1979; Lou & Clausen, 1968). Additionally, epidemiological and clinical studies have provided evidence that changes in arachidonic acid (AA)[1] metabolism, at least through the cyclooxygenase pathway, plays an important role in human cancers (Marnett & Honn, 1994; Schneider et al., 1994). Prostaglandins are produced at high concentrations by several neoplastic cells, including mammary and lung tumors, when compared to control tissue or nonmalignant tumors (McLemore et al., 1988; Nigam et al., 1985; Vergote et al., 1985). The synthesis of prostaglandins such as prostaglandin E_2 has been proposed to be immunosupressive, thereby providing a mechanism for enhanced tumor cell growth, whereas other cyclooxygenase products such as prostaglandin I_2 and thromboxane A_2 produced in the vasculature have been postulated to regulate the metastatic potential of tumor cells (Chen et al., 1992). More recently, AA and linoleic acid metabolites derived from the lipoxygenase pathway have also been shown to have both metastatic and mitotic potential. (Eling & Glasgow, 1994; Glasgow et al., 1992; Honn et al., 1994a,b; Liu et al., 1994; Tang et al., 1994, 1995). Finally, AA itself as well as prostaglandins have recently been demonstrated to regulate gene expression in a variety of cells (Cowlen & Eling, 1993; Jurivich et al., 1994; Rao et al., 1994; Tebbey et al., 1994). These studies suggest that the control of gene expression may be an important mechanism by which fatty acids or their metabolites control cell growth.

In spite of the aforementioned studies showing the importance of free AA and its metabolites in neoplastic cells, almost nothing is known about the turnover of arachidonate within phospholipid molecular species during cell proliferation. Mammalian cells generally contain as many as 20 different arachidonate-containing phospholipids, and arachi-

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* Address correspondence to this author at Bowman Gray School of Medicine, Medical Center Boulevard, Winston-Salem, NC 27157-1054. Telephone: (910) 716-3923. Fax: (910) 716-7277.

[‡] Recipient of a Centennial Fellowship awarded by the Medical Research Council of Canada.

[§] Bowman Gray School of Medicine.

[||] SmithKline Beecham Pharmaceuticals.

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[1] Abbreviations: CoA-IT, CoA-independent transacylase; AA, arachidonic acid; LA, linoleic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; NL, neutral lipids; ET-18-O-CH_3, 1-*O*-octadecyl-2-*O*-methylphosphocholine; HSA, human serum albumin;, FBS, fetal bovine serum; HBSS, Hanks balanced salt solution; TLC, thin-layer chromatography.

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donate is moved through different phospholipids in a sequential fashion (Chilton et al., 1996; Chilton & Murphy, 1986; MacDonald & Sprecher, 1991). For example, AA as a free acid in circulation is moved into most cells and rapidly converted to arachidonoyl-CoA (Bakken & Farstad, 1989; Laposata et al., 1985; Wilson et al., 1982). This arachidonoyl-CoA is then incorporated into 1-acyl-linked phospholipids by a CoA-dependent acyl transferase reaction (Chilton et al., 1987; Hill & Lands, 1968; Irvine, 1982; Waku, 1992; Waku & Lands, 1968). Once AA is placed into 1-acyl-linked phospholipids such as 1-acyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (PC), it is then transferred to 1-alkyl-2-lyso-PC and more slowly to 1-alk-1-enyl-2-lyso-*sn*-glycero-3-phosphoethanolamine (PE) by the enzyme CoA-independent transacylase (CoA-IT) (Chilton et al., 1995; Chilton & Murphy, 1986; Fonteh & Chilton, 1992; Kramer & Deykin, 1983; MacDonald & Sprecher, 1989; Masuzawa et al., 1989; Robinson et al., 1985; Sugiura et al., 1984). This enzyme is believed to be important not only in the maintenance of homeostatic arachidonate levels within cellular phospholipid species but also in the rapid redistribution of arachidonate into releasable pools following stimulation of inflammatory cells (Colard et al., 1986; Fonteh & Chilton, 1992; Venable et al., 1993).

We have recently discovered two structurally distinct molecules (SK&F 45905 and SK&F 98625) which are capable of inhibiting CoA-IT (Chilton et al., 1995). When provided acutely to neutrophils, these inhibitors attenuate the movement of labeled AA from 1-acyl-2-AA-PC to 1-alkyl-2-lyso-PC (Chilton et al., 1995; Winkler et al., 1995b). In addition to SK&F 45905 and SK&F 98625, we have established that the antineoplastic agent 1-*O*-octadecyl-2-*O*-methyl-phosphocholine (ET-18-O-CH_3) is a potent inhibitor of the enzyme CoA-IT (Winkler et al., 1995a). Moreover, these studies revealed that the CoA-IT inhibitors SK&F 45905 and SK&F 98625 possess antiproliferative properties. More specifically, all CoA-IT inhibitors including ET-18-O-CH_3 induce apoptosis in the human promyelocytic cell line HL-60. Structurally related compounds which possess no inhibitory activity toward CoA-IT do not induce apoptosis. In addition, inhibitors of phosholipase A_2, 5-lipoxygenase, and cyclooxygenase do not induce apoptosis suggesting that free AA or its metabolites are not responsible for this process.

The objective of the current study was to better elucidate how arachidonate−phospholipid remodeling relates to apoptosis. These studies reveal that long-term CoA-IT inhibition blocks the capacity of HL-60 cells to transfer arachidonate from 1-acyl-2-arachidonoyl-PC into ethanolamine-containing phospholipids. This results in a dramatic increase in the mole quantities of arachidonate in choline-containing phospholipids and concomitant reduction of the mole quantities of arachidonate in ethanolamine-containing phospholipids. Neither apoptosis nor the change in arachidonate-containing lipids can be reversed by exogenous ethanolamine or arachidonic acid supplementation. Taken together, these data reveal that maintenance of cell growth and attenuation of apoptosis requires the CoA-IT-dependent remodeling of arachidonate between phospholipids.

MATERIALS AND METHODS

Materials. Phospholipid standards phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylethanolamine (PE), lyso-PE and lyso-PC were obtained from Avanti Polar Lipids, Inc. (Birmingham, AL). Fatty acid standards were obtained from Cayman Chemical Co. (Ann Arbor, MI). Pyridine and acetic anhydride were purchased fron Alltech Associates Inc. (Deerfield, IL) . Phospholipase C from *Bacillus cereus*, essentially fatty acid-free human serum albumin (HSA), ethanolamine, fetal bovine serum (FBS), penicillin-streptomycin, and common laboratory chemicals were obtained from Sigma Chemical Co. RPMI 1640 culture media and Hanks balanced salt solution (HBSS) were obtained from Gibco (Grand Island, NY). [5,6,8,9,12,14,15-^{3}H]arachidonic acid, [CH_3-^{3}H]choline chloride and [^{14}C]ethanolamine were obtained from American Radiolabelled Chemicals Inc. (St. Louis, MO). Uniplate silica gel G thin-layer chromatography (TLC) plates were obtained from Analtech Inc. (Newark, DE). Silica gel columns were from Baker (Philipsburg, NJ). ET-18-O-CH_3, octadeuterated AA ($^2H_8$-AA) and trideuterated stearic acid ($^2H_3$-SA) were purchased from Biomol Research Laboratories (Plymouth Meeting, MA). SK&F 98625 is diethyl 7-(3,4,5-triphenyl-2-oxo-2,3-dihydroimidazol-1-yl)hepatine phosphonate, and SK&F 45905 is 2-[2-(3,4-chloro-3-(trifluoromethyl)-phenyl)ureido]-4-(trifluoromethylphenoxy)-4,5-dichlorobenzene sulfonic acid (Chilton et al., 1995). Pentafluorobenzyl bromide (20% in acetonitrile) and diisopropanolamine (20% in acetonitrile) were purchased from Pierce (Rockford, IL). All solvents (HPLC grade) were purchased from Fisher Scientific (Silver Spring, MD).

Cells. The human promyelocytic leukemia cell line HL-60 was obtained from the American Type Culture Collection and was maintained in RPMI media containing penicillin (250 units/mL) and streptomycin (250 μg/mL) and supplemented with 10% FBS at 37 °C. In all experiments, cells were incubated at a starting density of 5×10^5 cells/mL of media.

Extraction and Analysis of Lipids. Experiments were terminated by washing cells (2×) by centrifugation with ice-cold HBSS followed by the extraction of lipids (Bligh & Dyer, 1959). A fraction of the lipid extract containing $^2H_8$-AA and $^2H_3$-SA as internal standards was submitted to methanolic base hydrolysis by heating in methanol/water (75:25, v/v) containing 2 N KOH at 60 °C for 30 min. The solution was diluted with eight volumes of water and acidified with one volume of 6 N HCl, and the lipids were extracted by loading the solution onto octadecyl columns. The fatty acids were then eluted from the column with methanol. Pentafluorobenzyl ester derivatives of fatty acids were prepared, and the quantities of arachidonate or linoleic acid (LA) were determined by negative ion chemical ionization gas chromatography/mass spectrometry as previously described (Chilton & Murphy, 1987).

Another fraction of the extracted lipids was used to separate glycerolipid classes by HPLC using a silica column (Ultrasphere, 4.6 × 250 mm;Rainin Instrument Co, Inc., Woburn, MA) with a hexane/2-propanol/ethanol/50 mM phosphate buffer (pH 7.4)/acetic acid (490:367:100:30:0.6, v/v/v/v/v) mobile phase (Chilton, 1990). Fractions containing neutral lipids (NL), PE, phosphatidylinositol + phosphatidylserine (PI/PS), and PC were collected. The AA and/or LA content of each fraction was determined as described above.

For phospholipid subclass analysis, the PE and PC fractions isolated by HPLC were dried under a stream of

nitrogen and vortexed in 1 mL of 100 mM Tris buffer (pH 7.4). Phospholipase C, 40 and 20 units, was added to the PE and PC fractions, respectively, followed by 2 mL of ethyl ether, and the solutions were incubated for 6 h at 37 °C in a shaking water bath. The resulting diglycerides were extracted with 2 mL of hexane and then again with 2 mL of hexane/ether (1:1, v/v). The extracts were dried under a stream of nitrogen, and acetylated derivatives were prepared by incubating overnight in pyridine/acetic anhydride (1:5, v/v) at 37 °C in a shaking water bath. The solution was then dried under N_2 and extracted twice with ether/hexane (1:1, v/v). The extract was washed once with 1 mL of water, and the subclasses were separated by TLC using a benzene/hexane/ether (50:45:4, v/v/v) mobile phase. The areas containing 1-acyl, 1-alkyl, and 1-alk-1-enyl-linked lipids were identified by comigration of standards which were visualized with iodine. The TLC scrapings were extracted with ether/methanol (9:1, v/v) and were prepared for GC/MS analysis as described above.

Total lipid phosphorus was determined colorimetrically (Rouser et al., 1966). In some experiments, phosphorus was assayed directly on TLC scrapings following the separation of lipid classes by TLC using a chloroform/methanol/acetic acid/water (50:25:8:3, v/v/v/v) mobile phase.

Incubation of Cells with Labeled Lipids. In experiments where cells were labelled with [^{3}H]AA (200Ci/mmol), cells were washed and resupended at 20×10^6 cells per mL in Ca^{2+}-free HBSS. [^{3}H]AA (1 μCi/2×10^7 cells) was added in 200 μL of HBSS containing HSA (250 μg/mL), and the cells were incubated for 15 min at 37 °C. The cells were then washed by centrifugation at 4 °C with HBSS containing 250 μg of HSA/mL (2×), resuspended in culture media, and immediately incubated at 37 °C with the indicated concentrations of inhibitors. Lipids were extracted and analyzed as described above.

In some experiments, a neutral lipid fraction collected by HPLC was subjected to TLC using a hexane/ether/formic acid (90:60:6, v/v/v) mobile phase to separate the cholesterol ester, triglyceride, diglyceride, and free fatty acid fractions. The areas corresponding to each fraction were identified by comigration of standards which were visualized with iodine. TLC plates were also scanned for radiolabel with a Bioscan system 2000 Imaging Scanner (Bioscan Inc., Washington, DC). The areas corresponding to each neutral lipid class were scraped, and the associated radioactivity was determined by liquid scintillation counting.

For the determination of lysophospholipid quantities, cells (1.5×10^6/mL) were labelled with 10 μCi of [^{3}H]choline or 2 μCi of [^{14}C]ethanolamine for 36 h in culture media, washed 2× with media, and resuspended at 5×10^5 cells/mL. The cells were then incubated at 37 °C in the presence or absence of CoA-IT inhibitors. At the indicated times, cells were washed with HBSS followed by centrifugation (2×) and the lipids extracted as described above. The extracted lipids were then separated by TLC using a chloroform/methanol/ammonium hydroxide (65:30:8, v/v/v) mobile phase and the radioactivity comigrating with lyso-PC or lyso-PE was assessed by liquid scintilation counting of the TLC scrapings.

Electron Microscopy. Following the treatment of cells with inhibitors, cells were pelleted and fixed with 2.5% glutaraldehyde in 100 mM phosphate buffer, pH 7.3. Samples were then prepared for transmission electron microscopy, and samples to be sectioned were embedded in Spurr Resin (Polysciences, Warrington, PA). Thin sections (0.1 μm) were visualized at 80 keV in a Phillips EM-400.

Determination of Apoptosis by Flow Cytometry. Apoptosis was measured by quantitating the amount of broken DNA fragments using a fluorescent end-labeling method using the apotag kit (Oncor, Gaithersburg, MD) (Chapman et al., 1995; Gorczyca et al., 1993). Flow cytometric analysis was done on a Becton-Dickenson FACScan instrument using CellQuest software.

RESULTS

As mentioned in the introduction, very recent studies have identified three structurally distinct classes of CoA-IT inhibitors including ET-18-O-CH_3. SK&F 98625, SK&F 45905, and ET-18-O-CH_3 inhibit CoA-IT activity in microsomes of U937 cells with IC_{50}s of 9 μM, 6 μM and 0.5 μM, respectively (Winkler et al., 1995a,b). In addition to blocking CoA-IT activity, all three inhibitors prevent proliferation of the neoplastic cell line HL-60. We have also recently demonstrated that reduction in HL-60 cell numbers is due, in large part, to induction of cellular apoptosis. Although the compound ET-18-O-CH_3 is a known inhibitor of protein kinase C at concentrations higher than those used in the present study, the other two compounds, SK&F 98625 and SK&F 45905, were shown to not inhibit the activity of partially purified protein kinase C isozymes from rat brain (data not shown). These data raised fundamental questions as to how blocking an enzyme that remodels arachidonate between phospholipids influences apoptosis.

Influence of CoA-IT Inhibitors on the Movement of Labeled Arachidonate between Phospholipids. Since CoA-IT inhibitors had never been provided long-term to cells in culture, initial studies examined the influence of SK&F 98625, SK&F 45905, and ET-18-O-CH_3 on the remodeling of labeled arachidonate between phospholipid subclasses. HL-60 cells were removed from culture, pulse-labeled with [^{3}H]arachidonic acid, and placed back into culture in the presence and absence of each inhibitor. After 24 and 48 h in culture, the movement of labeled arachidonate between phospholipid classes and subclasses was determined. During the pulse-labeling period, HL-60 cells incorporated most of the exogenously added [^{3}H]arachidonic acid into PC, PI, and PE (Figure 1). When these cells were placed back in culture, there was a time-dependent movement of labeled arachidonate from PC into ethanolamine-containing molecular species (Figure 1A). For example, PE contained only 29% of labeled arachidonate after the initial pulse. However, 57% of the labeled arachidonate was associated with PE after a 48 h chase period. SK&F 98625, SK&F 45905, and ET-18-O-CH_3 all blocked the increase of labeled arachidonate in PE (Figure 1B−D). Labeled arachidonate in PE never exceeded 42% of the total in the presence of any of the three CoA-IT inhibitors. The treatment of cells with CoA-IT inhibitors blocked the time-dependent decrease in label associated with PC species, but the effects were not as apparent as with PE species. This is likely because cellular PC contains both a major donor of arachidonate (1-acyl-2-arachidonoyl-PC) as well as a major acceptor (1-alkyl-2-arachidonoyl-PC) of arachidonate from CoA-IT, and thus inhibition of CoA-IT would result in little overall change in radioactivity associated with PC.

Influence of CoA-IT Inhibitors on Endogenous Quantities of Arachidonate in Phospholipids. Although the aforemen-

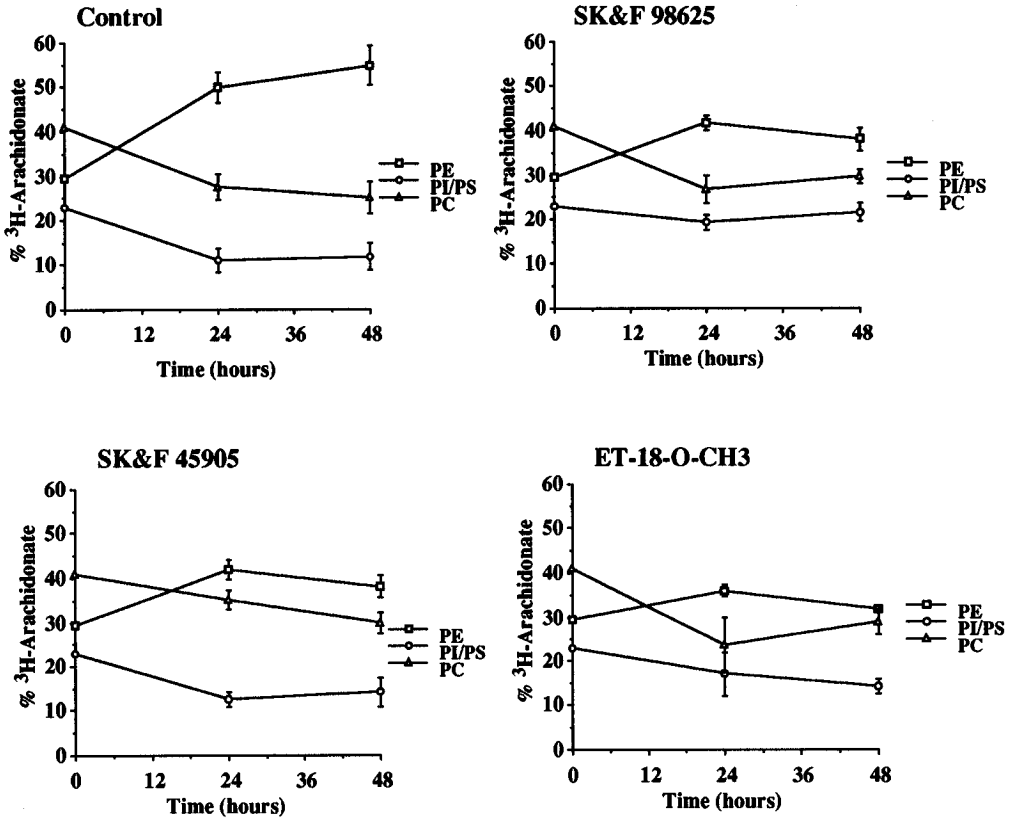

FIGURE 1: Movement of [^{3}H]arachidonate between phospholipids of HL-60 cells incubated with different CoA-IT inhibitors. HL-60 cells were labeled with [^{3}H]arachidonate for 15 min at 37 °C, were washed twice with HSA (250 μg/mL) in HBSS, and were then incubated at 37 °C for the indicated times in RPMI-1640 with 10% FCS in the presence of 0.05% DMSO (v/v) (control), 50 μM SK&F 45905, 25 μM SK&F 98625, or 25 μM ET-18-OCH$_3$. At the indicated times, cells were washed twice by centrifugation in HBSS, and cellular lipids were extracted. Lipid classes were separated by normal phase HPLC, and the radioactivity associated with each class was determined by liquid scintillation counting. Values are expressed as the percentage the total radioactivity that is associated with each class and represent the means of three different experiments ± SEM.

tioned studies demonstrated that CoA-IT inhibitors could block the remodeling of newly incorporated arachidonate between HL-60 cell phospholipids, it was not apparent whether this treatment would have an impact on mole quantities of endogenous arachidonate in phospholipid classes and subclasses. To determine this, cells were incubated with various doses of CoA-IT inhibitors for 48 h, and the arachidonate content of various phospholipid classes was measured by GC/MS. In these experiments, the amount of arachidonate in each phospholipid class was expressed in terms of the amount of total phospholipid (determined by lipid-phosphorus assay) in each class. This was done to ensure that losses were specific to arachidonate-containing phospholipids and not a general loss of all phospholipids from cellular membranes. In the absence of any inhibitor, the bulk of all endogenous arachidonate within the HL-60 cell is found within ethanolamine-containing phospholipids (Figure 2). All three inhibitors caused a similar dose-dependent loss of arachidonate from PE with a concomitant increase in arachidonate found in PC (Figure 2). For example, arachidonate in ethanolamine-containing phospholipids decreased by greater than 30% with all inhibitors and as much as 50% with ET-18-O-CH$_3$. In fact, levels of arachidonate in PC were comparable to those found in PE at the highest concentrations of ET-18-O-CH$_3$. In addition to PC, the content of arachidonate in neutral lipids increased as a function of the dose of CoA-IT inhibitor provided to the cells. This product was further isolated and determined to be arachidonate-containing triacylglycerides (data not shown). In contrast to PE, PC, and NL, the cellular content of arachidonate in PI remained unchanged following treatment with CoA-IT inhibitors. When cells were treated with inhibitors for 24 h, similar but slightly less pronounced alterations in arachidonate content were observed (data not shown).

In light of the dramatic shift in the distribution arachidonate-containing phospholipids, it was critical to determine whether the total arachidonate content of the cells was altered during CoA-IT treatment. Table 1 shows that the total quantity of arachidonate associated with glycerolipids was not altered by CoA-IT treatment. It was also important to determine if the shift in the distribution of arachidonate in phospholipids was specific to arachidonate-containing phospholipids or represented a general shift in the distribution of all phospholipids. Two sets of experiments were performed to examine this question. First, phospholipid classes were separated and the total amount of phosphorus in each class determined. Table 1 shows that PC was the largest phospholipid class in HL-60 cells followed by PE and PS/PI. CoA-IT inhibitors did not alter the distribution of phospholipid classes in HL-60 cells. A second means to ensure that CoA-IT inhibitors were affecting only arachidonate-containing phospholipids was to measure the quantity of another unsaturated fatty acid in HL-60 phospholipids. Table 2 shows that the distribution of linoleate in phospholipid classes after CoA-IT treatment. In contrast to the marked changes in arachidonate-containing PE and PC classes, CoA-IT inhibitors did not influence the distribution

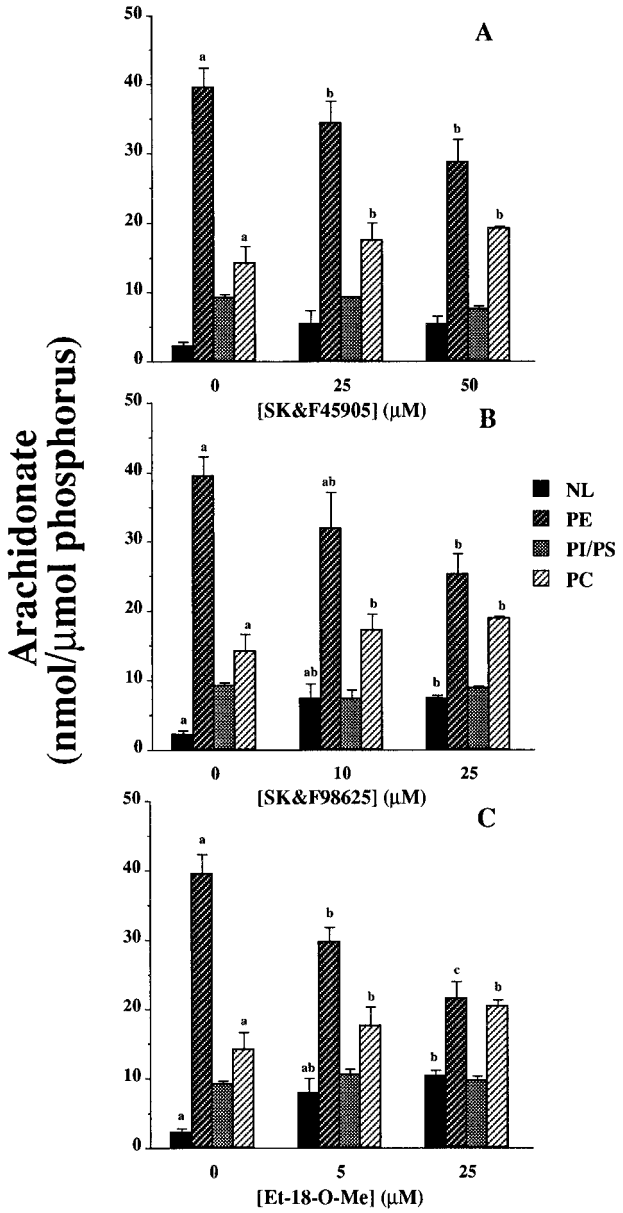

FIGURE 2: Distribution of arachidonate in phospholipid classes of HL-60 cells incubated with different CoA-IT inhibitors. HL-60 cells were incubated in the presence of different concentrations of SK&F 45905 (A), SK&F 98625 (B), or ET-18-OCH_3 (C) for 48 h at 37 °C in RPMI-1640 with 10% FCS. The cells were washed twice by centrifugation in HBSS, and the cellular lipids were extracted. The lipid classes were separated by normal phase HPLC, and the arachidonate content of NL, PE, PI/PS, and PC was determined by GC-MS analysis as described in Materials and Methods. The data are the mean ± SEM of three separate experiments. Values within each class without a common superscript are significantly different $p < 0.05$ as determined by a two-sided paired student's *t*-test.

of linoleate in PE or PC classes. However, the LA content in the NL fraction increased, likely reflecting an overall increase in cellular triglyceride mass. Taken together, these studies indicate that the redistribution in phospholipids observed above with CoA-IT inhibitors is specific to arachidonate-containing phospholipids and does not represent a general loss of cellular membranes.

Experiments were next performed to determine whether the changes in the distribution of PC and PE were reflected in all subclasses or were the result of losses or gains in specific subclasses. Figure 3 illustrates that all CoA-IT inhibitors caused a marked reduction in the quantity of

Table 1: Arachidonate Content and Phospholipid Composition of HL-60 Cells Treated with CoA-IT Inhibitors[a]

treatment	nmol of AA/μmol of phosphorus	lipid phosphorus (%)		
		PC	PI/PS	PE
control	70 ± 7.7	56.9 ± 1.3	12.0 ± 0.2	31.2 ± 1.3
SK&F 45905	80 ± 11	58.1 ± 1.1	12.3 ± 1.9	29.6 ± 0.8
SK&F 98625	68.5 ± 2	55.2 ± 1.6	14.8 ± 3.4	29.9 ± 1.9
ET-18-OCH_3	77 ± 10	49.2 ± 3.3	16.7 ± 4.0	34.0 ± 0.7

[a] HL-60 cells were incubated with SK&F 45905 (50 μM), SK&F 98625 (25 μM), ET-18-OCH_3 (25 μM), or diluent for 48 h. Cellular lipids were extracted, and total AA and lipid phosphorus were determined. Phospholipid classes were separated by TLC, and the lipid phosphorus content of each class was measured. Values are expressed as the means ± standard errors, $n = 4$ for AA per lipid phosphorus values and $n = 3$ for percent phosphorus values. There were no significant differences between treatments as determined by paired student's *t*-test analyses.

Table 2: Effect CoA-IT Inhibitors on the Distribution of Linoleic Acid in Cellular Lipid Classes[a]

treatment	nmol of linoleate per μmol of phosphorus			
	NL	PE	PI/PS	PC
diluent	5.7 ± 1.2[a]	8.8 ± 0.8	7.7 ± 1.4	9.4 ± 1.0
SK&F 45905	11.3 ± 2.7[b]	11.3 ± 2.8	8.1 ± 2.5	10.3 ± 1.4
SK&F 98625	9.4 ± 2.3[b]	6.7 ± 1.0	9.7 ± 2.3	11.5 ± 1.7
ET-18-OCH_3	12.1 ± 1.2[b]	7.3 ± 1.2	8.7 ± 2.4	11.9 ± 2.3

[a] HL-60 cells were incubated with SK&F 45905 (50 μM), SK&F 98625 (25 μM), ET-18-OCH_3 (25 μM), or diluent for 48 h. Cellular lipids were extracted, and total linoleate and lipid phosphorus were determined. Phospholipid classes were separated by HPLC, and the linoleate content in each class was measured. Values are expressed as the means ± standard errors, $n = 3$. Values in the same column without a common superscript are significantly different as determined by paired student's *t*-test analyses.

arachidonate in 1-acyl-2-arachidonoyl-PE. In addition, arachidonate was reduced in the largest PE subclass, 1-alk-1-enyl-2-arachidonoyl-PE. In contrast to PE subclasses, the arachidonate content of 1-acyl-linked PC increased following treatment with all three inhibitors. This increase was specific to 1-acyl-2-arachidonoyl-PC and was thus responsible for the observed overall increase in arachidonate-containing PC.

Accumulation of Lysophospholipids during CoA-IT Treatment. In addition to preventing arachidonate "loading" into PE, a potential result of CoA-IT inhibition would be the accumulation of one of the substrates for CoA-IT, lysophospholipids, which could not be acylated. Because of their detergent effects, these could be cytotoxic to HL-60 cells. Alternatively, they could serve as signals for cellular events (Kume & Gimbrone, 1994; Nakano et al., 1994). To determine whether any lyso-PC or lyso-PE were formed, cells were labeled with [^{3}H]choline chloride or [^{14}C]ethanolamine and were then treated with SK&F 45905 (50 μM), SK&F 98625 (25 μM), or ET-18-O-CH_3 (25 μM) for 24 h. No changes in radioactivity associated with lyso-PC or lyso-PE were observed in lipid extracts from cells incubated with CoA-IT inhibitors compared to untreated control cells (data not shown).

Supplementation of HL-60 Cells with Exogenous Ethanolamine or Arachidonic Acid. Since the redistribution of cellular arachidonate and more specifically the loss of arachidonate from PE were major changes induced by CoA-IT inhibitors, supplementation experiments were performed in an attempt to reverse the inhibition of cell proliferation.

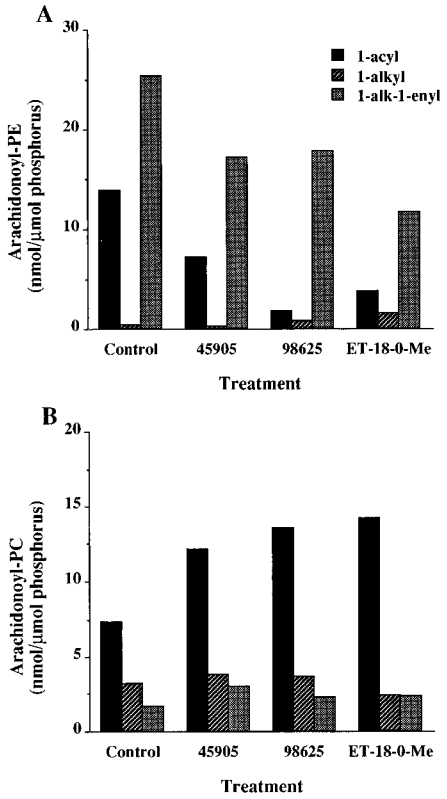

FIGURE 3: Distribution of arachidonate in PE (A) and PC (B) subclasses from HL-60 cells incubated with different CoA-IT inhibitors. HL-60 cells were incubated for 48 h at 37 °C in RPMI-1640 with 10% FCS in the presence of 0.05% DMSO (v/v) (control), 50 μM SK&F 45905, 25 μM SK&F 98625, or 25 μM ET-18-O-CH_3. The cells were washed twice by centrifugation in HBSS, and the cellular lipids were extracted. The lipid classes were separated by normal phase HPLC, and the PC and PE subclasses (1-acyl-, 1-alkyl-, and 1-alk-1-enyl-) were further isolated by TLC as described in Materials and Methods. The arachidonate content of each subclass was determined by GC-MS analysis. The values are those from one experiment which is representative of two separate experiments.

Specifically, HL-60 cells were cultured with exogenous arachidonic acid (50 μM) or ethanolamine (100 μM) or both together with CoA-IT inhibitors. It was reasoned that if the loss of arachidonate in PE was blocking the ability HL-60 cells to mobilize arachidonic acid during the cell cycle, supplementation with exogenous arachidonic acid would overcome this potential effect. In the case of ethanolamine supplementation, this was attempted to increase the overall PE content in the cell and overcome the loss of arachidonate-containing PE. The inhibitory effect of SK&F 45905, SK&F 98625, or ET-18-O-CH_3 on cell proliferation were unaffected by supplementing cells with either arachidonic acid or ethanolamine or both (Table 3).

Influence of CoA-IT Inhibitors on the Membrane Structure of HL-60 Cells and the Induction of Apoptosis. In order to better understand the observed effects of alterations in lipid composition on the changes in the membrane structure of the HL-60 cell, transmission electron microscopy of the cells was performed 24 h after the addition of CoA-IT inhibitors. As can be seen in Figure 4, cells treated with CoA-IT inhibitors show a number of the classical ultrastructural features of apoptosis (Falcieri et al., 1994; Wyllie et al., 1980) when compared to their nontreated counterparts. For example, there is extensive cytoplasmic vacuolation and characteristically condensed chromatin in cells treated with

Table 3: Effect CoA-IT Inhibitors on Cell Proliferation in the Presence or Absence of Exogenous Arachidonic Acid or Ethanolamine[a]

	cell number × 10^6			
treatment	control	arachidonate	ethanolamine	ethanolamine + arachidonate
diluent	1.25 ± 0.20	0.92 ± 0.19	1.18 ± 0.18	1.28 ± 0.15
SK&F 45905	0.78 ± 0.07	0.71 ± 0.20	0.83 ± 0.02	ND
SK&F 98625	0.84 ± 0.20	0.71 ± 0.07	0.74 ± 0.20	ND
ET-18-OCH_3	0.75 ± 0.10	0.64 ± 0.08	0.79 ± 0.09	0.73 ± 0.09

[a] HL-60 cells were at 0.5 × 10^6 cells/mL were incubated with SK&F 45905 (50 μM), SK&F 98625 (25 μM), ET-18-OCH_3 (25 μM), or diluent for 48 h. Cells were supplemented with 0.1% ethanol (control), 50 μM arachidonic acid, or 100 μM ethanolamine. Values are expressed as the means ± standard errors, $n = 3$. There were no significant differences in cell numbers between control and supplemented cells within each treatment as determined by paired student's *t*-test analyses.

CoA-IT inhibitors. Additionally, numerous micronuclei containing homogenously condensed chromatin can be observed. The integrity of the plasma membrane appears to be intact except for the SK&F 98625-treated cell which appears to be in the latter stages of dissolution when necrotic changes may be taking place.

In addition to morphological criteria, apoptosis was assessed by a fluorescent DNA end-labeling method in HL-60 cells incubated with various concentrations of CoA-IT inhibitors. Table 4 shows that the percentage of apoptotic cells increases in a dose-dependent fashion when cells are incubated with CoA-IT inhibitors and this induction of apoptosis parallels the observed changes in the distribution of arachidonate shown in Figure 2.

Correlation between the Quantities of Arachidonate in PE and 1-Acyl-2-arachidonoyl-PC and the Capacity of HL-60 Cells to Proliferate. In a final set of experiments, the arachidonate content of PE or 1-acyl-2-arachidonoyl-PC under a variety of conditions was determined and plotted as a function of the capacity of HL-60 cells to proliferate. As shown in Figure 5, there is a significant positive correlation between the proliferation of cells and the cellular content of arachidonyl-PE after 48 h incubation. In contrast, there is an inverse relationship between the cellular quantities of 1-acyl-2-arachidonoyl-PC and the capacity of HL-60 cells to proliferate.

DISCUSSION

The current results support the hypothesis that blockage of the enzyme CoA-IT in whole cells results in a redistribution of cellular arachidonate from 1-acyl- and 1-alk-1-enyl-2-arachidonyl-PE molecular species to 1-acyl-2-arachidonyl-PC species. These results in conjunction with earlier findings (Winkler et al., 1995a), which show that the incubation of HL-60 cells with CoA-IT inhibitors leads to the induction of apoptosis, point to a direct linkage between arachidonate−phospholipid remodeling and apoptosis. The following lines of evidence support this nexus. First, three structurally distinct compounds share the ability to inhibit CoA-IT activity and arachidonate−phospholipid remodeling and to induce apoptosis. Furthermore, closely related structural analogues and isomers of these compounds which possess no inhibitory action toward CoA-IT do not induce apoptosis. Taken together, these data strongly support the contention that induction of apoptosis is mediated by the blockage of

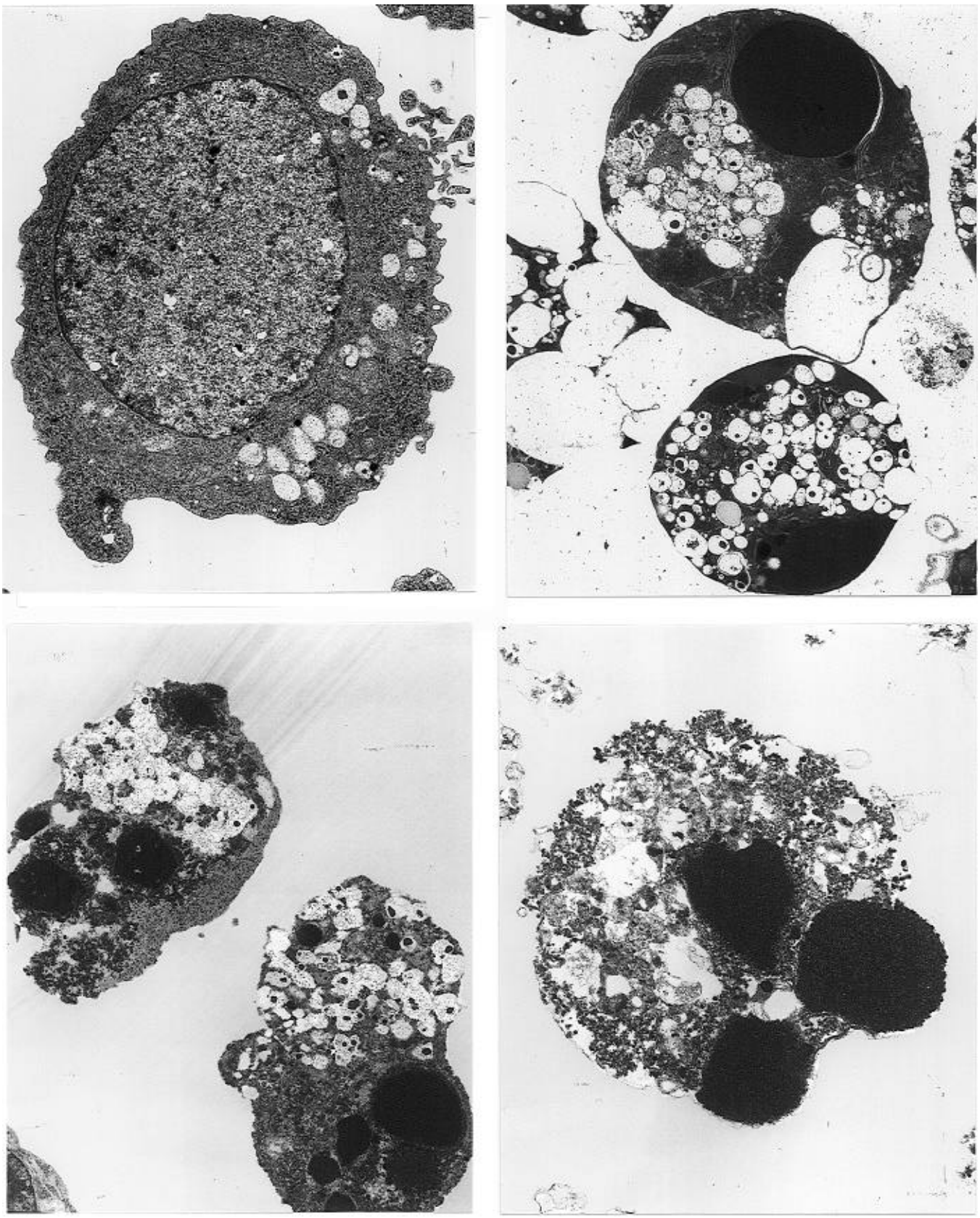

FIGURE 4: Transmission electron microsopy of HL-60 cells incubated with 0.05% DMSO (top left, magnification 13500×), SK&F 45905 (50 μM) (top right, magnification 10140×), SK&F 98625 (25 μM) (bottom left, magnification 18666×), or ET-18-O-CH_3 (25 μM) (bottom right, magnification 7835×) for 24 h.

remodeling and not that apoptosis is caused by some nonspecific toxic effect of the compounds. Secondly, the blockage of CoA-IT by these compounds does not cause cellular necrosis but rather specifically induces apoptosis. Three different methods including DNA ladder formation, fluorescent end labeling of DNA with subsequent FACS analysis, and morphological criteria using electron microscopy have been used to document apoptosis. Thirdly, compounds which inhibit 5-lipoxygenase, cyclooxygenase, secretory PLA_2, or cytosolic PLA_2 do not induce apoptosis

Table 4: Percentage of Apoptotic Cells following Treatment of HL-60 Cells with Various Concentrations of CoA-IT Inhibitors[a]

treatment	% apoptotic
untreated	0.46 ± 0.01
SK&F 98625 (10 μM)	1.8 ± 0.2
SK&F 98625 (25 μM)	50.3 ± 0.4
ET-18-OCH_3 (5 μM)	16.5 ± 0.9
ET-18-OCH_3 (25 μM)	44.6 ± 0.3

[a] HL-60 cells were incubated with the indicated concentrations of inhibitors for 24 h, and DNA fragmentation was measured by flow cytometry as described in Materials and Methods. The control (untreated) cells were treated with and without terminal deoxynucleotidyl transferase to assess nonspecific fluorescence. Areas were set on the control cells to exclude >99% of the cells from the apoptotic window. The number shown is the percent of cells falling into the apoptotic window. The results are the means ± standard errors for three separate experiments.

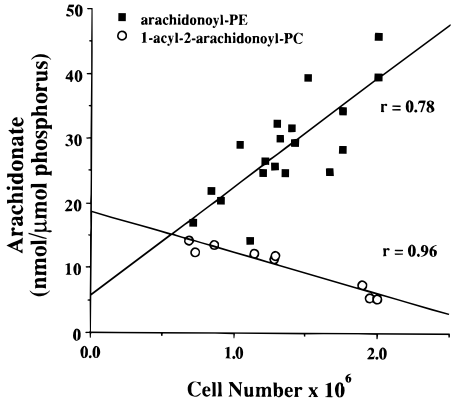

FIGURE 5: Correlation between the quantity of arachidonate in PE and 1-acyl-2-arachidonoyl-GPC and HL-60 cell proliferation. HL-60 cells were incubated at 37 °C with various concentrations of different CoA-IT inhibitors for 48 h in RPMI-1640 with 10% FCS. The cells were counted with a hemocytometer and then washed twice by centrifugation in HBSS, and the cellular lipids were extracted and PE or PC was isolated by HPLC as described in Materials and Methods. The arachidonate content of PE and 1-acyl-2-arachidonoyl-GPC were determined by GC-MS as described in Materials and Methods and the values were plotted against the cell numbers.

(Winkler et al., 1995a). This suggests that the effect of the CoA-IT inhibitors is not associated with a blockage of the release or further metabolism of arachidonic acid but with the remodeling process itself. Although the overexpression of cyclooxygenase-2 is protective against butyrate-induced apoptosis in epithelial cells and this protection can be reversed by nonsteroidal antiinflammatory drugs (Tsujii & DuBois, 1995), the inability of cyclooxygenase inhibitors to induce apoptosis in undifferentiated HL-60 cells is likely due to the limited ability of these cells to synthesize prostaglandins. Forthly, CoA-IT inhibitors do not change the overall distribution of phospholipid classes nor do they alter the phospholipid distribution of another polyunsaturated fatty acid, linoleate. Linoleate is not a substrate for the transacylation reaction. Therefore, CoA-IT inhibitors do not cause a general redistribution of fatty acids in phospholipids but affect only arachidonate-containing phospholipids. Finally, the observed changes in the distribution of cellular arachidonate following treatment with CoA-IT inhibitors are those which would be predicted on the basis of the phospholipid specificity for CoA-IT as determined previously in cell-free assays (Chilton & Murphy, 1986; MacDonald & Sprecher, 1989; Sugiura et al., 1984).

The critical finding that initiated the current study was that the antiproliferative agent ET-18-O-CH_3 is a very potent CoA-IT inhibitor (Winkler et al., 1995a). This observation raised a fundamental question as to the role of arachidonate−phospholipid remodeling in cell proliferation. The importance of this question was further emphasized when it was discovered that other structurally distinct CoA-IT inhibitors have antiproliferative properties and that all CoA-IT inhibitors (including ET-18-O-CH_3) induce cellular apoptosis. However, since long-term studies had not been performed with CoA-IT inhibitors, it was not apparent what remodeling events were being affected by long-term CoA-IT inhibition. The current study addressed this question to better elucidate the lipid-associated signaling events which lead to cellular apoptosis.

CoA-IT has been shown in broken cell assays to catalyze the transfer of 20 carbon highly unsaturated fatty acids, like arachidonate, from the *sn*-2 positions of certain donor phospholipids to the *sn*-2 position of certain acceptor lysophospholipids (Chilton & Murphy, 1986; MacDonald & Sprecher, 1989; Sugiura et al., 1984). On the basis of indirect evidence in whole cells, this transfer event has been proposed to occur between 1-acyl-2-arachidonoyl-PC and 1-alkyl-2-arachidonoyl-PC and more slowly with ethanolamine-containing phospholipids (Fonteh & Chilton, 1992; MacDonald & Sprecher, 1989; Tou, 1985). However, without inhibitors of this enzyme, it has been extremely difficult to test this hypothesis. In the current study, we demonstrate that blocking CoA-IT with several structurally unrelated compounds results in a dramatic redistribution of arachidonate in cellular phospholipids over a 24−48 h period. This time corresponds to the onset of programmed cell death with the characteristic nuclear, cytoplasmic, and membrane changes. Pulse−chase experiments with labeled arachidonate support the conclusion that arachidonate cannot be moved from 1-acyl-linked phospholipids into predominantly ethanolamine-containing phospholipids. Experiments measuring the endogenous quantities of arachidonate revealed that arachidonate accumulates in choline-containing phospholipids and specifically 1-acyl-2-arachidonoyl-PC. These data support the earlier studies in broken cell preparation which reveal that 1-acyl-2-arachidonoyl-PC is a key substrate that provides arachidonate to the enzyme CoA-IT (Chilton & Murphy, 1986; Hazen et al., 1990; Robinson et al., 1985; Sugiura et al., 1987). PE on the other hand was the phospholipid class which showed a dramatic loss of endogenous arachidonate after CoA-IT treatment. Specifically, 1-acyl-2-arachidonoyl-PE and 1-alk-1-enyl-2-arachidonoyl-PE lost large quantities of arachidonate. Cell-free studies have demonstrated both 1-acyl and 1-alk-1-enyl molecular species of PE to be good, but not exclusive, acceptors of AA from CoA-IT (Chilton & Murphy, 1986; MacDonald & Sprecher, 1989; Sugiura et al., 1987); the present study indicates that in intact HL-60 cells, these are the two most important acceptor substrates for CoA-IT-directed AA movement. These data support the concept that arachidonate moves through cellular phospholipids of HL-60 cells in a sequential fashion.

Arachidonate-containing phospholipids represent a minor proportion of all phospholipid molecular species in phospholipid classes such as PC and PE. Therefore, it was extremely important in these studies to determine whether the effects of the CoA-IT inhibitors were specific for

arachidonate-containing phospholipids or represented a disruption in the biosynthesis of an entire phospholipid class. Moreover, ET-18-O-CH_3 has recently been shown to inhibit PC biosynthesis and thus may alter the distribution of cellular phospholipids (Boggs et al., 1995). However, lipid phosphorus measurements of isolated phospholipids indicated that there was no change in the distribution of total phospholipids in the cell after CoA-IT treatment. In addition, CoA-IT inhibitors did not alter the distribution of linoleate among phospholipid classes. Although there are marked changes in the distribution of arachidonate-containing phospholipids, the total nanomole quantities of arachidonate within the HL-60 cell did not change. Again, these findings emphasize the apparent importance of the distribution and not the total content of arachidonate in controlling cell proliferation.

The mechanism by which a redistribution of arachidonate leads to programmed cell death is not known. One hypothesis is that arachidonate must be in PE before it can be released, and released AA or its metabolites control apoptosis. Two lines of evidence suggest that this is not the case. First, inhibitors of arachidonic acid release and metabolism do not induce apoptosis. Second, attempts to rescue the treated cells with high concentrations of exogenously provided arachidonic acid were not successful. A second postulate is that inhibition of CoA-IT leads to the accumulation of lysophospholipid acceptors. Lysophospholipids may then act as cytotoxic detergents, trigger gene regulation (Kume & Gimbrone, 1994; Nakano et al., 1994), or disrupt phospholipid synthesis (Boggs et al., 1995). However, no change in lyso-PC or lyso-PE levels were observed after 24 h of treatment with inhibitors, suggesting that lysophospholipid accumulation is not the mechanism. This may not be surprizing since lyso-PC has been shown to be rapidly converted to PC in metabolic labeling experiments (Boggs et al., 1995). A third hypothesis centers around the idea that cellular PE is critical for the maintenance of cell proliferation. However, there was no change in the quantity of total cellular PE after inhibitor treatment, and attempts to rescue HL-60 cells with exogenously provided ethanolamine were unsuccessful.

A fourth postulate is that the lack of remodeling between phospholipid subclasses results in a preferential loss or gain of arachidonate from a subcellular compartment that is key to signal transduction. Preliminary studies in our laboratory suggest that individual arachidonate-containing phospholipid subclasses are located in distinct subcellular compartments. For example, 1-acyl-2-arachidonoyl-PC and 1-acyl-2-arachidonoyl-PI are preferentially associated with the nucleus while ethanolamine-containing phospholipids are located in other membranes such as the plasma membrane. Studies are currently underway in our laboratory to determine the relevance of the localization of arachidonate-containing phospholipid subclasses to the generation of apoptotic signals. This subcellular distribution is extremely interesting in light of studies by Ishihara and colleagues (Ishihara et al., 1991) which demonstrate that there is an inverse relationship between cell proliferation and the content of arachidonate in nuclei of hepatic cells, and the recent finding that the overexpression of cyclooxygenase 2, which has been localized to the nucleus (Morita et al., 1995), leads to an apoptosis-resistant phenotype in intestinal epithelial cells (Tsujii & DuBois, 1995).

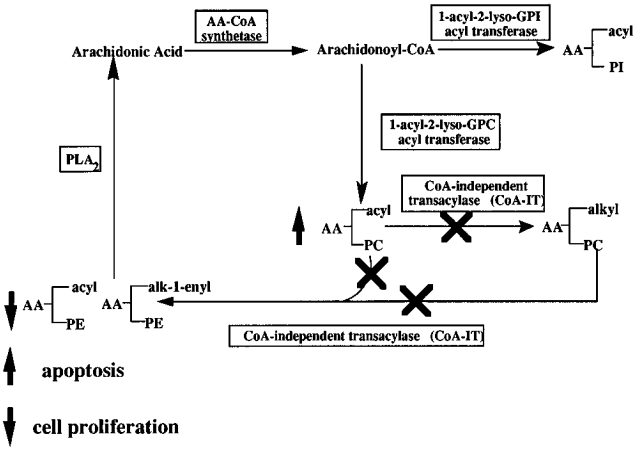

FIGURE 6: Proposed pathway for the remodeling of arachidonate between phospholipid subclasses and consequences of inhibiting CoA-independent transacylase. The Xs represent pathways blocked by CoA-IT inhibitors, and the dark arrows represent changes that occur following inhibition of CoA-IT.

In conclusion, it appears that CoA-IT inhibition can disrupt the ratio of arachidonate in phospholipids and this disruption can represent an important signal leading to cellular events such as apoptosis. The fact that three structurally distinct CoA-IT inhibitors alter the distribution of arachidonate in a manner that would be predicted from previous studies provides strong evidence that the inhibitors themselves are not directly inducing apoptosis, but that apoptosis results from the changes in arachidonate-phospholipid remodeling. In light of the data presented here, the scheme presented in Figure 6 summarizes the role of CoA-IT in arachidonate remodeling within the cell and the consequences of blocking this remodeling pathway on cell proliferation and death. Currently, it is not understood how the change in the arachidonate distribution generates an apoptotic signal. Although the signal generated by the redistribution of arachidonate following the inhibition of CoA-IT has not been identified, it is possible that this signal will ultimately lead to the production of known apoptotic signaling molecules like ceramide (Bose et al., 1995; Obeid et al., 1993) or changes in the expression of protein(s) involved in cell death signaling pathways such as the cysteine proteases of the interleukin-1β converting enzyme (ICE) family of enzymes or the induction of genes encoding peptides containing death domains such as TRADD or FADD (Cleveland & Ihle, 1995). Future studies will be necessary to determine where arachidonate-remodeling fits into the signaling events known to be important to apoptosis.

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