Modification of CaCo-2 cell membrane fatty acid composition by eicosapentaenoic acid and palmitic acid: effect on cholesterol metabolism

Shubha Murthy, Ella Albright, Satya N. Mathur, and F. Jeffrey Field

Department of Internal Medicine, University of Iowa, Iowa City, IA 52242

Abstract Membrane fatty acid composition of CaCo-2 cells was modified by incubating the cells for 8 days in medium containing 100 μM eicosapentaenoic acid or palmitic acid. The effect of membrane fatty acid changes on cholesterol metabolism was then studied. Cells incubated with eicosapentaenoic acid had significant changes in membrane fatty acid composition with an accumulation of 20:5 and 22:5 and a reduction in monoenoic fatty acids compared to cells grown in palmitic acid. Intracellular cholesteryl esters could not be detected in CaCo-2 cells grown in the presence of the n-3 polyunsaturated fatty acid. In contrast, cells incubated with the saturated fatty acid contained 2 μg/mg protein of cholesteryl esters. Cells grown in eicosapentaenoic acid, however, accumulated significantly more triglycerides compared to cells modified with palmitic acid. The rate of oleic acid incorporation into triglycerides was significantly increased in cells incubated with eicosapentaenoic acid. CaCo-2 cells modified by eicosapentaenoic acid had lower rates of HMG-CoA reductase and ACAT activities compared to cells modified with palmitic acid. The incorporation of the two fatty acids into cellular lipids also differed. Palmitic acid was predominantly incorporated into cellular triglycerides, whereas eicosapentaenoic acid was preferentially incorporated into phospholipids with 60% of it in the phosphatidylethanolamine fraction. The data indicate that membrane fatty acid composition is significantly altered by growing CaCo-2 cells in eicosapentaenoic acid. These modifications in membrane fatty acid saturation are accompanied by a decrease in the rates of cholesterol synthesis and cholesterol esterification.—**Murthy, S., E. Albright, S. N. Mathur, and F. J. Field.** Modification of CaCo-2 cell membrane fatty acid composition by eicosapentaenoic acid and palmitic acid: effect on cholesterol metabolism. *J. Lipid Res.* 1988. **29:** 773–780.

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CaCo-2 cells are derived from a human colon adenocarcinoma. When fully differentiated these cells have the morphological and biochemical characteristics of small intestinal absorptive cells (1). These cells have been shown to have microvillar brush border membranes; they form tight junctions at confluency; and they maintain their polarity when cultured on plastic or cellulose filters (2). These cells are proving to be useful models for the study of many parameters of enterocyte function such as glucose and ion transport (3), sodium-dependent P_i transport (4), and lipoprotein secretion (5). Recently, we have used these cells to study the regulation of acyl coenzyme A:cholesterol acyltransferase (ACAT) activity by cholesterol which is absorbed into the cell via bile salt micelles (6). The results suggested that ACAT activity was stimulated by the absorbed cholesterol and this regulation was secondary to the expansion of the ACAT substrate pool by the incoming cholesterol. This supported previous data whereby intestinal ACAT activity was stimulated in the intestines of animals ingesting a diet supplemented with cholesterol (7).

Our laboratory has also been interested in the effects of dietary fat saturation on cholesterol metabolism in the intestine. In an earlier study, intestinal ACAT activity was significantly increased in rabbits fed a diet enriched in polyunsaturated fatty acids compared to the activity in intestines from animals ingesting a diet supplemented with saturated fat (8). In a more recent study, intestinal ACAT activity was increased and 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase activity was decreased in rabbits fed menhaden oil (a fish oil enriched in n-3 polyunsaturated fatty acids) compared to the activities observed in the intestines of animals ingesting a diet supplemented with cocoa butter, a saturated fat (9). In both of these dietary studies, the alterations in the intestinal microsomal membrane fatty acid compositions reflected the composition of the ingested oils. It was postulated that the changes in membrane fatty acid saturation, independently of the membrane cholesterol content, regulated the activities of these two enzymes (8,9). This is not a new concept. Changes in cellular membrane fatty acid saturation have been correlated with the regulation of many membrane-bound enzymes and other cellular functions (10, review). The

Abbreviations: HMG, 3-hydroxy-3-methylglutaryl; ACAT, acylcoenzyme A:cholesterol acyltransferase; TLC, thin-layer chromatography; PBS, phosphate-buffered saline.

difficulty in interpreting the results from the above dietary studies, however, is that the dietary oils contain a mixture of fatty acids, all of which may have diverse effects on the parameters being studied, i.e., cholesterol metabolism. Using CaCo-2 cells as a model for the small intestinal absorptive cell, we studied whether membranes could be modified by an individual fatty acid and whether this, in turn, could affect intracellular cholesterol metabolism.

Since there has been recent enthusiasm for the ingestion of fish oil (an oil rich in eicosapentaenoic acid) for its hypolipidemic effect (11), we investigated the effect of this fatty acid on membrane fatty acid composition and cholesterol metabolism in CaCo-2 cells and compared this to the effect of a saturated fatty acid, palmitic acid. The results suggest that membrane fatty acid saturation can be significantly modified by growing CaCo-2 cells in the presence of eicosapentaenoic or palmitic acid. These modifications are associated with the regulation of HMG-CoA reductase and ACAT activities.

METHODS

Materials

[9,10-^{3}H]Oleic acid,[5,6,8,9,11,12,14,15,17,18-^{3}H]eicosapentaenoic acid, [1-^{14}C]palmitic acid, [2-^{3}H]glycerol, [2-^{14}C]acetate, [4,-^{14}C]cholesterol, [1-^{14}C]oleoyl coenzyme A, [5-^{3}H]mevalonic acid, 3-hydroxy-3-methyl [3-^{14}C]glutaryl CoA, and [1,2-^{3}H]cholesterol were from New England Nuclear (Boston, MA). Oleic acid, eicosapentaenoic acid, palmitic acid, oleoyl coenzyme A, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and nucleotide adenine diphosphate were purchased from Sigma Chemical Co. (St. Louis, MO). HMG-CoA was purchased from P-L Biochemicals, Inc. (Milwaukee, WI). Fatty acid standards for gas-liquid chromatography were purchased from Supelco Inc. (Bellefonte, PA) and Nu-Chek-Prep (Elysian, MN). All other chemicals were reagent grade.

Cell culture

CaCo-2 cells were a generous gift from Drs. E. Schaeffer and J. Ordovas, Tufts University School of Medicine, New England Medical Center, Boston, MA. CaCo-2 cells were grown in T-75 plastic flasks (Corning Glassworks, Corning, NY) in Dulbecco's modified Eagle's minimum essential medium (DMEM) (Gibco, Grand Island, NY) with 4.5 g/liter glucose, supplemented with 20% fetal calf serum (FCS) (Hyclone Laboratories, Inc., Logan, UT), 4 mM glutamine, 10 μg/ml insulin, 50 μg/ml penicillin, 50 μg/ml streptomycin, and 1% nonessential amino acids. Prior to confluency, the cells were split according to the method of Mohrmann et al. (4). Monolayers were grown on 60-mm plastic Petri dishes in 5 ml of supplemented DMEM, but the FCS concentration was changed to 10%. Fresh medium was supplied every 2 days. Cells were used 12 days after plating.

To enrich the cell membranes in the respective fatty acid, the medium was removed and replaced with DMEM containing 2.5% FCS and 100 μM of the fatty acid attached to fatty acid-poor albumin (3:1, molar ratio) 3 days after plating. The medium containing the fatty acid was changed every other day until the experiments were carried out on day eleven. On the day of the experiment, the medium was changed to M199, 10 mM HEPES, without FCS. Cell viability, as ascertained by erythrocin B exclusion, was unaffected by the presence of the fatty acid and was 90% on day 11. Cell growth, as determined by cell number and the amount of cell protein per dish, was similar in the two groups containing the fatty acids.

Fatty acid-albumin preparation

Stock solutions in 95% ethanol of eicosapentaenoic and palmitic acid were kept under nitrogen at 4°C. The necessary aliquot of the stock solution was taken and the sodium salt was prepared with excess NaOH. After the solvent was completely evaporated under nitrogen, the fatty acid salt was dissolved in 1.5 ml of hot distilled water and added rapidly to a small amount of a stirring solution of DMEM supplemented with 2.5% FCS and the required amount of albumin. The pH was adjusted to 7.4 and the volume of the medium was adjusted so that the final concentrations of the fatty acids were 100 μM. The medium was filtered through a 0.2-μm Millipore filter and used within 5 days.

Measurement of lipid synthesis

[^{3}H]Oleic acid (2.6 Ci/mmol), purified by TLC, was mixed with unlabeled oleic acid and NaOH (2 mol of NaOH/mol of oleic acid). The mixture was dried under nitrogen and 0.025 ml of 95% ethanol was added. Water, 0.2 ml, was then added followed by fatty acid-poor albumin so that the ratio of oleic acid to albumin was 1:1 (mol/mol). This solution was stirred well and M199 with 10 mM HEPES was added to make the final conentration of oleic acid 50 μM with a specific activity of 5,300 dpm/nmol.

CaCo-2 cells were incubated with 1 ml of the labeled oleic acid-BSA solution for 30 min at 37°C in 95% air-5% CO_2. After the incubation, the cells were washed thoroughly with ice-cold phosphate-buffered saline. The cells were scraped from the dish in 2 ml of 0.2 N NaOH and the lipids were extracted in chloroform-methanol 2:1(v/v) after neutralizing the solution with HCl. [^{14}C]Cholesteryl oleate (10,000 dpm) was added to adjust for recoveries. The chloroform phase was then evaporated to dryness under nitrogen and the residue was taken up in 0.125 ml of chloroform. The lipids were separated by TLC using hexanes-diethyl

ether–methanol–acetic acid, 85:15:1:1 (v/v) as the developing solvent. The lipids were visualized by iodine vapors and scraped into 4 ml of liquid scintillation fluid and counted. Quenching was determined by an external standard and the spillover of the ^{14}C counts into the ^{3}H channel was corrected for each sample.

Measurement of cholesterol synthesis

[^{14}C]Sodium acetate (56 mCi/mmol) was mixed with unlabeled sodium acetate in M199 containing HEPES, pH 7.4. The acetate was then diluted so the final concentration was 5 mM and the specific activity was 75 dpm/nmol.

CaCo-2 cells were incubated with 1 ml of the labeled acetate solution at 37°C in 95% air–5% CO_2. At the indicated time, the medium was removed and the cells were rinsed thoroughly with ice-cold PBS and scraped. The lipids were extracted with chloroform–methanol 2:1 (v/v) and separated by TLC as described. The band corresponding to free cholesterol was scraped into scintillation vials and counted. Recoveries were similar in all experiments (85%) as determined by recoveries of [^{3}H]cholesterol.

Measurement of palmitic and eicosapentaenoic acid incorporation into cellular lipids

[^{3}H]Eicosapentaenoic acid (79 Ci/mmol) and [^{14}C]palmitic acid (58 mCi/mmol) were mixed with the respective unlabeled fatty acids. Solutions of 100 μM in M199 were prepared as described for the culture medium. The specific activities of eicosapentaenoic acid and palmitic acid were 5,500 dpm/nmol and 12,300 dpm/nmol, respectively.

The fatty acids were incubated with the CaCo-2 cells for 4, 8, and 22 hr. The cells were then washed with cold PBS, scraped as before, and the lipids were extracted. The chloroform phase was aliquoted to determine total uptake of the fatty acids into cellular lipids and incorporation into the major lipid classes as well as individual phospholipids. The phospholipids were separated by TLC using two solvent systems: chloroform–methanol–methylamine–water 60:50:3:3 (v/v) and chloroform–methanol–acetic acid–water 50:40:6:2 (v/v). The bands corresponding to known phospholipid standards were scraped into scintillation vials and counted.

Enzyme assays

Acyl coenzyme A:cholesterol acyltransferase activity was measured as previously described (8); the specific activity of oleoyl-CoA was 19,250 dpm/nmol. 3-Hydroxy-3-methylglutaryl coenzyme A reductase activity was measured as described (12); the specific activity of HMG-CoA was 23,800 dpm/nmol.

Chemical analysis

Protein was determined according to the method of Lowry et al. (13). Cholesterol was measured by gas–liquid chromatography as described previously (14). Phospholipids were measured according to the method of Chalvardjian and Rudnicki (15). Triglycerides were measured fluorometrically (16). Fatty acids were determined as previously described (8).

RESULTS

Microsomal fatty acid composition

CaCo-2 cells were grown in medium containing 10% fetal calf serum for 3 days. The medium was then changed to 2.5% fetal calf serum containing 100 μM of palmitic acid or eicosapentaenoic acid attached to albumin (3:1, molar ratio). After another 8 days in culture, the fatty acid composition of microsomes prepared from the two populations of cells was estimated (**Table 1**). In microsomes obtained from cells grown in the presence of eicosapentaenoic acid, there was a marked decrease in the percent of monoenoic fatty acids, 16:1 and 18:1, compared to microsomes from cells exposed to palmitic acid. There was also a significant increase in the percent of eicosapentaenoic acid and its elongation product 22:5 in membranes from cells grown in media supplemented with eicosapentaenoic acid. Although the percent of saturated fatty acids remained similar in the two groups, the substantial increase in the polyenoic fatty acids in microsomes prepared from cells grown in eicosapen-

TABLE 1. Microsomal fatty acid composition in CaCo-2 cells

Fatty Acids	Eicosapentaenoic Acid	Palmitic Acid
14:0	2.9 ± 0.8	2.7 ± 0.4
16:0	28.2 ± 5.5	37.2 ± 2.5
16:1		9.7 ± 0.5[a]
18:0	18.4 ± 1.0	13.7 ± 1.6
18:1	5.0 ± 0.3[a]	28.5 ± 2.0
18:2		1.2 ± 0.2
18:3		
20:4	1.7 ± 0.2	2.0 ± 0.2
20:5	19.2 ± 2.5[a]	
22:5	22.6 ± 3.5[a]	
22:6		
Saturated	50.0	53.6
Monoenoic	5.0[a]	38.2
Polyenoic	43.5[a]	3.2
P/S	0.87	0.06

CaCo-2 cells were grown in medium containing 10% fetal calf serum for 3 days. From day 4 to day 11, the cells were grown in medium containing 2.5% fetal calf serum and 100 μM of either palmitic or eicosapentaenoic acid attached to albumin (3:1, mol/mol). The cells were then scraped from the dishes in phosphate-buffered saline and homogenized by sonication. The whole homogenates were centrifuged at 10,000 rpm for 20 min and microsomes were prepared by centrifuging the supernatant at 40,000 rpm for 1 hr. Lipids were extracted from the microsomes with chloroform–methanol 2:1 (v/v). The chloroform phase was evaporated under nitrogen and methyl esters of the fatty acids were prepared by adding BF_3. The fatty acids were separated by gas–liquid chromatography as described in Methods.

[a] $P < 0.001$.

taenoic acid resulted in a large difference in the polyunsaturated to saturated fatty acid ratio between the two membrane preparations.

Lipid content of CaCo-2 cells

To determine what effect growing CaCo-2 cells in the presence of 100 μM of the two different fatty acids had on the accumulation of intracellular lipids, the amount of cholesterol, triglyceride, and phospholipids was measured in the two groups. The results are shown in **Table 2.** There was a significant increase in total cholesterol in cells modified with palmitic acid compared to the total cholesterol content of cells grown in eicosapentaenoic acid. This increase in cholesterol was secondary to an increase in the amount of cholesteryl esters. No cholesteryl esters could be detected in cells modified by eicosapentaenoic acid. CaCo-2 cells grown in the presence of the n-3 polyunsaturated fatty acid accumulated significantly more triglycerides than cells grown in the saturated fatty acid. Similar amounts of phospholipids were observed in both groups of cells.

Microsomal cholesterol and phospholipid contents were similar in the two groups. The cholesterol content in microsomes prepared from cells exposed to eicosapentaenoic acid was 89 ± 6 nmol/mg protein compared to 87 ± 5 nmol/mg protein in microsomes from cells grown in palmitate. The microsomal phospholipid contents were 667 ± 50 nmol/mg protein and 667 ± 35, respectively.

Lipid synthesis in CaCo-2 cells

The incorporation of labeled oleic acid into cholesteryl oleate, triglycerides, and phospholipids was used to estimate the synthetic rates of the three lipid classes in CaCo-2 cells. The results are shown in Figs. 1 and 2. The rate of incorporation of oleic acid into the three lipids was linear over the 30-min assay **(Fig. 1).** The rate of oleic acid incorporation into triglycerides was greater than the rate of incorporation of the fatty acid into phospholipids or cholesteryl esters in both groups. Cells grown in medium containing the saturated fatty acid had increased incorporation of oleate into cholesteryl oleate and phospholipids compared to the rates observed in cells grown in eicosapentaenoic acid. In contrast, compared to palmitate cells, the rate of oleic acid incorporation into triglycerides was higher in cells cultured with eicosapentaenoic acid **(Fig. 2).** The rates of triglyceride and phospholipid synthesis were also estimated using labeled glycerol as substrate instead of oleic acid. The results were similar (data not shown).

Microsomal ACAT activity

Although oleic acid incorporation into cholesteryl oleate has been used in other cell types as a measurement of ACAT activity (17, 18), an expansion of the substrate fatty acid pool by the supplemented fatty acids may have adverse effects on the availability of the labeled oleic acid for esterification. To investigate whether the changes observed in the rates of oleic acid incorporation into cholesteryl oleate reflected changes in ACAT activity, microsomes were prepared from the cells in both groups and ACAT activities were measured. Activities were determined using endogenous membrane cholesterol as substrate and after adding exogenous cholesterol solubilized in Triton WR-1339 to ensure saturation of the enzyme with substrate. As shown in **Fig. 3,** both in the presence and absence of exogenous cholesterol, ACAT activities were significantly lower in microsomes prepared from cells modified by eicosapentaenoic acid. The addition of cholesterol to the assays did not significantly alter ACAT activities in microsomes from either groups suggesting that the endogenous cholesterol was at saturation for the enzyme.

Cholesterol synthesis

The synthetic rate of cholesterol was estimated by measuring labeled acetate incorporation into cholesterol as well as measuring microsomal HMG-CoA reductase activity. **Fig. 4** shows that the rate of acetate incorporation into cholesterol was significantly lower in CaCo-2 cells modified by eicosapentaenoic acid compared to the rate observed in cells grown in palmitic acid. Microsomal HMG-CoA reductase activities confirmed these results. Reductase activities were 60 ± 4 and 90 ± 5 pmol of mevalonate

TABLE 2. Lipid content in CaCo-2 cells

	Cholesterol		Triglyceride	Phospholipid
	Free	Ester		
	μg/mg		*μg/mg*	*nmol/mg*
Eicosapentaenoic acid	22.9 ± 0.7	nd[a]	232.6 ± 4.5[b]	1326 ± 70
Palmitic acid	22.9 ± 0.5	2.0 ± 0.1	151.1 ± 3.3	1070 ± 47

CaCo-2 cells were cultured as described in Table 1. Lipids were extracted and separated by thin-layer chromatography as described in Methods; n = 12 separate dishes.
[a]None detected.
[b]$P < 0.001$.

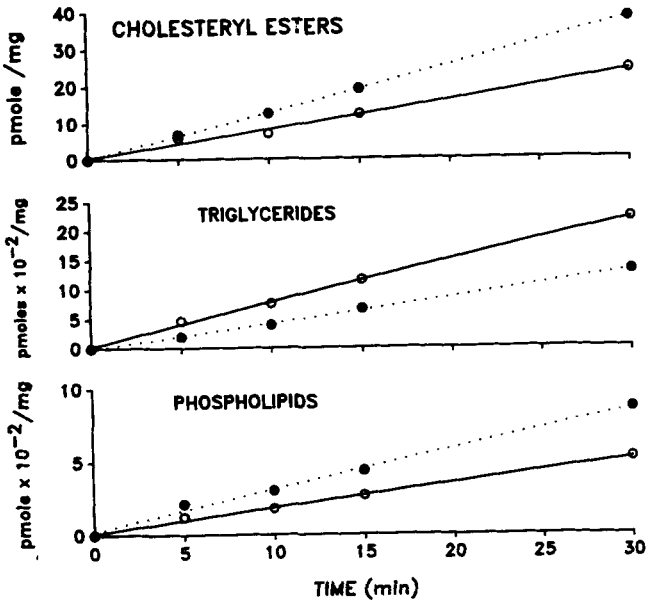

Fig. 1 [^{3}H]Oleate incorporation into cellular lipids in CaCo-2 cells. CaCo-2 cells modified with either eicosapentaenoic acid or palmitic acid were incubated with 50 μM labeled oleic acid for 30 min at 37°C in 95% air–5% CO_2. The cells were then washed thoroughly, scraped from the dish, and the lipids were extracted in chloroform–methanol 2:1 (v/v). The lipids were separated by thin-layer chromatography as described in Methods; (○) 20:5; (●) 16:0.

formed/mg per min in microsomes prepared from cells incubated with eicosapentaenoic and palmitic acids, respectively.

Incorporation of the fatty acids into cellular lipids

The effects of the two fatty acids on cholesterol metabolism in CaCo-2 cells may also be related to their intracellular distribution. If a specific fatty acid is incorporated preferentially into neutral lipids rather than polar lipids within a cell, membrane modifications may not be as extensive as in cells in which the fatty acid becomes incorporated predominantly into phospholipids. To investigate the incorporation of eicosapentaenoic and palmitic acids into cellular lipids, CaCo-2 cells were incubated with 100 μM of the labeled fatty acid for 4, 8, and 22 hr. Lipids were then extracted from the cells and separated by thin-layer chromatography. **Fig. 5** shows the distribution of the labeled fatty acids as a percentage of the total that was incorporated into cellular lipids at 22 hr. The data for the 4- and 8-hr time points showed similar differences between the two fatty acids. Eicosapentaenoic acid was preferentially incorporated into phospholipids as 70% of the label was found in this lipid class. The remaining activity was detected in triglycerides with smaller amounts in free fatty acids and cholesteryl esters. In contrast, the reverse was true for palmitic acid. Sixty percent of the palmitate label was incorporated into triglycerides with the remainder found in phospholipids and fatty acids predominantly.

Fig. 6 shows the incorporation of the two fatty acids into the individual phospholipids. Phosphatidylethanolamine and phosphatidylcholine contained approximately 90% of the fatty acid labels in both groups. Seventy percent of eicosapentaenoic acid was incorporated into phosphatidylethanolamine with 18% in phosphatidylcholine. In contrast, 30% of palmitic acid was incorporated into phosphatidylethanolamine whereas 54% was found in phosphatidylcholine. Smaller amounts of both labels were incorporated into phosphatidylserine, phosphatidylinositol, and other phospholipids not shown including lysophospholipids and sphingomyelin.

DISCUSSION

This is the first study that demonstrates the regulation of intracellular cholesterol metabolism by the modification of cell membrane fatty acid saturation in cultured intestinal cells. The implication, as suggested by previous dietary studies (8, 9), is that dietary fat saturation independently of dietary cholesterol can alter the rates of cholesterol synthesis and esterification in this organ. Dietary fat saturation has long been recognized as an important factor in modifying plasma cholesterol levels (19). Fatty acid modification of cell membranes in the intestine may play a role in this effect. Since intestinal mucosa is a major site of cholesterol synthesis, being second only to the liver (20), a decrease in the rate of cholesterol synthesis in this organ by fatty acid modification of its cellular membranes could significantly reduce the contribution of the small intestine to the total body cholesterol stores.

The rate-controlling enzymes for cholesterol synthesis and esterification in the intestine are HMG-CoA reductase and ACAT, respectively. From previous dietary studies, we have suggested that the activities of these two enzymes in the intestine are regulated by alterations in membrane fatty acid composition (8, 9). In CaCo-2 cells cultured in

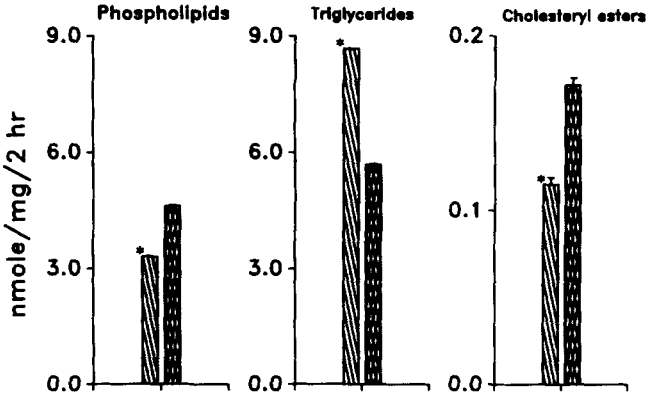

Fig. 2. [^{3}H]Oleate incorporation into cellular lipids in CaCo-2 cells; (▨) 20:5; (■) 16:0; *$P < 0.001$ vs. 16:0 cells.

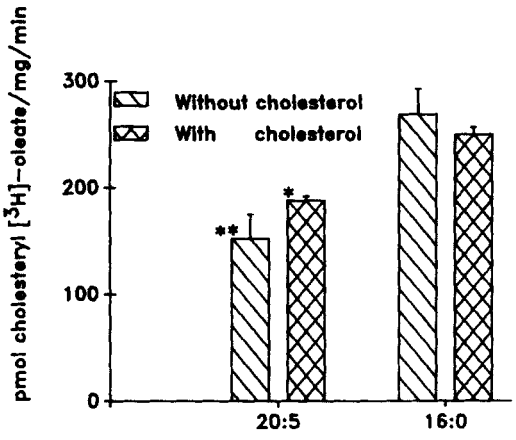

Fig. 3. Microsomal ACAT activity in CaCo-2 cells. Microsomes were prepared from the modified cells. ACAT activities were measured using labeled oleoyl-CoA as substrate in the presence and absence of added cholesterol (20 μg/assay) solubilized in Triton WR-1339 (600 μg/assay); $^{*}P < 0.001$ vs. 16:0 cells (with cholesterol); $^{**}P < 0.02$ vs. 16:0 cells (without cholesterol).

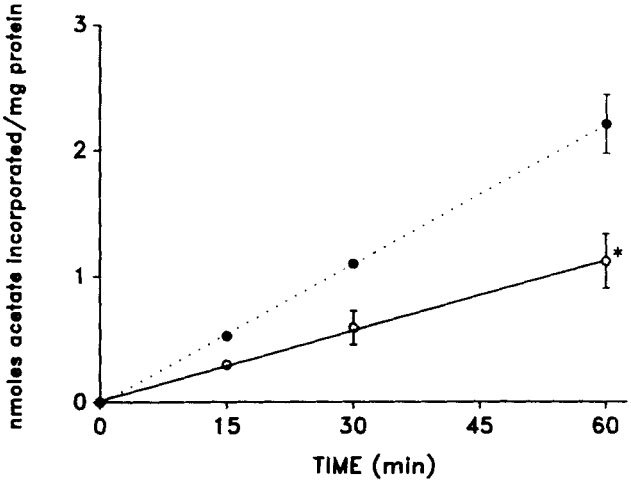

Fig. 4. Cholesterol synthesis in CaCo-2 cells. Modified cells were incubated with 5 mM of [^{14}C]acetate for 1 hr at 37°C in 95% air–5% CO_2. The cells were then washed thoroughly, scraped from the dish, and the unesterified cholesterol was isolated by thin-layer chromatography after lipid extraction as described in Methods; (○) 20:5; (●) 16:0; $^{*}P < 0.001$ vs. 16:0.

the presence of palmitic and eicosapentaenoic acids, the fatty acid composition of the cell membranes was significantly different in the two cell populations. Membrane cholesterol contents were similar. Cells grown in eicosapentaenoic acid accumulated significant amounts of this fatty acid and its elongation product 22:5 in their membranes. Apparently CaCo-2 cells do not have the capacity to desaturate eicosapentaenoic acid as 22:6 did not accumulate. The monoenoic fatty acids, 16:1 and 18:1, were also substantially lower in membranes prepared from cells modified with eicosapentaenoic acid. The membrane polyunsaturated to saturated fat ratio was substantially higher in this population of cells compared to CaCo-2 cells grown in the saturated fatty acid. Activities of the membrane-bound enzymes, HMG-CoA reductase and ACAT, were substantially reduced in the cells modified by eicosapentaenoic acid.

In two previous dietary studies that compared the effects of a diet supplemented with a fish oil to a diet supplemented with cocoa-butter oil on hepatic and intestinal ACAT activities, animals ingesting the fish oil had higher ACAT activities compared to the activities in animals ingesting the diet containing the more saturated oil (9, 21). These results are in contrast to the present study which demonstrates a decrease in ACAT activity in CaCo-2 cells modified by eicosapentaenoic acid. An earlier study, however, showed that ACAT activities were higher in mouse Ehrlich ascites tumor cells that were prepared from animals ingesting a diet enriched in saturated fats compared to ACAT activities in cells obtained from mice fed a diet supplemented with a polyunsaturated fat (22). It is difficult, therefore, to compare the results of dietary studies to results obtained by cell culture. By using cell culture, the many variables that can occur in dietary studies are rigidly controlled. In dietary studies, for example, the oils that are ingested by the animals are mixtures of fatty acids which by themselves could affect ACAT activity differently. Other factors such as the absorption of biliary cholesterol by the intestine, changes in lipoprotein cholesterol levels (23), the fed-state of the animals at the time of killing (24), and hormonal changes (25) have all been shown to regulate ACAT activity independently of the lipid ingested in the intact animal. It is clear from the present study that the enrichment of membranes with eicosapentaenoic acid results in a decrease in ACAT activity in CaCo-2 cells. Since this is in contrast to the increase in intestinal ACAT activity observed in animals ingesting a fish oil, it suggests that factors other than fatty acid saturation may play a role in the regulation of intestinal ACAT activity in vivo.

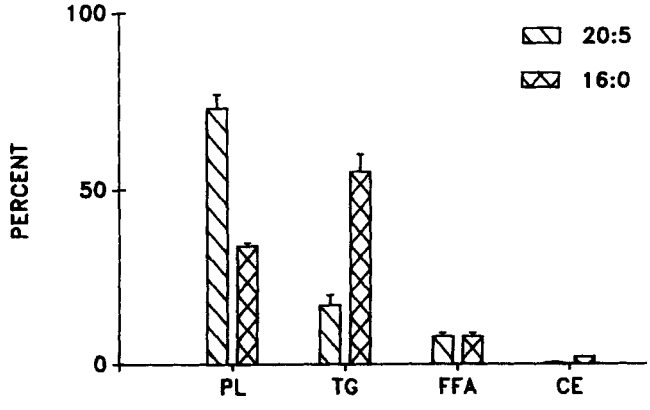

Fig. 5. Incorporation of 20:5 and 16:0 into cellular lipids of CaCo-2 cells. Unmodified cells were incubated with either 100 μM [^{3}H]eicosapentaenoic acid or [^{14}C]palmitic acid for 22 hr at 37°C in 95% air–5% CO_2. The cells were then washed thoroughly, scraped from the dish, and the lipids were extracted. The individual lipids were separated by thin-layer chromatography as described in Methods.

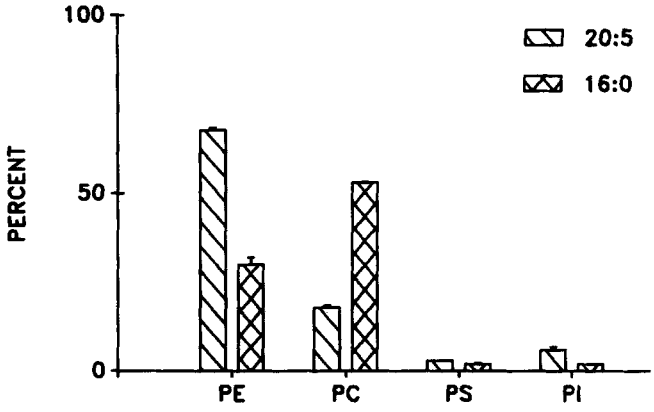

Fig. 6. Incorporation of 20:5 and 16:0 into individual phospholipids. The methodology was the same as that described for Fig. 6 except the phospholipids were separated by thin-layer chromatography using two different solvent systems described in Methods.

A major determinant of microsomal ACAT activity is the availability of membrane cholesterol as substrate (14). In the present study, however, the regulation of ACAT activity by membrane fatty acid modification was not related to the microsomal cholesterol content. Although the total microsomal cholesterol content is a gross estimate of the amount of cholesterol available to ACAT, it was similar in microsomes obtained from cells from both groups. Moreover, despite the addition of exogenous cholesterol to the microsomes ensuring saturation of the enzyme (26), ACAT activity remained significantly lower in microsomes prepared from CaCo-2 cells modified by eicosapentaenoic acid. Since ACAT activities were not significantly altered after the addition of exogenous cholesterol, it suggests that the enzyme was already saturated in these microsomal preparations. There was a lesser amount of intracellular cholesteryl esters in cells grown in eicosapentaenoic acid compared to the amount observed in cells modified by palmitic acid, which further supports a lower ACAT activity in these cells.

The activity of HMG-CoA reductase and the incorporation of labeled acetate into cholesterol were also lower in CaCo-2 cells modified by eicosapentaenoic acid. It is interesting that the changes in activities of both HMG-CoA reductase and ACAT were in the same direction in these cells. In dietary studies in which both enzymes have been studied (9) or in other cell culture systems where the cells are actively processing cholesterol (17, 18), the regulation of these two enzymes is usually reciprocal. This makes good sense. If the intracellular unesterified cholesterol compartment becomes expanded, the cell responds appropriately by down-regulating HMG-CoA reductase activity and up-regulating ACAT activity. Under the conditions of the present experiment, in the absence of cholesterol and therefore the need to metabolize this sterol, the cell is responding only to the modifications produced by the single fatty acid. As already mentioned, the effect of the fatty acids appears to be independent of cholesterol, and the observation that HMG-CoA reductase and ACAT activities were concurrently decreased in cells incubated with eicosapentaenoic acid supports this. It is more likely that both enzymes are sensitive to the structural physical properties of the lipids in the membrane which surrounds them. Modification of this lipid "microenvironment" resulting in the alteration of the active sites of the enzymes could lead to the regulation of activities observed in this study.

The evidence that the activities of HMG-CoA reductase and ACAT are regulated by changes in membrane fatty acid saturation is one of correlation only. It is difficult, if not impossible, to prove. When cells are exposed to high concentrations of fatty acids, triglycerides accumulate in the form of intracytoplasmic lipid droplets (27). These lipid droplets are in a cellular compartment so separate from membrane-bound enzymes that they should not affect the activities of these enzymes. However, in CaCo-2 cells as in HepG2 and hepatocytes, the triglycerides are being mobilized for secretion in lipoprotein particles (28). Lipoprotein synthesis and secretion in hepatocytes exposed to fatty acids have been shown to regulate the rate of intracellular cholesterol synthesis (29). If this is occurring in CaCo-2 cells that are cultured in the presence of these two fatty acids, then it is likely that this may also affect ACAT and HMG-CoA reductase activities. This is presently being pursued.

Another factor that may play a role in the regulation of these enzymes is the difference observed between the incorporation of palmitic acid and of eicosapentaenoic acid into the CaCo-2 cellular lipids. Eicosapentaenoic acid had a greater propensity for being incorporated into phospholipids compared to palmitic acid. This could certainly result in more significant membrane changes in those cells exposed to this fatty acid compared to cells modified by palmitic acid in which the greatest percentage of the fatty acid was incorporated into triglycerides. Even the distribution into the different phospholipid classes was not alike. The majority of eicosapentaenoic acid was found in phosphatidylethanolamine, whereas palmitic acid was predominantly incorporated into phosphatidylcholine. These differences in cellular distribution between the two fatty acids, independently of whether they are saturated or unsaturated, could play a role in the regulation of the two enzymes.

Lastly, cell proliferation and growth have been shown to be adversely affected by modification with unsaturated fatty acids (30). In the present study, however, cell viability, cell number, and total protein on the day of the experiment were similar in the two cellular groups. Differences in the rates of cholesterol synthesis and esterification between the two cell populations, therefore, were not related to adverse effects of the two fatty acids on the cells.

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