# Influence of eicosapentaenoic acid (20:5, n-3) on secretion of lipoproteins in CaCo-2 cells

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**Abstract** CaCo-2 cells, grown on filter membranes, were used to study the effects of fatty acids on cellular metabolism of triacylglycerol and phospholipids. The rate of triacylglycerol secretion was enhanced more than 2-fold, from 1 to 2 weeks after reaching confluency, in the presence of 0.6 mM fatty acids. Triacylglycerol secretion and oxidation of oleic acid increased 2- and 9-fold, respectively, with this culture system, as compared to cells grown on conventional plastic dishes. Eicosapentaenoic acid (20:5 n-3), when compared to oleic acid, did not reduce formation of triacylglycerol or enhance phospholipid synthesis in CaCo-2 cells during short term (<24 h) experiments, when the cells resided on membranes, regardless of what type of radioisotopes were used as precursors in the incubation media. However, the n-3 fatty acid was preferentially incorporated into phosphatidylinositol, lysophosphatidylcholine, and sphingomyelin, as compared to oleic acid. The disappearance from the apical medium and cellular uptake of labeled eicosapentaenoic and oleic acid were similar during incubations up to 24 h, and the metabolism of these fatty acids to acid-soluble materials and $CO_2$ was equal. Light scattering analysis indicated that secreted lipoproteins of density <1.006 g/ml were in the same size-range as chylomicrons derived from human plasma. Assessment of secreted apolipoprotein B showed that by incubating CaCo-2 cells with oleic acid, apolipoprotein B levels increased approximately 1.4-fold when compared to cells incubated with eicosapentaenoic acid, whereas the amount of triacylglycerol and size-range of particles were similar for the two fatty acids. Our data indicate that CaCo-2 cells grown on filter membranes exhibit enterocyte-like characteristics with the ability to synthesize and secrete chylomicrons. Eicosapentaenoic acid and oleic acid are absorbed, metabolized, and influence secretion of lipoprotein particles in a similar way, except for some differences in incorporation of the fatty acids into certain phospholipid classes and a reduced secretion of apolipoprotein B. The culture conditions, including time after confluency and cellular support, are critical for the rate of secretion in the presence of eicosapentaenoic acid and oleic acid.—**Ranheim, T., A. Gedde-Dahl, A. C. Rustan, and C. A. Drevon.** Influence of eicosapentaenoic acid (20:5, n-3) on secretion of lipoproteins in CaCo-2 cells. *J. Lipid Res.* 1992. **33:** 1281-1293.

**Supplementary key words** triacylglycerol • phospholipids • intestine • fatty acids • oleic acid

The significance of total plasma cholesterol and LDL-cholesterol as risk factors for development of atherosclerosis and ischemic heart disease is well established, whereas the role of triacylglycerol as an independent risk factor is less clear (1). Fish oils rich in very long-chain n-3 fatty acids induce a wide range of biochemical changes in mammals, of which the plasma lipid-lowering effect is one of the most pronounced (2). From observations in Greenland Eskimos, with their low incidence of death from ischemic heart disease and high intake of seafood, a renewed interest in polyunsaturated fatty acids (PUFAs) has thrived (3).

The beneficial effect of n-3 fatty acids on serum lipids and lipoprotein levels observed in both normolipidemic and hyperlipidemic subjects fed diets supplemented with fish oil has been attributed to decreased synthesis and secretion of hepatic triacylglycerol or apolipoprotein B in very low density lipoprotein (VLDL) (4-9). Fish oil may also attenuate the increase in serum lipids observed after meals rich in cholesterol, probably by inhibiting VLDL synthesis (10, 11). Reduced esterification of fatty acids to cholesterol and diacylglycerol (7, 12), increased fatty acid oxidation (10, 13, 14), increased incorporation into phospholipids (15), and decreased availability of free fatty acids (16) have been suggested as reasons for decreased synthesis and secretion of VLDL from the liver. The fish oil-induced reduction in postprandial lipemia after long term ingestion may reflect a more rapid removal of chylomicrons, because less VLDL from the liver is present to compete for hydrolysis of triacylglycerol by lipoprotein lipase (2, 5, 17). Another possibility is that very long-chain n-3 fatty acids cause a reduced formation

Abbreviations: BSA, bovine serum albumin; EPA, eicosapentaenoic acid; OA, oleic acid; CM, chylomicron; VLDL, very low density lipoprotein; TG, triacylglycerol; FFA, free fatty acids; CE, cholesteryl ester; PL, phospholipid; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine; LPC, lysophosphatidylcholine; S, sphingomyelin; LDL, low density lipoprotein; PUFA, polyunsaturated fatty acid; FCS, fetal calf serum; TLC, thin-layer chromatography.

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of chylomicrons (18). Thus, the intestine would contribute to the reduced plasma concentration of triacylglycerol in the nonfasting state. Fatty acid-induced changes in composition of chylomicrons may be due to decreased triacylglycerol content. This could affect the size of these lipoprotein particles.

Previous studies concerning the effect of n-3 fatty acids on lipid metabolism refer mostly to experiments with humans, intact animals, perfused liver, and rat hepatocytes. However, we have little knowledge in which way n-3 fatty acids are metabolized in the intestine.

There has emerged in recent years a cell line that originates from human colon cancer cells, CaCo-2, established by Fogh, Wright, and Loveless (19). These cells undergo enterocytic differentiation in culture, developing a number of small intestinal functions, described by Pinto et al. (20). The differentiation is a growth-related process starting after confluency, expressing apical brush-border hydrolase activity (21, 22), transport of ions (23, 24), tight junctions, and maintenance of polarity when plated on filter membranes or plastic surfaces (21, 25). Of relevance to our study is that CaCo-2 cells cultured on plastic supports synthesize lipoproteins (26), and that nascent lipoproteins are preferentially secreted from the basolateral side of cells grown to confluence on filters (27, 28).

In the present study, we have used the CaCo-2 cell line cultured on filter membranes as an in vitro model for intestinal lipoprotein metabolism to study regulation of triacylglycerol and phospholipid metabolism in response to eicosapentaenoic acid and oleic acid.

## EXPERIMENTAL PROCEDURES

### Chemicals

[1,2,3-$^{3}$H]glycerol (200 Ci/mol), [methyl-$^{14}$C]bovine serum albumin (1863 Ci/mol), [1-$^{14}$C]eicosapentaenoic acid (52 Ci/mol), [1-$^{14}$C]oleic acid (58 Ci/mol), and [$^{3}$H]water (18 Ci/mol) were obtained from Du Pont, NEN Products, Boston, MA. Essentially fatty acid-free bovine serum albumin (BSA), oleic acid, and $\beta$-hydroxybutyrate dehydrogenase (product no. H-5132) were purchased from Sigma Chemical Co., St. Louis, MO. Eicosapentaenoic acid (95% pure) was generously provided by Norsk Hydro, Porsgrunn, Norway. TLC plates (Silica gel F 1500) were purchased from Schleicher and Shuell, Dassel, Germany. Fatty acids (0.6 mM, sodium salts) were added to albumin (0.24 mM) and dissolved in serum-free cell medium (see below). The ratio of fatty acids to albumin was 2.5:1.

### Cell culture

Monolayer cultures of CaCo-2 cells were obtained from American Type Culture Collection, Rockville, MD. At passage no 17 they were grown in 75-cm$^2$ plastic flasks (Costar, Cambridge, MA) at 37°C in air and 5% $CO_2$ in Dulbecco's Modified Eagle's medium (DMEM) (4.5 g/l glucose and 3.7 g/l sodium bicarbonate) (Flow Laboratories, Irvine, Ayrshire, UK). The medium was supplemented with 20% fetal calf serum (FCS), (Gibco, Paisley, UK), L-glutamine (2 mM), insulin (10 $\mu$g/ml) (Sigma), penicillin (100 IU/ml), streptomycin (100 $\mu$g/ml), and 1% nonessential amino acids, from Flow Laboratories. Grown under these conditions the doubling time of the CaCo-2 cells was about 70 h. The culture medium was changed every other day, and the day before an experiment.

For subculture the medium was removed, and the cells were detached from the culture flasks with 0.25% trypsin (Difco Laboratories, Detroit, MI) in a $Ca^{2+}$-, $Mg^{2+}$-free phosphate-buffered saline containing 0.2 g/l EDTA. Culture medium with FCS was added to stop trypsinization. Cells were suspended and seeded at approximately $4 \times 10^4$ cells/cm$^2$ in new flasks according to the method of Mohrmann et al. (22).

Cells were grown to confluency in 25-cm$^2$ plastic flasks (Costar) in 2.0 ml complete DME medium, or in 1.5 ml of complete DME medium containing approximately $10^6$ cells plated on the apical side of presoaked membrane filters of 3.0 $\mu$m pore size and 24.5 mm diameter (Transwell-COL, Costar). We used this large pore size of filter membranes to allow all the secreted chylomicron particles to diffuse freely through the membrane (see Table 1). The lower well contained 2.6 ml of complete DME medium. Monolayers were harvested 2 weeks after reaching confluency. Cell viability, as evaluated by trypan blue exclusion test, was never less than 90%. The cells were mycoplasma negative as determined with Hoechst 33258 (29).

### Electrical resistance of filter-grown cells

Measurements of electrical resistance across the filter membrane (Millicell-ERS, Millipore Corp., Bedford, MA) were performed routinely before any experiment, to make sure that the cell monolayers were confluent and resistant to ionic diffusion and to penetration of large molecules. The background resistance of the filter was approximately 35 ohm/cm$^2$. When electrical resistance of >100 ohm/cm$^2$ (background resistance subtracted) was obtained, the cells formed a tight monolayer. At this level of electrical resistance, the monolayers prevented diffusion of [methyl-$^{14}$C]bovine serum albumin. After 24 h at 37°C less than 5% of the radiolabeled albumin was found on the basolateral side of the cells.

### Morphology

Cells plated on membrane filters and cultured for 2 weeks after confluency were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.4. After fixation the cells

were postfixed in 1% osmium tetroxide in cacodylate buffer, dehydrated in graded ethanols, embedded in epoxy resin and thin-sectioned by an LKB Ultramicrotome. Samples were examined by a Jeol 100CX transmission electron microscope.

Cell cultures prepared for scanning electron microscopy were fixed, postfixed, and dehydrated as in transmission electron microscopy, dried in critical point drier (Balzers Union), stained with gold/palladium (40/60) using a sputter coater (Polaron E5000), and examined by a Jeol 35C electron microscope.

### Measurement of cell-associated and secreted lipids

The culture medium was removed and the remaining cells were rinsed twice by serum-free DME medium; unattached and damaged cells were thereby washed off. The cells were incubated in serum-free DME medium containing HEPES (10 mM), different albumin-bound fatty acids (concentrations and incubation times are indicated in legends to tables and figures), [1,2,3-$^{3}$H]glycerol (13 $\mu$Ci/ml), [1-$^{14}$C]eicosapentaenoic acid (1 $\mu$Ci/ml), [1-$^{14}$C]oleic acid (1 $\mu$Ci/ml), or [$^{3}$H]water (4 mCi/ml). The counting efficiencies for $^{3}$H and $^{14}$C were 65% and 92%, respectively, calculated by means of external standards. For cells growing on microporous filter membranes, the radioisotope was added to the upper chamber in the cell culture system. The cells were scraped off the filter membranes into phosphate-buffered saline. The medium was collected from the inside and outside of the tissue culture inserts and treated further as explained below. Samples were taken for protein determination and measured, using BSA as a reference protein for the assay (30).

### Lipid extraction and thin-layer chromatography

Lipids from cells and media were extracted with chloroform–methanol 2:1 (v/v) (31). The homogenized cell fraction was mixed with 20 vol of chloroform–methanol 2:1 (v/v). Four volumes of a 0.9% phosphate-buffered saline of pH 2 was added and the mixture was allowed to separate into two phases. The organic phase was dried under a stream of nitrogen at 40°C. Medium devoid of cellular debris was added to 4 volumes of chloroform–methanol 2:1 (v/v) and 2% serum as unlabeled carrier for the lipids. The water phase of the medium extract was reextracted once with 4 vol chloroform–methanol 2:1 (v/v), and the combined organic phases were further treated in the same way as for the cells.

The residual lipid extract was redissolved in 200 $\mu$l hexane and separated by thin-layer chromatography (TLC) using hexane–diethyl ether–acetic acid 80:20:1 (v/v/v) as developing solvent. In other experiments, where it was important to separate diacylglycerol and unesterified cholesterol, hexane–diethyl ether–acetic acid 65:35:1 (v/v/v) followed by hexane as a second developing solvent was used. Phospholipids were separated according to Parker and Peterson (32), using chloroform–methanol–acetic acid–water 100:60:16:8 (v/v/v/v).

The various lipids were finally identified by iodine or 2',7'-dichlorofluorescein (Sigma), and the TLC foils were cut into 8 ml of liquid scintillation fluid and counted in a scintillation spectrometer.

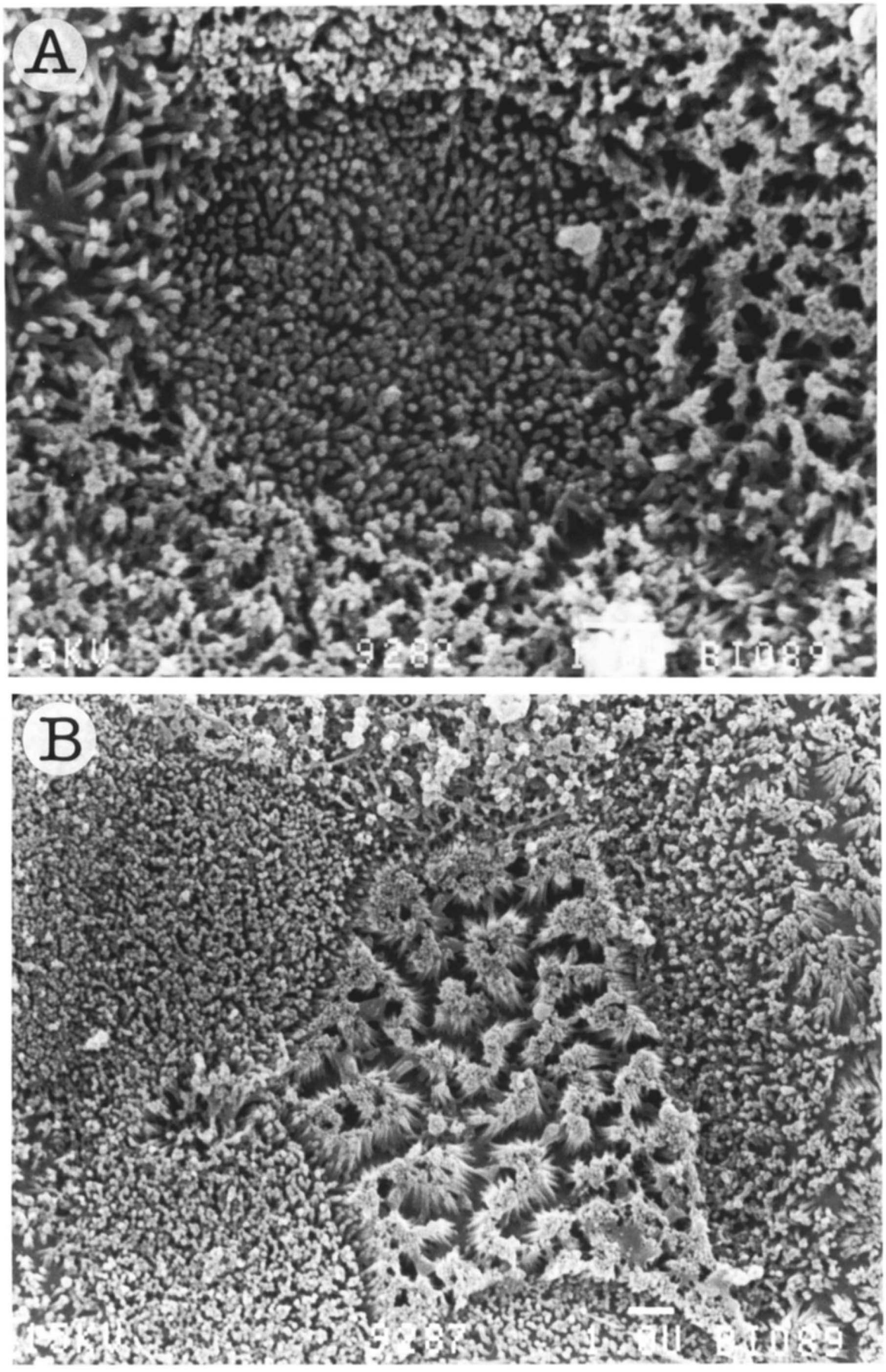
### Uptake and degradation of fatty acids

Cellular uptake and formation of acid-soluble products of [1-$^{14}$C]eicosapentaenoic acid (1 $\mu$Ci/ml) or [1-$^{14}$C]oleic acid (1 $\mu$Ci/ml) were determined. Medium (0.5 ml) and 0.25 ml of cell homogenate were treated with equal volumes of $HClO_4$ (1 M). The mixture was centrifuged at 3500 rpm for 10 min and samples from the supernatants were counted. The precipitates were resuspended, washed with 2 ml 1 M $HClO_4$ and resolubilized in 1.0 ml of saline containing sodium dodecyl sulfate (SDS) (70 mM) and Triton X-100 (10%). More than 98% of intact fatty acids added were precipitated with this method. A set of no-cell controls was analyzed together with the experiment samples.

### Fatty acid oxidation

Measurements of oxidation of fatty acids to acid-soluble products and $CO_2$ were performed as described by Christiansen, Borrebaek, and Bremer (33). Acid-soluble products were mostly ketone bodies, Krebs cycle intermediates, and acetylcarnitine.

Medium containing 1.5 ml serum-free DME medium, HEPES (20 mM), either [1-$^{14}$C]eicosapentaenoic acid (1 $\mu$Ci/ml, 0.6 mM) or [1-$^{14}$C]oleic acid (1 $\mu$Ci/ml, 0.6 mM), L-carnitine hydrochloride (0.5 mM), and gentamicin (50 $\mu$g/ml), was added to the apical side of CaCo-2 cells and incubated for 24 h. The basolateral membrane surface was exposed to 4 ml of the incubation media without fatty acids. A special tight plexiglass bell was developed to measure formation of $CO_2$ released from CaCo-2 cells cultured on filters. The bells had a center well (Kontes, Vineland, NJ) containing 300 $\mu$l phenylethylamine–methanol 1:1 (v/v) and a folded filter paper, and were closed with stopper tops (Kontes). Four hundred $\mu$l perchloric acid (2.8 M) was added to the cell monolayer in the apical well and then incubated at room temperature for 60 min to trap all $^{14}CO_2$. At the end of the incubation the well was cut off and counted (liquid scintillation spectrometry). The acid-soluble radioactivity was measured in the $HClO_4$ extract. The mixture was centrifuged at 3500 rpm for 10 min and samples of 1.0 ml of the supernatants were counted. The supernatant excess was neutralized with potassium phosphate buffer (0.5 M, pH 6.5), and ketone bodies ($\beta$-hydroxybutyrate and acetoacetate) were measured according to Williamson and Mellanby (34).



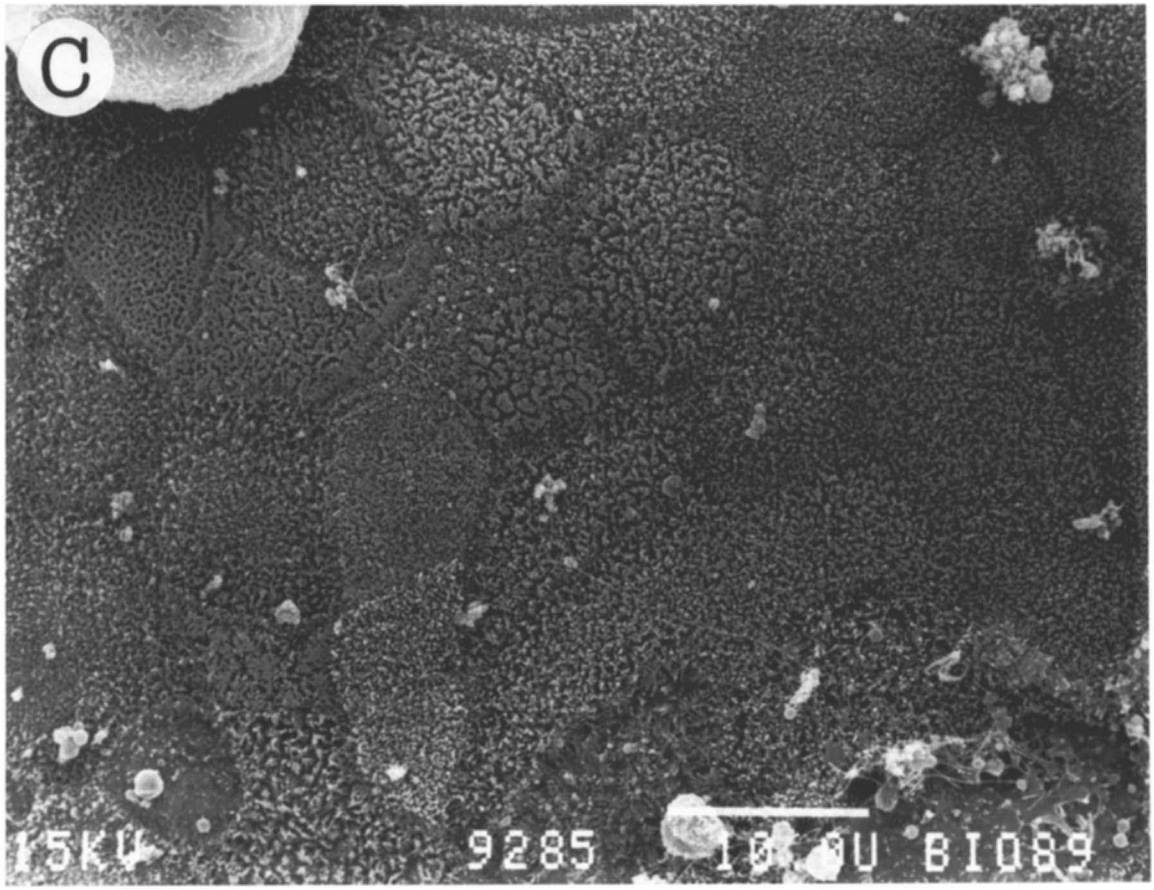



**Fig. 1.** Scanning electron micrographs of monolayer CaCo-2 cells. The cells were plated on collagen-treated polycarbonate membranes and cultured for 2 weeks after confluency. Two different patterns of brush border organization are seen. A: "thick carpet" pattern (bar, 1 $\mu$m), B: "flower-like" pattern (bar, 1 $\mu$m), C: combination of the two patterns (bar, 10 $\mu$m).

### Lipoprotein isolation, characterization and assessment of apolipoprotein B

CaCo-2 cells were incubated in DME medium with different albumin-bound fatty acids. At given times the medium was collected, and the triacylglycerol-rich lipoproteins of density less than 1.006 g/ml were isolated by ultracentrifugation in a Sorvall TFT 45.6 fixed angle rotor at 38.000 rpm for 20 h (35). The top containing the chylomicron fraction was collected by tube-slicing and further examined by analysis of lipid composition, apolipoprotein B, and particle size. When CaCo-2 cells were incubated with [$^3$H]glycerol, we observed that approximately 97% of the secreted radioactive triacylglycerol was recovered in the basolateral fraction of d $<$1.006 g/ml. The secretory rate of triacyl[$^3$H]glycerol was therefore used as an estimate of chylomicron secretion. A radioimmunoassay (Pharmacia Diagnostics AB, Uppsala, Sweden) was used for apoB analysis. Dynamic light scattering or photon correlation spectroscopy (36) and single-particle dynamics by resistive pulse technique (37) were used to measure particle size of lipoproteins (d $<$1.006 g/ml) obtained from cells incubated with different fatty acids.

### Statistical analysis

All values are reported as the mean $\pm$ SD of at least triplicate samples representative of not less than three separate experiments, if not otherwise indicated. Comparison of different treatments was evaluated by the Mann-Whitney non-parametric test (two-tailed).

## RESULTS

### Morphology of cultured CaCo-2 cells

Our cell line developed patterns of mature enterocytes as reported by Pinto et al. (20). During proliferation, the cells grew in semi-spheroid clusters as observed by phase contrast microscopy. Dome formations (hemicysts), randomly distributed, appeared when confluency was achieved and were probably due to fluid and electrolyte accumulation between the culture membrane and the cell monolayer (23).

Confluent CaCo-2 cells appeared as a monolayer on filter membranes, and scanning electron microscopy revealed two morphologically different patterns of brush border microvilli (**Fig. 1**). Antibodies against proteins in

microvilli membranes give the same two distribution patterns, and this is probably a growth-related phenomenon (20, 21).

By transmission electron microscopy (**Fig. 2**) it was observed that the cells formed a polarized monolayer with a morphological asymmetry. The apical surface covered with microvilli extended upward into the medium, and the basolateral surface interdigitated with the membrane filter (not shown on this micrograph). In confluent cultures intercellular junctional complexes composed of desmosomes and tight junctions appeared (Fig. 2).

### Effect of time post-confluency and growth support of CaCo-2 cells, on secretion of triacyl[$^3$H]glycerol

As cell differentiation progressed on filter membranes, the rate of secretion of labeled triacylglycerol into the medium increased approximately twofold from 1 to 2 weeks after confluency (**Fig. 3A**). At that time, the rate of secretion had reached a maximum, and decreased after 3 weeks. Secretion of triacylglycerol in cells cultured on plastic increased fivefold from 1 to 3 weeks post-confluency (Fig. 3B). Incorporation of [$^3$H]glycerol into triacylglycerol secreted to basolateral media was similar when eicosapentaenoic acid or oleic acid was added to the apical well (Fig. 3A).

When CaCo-2 cells were grown on filter membranes with pore-size of 3 $\mu$m, secretion of triacylglycerol and rate of $\beta$-oxidation increased two- and ninefold, respectively (**Fig. 4**), suggesting that the cells grown on filters are more metabolically active than cells grown on plastic. Further experiments were carried out on filters at 2 weeks after reaching confluency.

### Time course for the effect of fatty acids on synthesis and secretion of labeled glycerolipids

Synthesis and secretion of glycerol-labeled triacylglycerol and phospholipid indicated that there was no reduction at any time point in the presence of eicosapentaenoic acid, as compared to oleic acid (**Fig. 5**). After 2 h incubation the rate of triacylglycerol synthesis decreased, whereas the secretory rate was linear from 2 to 12 h. Both fatty acids showed a slight delay for secretion

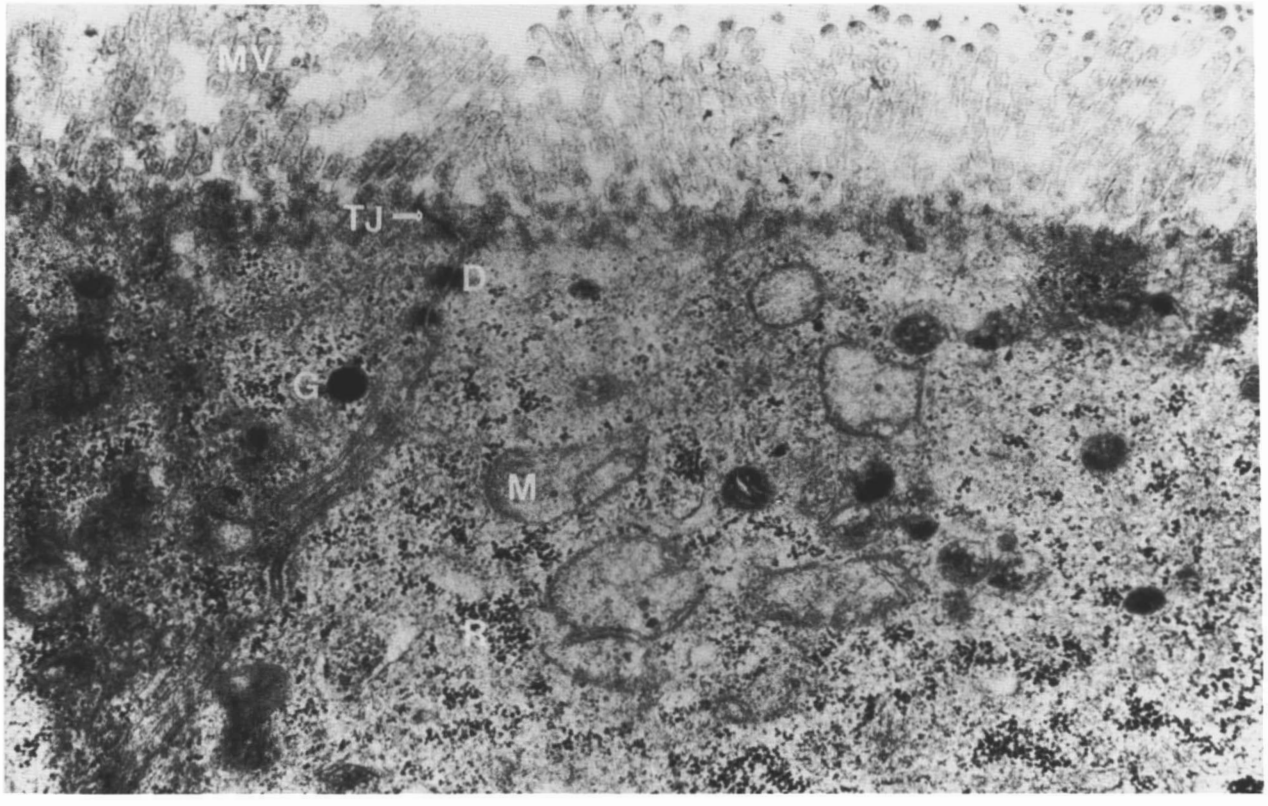



**Fig. 2.** Transmission electron micrograph of monolayer CaCo-2 cells. The cells were plated on filter membranes and cultured for 2 weeks after confluency. Brush border microvilli (MV) on the apical surface of the cells, tight junctions (TJ) between adjacent cells, intercellular desmosomes (D), glycogen (G), two nuclei (N), numerous small polyribosomes (R), and mitochondria (M) with closely packed cristae (× 10000).

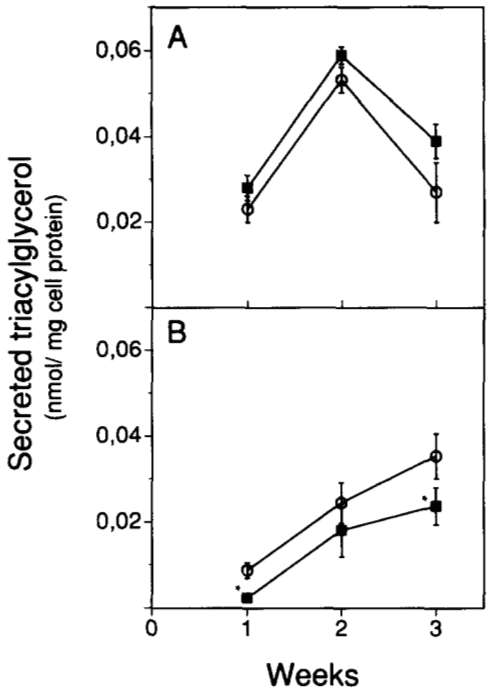



**Fig. 3.** Secreted triacyl[$^3$H]glycerol from 1- to 3-weeks postconfluent CaCo-2 cells in the presence of different fatty acids. Cells grown on filter membranes (panel A) and plastic supports (panel B) were incubated in complete DME medium for 1, 2, and 3 weeks after reaching confluency. The medium was replaced with serum-free DME medium containing [1,2,3-$^3$H]glycerol (13 $\mu$Ci/ml, sp act 21.7 Ci/mol) and 0.6 mM of either eicosapentaenoic acid (■) or oleic acid (○) for 5 h. Data represent means ± SD from three cultures; *represents significant difference between eicosapentaenoic acid and oleic acid at $P < 0.03$.

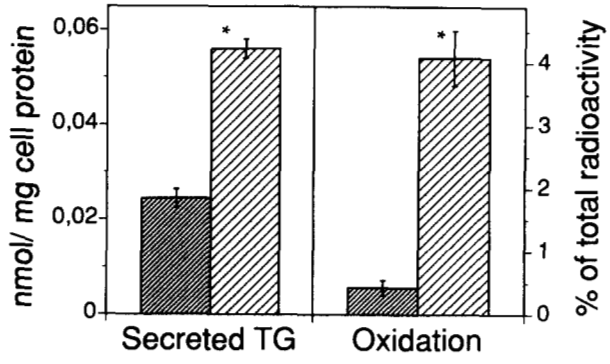



**Fig. 4.** Triacylglycerol secretion and fatty acid oxidation in CaCo-2 cells cultured on plastic surfaces versus filter membranes. CaCo-2 monolayers cultured for 2 weeks after confluency either on plastic supports (Petri dishes, 60 mm or 25-cm$^2$ flasks for oxidation) (dark hatched bars) or on filter membranes (light hatched bars) were incubated with oleic acid (0.6 mM) in serum-free DME medium, containing [1,2,3-$^3$H]glycerol (13 $\mu$Ci/ml, sp act 21.7 Ci/mol) for 5 h to measure secretion of labeled triacylglycerol into the media. To obtain fatty acid-soluble products and $CO_2$, the cells (culturing conditions as described above) were incubated with [1-$^{14}$C]oleic acid (1 $\mu$Ci/ml, sp act 1.7 Ci/mol, 0.6 mM) for 24 h. Data represent means ± SD of six cultures given as nmol/mg cell protein (left panel) and % of added radioactivity (right panel); *represents significant difference between plastic supports and filter membranes at $P < 0.01$.

of lipoproteins during the first hours of incubation. The rate of synthesis and secretion increased during the whole incubation period, and indicated no definite plateau.

Most of the secreted lipids were recovered on the basolateral side of the filter membrane (Fig. 5, insert). Approximately 10% of secreted triacyl[$^3$H]glycerol was found in the apical medium after 12 h incubation, suggesting that the cell monolayer was capable of secreting lipoproteins in a polarized fashion. This also indicates that the monolayer was not leaky to macromolecules.

The molar ratio between fatty acid and albumin (2.5:1) was kept constant in all incubations. For synthesis as well as secretion of labeled triacylglycerol, saturation was achieved at a concentration of 0.6 mM of both fatty acids (data not shown). Thus, this concentration was chosen for further experiments.

### Time course of uptake and metabolism of radioactive fatty acids

Either [1-$^{14}$C]eicosapentaenoic acid or [1-$^{14}$C]oleic acid was added to CaCo-2 cells in the apical chamber. The disappearance of labeled fatty acids from the media and the cellular uptake were similar during the incubation period

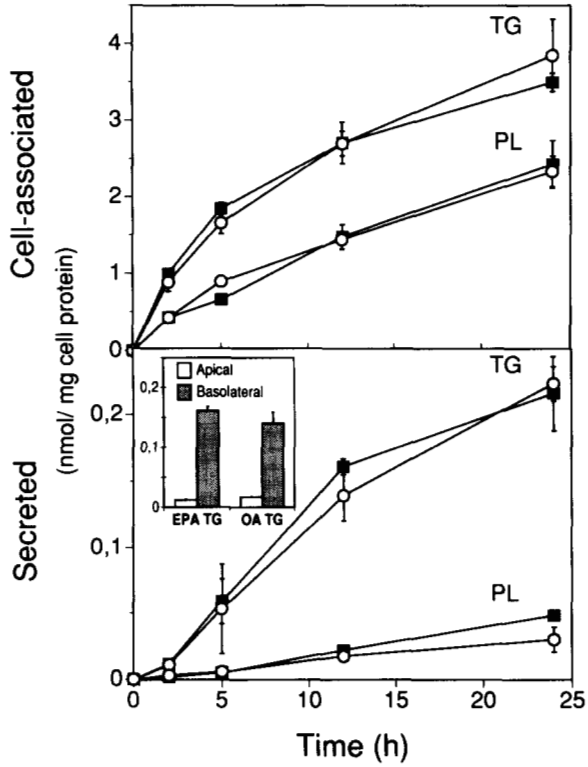



**Fig. 5.** Effect of eicosapentaenoic acid and oleic acid on synthesis and secretion of triacyl[$^3$H]glycerol (TG) and [$^3$H]phospholipid (PL) in CaCo-2 cells. The cells plated on filter membranes and cultured for 2 weeks after confluency were incubated with eicosapentaenoic acid (0.6 mM) (■) or oleic acid (0.6 mM) (○) in serum-free DME medium containing [$^3$H]glycerol (13 $\mu$Ci/ml, sp act 21.7 Ci/mol). Insert: Secretion of triacyl[$^3$H]glycerol into the apical versus the basolateral media after 12 h of incubation. Data represent means ± SD for six cultures.

(**Fig. 6**, Apical medium and Cells). During the initial 2 h approximately 20% of the fatty acids had entered the cells. The cells contained hardly any acid-soluble degradation products (<1% of total radioactivity added). Acid-precipitable products in the basolateral medium reflect the amount of radioactive fatty acids secreted from the CaCo-2 cells (Fig. 6, Basolateral medium). The content of acid-precipitable or lipid-extractable products in basolateral media was similar in cells incubated with eicosapentaenoic acid or oleic acid (**Fig. 7**, lower panel). During incubation the labeled fatty acids stuck to the filter membrane and plastic wells, and full recovery was therefore not achieved. Approximately 4% of the added labeled fatty acids was lost after an incubation period of 30 min.

In the cells and media there were similar appearances

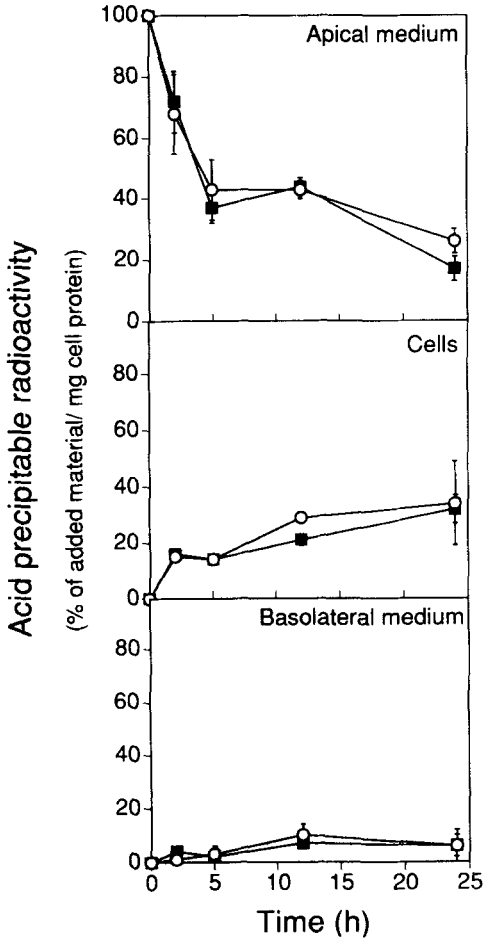



**Fig. 6.** Acid-precipitable products in CaCo-2 cells incubated with [1-$^{14}$C]eicosapentaenoic acid and [1-$^{14}$C]oleic acid. CaCo-2 cells plated on filter membranes and cultured for 2 weeks after confluency were incubated for 24 h in serum-free DME medium containing HEPES (10 mM), eicosapentaenoic acid (1 $\mu$Ci/ml, sp act 1.7 Ci/mol, 0.6 mM) (■) or oleic acid (1 $\mu$Ci/ml, sp act 1.7 Ci/mol, 0.6 mM) (○). The results are expressed as % of added acid-precipitable radioactivity/mg cell protein. Data represent means ± SD of six to nine cultures.

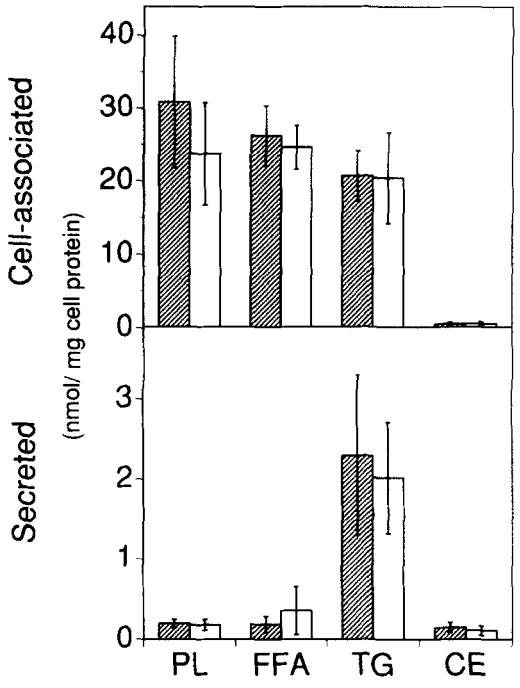



**Fig. 7.** Incorporation of [1-$^{14}$C]eicosapentaenoic acid and [1-$^{14}$C]oleic acid into cell-associated and secreted lipids. CaCo-2 cells plated on filter membranes and cultured for 2 weeks after confluency were incubated with eicosapentaenoic acid (hatched bars) or oleic acid (open bars) (1 $\mu$Ci/ml, sp act 1.7 Ci/mol, 0.6 mM) for 5 h. Media from the basolateral chambers and the cells were collected and treated as described in Experimental Procedures. Data represent means ± SD from eight to twelve cultures.

of radiolabeled eicosapentaenoic acid and oleic acid in phospholipids, triacylglycerol, and cholesteryl ester after 5 h incubation (Fig. 7). At this time the incorporation of labeled fatty acids into cellular lipids probably reflects the fatty acid distribution within the cells, since the labeled fatty acids have not reached equilibrium, and thus the fatty acids have not been reesterified.

### Oxidation of labeled eicosapentaenoic acid and oleic acid to acid soluble products and $CO_2$

When incubating CaCo-2 monolayers for 24 h with [1-$^{14}$C]eicosapentaenoic acid or [1-$^{14}$C]oleic acid, a similar amount of radioactivity was released as $CO_2$ and acid-soluble products into the medium, although less than 5% of the lipids were oxidized (**Fig. 8**). Ketone body formation may be used as a measurement of mitochondrial $\beta$-oxidation rate, and both acetoacetate and $\beta$-hydroxybutyrate were measured enzymatically. It was demonstrated that acetoacetate formation was similar in cells incubated with eicosapentaenoic acid or oleic acid, 0.4 ± 0.08 and 0.3 ± 0.1 nmol/h per mg cell protein, respectively (no-cell filters incubated with identical media were subtracted). No $\beta$-hydroxybutyrate could be detected in the media of CaCo-2 cells.

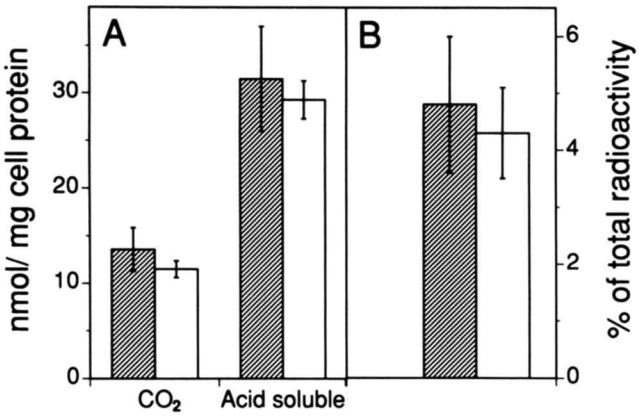



**Fig. 8.** Oxidation of eicosapentaenoic acid and oleic acid in CaCo-2 cells. The cells plated on filter membranes and cultured for 2 weeks after confluency were incubated in serum-free DME medium containing HEPES (20 mM), L-carnitine hydrochloride (0.5 mM), gentamicin (50 μg/ml), [1-$^{14}$C]eicosapentaenoic acid (hatched bars) or [1-$^{14}$C]oleic acid (open bars) (1 μCi/ml, sp act 1.7 Ci/mol, 0.6 mM) for 24 h. Panel A: $CO_2$ was collected and acid-soluble radioactivity in the incubation medium was measured as described in Experimental Procedures. Panel B: The sum of $CO_2$ and acid-soluble radioactivity as % of total added radioactivity is given. Data represent means ± SD of six cultures.

### Incorporation of labeled fatty acids into cellular phospholipids

[1-$^{14}$C]eicosapentaenoic acid or [1-$^{14}$C]oleic acid was incorporated into phosphatidylcholine, phosphatidyletanolamine, phosphatidylinositol, and phosphatidylserine in decreasing order (**Fig. 9**). Approximately 80% of the labeled fatty acids in the phospholipid fraction was incorporated into phosphatidylcholine and phosphatidylethanolamine. The incorporation of labeled oleic acid was significantly higher in phosphatidylcholine and significantly lower in phosphatidylinositol, lysophosphatidylcholine, and sphingomyelin, as compared to eicosapentaenoic acid.

### Synthesis and secretion of lipids using tritiated water as precursor

[$^{3}$H]water is incorporated into lipids through hydride transfer reduction. Thus, potential problems encountered with labeling precursor pools should be avoided, and incorporation of tritiated water closely reflects actual rates of synthesis. Incorporation into phospholipids, triacylglycerol, and cholesteryl ester in cells and basolateral media was similar up to 24 h, when 0.6 mM eicosapentaenoic acid or oleic acid was added to the apical well (**Fig. 10**). Although there was a tendency for eicosapentaenoic acid to promote a lower secretion of triacylglycerol, as compared to oleic acid (Fig. 10), the difference was not significant. Further unpublished experiments (T. Ranheim, A. Gedde-Dahl, A. C. Rustan, and C. A. Drevon) show that there is a similar triacylglycerol secretion in the presence of these two fatty acids.

### Determination of lipoprotein particle size and apolipoprotein B

The size of secreted lipoprotein particles of d <1.006 g/ml corresponds to human plasma chylomicrons (**Table 1**). The fractions were heterogenous in size, ranging in diameter from 100 to 1000 nm as determined by light scattering (36). The particle size of chylomicrons secreted by cells cultured on filter membranes was also measured by a resistive pulse technique (37). These measurements confirmed the heterogeneity of the lipoprotein particles (data not shown).

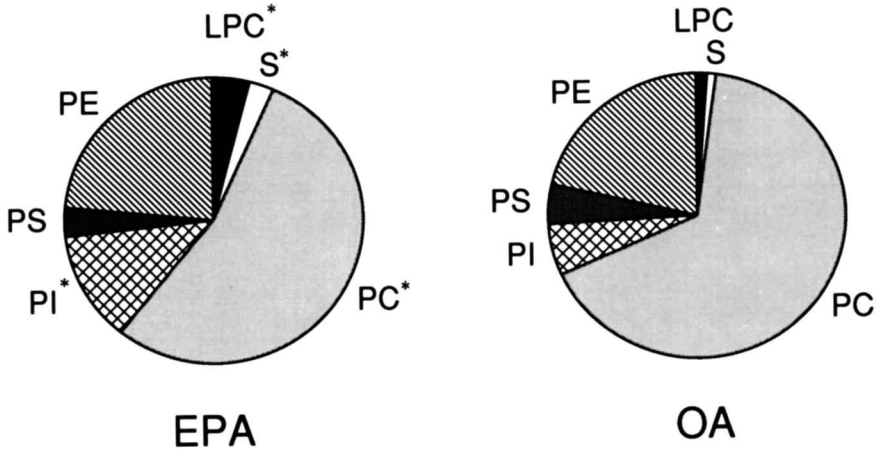



**Fig. 9.** Incorporation of [1-$^{14}$C]eicosapentaenoic acid and [1-$^{14}$C]oleic acid into separate phospholipids. CaCo-2 cells plated on filter membranes and cultured for 2 weeks after confluency were incubated for 24 h in serum-free DME medium containing HEPES (10 mM), [1-$^{14}$C]eicosapentaenoic acid or [1-$^{14}$C]oleic acid (1 μCi/ml, sp act 1.7 Ci/mol, 0.6 mM). Separation of phospholipids was performed by thin-layer chromatography as described in Experimental Procedures. The 100% values obtained from cells correspond to 68.1 and 65.3 nmol/mg cell protein for eicosapentaenoic acid and oleic acid, respectively. Data represent means ± SD of three cultures; *represents significant difference between the fatty acids at $P < 0.05$.

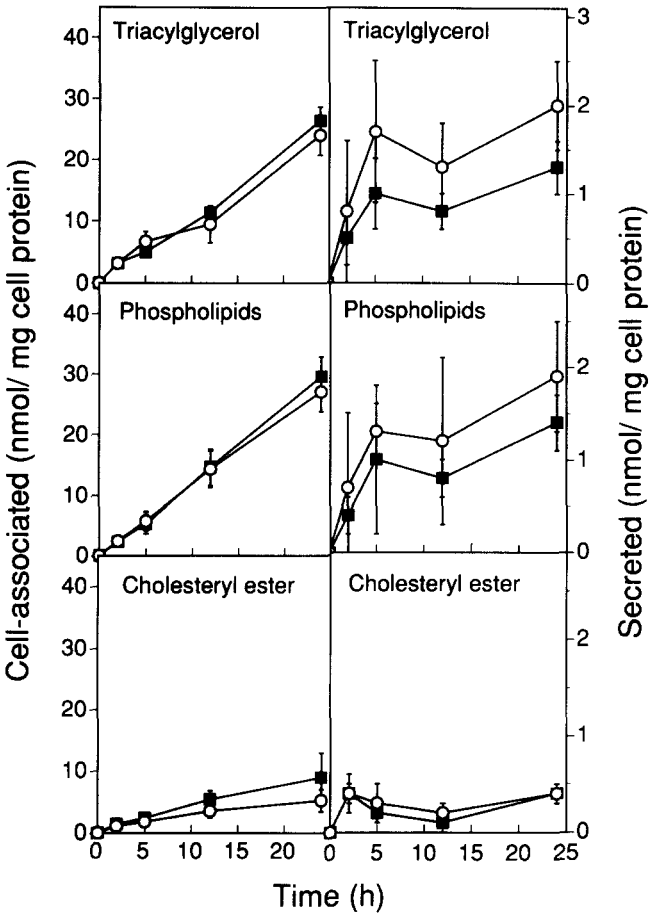



**Fig. 10.** Incorporation of [$^3$H]water into synthesized and secreted triacylglycerol, phospholipids, and cholesteryl esters. CaCo-2 cells plated on filter membranes and cultured for 2 weeks after confluency were incubated in serum-free DME medium containing [$^3$H]water (4 mCi/ml, sp act 6.7 mCi/μmol), HEPES (20 mM) and either eicosapentaenoic acid (■) or oleic acid (○) for 24 h. Data represent means ± SD of six cultures.

Assessment of secreted apoB showed that by incubating CaCo-2 cells with oleic acid, apoB levels increased approximately 1.4-fold, as compared to cells incubated with eicosapentaenoic acid (Table 1).

## DISCUSSION

Ultrastructural studies revealed that CaCo-2 cells are polarized and possess morphological and functional features of intestinal epithelial cells. Evaluation by electron microscopy, electrical measurements, and labeled bovine serum albumin demonstrated that cells grown on filter membranes formed tight and resistant monolayers.

Our results suggest a strong correlation between the rate of triacylglycerol secretion and postconfluent differentiation. This is in accordance with studies of brush-border enzyme activities, development of tight junctions, and dome formations of CaCo-2 cells (20, 25, 38–40). With respect to their long period of postconfluent differentiation, CaCo-2 cells do not resemble human enterocytes which are replaced every 36 h (41).

The preference for basolateral secretion in CaCo-2 cells may reflect the pattern of vectorial functions in absorption, transcytosis, and secretion (42). Eicosapentaenoic acid and oleic acid influenced synthesis and secretion of triacylglycerols whether the cells were grown on plastic or filter membranes. Grown on filter supports, lipoprotein secretion from the basolateral side of the cells should not be restricted (27). The growth of CaCo-2 cells on filters may also promote their differentiation due to presentation of nutrients to the basolateral, as it occurs in vivo (24). Our study indicates that CaCo-2 cells are more metabolically active when grown on filter membranes compared to cells cultured on plastic surfaces, as evaluated by triacylglycerol secretion and rate of fatty acid oxidation (Fig. 4). When CaCo-2 cells were grown on filters, they secreted a greater proportion of apoB-48 relative to apoB-100 when compared to cells grown on plastic, suggesting that the cells cultured on filter supports may be further differentiated (28).

CaCo-2 cells express several digestive microvilli membrane hydrolases confined to the apical surface (21). In accordance with these data it is possible that the postsecretory remodeling of lipoproteins by the cells, could be avoided growing them on filter membranes. Field, Albright, and Mathur (43) demonstrated that 50% of the secreted lipoproteins were hydrolyzed by incubating conventionally grown cells with VLDL-containing labeled triolein.

There is a certain disagreement concerning the influence of molecular chain-length and degree of unsaturation on absorption of eicosapentaenoic acid compared to oleic acid. Reports by Nelson and Ackman (44) and Chernenko et al. (45) indicated that eicosapentaenoic acid is absorbed as efficiently as oleic acid in rats. Our data

TABLE 1. Effect of eicosapentaenoic acid (EPA) and oleic acid (OA) on lipoprotein particle size (diameter) and apoB

| Treatment | Particle size | ApoB |
|---|---|---|
| | *nm* | *ng/mg cell protein* |
| BSA (control), 0.24 mM | 485 ± 190 | 101 ± 2 |
| EPA, 0.6 mM | 397 ± 214 | 89 ± 1[a] |
| OA, 0.6 mM | 415 ± 262 | 127 ± 1 |

CaCo-2 cells, plated on filter membranes and cultured for 14 days after confluency, were incubated in serum-free DME medium for 24 h with or without fatty acids complexed to albumin. The media were harvested, and the lipoprotein fractions were isolated by ultracentrifugation at d < 1.006 g/ml. Data represent means ± SD of four cultures.

[a]Represents significant difference between eicosapentaenoic acid and oleic acid at $P < 0.03$.

support this observation as no significant difference in absorption throughout 24 h was observed between eicosapentaenoic acid and oleic acid in CaCo-2 cells (Fig. 6).

The amount of newly secreted triacylglycerol was similar in cells incubated with oleic acid or eicosapentaenoic acid, regardless of different labels used as precursors. Secreted lipoproteins with d <1.006 g/ml consisted of particles with mean diameter close to 400 nm, suggesting that incubation of cells with fatty acids had no measurable influence on lipoprotein particle size. The secreted amount of apoB was significantly increased in media from cells incubated with oleic acid, as compared to cells incubated with eicosapentaenoic acid (Table 1), which may suggest that the number of chylomicrons is increased in the presence of oleic acid. Estimates show that the mean particle size of chylomicrons secreted from oleic acid-treated cells is approximately 370 nm in diameter, well within the range of lipoprotein particle size in this study, because of the great heterogenity of chylomicrons. This is in contrast to findings reported by Dashti, Smith, and Alaupovic (46), who demonstrated that oleate induced increased particle size of lipoproteins secreted by CaCo-2 cells, as evaluated by measuring the content of apolipoprotein and triacylglycerol in the lipoprotein particles. From our results it is possible that there is a discrepancy of the effect of eicosapentaenoic acid on secretion of triacylglycerol and apoB. This might be due to the fact that the precision of measurement of apoB is markedly better than for evaluation of triacylglycerol synthesis and secretion. However, we think that the most likely explanation is that eicosapentaenoic acid specifically reduces apolipoprotein B secretion.

Dietary fatty acids significantly influence levels of plasma lipoproteins, and a major contribution to the effect of chronic fish oil consumption on serum triacylglycerol concentration may be due to inhibition of postprandial secretion of chylomicrons from the enterocytes (5, 47). It is also possible that a low secretion of VLDL from the liver would cause a more rapid removal of chylomicrons due to less competition for hydrolysis of triacylglycerol by lipoprotein lipase (2). Our results suggest that there was no reduction of triacylglycerol formation and secretion from CaCo-2 cells incubated with eicosapentaenoic acid as compared to oleic acid (Figs. 5 and 10). These observations are inconsistent with previous findings in CaCo-2 cells, where eicosapentaenoic acid decreased newly synthesized and secreted triacylglycerol (43, 48). Discrepancies between the previous findings and the present study may be ascribed to variations in cell clones, but most likely to stages of differentiation and culturing conditions (Figs. 3 and 4). Experiments carried out in our laboratory on cells grown on plastic surfaces indicated that oleic acid promoted a significantly higher intracellular accumulation and secretion of triacyl[$^3$H]glycerol than eicosapentaenoic acid (Fig. 3).

Nestel et al. (10) suggested that decreased VLDL production induced by feeding fish oil to rats partly reflects diversion of polyenoic fatty acids into pathways of oxidation and ketogenesis. Unsaturated fatty acids might be converted to ketone bodies rather than being incorporated into triacylglycerol (49, 50). In the present study we have demonstrated that eicosapentaenoic acid and oleic acid are oxidized to a similar extent in CaCo-2 cells, although only approximately 5% of the lipids were diverted into this pathway. This fraction of oxidized fatty acids is so small that it probably does not contribute significantly to alteration of lipoprotein production. Nossen et al. (6) showed no increase in formation of acid-soluble radioactivity from labeled eicosapentaenoic acid as compared to oleic acid in cultured rat hepatocytes. On the other hand, Aarsland et al. (14), Rustan, Christiansen, and Drevon (13), and Halminski, Marsh, and Harrison (51) have shown that peroxisomal $\beta$-oxidation is increased after fish-oil feeding for several days to weeks in rats. However, these studies were long-term experiments and therefore not comparable with the present findings.

Our experimental conditions might mimic an acute fat load, and may be in accordance with results obtained by Harris et al. (5). They observed that postprandial levels of triacylglycerol in subjects given an acute fat load containing very long-chain n-3 fatty acids were similar to postprandial triacylglycerol levels seen in human subjects given an acute fat load of saturated fatty acids. However, Harris et al. (5) also found that chronic supplementation of n-3 fatty acids for 4 weeks caused a significant lowering of plasma postprandial triacylglycerol concentrations after an acute fat load. Thus, if CaCo-2 cells were incubated with very long-chain n-3 fatty acids for an extensive period of time (several days), the cells might reduce synthesis and secretion of chylomicrons as suggested by Hall et al. (52). Intestinal cells incorporate fatty acids into their membranes, and it is feasible that polyenes could decrease synthesis of triacylglycerol via lipid-protein interactions in membranes (5, 53). In the liver, n-3 fatty acids acutely inhibit synthesis of triacylglycerol and cholesteryl ester, probably by inhibiting acyl-CoA:diacylglycerol acyltransferase (7), phosphatidate phosphohydrolase (51), and acyl-CoA:cholesterol acyltransferase, respectively (12, 54). From chronic feeding experiments with rats, data suggest that reduced formation of triacylglycerol is conveyed via reduced acyl-CoA:diacylglycerol acyltransferase activity, and this could be due to altered membrane composition (13).

Culture conditions can affect cell polarity and alter cell functions, as discussed by Trotter and Storch (55). Provided that the cells used in our study are representative of human enterocytes in vivo, we can conclude that the CaCo-2 cells exhibit intestinal, mucosal, and morphological properties, including absorption and oxidation of fatty acid. The cells secrete chylomicrons within the nor-

mal size-range. Eicosapentaenoic acid and oleic acid are taken up, metabolized, and promote similar secretion of triacylglycerol. However, some differences in incorporation of the fatty acids into certain phospholipid classes and in secretion of apoB were found. The culture conditions, including time after confluency and cellular support, are critical for the rate of secretion in the presence of eicosapentaenoic acid and oleic acid.

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