

# Palmitic acid and linoleic acid metabolism in Caco-2 cells: different triglyceride synthesis and lipoprotein secretion

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**Abstract** Polarized monolayers of intestinal Caco-2 cells were used to study the effects of saturated palmitic acid (16:0) and polyunsaturated linoleic acid (18:2) on triglyceride synthesis and lipoprotein secretion. Monolayers were incubated for 24 h, at the apical or luminal side, with palmitic acid (16:0) or linoleic acid (18:2) in physiological concentrations. Incubation with 1.0 mM 16:0 or 18:2 resulted in differences in the composition and amount of secreted lipoproteins. Radiolabeled lipids in the lipoproteins secreted during incubation with 18:2 were found in the chylomicron/VLDL (very low density lipoprotein) density whereas with 16:0 the secreted lipoproteins were in the intermediate density/low density lipoprotein (IDL/LDL) density range. More triglyceride was secreted into the (basolateral) medium during incubation with 1.0 mM 18:2 ( $41 \pm 12\%$  of total triglyceride synthesized) than with 1.0 mM 16:0 ( $18 \pm 3\%$  of total). The biochemical findings correlate with conspicuous morphological changes in the cells in the presence of 16:0, but not 18:2. Increasing concentrations of 16:0 (0.1–1.0 mM) caused gradual accumulation of intracellular membrane. Microvilli became strongly reduced in number. With 1.0 mM palmitic acid we found an increased incorporation of [<sup>14</sup>C]palmitic acid into phosphatidic acid (14.8% of total incorporation into phospholipid with 16:0 vs. < 0.5% with 18:2) and diacylglycerol (12.5% with 16:0 vs. 0.5% with 18:2) and the amount of intracellular phospholipid doubled. The morphological changes were completely reversed after 24 h with 1.0 mM 18:2. ■ We conclude from our results that, compared to 18:2, 16:0 is not efficiently incorporated into triglycerides. 16:0 is incorporated into cellular phospholipids in a greater proportion than 18:2, causing accumulation of intracellular phospholipid and the precursors phosphatidic acid and diacylglycerol. Different processing of 18:2 and 16:0 by Caco-2 cells resulted in profound differences in triglyceride synthesis and lipoprotein composition and secretion.—van Greevenbroek, M. M. J., W. F. Voorhout, D. W. Erkelens, G. van Meer, and T. A. W. de Bruin. Palmitic acid and linoleic acid metabolism in Caco-2 cells: different triglyceride synthesis and lipoprotein secretion. *J. Lipid Res.* 1995. 36: 13–24.

**Supplementary key words** fatty acids • electron microscopy • enterocytes • morphology • polarized cells

In enterocytes and hepatocytes, apolipoprotein B is translocated across the membrane of the endoplasmic reticu-

lum and subsequently secreted as structural protein of lipoproteins. ApoB mRNA in these cells is abundantly translated into apoB protein. A generally accepted hypothesis on the mechanism of regulation of lipoprotein synthesis in intestinal cells and hepatocytes is that it occurs at the level of protein degradation. In this process, microsomal transfer protein is the obligatory protein for co-translational transfer of triglycerides into the lipoprotein particle (1). In case of incomplete protein translocation or improper assembly of the particle, apoB is degraded intracellularly at the cytoplasmic side of the endoplasmic reticulum membrane (2–4). In the past 8 years the effect of fatty acids on the secretion of lipoproteins has been studied in HepG2 cells (2, 5, 6), in Caco-2 cells cultured on plastic supports (7–10), and in polarized Caco-2 cells cultured on permeable supports (11–13). Caco-2 cells, a human cell line derived from a colon carcinoma, differentiate in culture into enterocytes (14). Monolayers grown on permeable supports act as polarized permeability barriers between two compartments and show important functional properties of transporting epithelium. Caco-2 cells generate a polarized distribution of membrane lipids (15) and proteins (16), show polarized protein secretion (16), uptake and metabolism of fatty acids (17) and drugs (18), and uptake and transport of bile acids (19). Confluent monolayers of Caco-2 cells on filters have well-developed microvilli and express a variety of cell surface markers that are also found in enterocytes of the intestinal villus. They exhibit well-developed tight junctions providing a monolayer unpenetrable for

Abbreviations: DMEM, Dulbecco's modified Eagle's minimum essential medium; FCS, fetal calf serum; BSA, bovine serum albumin; d, density; LDL, low density lipoprotein; IDL, intermediate density lipoprotein; VLDL, very low density lipoprotein; LPFS, lipoprotein-free serum; HP-TLC, high performance thin-layer chromatography; HRPO, horseradish peroxidase; 16:0, palmitic acid; 18:2, linoleic acid.

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macromolecules (20) and thus provide an isolated model of the human intestinal cell. Caco-2 cells can synthesize both apoB-48 and apoB-100, secreted as the structural protein of lipoproteins (12) and the apolipoproteins A-I, A-IV, C-III, and E (8, 12). The results published concerning the regulation of lipoprotein synthesis in Caco-2 cells are somewhat conflicting. This may be due to varying differentiation levels of the cells in various studies. Differentiation of the Caco-2 cells depends on the culture supports and culture conditions used, and on time after confluence of the monolayers. It may also depend on the source and passage number of the cells. It has been suggested that fatty acids in the culture media of Caco-2 cells determine the amount of lipoproteins secreted and the composition of these particles. Addition of oleic acid stimulates the secretion of lipoproteins by Caco-2 cells compared with fat-free incubations (8). In 1988, Field, Albright, and Mathur (7) studied the regulatory effect of saturated and unsaturated fatty acids on synthesis and secretion of triglyceride-rich lipoproteins by Caco-2 cells. They reported that oleic acid potentially stimulated the triglyceride secretion by Caco-2 cells grown on petri dishes, followed in descending order by linoleic, linolenic, palmitic, and myristic acids. Eicosapentaenoic acid, from fish oil, has been described as stimulating triglyceride secretion by Caco-2 cells grown on filters in a similar way as oleic acid (13). Eicosapentaenoic acid caused a slight decrease in secretion of apoB compared with oleic acid. Murthy et al. (10), on the other hand, reported that oleic acid was predominantly incorporated into cellular triglycerides whereas eicosapentaenoic acid was preferably incorporated into cellular phospholipids.

Not many data are available on the effects of palmitic acid (16:0) and linoleic acid (18:2) on lipoprotein secretion by Caco-2 cells, despite their abundance in the human diet. In a preliminary abstract regarding the effects of fatty acids on lipoprotein secretion, we reported that palmitic acid decreased the secretion of apoB by the Caco-2 cells (21). Under the same circumstances linoleic acid increased apoB output to even higher levels than caused by oleic acid, a commonly used fatty acid in a number of studies on lipoprotein synthesis (7, 8, 22). Most studies on the effects of fatty acids on lipoprotein secretion focus specifically on the lipoprotein synthesis pathway. In the present study we compared the uptake and metabolism of palmitic acid and linoleic acid by polarized Caco-2 cells, their incorporation into cellular lipids, their effects on cell morphology, and on lipoprotein secretion.

## EXPERIMENTAL PROCEDURES

### Materials

[1-<sup>14</sup>C]palmitic acid (57 mCi/mmol) and [1-<sup>14</sup>C]linoleic acid (53 mCi/mmol) were purchased from New England

Nuclear (Boston, MA). Palmitic acid and linoleic acid (both 99% pure by gas chromatography) and essentially fatty acid-free BSA were obtained from Sigma (St. Louis, MO). HP-TLC plates (Silica 60 F<sub>254</sub>) were purchased from Riedel de Haën (Seelze, Germany) and autoradiograms were made on Fuji Medical X-ray film (Fuji, Tilburg, Netherlands).

### Cell culture

Caco-2 cells (14) of intermediate passages (69–92) were grown in DMEM supplemented with 20% heat-inactivated FCS, 1% nonessential amino acids, 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco BRL, Grand Island, NY). The Caco-2 cells were maintained in a hydrated atmosphere at 37°C/5% CO<sub>2</sub> and subcultured weekly at a surface dilution of 1:25 using trypsin-EDTA (15). Stock cultures of cells were grown in 75-cm<sup>2</sup> culture flasks (Costar, Cambridge, MA) and medium was changed on days 3, 5, and 6 after plating.

For experiments, cells were cultured on microporous membranes of 0.45 μm pore size (Transwell COL<sup>TM</sup>, Costar, Cambridge, MA). The filter diameter and surface area were 24.5 mm and 4.7 cm<sup>2</sup>, respectively. Filter inserts were suspended in six 20-mm high polypropylene rings, and placed into a 150-mm diameter glass petri dish with 90 ml medium. Caco-2 cells were plated on filters at a surface dilution of 1:25 from stock cultures in 75-cm<sup>2</sup> flasks. Apical medium was changed every other day starting at day 5 after plating. Basolateral medium was changed on days 7 and 11 after plating. Confluence of the cell monolayers was visually determined by phase contrast microscopy. The filters were transferred from the petri dishes to 6-well cluster dishes 1 day before the experiments which were performed on days 14–18 after plating. Monolayers were than 8–10 days postconfluent.

### BSA-fatty acid complex preparation

Fatty acids were complexed to essentially fatty acid-free BSA in serum-free DMEM according to Van Harken, Dixon, and Heinberg (23). Two-hundred μl 50 mM linoleic acid in 96% ethanol was added, during vortexing, to 400 μl 100 mM NaOH in 96% ethanol. In the case of palmitic acid, 2 ml 5 mM in 96% ethanol was added to 400 μl of the NaOH stock solution to prevent formation of precipitate. The ethanol mixture was evaporated to dryness using N<sub>2</sub> and the pellet was dissolved in 0.5 ml hot bidistilled water and added to 9.5 ml of stirring cold serum-free DMEM/1% BSA solution. The fatty acid concentration of the fatty acid-BSA solutions was 1 mM. The ratio of fatty acid to BSA was 7.5:1 on a molar basis. Fatty acid-BSA solutions were warmed to 37°C before addition to the Caco-2 cells. When used, [1-<sup>14</sup>C]palmitic acid or [1-<sup>14</sup>C]linoleic acid was added to the corresponding fatty acid stock solution in ethanol.

## Incubation of cells with palmitic acid or linoleic acid

Monolayers on filters were rinsed 3 times with serum-free DMEM before adding 1.5 ml fatty acid-BSA complexes to the apical cell surface. Three ml of serum-free DMEM without BSA or fatty acid was applied to the basolateral side of the filters. Cells were incubated with fatty acids for 24 h at 37°C unless stated otherwise. After the incubation, apical and basolateral media were collected and centrifuged at 1,000 rpm (TJ-6 centrifuge, Beckman Inc., Palo Alto, CA) for 10 min to remove cell debris. The lipid content of cells and media was analyzed on one- and two-dimensional TLC. Total cellular cholesterol and triglyceride were determined with an enzymatic color reaction (Boehringer Mannheim, Germany). Density of lipoproteins secreted by Caco-2 cells was determined in a KBr density gradient according to Redgrave, Roberts, and West (24).

## Neutral lipids and phospholipids in Caco-2 cells and media

Apical and basolateral media were collected in glass tubes on ice at the end of each incubation. Caco-2 monolayers were rinsed twice with serum-free DMEM. The filters were cut out of the insert and transferred into 0.5 ml ice-cold PBS. Lipids were extracted from cells and media according to the method of Bligh and Dyer (25). The chloroform phase was dried under N<sub>2</sub> and extracts were analyzed by one-dimensional and two-dimensional TLC, respectively.

**Neutral lipids.** Aliquots of the extracts were applied to HP-TLC plates using [<sup>14</sup>C]cholesteryl oleate and [<sup>14</sup>C]trioleoyl glycerol as standards. The solvent system used for the one-dimensional HP-TLC was hexane-diethylether-acetic acid 90:10:1 (vol/vol) (26). After collecting triglyceride and cholesteryl ester, an additional run was performed using benzene-diethylether-ethyl acetate-acetic acid 80:10:10:0.2 (vol/vol) to further separate phospholipids and diglycerides.

**Phospholipids.** Two-dimensional TLC was performed according to the method of Renkonen et al. (27). The lipid spots on the TLC plates were visualized with iodine vapor or autoradiography. The lipid-containing spots were scraped directly into counting vials. Ultima Gold (Packard Instrument Company, Downers Grove, IL) was added before scintillation counting and radioactivity was measured with a Packard 1900 CA Tri-Carb liquid scintillation counter. In unlabeled cells, cellular phospholipid phosphate was determined according to Rouser, Fleischer, and Yamamoto (28).

## Density gradient ultracentrifugation of secreted lipoproteins

The lipoproteins secreted into the basolateral media were separated on a KBr density gradient (24, 29). Three

ml of basolateral medium was adjusted to d 1.250 g/ml with solid KBr in polyallomer tubes and overlaid with 2.8 ml of KBr/NaCl, 1 mM EDTA solutions of d 1.063, d 1.019, and d 1.006 g/ml, respectively. The gradients were centrifuged for 24 h at 32,000 rpm in a Beckman SW 41 rotor. Fractions of 0.5 ml were aspirated and 75 μl was counted in a Packard 1900 CA Tri Carb liquid scintillation counter. A control KBr gradient was processed identically in each run to determine the actual density of each 0.5-ml fraction.

## Vesicular transport in the fatty acid-incubated cells

After incubation with 1.0 mM palmitic acid, 1.0 mM linoleic acid, or 1% BSA, the ability of the cells to synthesize lipids and transport these via vesicular transport was assayed as described before (15). Fluorescent ceramide (C6-[7-nitro-2,1,3-benzoxadiazol-4-yl]aminocaproyl-ceramide), was applied to the apical side of the monolayers at the end of the 24-h experiments. It was converted to glucosylceramide and sphingomyelin. Arrival of these short chain analogs of native membrane lipids at the cell surface is a measure of vesicular transport in the cells.

## Cell morphology

Monolayers of Caco-2 cells grown on filters were fixed in 0.1 M PIPES containing 2% paraformaldehyde and 0.2% glutaraldehyde. Ultrastructural morphology of the cells was studied after freeze substitution according to the method described by van Genderen et al. (30).

## ApoB ELISA

Total amount of apoB secreted by the cells to the basolateral medium during 24 h incubation was determined using a sandwich ELISA for apoB adapted from Ordovas et al. (31). Sheep polyclonal anti-human-apoB antibody (Boehringer Mannheim, Germany) 1:5,000 was used as capturing antibody. The same antibody was purified over an LDL-coupled Sepharose 4B column (Pharmacia, Uppsala, Sweden). The purified antibody was coupled to HRPO and used as detecting antibody. We used purified human LDL (0–10,000 ng/ml) for a standard curve. No cross-reactivity occurred with HDL, apoA-I, albumin, or lipoprotein-free serum. Intra- and interassay coefficients of variation were 7.5% and 10.6%, respectively.

## Statistical analysis

All values are expressed as mean ± standard deviation (SD). Mean differences between groups (palmitic acid vs. linoleic acid) were calculated by unpaired Student's *t*-tests. Statistical significance is reached when *P* < 0.05 (two-tailed).

## RESULTS

### Uptake of [1-<sup>14</sup>C]palmitic acid and [1-<sup>14</sup>C]linoleic acid by Caco-2 cells

Free fatty acids were efficiently taken up by the cells: 85% of the apical [1-<sup>14</sup>C]palmitic acid and 81% of apical [1-<sup>14</sup>C]linoleic acid was absorbed and metabolized by the Caco-2 cells. Total incorporation of label into lipid was equal with palmitic acid and linoleic acid with both 1.0 mM and 0.1 mM fatty acid ( $16 \pm 2$  KBq vs.  $15 \pm 3$  KBq, respectively, with 1.0 mM, and  $4.4 \pm 2.0$  KBq vs.  $5.4 \pm 2.7$  KBq, respectively, with 0.1 mM fatty acid). No more than 0.5% of the original labeled free fatty acid in the apical compartment had crossed the monolayer and the filter at the end of the incubation. This confirms that the monolayers were not permeable to fatty acids or fatty acid-BSA complexes. Less than  $2.5 \times 10^{-3}$  nmol free fatty acid/nmol phospholipid was present in the cells after any of the incubations with roughly 250 nmol of free fatty acid left in the apical medium. This shows that the apical cell membrane maintained its integrity with the concentrations of fatty acid used in the study.

### Incorporation of fatty acids into lipids

Fatty acids were equally incorporated into phospholipid and triglyceride with 0.1 mM palmitic acid and with 0.1 mM and 1 mM linoleic acid. After incubation with 1.0 mM palmitic acid, the incorporation of this fatty acid into phospholipids was 3-fold higher than the incorporation into triglycerides (Table 1). Total phospholipid mass was significantly higher in the cells incubated with 1.0 mM palmitic acid than with 1.0 mM linoleic acid or 1% BSA ( $580 \pm 20$  vs.  $370 \pm 10$  and  $380 \pm 30$  nmol phosphate/filter, respectively,  $P < 0.001$   $n = 4$ ). In addition, the total amount of newly synthesized triglyceride and phospholipid differed significantly between palmitic acid and linoleic acid at 1.0 mM concentration ( $P < 0.02$  and  $P < 0.025$ , respectively, Table 1). In all incubations, only

trace amounts of labeled cholesteryl esters were found in the cells. We found a significant increase in the relative amount of intracellular 16:0/16:0 phosphatidylcholine when monolayers were incubated with 1.0 mM palmitic acid (38% vs. 3% of total phosphatidylcholine). With 1.0 mM linoleic acid, an increase in 18:2/18:2 phosphatidylcholine was found: 25% of total phosphatidylcholine compared with 0.5% in the control incubation.

A higher incorporation of labeled fatty acid into diacylglycerol and phosphatidic acid was found in the incubations with 1.0 mM palmitic acid than with 1.0 mM linoleic acid. Relative incorporation of labeled fatty acid in diacylglycerol was 12.5% with palmitic acid versus 0.5% linoleic acid, and in phosphatidic acid incorporation was 14.8% versus not detectable, respectively. The cellular mass of phosphatidic acid was increased with palmitic acid versus linoleic acid (52 nmol/filter vs. 3 nmol/filter, respectively). Diacylglycerol and phosphatidic acid are precursors of phospholipid and triglyceride in the glycerol-3-P pathway, which is important in Caco-2 cells.

The cellular mass of cholesterol was  $167 \pm 19$  nmol/filter in all incubations. Intracellular triglyceride mass was  $215 \pm 35$  nmol/filter after incubation with 1.0 mM palmitic acid,  $323 \pm 8$  nmol/filter with linoleic acid, and it was  $90 \pm 3$  nmol/filter in the control incubations ( $n = 2$ ).

### Triglyceride secretion by Caco-2 cells

The basolateral/apical polarity of triglyceride secretion was high in all incubations. It was similar after incubations with 0.1 mM palmitic acid or linoleic ( $2.5 \pm 1.2$   $n = 7$  and  $2.8 \pm 1.2$   $n = 4$ , respectively). However, with 1.0 mM linoleic acid it was significantly higher than with 1.0 mM palmitic acid ( $4.7 \pm 1.4$   $n = 6$  vs.  $2.9 \pm 1.0$   $n = 7$ , respectively;  $P < 0.05$ ).

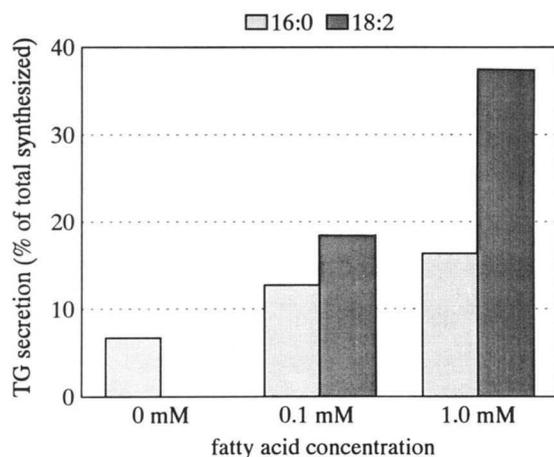
Triglyceride secretion was determined as a measure of lipoprotein secretion in the incubations with 0.1 mM and 1.0 mM palmitic acid or linoleic acid. With 1.0 mM fatty acid, a significant difference was found in basolateral secretion, i.e.,  $37.4 \pm 12.2\%$  of total synthesized triglyceride ( $n = 9$ ) with linoleic acid and  $16.3 \pm 6.7\%$  ( $n = 7$ ) with palmitic acid ( $P = 0.001$ ). In addition, also with low fatty acid concentrations (0.1 mM) we found a significant difference in the secretion capacity of the cells ( $18.3 \pm 2.5\%$  with linoleic acid,  $n = 4$ , vs.  $12.7 \pm 2.0\%$  with palmitic acid,  $n = 5$ ,  $P < 0.01$ ). In control incubations with 1% BSA, using trace amounts of [1-<sup>14</sup>C]palmitic acid, the basolateral triglyceride secretion was  $6.9 \pm 0.8\%$  (Fig. 1). Thus, the triglyceride secretion by the cells is lower with palmitic acid than with linoleic acid in both concentrations used.

To study the time-dependency of the effects of palmitic acid and linoleic acid on Caco-2 cells, we incubated the cells for 0, 3, or 9 h with 1.0 mM unlabeled palmitic acid or linoleic acid before incubation with the corresponding

TABLE 1. Total synthesized triglyceride and phospholipid after 24 h incubation with [1-<sup>14</sup>C]linoleic acid or [1-<sup>14</sup>C]palmitic acid

Fatty Acid		Triglyceride	Phospholipid
16:0, 0.1 mM	$n = 5$	$744 \pm 63$	$837 \pm 223$
18:2, 0.1 mM	$n = 5$	$996 \pm 25$	$1065 \pm 82$
		$P = 0.06$	$P = 0.06$
16:0, 1.0 mM	$n = 6$	$3289 \pm 1683$	$10291 \pm 3340$
18:2, 1.0 mM	$n = 9$	$6614 \pm 2767$	$5726 \pm 3054$
		$P < 0.02$	$P < 0.025$

Total incorporation of label into triglyceride and phospholipid recovered from the basolateral medium, apical medium, and cells after 24 h incubation with 0.1 or 1.0 mM [1-<sup>14</sup>C]palmitic acid or [1-<sup>14</sup>C]linoleic acid. Data are expressed as total Bq  $\pm$  SD. The total amount of radioactive label applied was 3,700 Bq with 0.1 mM and 37,000 Bq with 1.0 mM fatty acid.



**Fig. 1.** Triglyceride secretion during 24 h incubation with palmitic acid or linoleic acid. The basolateral triglyceride secretion was different between palmitic acid and linoleic acid with both 0.1 mM ( $P < 0.01$ ) and with 1.0 mM ( $P = 0.001$ ) fatty acid.

radiolabeled fatty acid (**Fig. 2**). After 0, 3, and 9 h preincubation with unlabeled fatty acid the transport of newly synthesized triglyceride to the basolateral side of the monolayer was significantly lower in cells incubated with palmitic acid than in the linoleic acid incubations. Longer preincubation times (3 and 9 h) with unlabeled palmitic acid reduced the percentage of newly synthesized triglyceride secreted into the basolateral compartment. The opposite was observed after preincubation of the cells with linoleic acid.

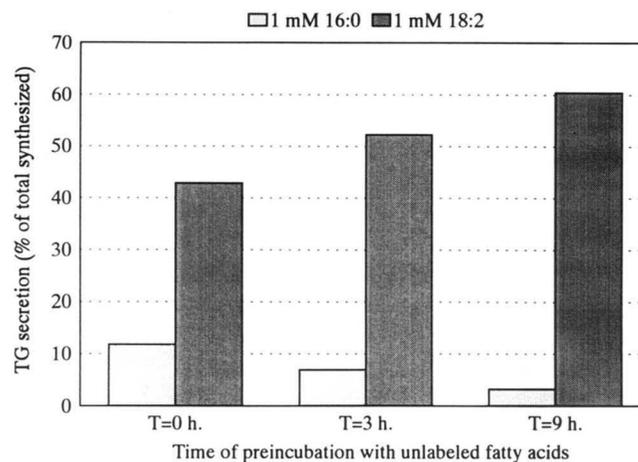
Diminished basolateral secretion was also observed when assayed independently by quantitating the transport of the membrane lipid sphingomyelin from the Golgi complex to the plasma membrane. Newly synthesized sphingomyelin is transferred to the plasma membrane by vesicular transport and it has been demonstrated that short chain sphingomyelin analogues can be used as a marker of secretion (32–34). At the end of the 24-h incubation, basolateral transport of sphingomyelin during 1 h 37°C was 12% of total synthesized after incubation with palmitic acid, compared to 27% and 26% with linoleic and BSA (control), respectively. This indicates that vesicular transport was affected after incubation with palmitic acid. The biochemical and functional differences found in the cells suggested that palmitic acid and linoleic acid might have different effects on the overall appearance of Caco-2 monolayers.

### Morphology of Caco-2 cells after incubation with fatty acid

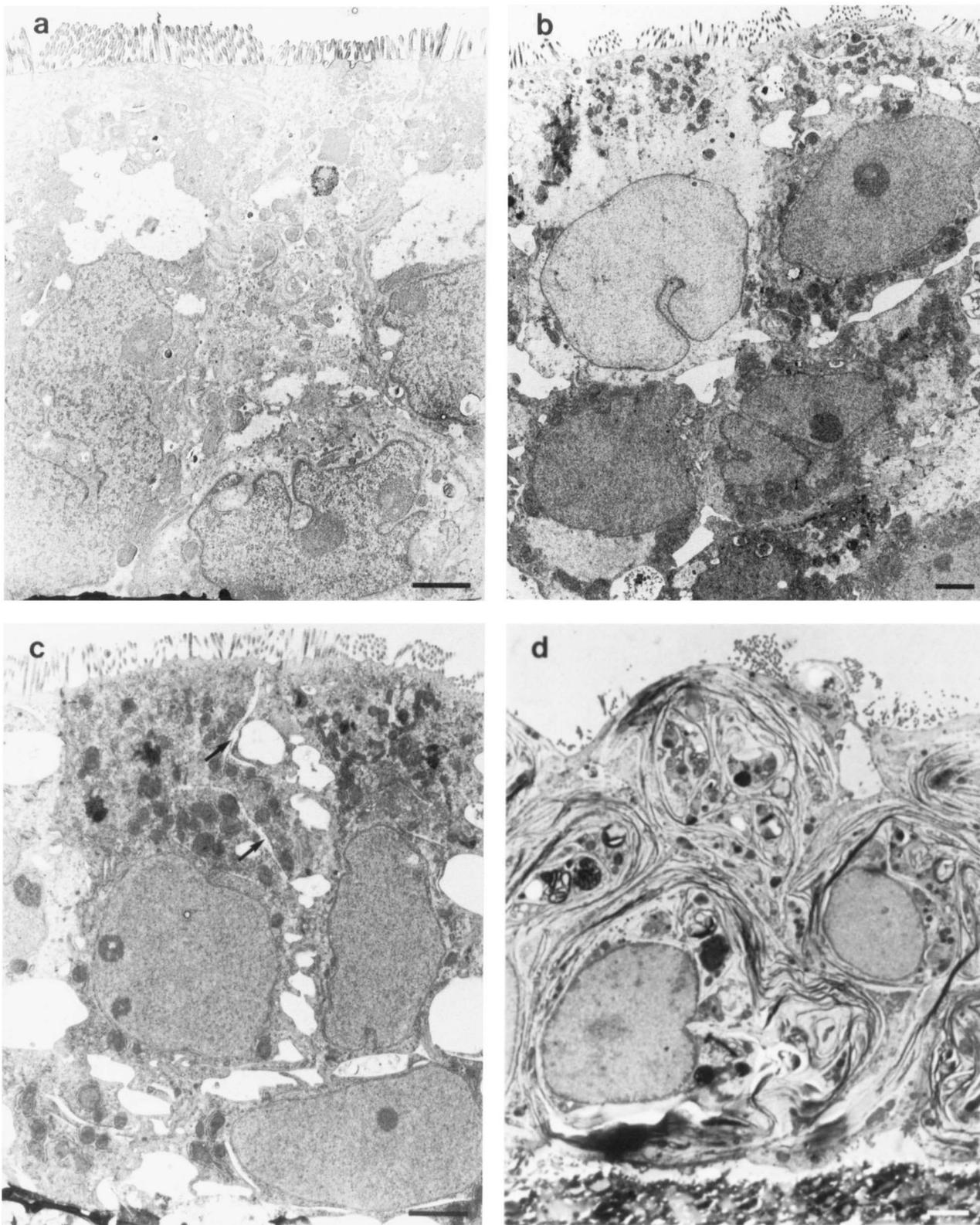
In two independent experiments, an ultrastructural investigation was performed on Caco-2 cell monolayers after 24 h incubation with palmitic acid, linoleic acid, or BSA as control. No morphological differences were observed between the 1.0 mM linoleic acid and control incu-

bations. After seeding, Caco-2 cells formed confluent monolayers with a clearly polarized morphology. The apical side of the cells was homogeneously covered with microvilli and no irregularities were observed in the confluent monolayers. Glycogen is visible mainly in the apical part of the polarized cells whereas the cell nuclei were predominantly seen in the basal half of the cells (**Fig. 3**, panel a).

With increasing concentrations of palmitic acid (0.1–0.25–0.5–1.0 mM) the ultrastructural morphology of the cells showed increasing changes compared to the linoleic acid and control incubations (**Fig. 3**, panels a–d); no differences were seen with 0.1 mM or 0.25 mM fatty acid. Polarized Caco-2 cells changed conspicuously with 0.5 mM and even more with 1.0 mM palmitic acid compared to linoleic acid or control incubations. These concentrations of palmitic acid strongly reduced the presence of microvilli at the apical side of the cells. Cells rounded off and an increase in the interstitial space between cells was seen whereas tight junctions remained intact. A pronounced accumulation of membranes occurred in the cells. The changes of the cells incubated with palmitic acid, however, had no effect on the functional confluence of the monolayers as no free fatty acid had crossed the filter membrane to the basolateral compartment during the incubation (see above). We observed that the palmitic acid-incubated cells were laden with membranes extending throughout the cells without accumulation of lipid droplets or lipoprotein-like particles in any distinct part of the cells (**Fig. 3**, panel d). These findings agree with both



**Fig. 2.** Transport of newly synthesized triglycerides to the basolateral side of the Caco-2 monolayer, measured after 0, 3, or 9 h preincubation with palmitic acid and linoleic acid prior to [ $^{14}\text{C}$ ]palmitic acid and [ $^{14}\text{C}$ ]linoleic acid incubation, respectively. The transport of triglycerides to the basolateral medium increased after prolonged preincubation with linoleic acid whereas it decreased after preincubation with palmitic acid. The total amount of triglyceride synthesized by the cells in 24 h after preincubation with palmitic acid for 0 h, 3 h, or 9 h was 4590 Bq, 4190 Bq, and 4700 Bq, respectively. After preincubation with linoleic acid these amounts were 7230 Bq, 6410 Bq, and 6400 Bq, respectively.



**Fig. 3.** Electron micrographs of monolayers of Caco-2 cells grown on Transwell-COL filters for 13 days. The cells were incubated for 24 h with 1% BSA, 1.0 mM linoleic acid, or increasing concentrations of palmitic acid. Panel a: morphology of cells incubated with 1.0 mM linoleic acid was equal to the control incubation with 1% BSA. A representative example is given in this panel. Panel b: With 0.1 mM palmitic acid, no morphological changes are seen. Panel c: with 0.5 mM palmitic acid some intracellular membrane accumulated in the cells (arrows). Panel d: With 1.0 mM palmitic acid the cells are laden with intracellular membranes; microvilli are strongly reduced.

the higher level of newly synthesized cellular phospholipids in the cells incubated with [1-<sup>14</sup>C]palmitic acid (Table 1) and with the increase in total cellular phospholipid.

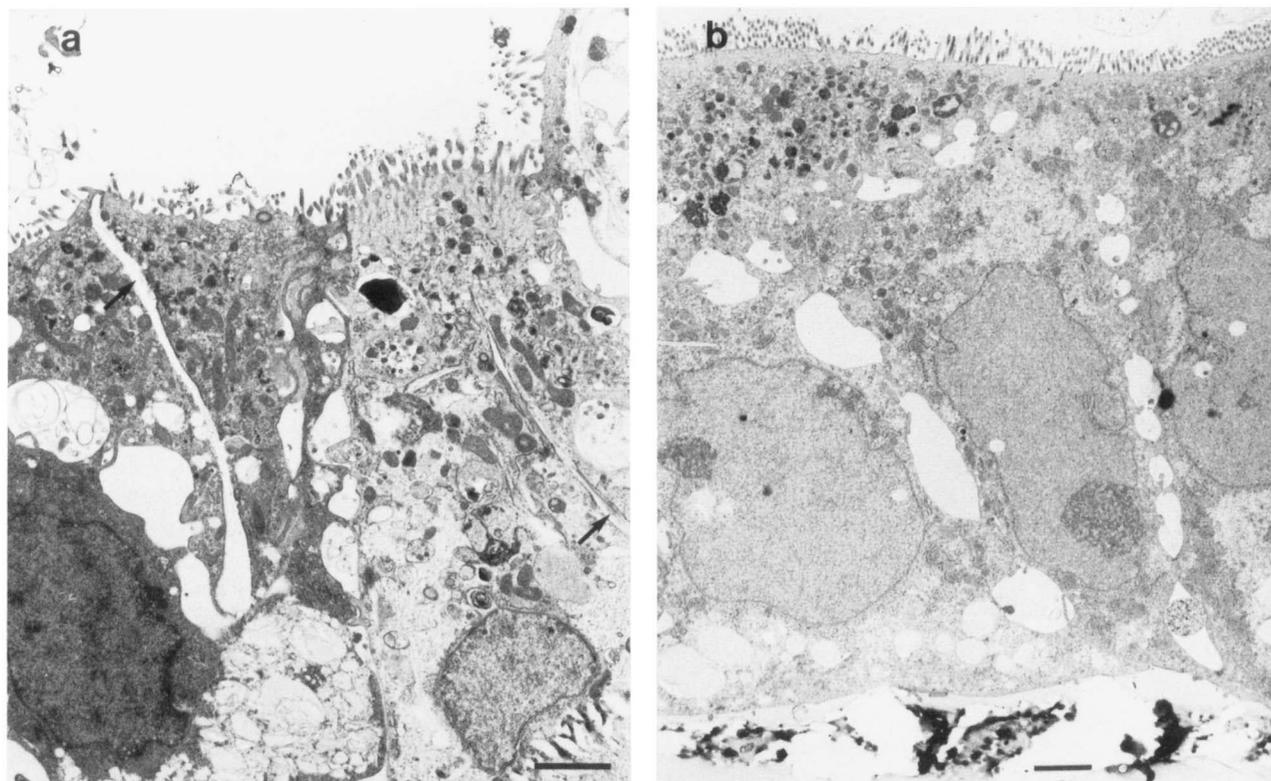
### Reversibility of the morphological effect of high palmitic acid concentrations

We determined whether the effects of 1.0 mM palmitic acid on Caco-2 cell morphology, i.e., the intracellular accumulation of membranes, were reversible. We extended the 24-h incubations with an additional incubation for 24 h with 0.5 or 1.0 mM linoleic acid, known to have no adverse effects on morphology and function. The effects of palmitic acid were reversed in part with 0.5 mM linoleic acid and completely reversed to normal morphology with 1.0 mM linoleic acid. The intracellular membrane accumulation disappeared and microvilli and morphological polarity were restored (Fig. 4).

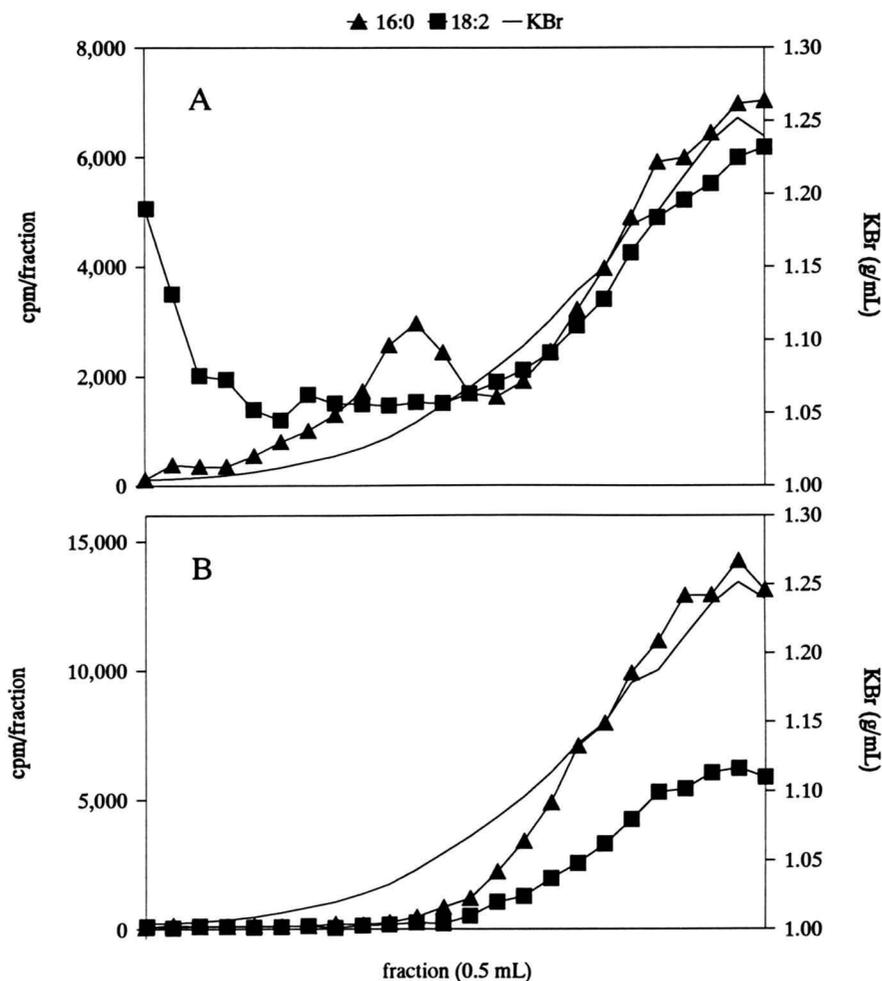
### Lipoprotein secretion and particle size after 1.0 mM palmitic acid or linoleic acid incubation

Subsequently, we studied whether palmitic acid, in addition to the cellular effects, would also influence the type of lipoprotein particles that were secreted. We fractionated basolateral media on 1.006–1.250 g/ml KBr density

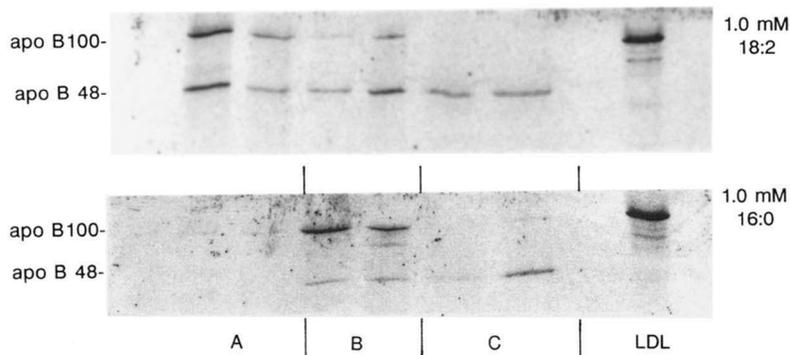
gradient (24). Lipoproteins secreted to the basolateral medium after incubation with 1.0 mM [1-<sup>14</sup>C]linoleic acid appeared mainly at chylomicron density,  $d < 1.006$  g/ml (Fig. 5, panel A). In contrast, lipoproteins secreted during [1-<sup>14</sup>C]palmitic acid incubation were present at  $d$  1.020–1.067 g/ml or LDL density, indicating secretion of relatively triglyceride-depleted or phospholipid-enriched lipoproteins. Lipoproteins secreted in the incubation with palmitic acid or linoleic acid, therefore, differed in composition. Immunoblotting of the three peak fractions was done to verify the presence of buoyant lipoproteins at the peak fractions of the Redgrave gradient (Fig. 6). ApoB-100 and apoB-48 were found at chylomicron and LDL density, in agreement with the presence of lipid (Figs. 5 and 6). The fraction with the highest density contained only a small amount of apoB-48 and no apoB-100. ApoB proteins secreted to the basolateral medium were measured using a sandwich ELISA for total apoB. Secreted apoB was  $680 \pm 300$  ng/filter ( $n = 16$ ) with BSA,  $700 \pm 310$  ng/filter ( $n = 21$ ) with palmitic acid, and  $1140 \pm 570$  ng/filter ( $n = 22$ ) with linoleic acid ( $P < 0.01$ ). The basolateral secretion of triglyceride was 20, 23, and 93 nmol/filter with BSA, 1.0 mM palmitic acid, and 1.0 mM linoleic acid, respectively. Thus, the



**Fig. 4.** The morphologic effects of 1.0 mM palmitic acid on the Caco-2 cells, as shown in Fig. 3, panel d, were reversed by linoleic acid. Panel a: after 24 h incubation with 1.0 mM palmitic acid the cells were incubated with 0.5 mM linoleic acid. Normal morphology of the cells is partly restored. Panel b: Cells after 24 h incubation with 1.0 mM palmitic acid followed by 24 h incubation with 1.0 mM linoleic acid. The normal morphology of the cells is almost completely restored. The accumulated membranes have disappeared from the cells and functional morphology is restored.



**Fig. 5.** Characteristics of secreted lipoproteins in density gradient analysis. Panel A: Density gradient profiles of lipoproteins in the basolateral medium after 24 h fatty acid incubation. Lipoproteins were recovered from the basolateral media of Caco-2 cells incubated in two independent experiments for 24 h with 1.0 mM [ $^{14}\text{C}$ ]palmitic acid or 1.0 mM [ $^{14}\text{C}$ ]linoleic acid, and separated on a 1.006–1.250 g/ml KBr density gradient. Panel B: MDCK cells were incubated with 1.0 mM [ $^{14}\text{C}$ ]linoleic acid and 1.0 mM [ $^{14}\text{C}$ ]palmitic acid as control for the shedding of membrane fragments into the basolateral medium.



**Fig. 6.** Apolipoprotein B-100 and B-48 in secreted lipoproteins. Immunoblot of apoB-100 and apoB-48 in the different density fractions of the basolateral medium after incubation with 1.0 mM 16:0 or 1.0 mM 18:2. The duplicates are the results of two independent experiments. Lane A:  $d < 1.007$ , lane B:  $1.020 < d < 1.068$ , lane C:  $d > 1.080$ , LDL: delipidated human LDL.

buoyant lipoproteins secreted during linoleic acid incubation contain 2.5-fold more triglyceride per apoB than in palmitic acid or BSA incubations. These data are consistent with the secretion of lipoproteins closely resembling chylomicrons in the incubation with linoleic acid. We estimate that the ratio of triglyceride over apoB was 3000:1 mol/mol in the lipoproteins secreted with linoleic acid, 1200:1 mol/mol with palmitic acid, and 1000:1 mol/mol in the BSA incubation, respectively.

In both the [ $^{14}\text{C}$ ]palmitic acid and the [ $^{14}\text{C}$ ]linoleic acid incubations, a considerable amount of total activity appeared in the most dense part of the gradient ( $d > 1.080$  g/ml) that contains mainly proteins (Fig. 5, panel A). As a control to the separation capacity of the KBr gradient, confluent monolayers of MDCK II cells were grown on polycarbonate filters and incubated with [ $^{14}\text{C}$ ]palmitic acid and [ $^{14}\text{C}$ ]linoleic acid under the same conditions as Caco-2 cells. MDCK II, a kidney-derived cell line, is known to release membrane fragments and is not capable of synthesizing lipoprotein particles. In the density gradient of the basolateral medium from cultures of MDCK II cells incubated with radiolabeled fatty acid, the label was found in the protein fraction (Fig. 5, panel B) and no radioactivity was present at lipoprotein densities. It is, therefore, most likely that the radioactivity appearing in the LPFS obtained from the Caco-2 incubations represents secreted or disrupted cellular membrane fragments or non-lipids. We conclude that the radioactive label appearing in lipoprotein densities in the basolateral media was not contaminated with membrane fragments but represented newly formed lipoprotein particles.

## DISCUSSION

In the present study, different effects of 0.1 mM and 1.0 mM palmitic acid and linoleic acid on polarized Caco-2 cells were found, with respect to metabolism of the fatty acids, incorporation into phospholipids and triglycerides, cell morphology, and the density of the secreted lipoproteins. Palmitic acid and linoleic acid are quantitatively important constituents of the human diet and the concentrations used in the present study are physiological in the human postprandial intestine. It should be realized that the Caco-2 cell line has its specific limitations compared to the *in vivo* situation of the human intestine. Caco-2 cells lack the intestinal fatty acid binding protein (FABP) but do express, like the normal enterocyte, the liver-FABP abundantly (35). The major intestinal pathway for synthesis of triglycerides is the monoacylglycerol pathway. Fatty acids absorbed by intestinal cells can, however, be assimilated to triglycerides via the phosphatidic acid pathway (36) and it has been established also that, in the absence of luminal 2-monoacylglycerol, fatty acids are effectively incorporated into triglycerides of

lymph chylomicrons (37). A specific characteristic of the Caco-2 cell line is that it uses the glycerol-3-phosphate pathway for the synthesis of triglycerides (17, 38). It is evident, however, from the results of other investigators (7, 11, 13, 39) and from our present results, that Caco-2 cells are able to synthesize and secrete lipoproteins at chylomicron density. Caco-2 monolayers cultured on filters are, despite their drawbacks, currently one of the best models available of the human intestine *in vitro* to study the synthesis and secretion of intestinal lipoproteins. The Caco-2 monolayers we used showed polarized triglyceride secretion directed toward the basolateral side of the monolayers. The control monolayers after 14 days in culture showed functional morphology with electron microscopy: the cells expressed a well-developed brush border (Fig. 3 panel a). Less than  $2.5 \times 10^{-3}$  nmol free fatty acid/nmol phospholipid was present in the cells after 24 h incubation and no difference was found between palmitic acid and linoleic acid. With roughly 250 nmol of free fatty acid left in the apical medium at the end of the incubation, it is very unlikely that the apical membrane had been damaged. No differences were observed in the absorption of linoleic acid or palmitic acid from the fatty acid-BSA complexes in any of the incubations.

With low (0.1 mM) concentrations of palmitic acid and linoleic acid, we found a significant difference in the basolateral triglyceride secretion of the cells ( $18.3 \pm 2.5\%$  with linoleic acid vs.  $12.7 \pm 2.0\%$  with palmitic acid). With this low fatty acid concentration, no difference was observed in cell morphology or in incorporation of fatty acids into triglyceride and phospholipid (Table 1; Fig. 3, panel b).

With high (1.0 mM) concentrations of linoleic acid and palmitic acid we found distinct differences in lipid synthesis. With 1.0 mM palmitic acid, a higher amount of phospholipid and a lower amount of triglycerides was synthesized compared to the incubation with linoleic acid (Table 1). Phospholipids synthesized in the 1.0 mM palmitic acid incubation were primarily retained in the cells. Both the intracellular amount of saturated phospholipid species (16:0/16:0-phospholipids) and the total mass of intracellular phospholipid were higher in the cells incubated with 1.0 mM palmitic acid compared to 1% BSA or 1.0 mM linoleic acid. Less lipoprotein was secreted with 1.0 mM palmitic acid than with 1.0 mM linoleic acid, measured as lower apoB concentrations in the basolateral media. The basolateral triglyceride secretion was increased compared to the low fatty acid concentration ( $37.4 \pm 12.2\%$  with linoleic acid and  $16.3 \pm 6.7\%$  with palmitic acid; Fig. 1).

Increasing concentrations of palmitic acid caused increasing changes in cell morphology (Fig. 3). With 1.0 mM palmitic acid this resulted in accumulation of intracellular membrane and strongly diminished microvilli. These abnormalities in cell morphology were partly rever-

sible with 0.5 mM linoleic acid. Normal morphology was completely restored with 1.0 mM linoleic acid (Fig. 4).

We hypothesize that in cells incubated with 0.1 mM palmitic acid the cause of the lower secretion capacity is the saturation of endoplasmic reticulum membranes with 16:0/16:0-phospholipids. Membranes composed of saturated lipid species may be abnormally rigid and are therefore likely to hamper vesicular transport. We used the delivery of a short chain sphingomyelin to the apical and basolateral plasma membrane as marker to assay the condition of the secretory pathway (15). In the presence of 1.0 mM palmitic acid, decreased delivery of sphingomyelin to the basolateral side of the Caco-2 cells was observed indicating decreased vesicular transport from the Golgi complex to the plasma membrane.

There may be two possible explanations for the effects of palmitic acid on the cells. First, palmitic acid may cause increased saturation of cellular membranes due to increased synthesis of phospholipids with excess saturated fatty acid species. These more rigid membranes may result in reduced vesicular transport and, therefore, reduced triglyceride and lipoprotein secretion. Synthesis of triglyceride may then be inhibited via a feed-back mechanism. The accumulating pool of free palmitic acid in the cells will subsequently be routed into the phospholipid synthesis pathway. This feed-back hypothesis is not supported directly by data from literature (40, 41) reporting that enterocytes are able to accumulate large amounts of intracellular triglyceride or lipoproteins if secretion is hampered. On the other hand, the tripalmitoylglycerol that is synthesized in the cells during incubation with palmitic acid is not abundantly present in normal physiology. Possibly, excess palmitic acid, accumulating in the cells as a result of reduced vesicular transport, cannot be stored as triglyceride as tripalmitoylglycerol is a solid crystal at 37°C and, as such, potentially toxic for the cell, explaining the preferential incorporation into phospholipids. Secondly, the effects caused by palmitic acid can be the result of a direct metabolic effect. The substrate affinity of palmitic acid for different enzymes involved in triglyceride and phospholipid synthesis is unknown. Nevertheless, our results suggest that palmitic acid may be preferentially incorporated into phospholipids rather than triglycerides. With low palmitic acid concentrations, these more saturated phospholipid species will then be synthesized and this results in a reduction in vesicular transport, explaining the observed low triglyceride secretion. With 1.0 mM palmitic acid, both the decrease in vesicular transport and triglyceride secretion, and the increase in cellular phospholipid can be explained. Eventually, this process results in the accumulation of intracellular membranes observed by electron microscopy.

Incubation of cells with linoleic acid increased the transport of triglycerides and resulted in the secretion of chylomicrons. In 1988, Murthy et al. (9) studied the effect

of chronic incubation of Caco-2 cells with low concentrations of palmitic acid. They found 60% of the radiolabeled palmitic acid in cellular triglycerides. In our experiments, palmitic acid added in a low concentration (0.1 mM) was incorporated equally into triglycerides and phospholipids. With high concentrations, incorporation of fatty acid into phospholipids was 3-fold higher than into triglycerides (Table 1). The differences in the effects of isolated fatty acids, palmitic acid, and linoleic acid, used in our experiments illustrate that the next step in this kind of experiment is to study the metabolic fate of radiolabeled fatty acids in a mixture of fatty acids resembling the human diet. This will provide a physiological situation of assessing lipoprotein secretion by Caco-2 cells.

More triglyceride was secreted into the basolateral than into the apical medium in all incubations. This is in agreement with the results of Trotter and Storch (17) in their 60-min incubations of Caco-2 cells with palmitic acid. Levin and co-workers (42) have reported lack of polarity of triglyceride secretion by Caco-2 cells.

Caco-2 cells incubated with linoleic acid secreted lipoproteins at chylomicron density. In contrast, after incubation with 1.0 mM palmitic acid, cells secreted more dense lipoproteins (Fig. 5, panel A). The amount of triglyceride/apoB (nmol/ng) is 2.5 times higher with linoleic acid than with palmitic acid. A higher triglyceride/apoB ratio implicates lower density of the lipoproteins and confirms the results of the density gradient. These results are in agreement with the results of Kalogeris and Story (43) who reported that chylomicrons isolated from lymph of rats fed butter oil were smaller than those of rats fed corn oil diets.

In the present study, we have compared the effects of 0.1 and 1.0 mM linoleic acid and palmitic acid on polarized monolayers of Caco-2 cells. We studied the uptake and metabolism of the two fatty acids and compared their effects on Caco-2 cell morphology and lipoprotein secretion. After a 24-h incubation with 1.0 mM of either fatty acid, palmitic acid had been preferentially used for phospholipid synthesis. By contrast, linoleic acid was incorporated in equal amounts into triglycerides and phospholipids. Phospholipids were retained in the cells whereas triglycerides were mainly secreted. Furthermore, cells incubated with increasing concentrations of palmitic acid showed increasing accumulation of membranes in the cells, whereas no morphological changes were seen in the cells incubated with linoleic acid and control cells. Vesicular transport was reduced in the Caco-2 cells incubated with 1.0 mM palmitic acid. The effects of 1.0 mM palmitic acid were reversible with 1.0 mM linoleic acid. The triglyceride secretion capacity of the cells was lower with palmitic acid than with linoleic acid with both 0.1 and 1.0 mM fatty acid. Lipoproteins secreted by the Caco-2 cells incubated with linoleic acid were present in the  $d < 1.006$  g/ml or chylomicron fraction whereas those

secreted during palmitic incubation were at LDL density. We conclude from our results that palmitic acid in low concentration induces reduced secretion capacity of the cells. With higher concentrations of palmitic acid this results in increased synthesis of phospholipid which is subsequently retained in the cells giving raise to the observed morphological effects. ■

This work was supported by a fund from the National Nutritional Science Network in the Netherlands and the Foundation of Lipid Research of the Utrecht University Hospital. T. W. A. de Bruin is Senior Clinical Investigator of the Dutch Heart Foundation.

Manuscript received 18 August 1993 and in revised form 14 July 1994.

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