Regulation of triglyceride-rich lipoprotein secretion by fatty acids in CaCo-2 cells

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Abstract The effect of fatty acids on secretion of triglyceride-rich lipoprotein (d < 1.006 g/ml) by CaCo-2 cells was studied. Of the fatty acids studied, oleic acid (18:1) was the most potent stimulator of newly synthesized triglyceride secretion in triglyceride-rich lipoproteins followed in descending order by 18:2, 18:3, and 16:0 = 14:0. All the fatty acids increased intracellular triglyceride synthesis. Fatty acids 14:0, 16:0, 18:2, and 18.3 caused similar increases; however, 18:1 caused the highest rates of triglyceride synthesis. Oleic acid (18:1) was used to further study the secretion of lipoproteins of density < 1.006 g/ml by CaCo-2 cells. There was a step-wise increase in cellular triglyceride synthesis with increasing oleic acid concentration. Above 250 μM of the fatty acid, however, newly synthesized triglyceride secretion in triglyceride-rich lipoproteins plateaued, suggesting saturation of the secretory pathway. After stimulating triglyceride synthesis by oleic acid, radiolabeled triglyceride secreted in triglyceride-rich lipoproteins was initially delayed resulting in a sigmoid-shaped curve for secretion. This was most pronounced in control cells, which were not incubated with the fatty acid. Over 6 hr, cells incubated with oleic acid secreted more newly synthesized triglyceride in triglyceride-rich lipoproteins compared to control cells. The secretion of lipoproteins of density < 1.006 g/ml was dependent upon protein synthesis and normal microtubular function in as much as cycloheximide and colchicine significantly decreased triglyceride transport without changing cellular triglyceride synthesis. Triglyceride and unesterified cholesterol mass in lipoproteins of density < 1.006 g/ml were increased 57 and 244%, respectively, in medium from cells incubated with oleic acid compared to control cells. By 24 hr, 0.17% of lipoproteins of density < 1.006 g/ml were taken up and degraded. Over the same period, approximately 50% of the lipoprotein triglyceride was hydrolyzed. Under conditions whereby lipoprotein secretion was stimulated fourfold by oleic acid, the activities of HMG-CoA reductase and ACAT were unchanged from activities in control cells. The data suggest that CaCo-2 cells secrete triglyceride-rich lipoproteins of density < 1.006 g/ml in response to fatty acids in the medium. Triglyceride-rich lipoprotein secretion is a saturable process and dependent on protein synthesis and normal microtubular function. An increase in triglyceride-rich lipoprotein secretion is accompanied by an increase in triglyceride mass in lipoproteins of density < 1.006 g/ml. By 24 hr, significant postsecretory remodeling of lipoproteins occurs. Short-term stimulation of triglyceride-rich lipoprotein synthesis does not change cholesterol synthesis or esterification in CaCo-2 cells.—**Field, F.J., E. Albright, and S.N. Mathur.** Regulation of triglyceride-rich lipoprotein secretion by fatty acids in CaCo-2 cells. *J. Lipid Res.* 1988. **29:** 1427–1437.

Supplementary key words lipoproteins • HMG-CoA reductase • ACAT

The absorption of free fatty acids by the small intestinal absorptive cell results in an increase in the synthesis of intracellular triglycerides (1, review). Like the liver, an influx of free fatty acids promotes triglyceride secretion in the form of triglyceride-rich lipoprotein particles (2). From the liver, very low density lipoproteins (VLDL) are secreted, whereas the intestine secretes both VLDL and chylomicrons (2). Judging by studies in the liver, not all fatty acids are equal in their ability to stimulate lipoprotein production. Goh and Heimberg (3) found that the equimolar quantities of linoleic and oleic acids caused the secretion of similar amounts of triglyceride in the perfused rat liver. However, a saturated fatty acid, such as palmitic acid, was much less potent. Davis and Boogaerts (4) observed in cultured rat liver cells that triglyceride-rich lipoprotein secretion was highest in cells incubated with oleic acid, followed in descending order by myristic, linoleic, linolenic, and palmitic acids, respectively.

Fatty acids also affect lipoprotein secretion in the intestine. Using a lymph-fistula rat model, Ockner, Hughes, and Isselbacher (2) observed that the fatty acid composition of the lipid that was infused into the duodenum regulated the distribution of lymph triglycerides between VLDL and chylomicrons. Linoleic acid, for example, caused an increase in chylomicron triglyceride. In contrast, palmitate resulted in an increase in both VLDL and chylomicron triglyceride.

Abbreviations: VLDL, very low density lipoproteins; HMG, hydroxymethylglutaryl; ACAT, acyl-CoA:cholesterol acyltransferase.

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The degree of fatty acid saturation, therefore, appeared to regulate intestinal lipoprotein secretion. All of the fatty acids that they tested, including oleic, linoleic, and palmitic acids, caused similar outputs of lymphatic triglyceride. It has also been demonstrated in this experimental model that new protein synthesis and normal microtubular function are important factors for lipoprotein secretion by the intestine (5,6).

In recent reports, CaCo-2 cells have been used to investigate cholesterol metabolism and apolipoprotein secretion (7–10). In the present study, we have utilized these cells to investigate the regulation of cholesterol metabolism and triglyceride-rich lipoprotein lipid secretion by the uptake of fatty acids. The results of this study suggest that the secretion of lipoproteins of density < 1.006 g/ml is regulated by the availability and type of fatty acid taken up by the CaCo-2 cell. The synthesis and secretion of triglyceride-rich lipoproteins by oleic acid do not alter the basal rates of HMG-CoA reductase or ACAT activities in these cells.

MATERIALS AND METHODS

Materials

[1,2-^{3}H]Cholesterol, [carboxyl-^{14}C]triolein, [5-^{3}H] mevalonolactone, [1-^{14}C]oleoyl-coenzyme A, 3-hydroxy-3-methyl[3-^{14}C]glutaryl-CoA, and [2-^{3}H]glycerol were purchased from New England Nuclear (Boston, MA). Iodine-125 was purchased from Amersham Corp. (Arlington Heights, IL). Oleic acid, mevalonolactone, cholesterol, colchicine, cycloheximide, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, nucleotide adenine diphosphate, oleoyl-coenzyme A, palmitic acid, myristic acid, linoleic, and linolenic acids were from Sigma Chemical Co., (St. Louis, MO). HMG-CoA was purchased from P-L Biochemicals, Inc. (Milwaukee, WI). All other chemicals were reagent grade.

Cell cultures

The methodology for culturing CaCo-2 cells has been previously described (7). CaCo-2 cells were used for experimentation on day 11 or 12 after plating in 60-mm plastic petri dishes. Cell viability was monitored by trypan blue exclusion. Viability was not significantly altered by any of the manipulations to be described below.

Fatty acid–albumin preparation

Stock solutions of the fatty acids in 90% ethanol 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 evaporated completely under nitrogen, the fatty acid salt was dissolved in 1.5 ml of hot distilled water and added rapidly to a small amount of M199 containing 10 mM HEPES, pH 7.4, and the appropriate amount of albumin to maintain a fatty acid–albumin ratio of 3:1. The volume was then adjusted with more M199 so that the final concentrations of the fatty acids were those necessary for the experiment.

Triglyceride-rich lipoprotein secretion

The methodology used to estimate triglyceride-rich lipoprotein production was a modification of the methodology described by Davis et al. (11) in rat hepatocytes. CaCo-2 cells were incubated with the respective fatty acid–albumin mixture (3:1, mol/mol) and [^{3}H]glycerol, 500 mCi/mmol (20 μM, 10 μCi/dish) in M199, 10 mM HEPES without fetal calf serum. At the times indicated, the medium was removed and cellular debris was eliminated by centrifugation at 2,000 rpm for 10 min. Two ml of human plasma, which had been heated to 60°C for 10 min, was added as a carrier. The addition of carrier did not alter the distribution of labeled glycerol between lipoproteins of density less than or greater than 1.006 g/ml. The density was adjusted, when necessary, to 1.006 g/ml and the triglyceride-rich lipoproteins were isolated by ultracentrifugation in a Ti-50 rotor at 105,000 *g* for 18 hr. Lipids from the cells and the isolated lipoproteins were extracted with chloroform–methanol 2:1 (v/v). The water phase of the lipoprotein extract was washed once with chloroform. The combined chloroform phases were washed three times with water. The resulting chloroform phases from cells and lipoproteins were dried under nitrogen and the lipids were separated by thin-layer chromatography 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. It was observed that 80% of the glycerol label was incorporated into lipoprotein triglyceride with the remainder in phospholipids. The incorporation of labeled glycerol into total lipoprotein lipids was used as a measure of lipoprotein secretion.

Lipid mass of lipoproteins of density < 1.006 g/ml

CaCo-2 cells were cultured in T-75 flasks. On day 10, the medium was changed to M199 containing 10 mM HEPES, pH 7.4, with or without 250 μM of oleic acid attached to albumin (3:1, mol/mol). The medium in control flasks contained the same amount of albumin as the experimental medium but without the fatty acid. The cells were incubated for 18 hr. The medium was removed and centrifuged at 2,000 rpm for 10 min

to remove cellular debris. The density was adjusted to 1.006 g/ml. Because no carrier could be used to isolate the small amount of secreted lipoproteins, triglyceride-rich lipoproteins were isolated by ultracentrifuging the medium for 30 hr at 105,000 *g* in a Ti-50 rotor. The lipids were extracted from the cells and the lipoprotein fraction with chloroform–methanol 2:1 (v/v). The chloroform phase was dried under nitrogen. Because of the small amount of lipoprotein cholesterol secreted per flask, lipids extracted from lipoproteins secreted by one flask of cells were analyzed for cholesterol only. Both phospholipids and triglycerides could be analyzed from the lipid extract of the lipoprotein fraction from cells in a single T-75 flask.

VLDL degradation and VLDL-triglyceride hydrolysis by CaCo-2 cells

Human VLDL was iodinated with ^{125}I according to the method of McFarlane (12). Ninety-eight percent of the label was precipitated by trichloroacetic acid. ^{125}I-Labeled VLDL (15 μg, 0.5 μCi) was added per dish. Empty dishes served as controls. The amount of ^{125}I-labeled VLDL that was degraded at 3, 6, and 24 hr was determined according to the method of Goldstein, Basu, and Brown (13).

To determine the extent of hydrolysis of VLDL triglyceride by CaCo-2 cells, [^{14}C]triolein, dissolved in a small amount of ethanol, was incubated with human VLDL for 18 hr at 4°C. The VLDL labeled with [^{14}C]triglyceride (175 μg protein, 10,000 cpm) was added to dishes of CaCo-2 cells for 3, 6, and 24 hr. Control dishes were maintained at 4°C and empty dishes served as a means for determining the amount of labeled triglyceride added. At the end of the incubation period, the medium was collected. Lipids were extracted from the medium and the amount of radioactivity remaining in triglycerides was determined by thin-layer chromatography.

Enzyme assays

Acylcoenzyme A:cholesterol acyltransferase activity was measured as previously described using oleoyl-CoA of specific activity 19,250 dpm/nmol (14). 3-Hydroxy-3-methylglutaryl coenzyme A reductase activity was measured as previously described with specific activity of HMG-CoA being 23,800 dpm/nmol (15). Activities were measured in total membrane preparations. CaCo-2 cells were scraped from the dishes and sonicated with sixty 60-watt-sec pulses to disrupt the cells. Excess buffered saline was added and the whole homogenate was centrifuged at 105,000 *g* for 1 hr. The total membrane preparations were washed once and used immediately for enzyme activity determinations.

Chemical analysis

Protein was determined according to the method of Lowry et al. (16). Cholesterol was measured by gas–liquid chromatography as described previously (17). Phospholipids were measured according to the method of Chalvardjian and Rudnicki (18). Intracellular triglycerides were measured fluorometrically (19). Triglyceride-rich lipoprotein triglycerides were measured with an enzymatic kit from Sigma.

Statistical analysis

All values are reported as the mean ± standard error. Differences were determined to be significant by the Student's *t* test using two-tailed *P* values.

RESULTS

Effect of fatty acids on cellular triglyceride synthesis and triglyceride-rich lipoprotein secretion

CaCo-2 cells were incubated with 250 μM of 14:0, 16:0, 18:1, 18:2, and 18:3, and [^{3}H]glycerol for 4 hr. Triglyceride-rich lipoproteins were isolated by ultracentrifuging the medium and 2 ml of human plasma for 18 hr at a density of 1.006 g/ml. Lipids were extracted from the lipoproteins and the cells as described in Methods. Of the labeled triglyceride found in the medium after the 4 hr incubation, 90% was recovered in the lipoprotein fraction of density less than 1.006 g/ml. Moreover, 80% of the glycerol label was incorporated into lipoprotein triglyceride with the remainder found in phospholipids. For simplicity, the incorporation of labeled glycerol into cellular triglycerides and triglyceride-rich lipoprotein lipids is shown (**Fig. 1**). All the fatty acids tested significantly increased the synthesis of triglycerides within CaCo-2 cells when compared to the synthesis of triglycerides observed in control cells incubated without a fatty acid. The effect of 14:0, 16:0, 18:2, and 18:3 on triglyceride synthesis was similar, whereas 18:1 significantly increased the rate of triglyceride synthesis more so than the other fatty acids. In contrast, the saturated fatty acids 14:0 and 16:0 did not significantly stimulate triglyceride-rich lipoprotein secretion above the amount secreted by control cells. Glycerol incorporation into triglyceride-rich lipoprotein lipids, however, was significantly stimulated by 18:2 and 18:3, even though these fatty acids caused increases in intracellular triglyceride synthesis equal to those produced by the saturated fatty acids, 14:0 and 16:0. Oleic acid was the most potent stimulator of secretion of lipoproteins of density less than 1.006 g/ml compared to the other fatty

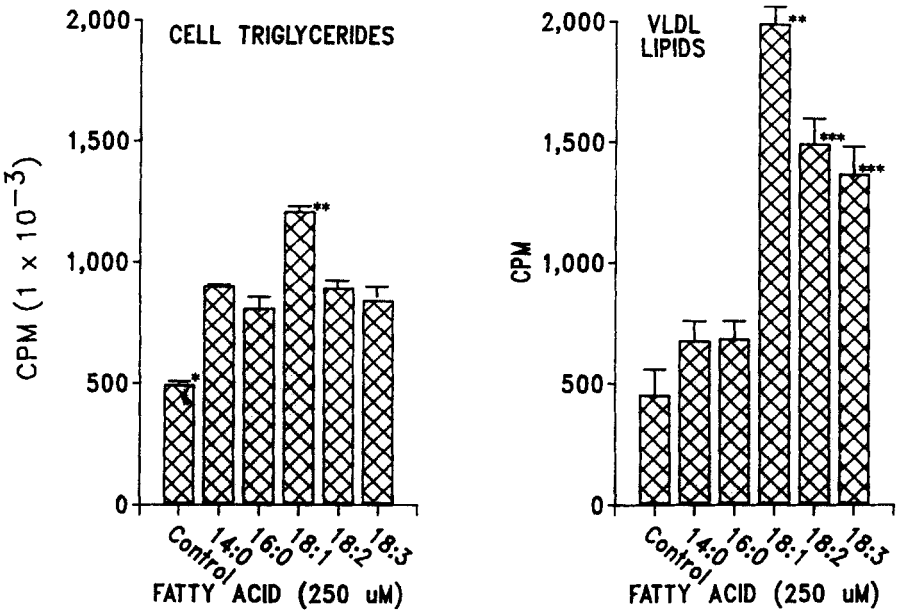

Fig. 1. Effect of fatty acids on cellular triglyceride synthesis and triglyceride-rich lipoprotein secretion. CaCo-2 cells were incubated for 4 hr with 250 μM 14:0, 16:0, 18:1, 18:2, and 18:3 attached to albumin in a 3:1, mol/mol ratio in M199 without serum. Control cells were incubated with 83 μM albumin in the absence of the fatty acid. [^{3}H]Glycerol was added at the same time as the fatty acid. At the end of the incubation period, triglyceride-rich lipoproteins were isolated from the medium after adding plasma as a carrier and ultracentrifuging at a density of 1.006 g/ml. Lipids were extracted from the cells and the lipoproteins, and the incorporation of labeled glycerol into cell triglycerides and lipoprotein lipids was determined. The ordinate is expressed as cpm of glycerol incorporated per dish. The data represent the mean ± SE of at least six dishes. *$P < 0.001$ vs all fatty acids; ** $P < 0.01$ vs 14:0, 16:0, 18:2, 18:3; ***$P < 0.001$ vs control, 14:0, 16:0.

acids. Oleic acid stimulated triglyceride-rich lipoprotein secretion four-fold more than the secretion of these lipoproteins by control cells. Oleic acid was used, therefore, to further study triglyceride-rich lipoprotein secretion by CaCo-2 cells.

Effect of oleic acid concentration on cellular triglyceride synthesis and triglyceride-rich lipoprotein secretion

CaCo-2 cells were incubated for 4 hr with increasing concentrations of oleic acid. The fatty acid to albumin ratio was kept constant in the medium of all dishes. Medium in control dishes contained 83 μM albumin without the fatty acid. As the concentration of oleic acid was increased in the medium, the incorporation of labeled glycerol into cellular triglycerides increased in step-wise fashion up to 1 mM of oleic acid (**Fig. 2**). In contrast, there was a limit to what the CaCo-2 cells could secrete. Triglyceride-rich lipoprotein secretion increased with increasing oleic acid concentrations up to 250 μM of the fatty acid. Above 250 μM of oleic acid, however, glycerol incorporation into triglyceride-rich lipoprotein lipids plateaued. The incorporation of glycerol into lipids of lipoproteins of density greater than 1.006 g/ml did not change with increasing oleic acid concentrations, suggesting that there was not a redistribution of the glycerol label into more dense lipoproteins at higher concentrations of oleic acid.

Effect of oleic acid on cellular triglyceride synthesis and triglyceride-rich lipoprotein secretion

A time course for the effect of oleic acid on triglyceride synthesis and lipoprotein secretion was then studied. CaCo-2 cells were incubated with or without 250 μM oleic acid for 6 hr and the incorporation of labeled glycerol into cellular triglycerides and triglyceride-rich lipoprotein lipids was measured. **Fig. 3** shows these results. The rate of triglyceride synthesis within CaCo-2 cells was significantly increased in cells incubated with oleic acid. The stimulation of intracellular triglyceride synthesis by oleic acid occurred within 1 hr and was linear for at least 4 hr before plateauing somewhat at 6 hr. Triglyceride synthesis in control cells was much slower but was clearly measurable 1 hr

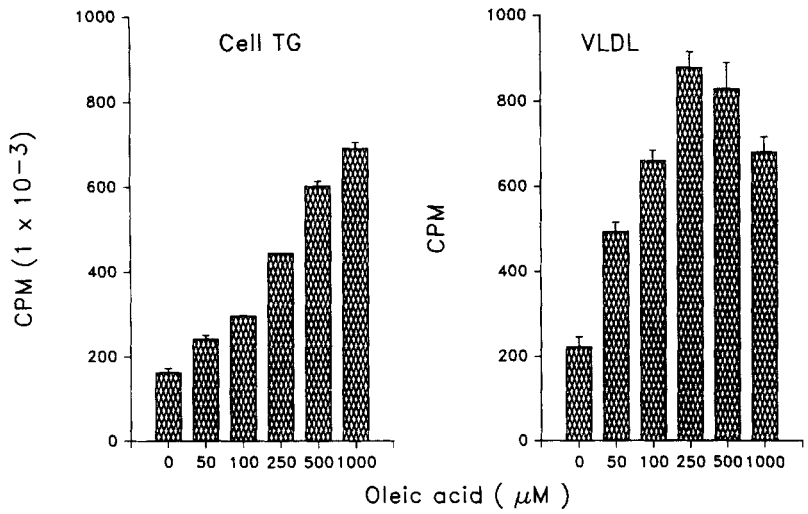

Fig. 2. Effect of oleic acid concentration on cellular triglyceride synthesis and triglyceride-rich lipoprotein secretion. CaCo-2 cells were incubated for 4 hr with increasing concentrations of oleic acid attached to albumin in a 3:1, mol/mol ratio. Control cells were incubated with 83 μM albumin without the fatty acid. The incorporation of labeled glycerol into cellular triglycerides and lipoprotein lipids was determined as described in Fig. 1. The data represent the mean ± SE of three dishes. All fatty acid concentrations are significantly different from control, $P < 0.02$.

after adding the labeled glycerol. In contrast, the appearance of the triglyceride label in triglyceride-rich lipoproteins was delayed, especially so in the control cells without the fatty acid. In control cells, detectable radiolabeled triglyceride secreted in lipoproteins of density less than 1.006 g/ml was not observed until 3 hr after adding the labeled glycerol. Moreover, in CaCo-2 cells incubated with oleic acid, the secretion of lipoproteins of density < 1.006 g/ml was detected at 1 hr. The rate of secretion increased rapidly over 4 hr before leveling off at 6 hr. At each time point, the amount of labeled triglyceride that was secreted was higher in cells incubated with the fatty acid.

Effect of oleic acid on triglyceride-rich lipoprotein lipid mass

CaCo-2 cells were grown in T-75 flasks. On day 10 after plating, the medium was changed to serum-free medium containing 250 μM of oleic acid attached to albumin. Control medium contained the same amount of albumin but without the fatty acid. After 18 hr, the medium was removed and lipoproteins of density less than 1.006 g/ml were isolated by ultracentrifugation as described in Methods.

Table 1 shows the values for the triglyceride-rich lipoproteins as well as intracellular lipids. In CaCo-2 cells incubated with oleic acid, triglyceride content was significantly increased compared to the triglyceride content of control cells. Total cholesterol within cells was not significantly different between cells incubated with or without the fatty acid. However, there was a tendency for more unesterified and less esterified cholesterol in CaCo-2 cells incubated with oleic acid. Phospholipid content was similar in the two groups of cells. CaCo-2 cells incubated with oleic acid secreted almost 60% more triglyceride into the medium in lipoproteins of density < 1.006 g/ml as compared to the triglyceride secreted by control cells. More cholesterol was secreted by cells incubated with oleic acid with the increase occurring in the unesterified fraction. The phospholipid content of the lipoproteins was significantly increased in cells incubated with oleic acid.

It is possible that after 18 hr of incubation, the lipids that are secreted in triglyceride-rich lipoproteins by CaCo-2 cells may not represent the composition of nascent lipoproteins. Postsecretory remodeling of the lipoproteins by the cells could have occurred. To investigate whether there was significant reuptake and degradation of secreted lipoproteins of density < 1.006 g/ml by CaCo-2 cells, the degradation of ^{125}I-labeled VLDL (15 μg, 0.5 μCi) was determined by incubating the cells with labeled VLDL for 3, 6, and 24 hr. The amount of VLDL that was degraded was 6.4 ± 0.3, 10.6 ± 0.3, and 26.4 ± 0.9 ng/mg cell protein, respectively. At 24 hr, this corresponds to 0.17% of the VLDL that was added.

To examine whether CaCo-2 cells hydrolyze triglyceride-rich lipoprotein triglyceride, cells were incubated with VLDL containing the labeled triolein for 3, 6, and 24 hr. The amount of triolein that was hydrolyzed was then determined. Control cells were incubated under identical conditions except that they were

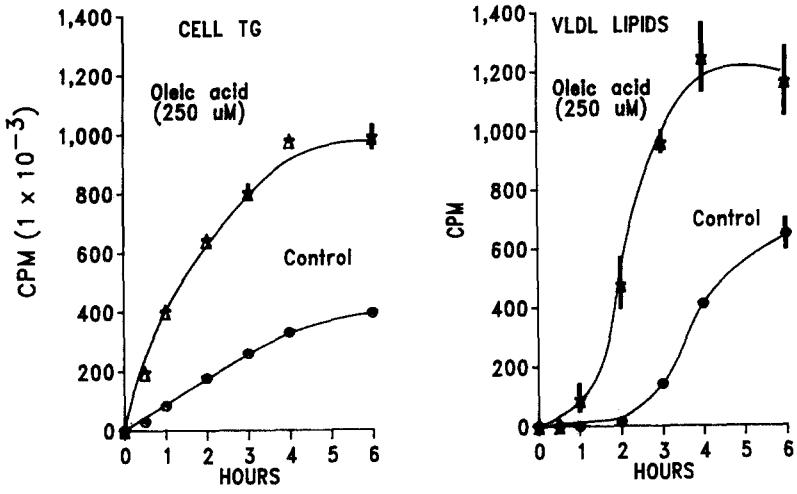

Fig. 3. Time course for cellular triglyceride synthesis and triglyceride-rich lipoprotein secretion. CaCo-2 cells were incubated with or without 250 μM oleic acid for 0.5, 1, 2, 3, 4, and 6 hr. [^{3}H]Glycerol was added at the same time as the fatty acid. The incorporation of labeled glycerol into cellular triglycerides and lipoprotein lipids was determined as described in Fig. 1. The data points represent the mean ± SE of at least six dishes at each time point. All points generated from dishes containing oleic acid were significantly different from control ($P < 0.01$) except for the 30-min time point in VLDL lipids.

kept at 4°C. **Table 2** shows these data. At 3 and 6 hr, a modest but significant amount of VLDL triglyceride was hydrolyzed. Moreover, compared to control cells at 24 hr, 50% of the triglyceride label was hydrolyzed. The data from this experiment and the one described above suggest that CaCo-2 cells can significantly modify the secreted lipoprotein lipid. The triglyceride mass measurements of lipoproteins of density < 1.006 g/ml as shown in Table 1 underestimate the amount of triglyceride secreted in nascent particles. This will be discussed later in more detail.

Effect of cycloheximide and colchicine on triglyceride synthesis and triglyceride-rich lipoprotein secretion

To investigate the need for new protein synthesis during triglyceride-rich lipoprotein secretion, CaCo-2 cells were preincubated for 30 min with 0.1 mM cycloheximide. Oleic acid was then added and labeled glycerol incorporation into cell triglycerides and triglyceride-rich lipoproteins was determined after 4 hr. **Fig. 4** shows these results. New protein synthesis, as

TABLE 1. Cell and triglyceride-rich lipoprotein lipids

	Cholesterol			
	Unesterified	Ester	Triglycerides	Phospholipids
	μg/mg protein per 18 hr			
Triglyceride-rich lipoproteins				
Control	0.016 ± 0.003	0.018 ± 0.005	0.65 ± 0.06	0.121 ± 0.010
Oleic acid	0.055 ± 0.005*	0.023 ± 0.003	1.02 ± 0.06*	0.175 ± 0.003*
	μg/mg protein			
Cells				
Control	22.9 ± 0.24	0.60 ± 0.16	58 ± 1	397 ± 16
Oleic acid	24.2 ± 0.44	nd	81 ± 1*	460 ± 23

CaCo-2 cells were grown in T-75 flasks for 10 days. The medium was then changed to M199 without serum containing 83 μM BSA or 250 μM oleic acid attached to albumin (3:1, mol/mol) for 18 hr. The medium was collected and lipoproteins of density less than 1.006 g/ml were isolated by ultracentrifugation for 30 hr as described in Methods. Lipids were extracted from the cells and lipoproteins and analyzed as described in Methods (n = 12 flasks); nd, none detected; *, $P < 0.001$ vs control.

TABLE 2. Hydrolysis of VLDL-[^{14}C]triolein by CaCo-2 cells

Hours	^{14}C-Labeled Triglycerides		% Hydrolysis
	4°C	37°C	
	cpm/dish		
3	11,226 ± 334	9,513 ± 195	15*
6	10,758 ± 140	8,497 ± 55	21*
24	9,019 ± 112	4,536 ± 84	50**

[^{14}C]Triolein was complexed to human VLDL as described in Methods. VLDL, 175 μg protein containing approximately 10,000 cpm, was added to the dishes. Control dishes were maintained at 4°C. At the end of the incubation period, the medium was collected. The lipids were extracted and labeled triglycerides were isolated by thin-layer chromatography. Labeled triglycerides isolated from the medium of empty dishes contained 10,050 ± 200 cpm. The data are expressed as the mean ± SE of three dishes at each time point. *, $P < 0.05$ vs control; **, $P < 0.001$ vs control.

measured by labeled leucine incorporation into TCA-precipitable protein, was inhibited by 93% (data not shown). The incorporation of labeled glycerol into cellular triglycerides was not appreciably affected by cycloheximide. The stimulation of triglyceride synthesis by oleic acid in cells incubated with cycloheximide was similar to the degree of stimulation observed in cells incubated without the protein inhibitor. In contrast, the secretion of triglyceride-rich lipoproteins was significantly decreased in cells incubated with cycloheximide.

To study the role of microtubules in triglyceride-rich lipoprotein secretion, colchicine, 100 μM, was added to the media of CaCo-2 cells for 18 hr prior to stimulating lipoprotein secretion with oleic acid. As shown in **Fig. 5,** cells incubated with colchicine had significantly more of the glycerol label in cellular triglycerides than did control cells without colchicine. The stimulation of triglyceride-rich lipoprotein secretion by oleic acid, however, was completely blocked in CaCo-2 cells preincubated with colchicine. Both experiments suggest that new protein synthesis and normal microtubular function are important in the secretion of lipoproteins of density < 1.006 g/ml by CaCo-2 cells.

Effect of oleic acid on HMG-CoA reductase and ACAT activities

Since cholesterol, both free and esterified, is an integral component of the triglyceride-rich lipoprotein, the effect of inducing lipoprotein synthesis and secretion by oleic acid on cholesterol metabolism was studied. Whole membrane preparations were prepared

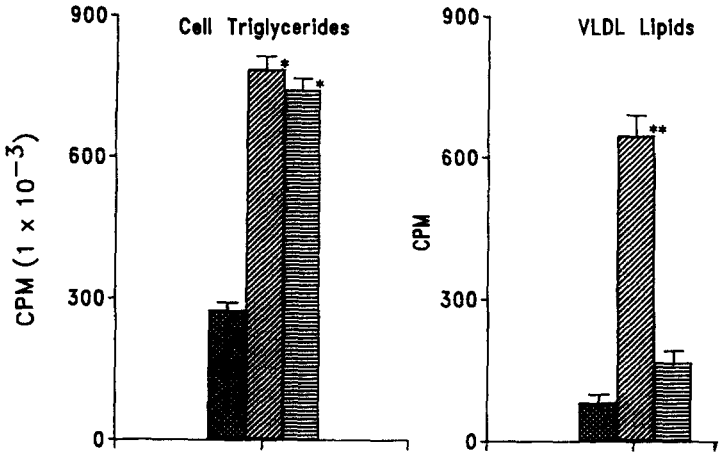

Fig. 4. Effect of cycloheximide on cellular triglyceride synthesis and triglyceride-rich lipoprotein secretion. CaCo-2 cells were preincubated with or without 0.1 mM cycloheximide for 30 min prior to additing 250 μM oleic acid and [^{3}H]glycerol for 4 hr. Cycloheximide was present in the dishes for the full length of the experiment. The incorporation of labeled glycerol into cellular triglycerides and lipoprotein lipids was determined as described in Fig. 1. (▩) control; (▨) oleic acid; (▤) oleic acid + cycloheximide. The data represent the mean ± SE of six dishes; *$P < 0.001$ vs control; **$P < 0.001$ vs control and oleic acid + cycloheximide.

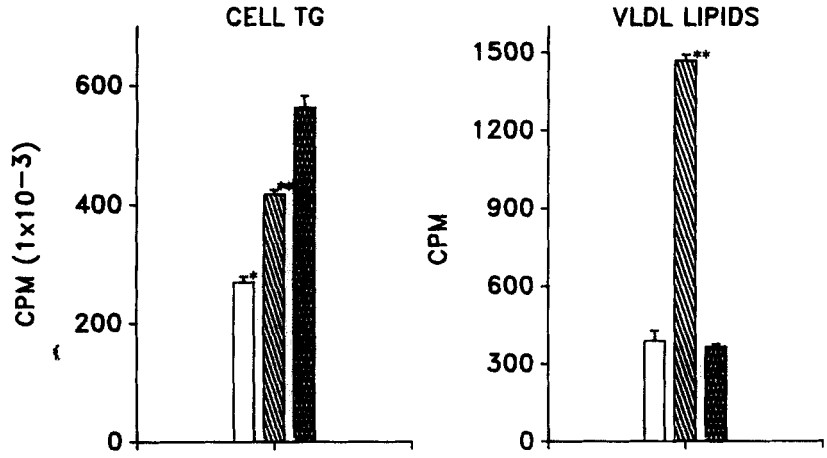

Fig. 5. Effect of colchicine on cellular triglyceride synthesis and triglyceride-rich lipoprotein secretion. CaCo-2 cells were preincubated for 18 hr in the presence or absence of 0.1 mM colchicine. [^{3}H]Glycerol and 250 μM oleic acid were then added for 4 hr and the incorporation of labeled glycerol into cellular triglycerides and lipoprotein lipids was measured. (□) Control; (▨) oleic acid; (■) oleic acid + colchicine. The data represent the mean ± SE of six dishes; *$P < 0.001$ vs oleic acid and oleic acid + colchicine; **$P < 0.001$ vs control and oleic acid + colchicine.

from CaCo-2 cells incubated in the presence or absence of 250 μM of oleic acid for 1, 2, and 4 hr. The activities of both HMG-CoA reductase and ACAT were measured. At no time point was there a significant difference between the activities of either enzyme from membranes prepared from control cells or cells incubated with the fatty acid.

DISCUSSION

The rates of intracellular triglyceride synthesis and the secretion of triglyceride-rich lipoproteins of density < 1.006 g/ml were significantly increased in CaCo-2 cells incubated with the unsaturated fatty acids, 18:1, 18:2, and 18:3. In this regard, CaCo-2 cells are very similar to cultured rat hepatocytes which have been shown to secrete triglyceride-rich lipoproteins in response to the addition of fatty acids to the culture medium (11). In both CaCo-2 cells and isolated rat hepatocytes, oleic acid was the most potent stimulator of triglyceride-rich lipoprotein secretion, whereas the saturated fatty acid, palmitic acid, was the least potent. In contrast to these findings, lymph triglyceride output was shown to be similar in lymph-fistula rats that had their duodenums perfused with palmitic or oleic and linolenic acids (2). A simple explanation for this difference may be related to the colonic origin of CaCo-2 cells. These cells may differ considerably from rat small intestinal absorptive cells as to their capacity for synthesizing and secreting lipoproteins. Aside from tissue or even species differences, however, there are other factors that can influence triglyceride output into lymph of lymph-fistula animals other than the fatty acids that are infused into the duodenum. Altering the rate of lymph flow has been shown to regulate triglyceride transport (20). Exogenous or biliary phosphatidylcholine can result in an increase in triglyceride output (21) as well as certain dietary measures prior to the infusion of different fatty acids (22). Under controlled conditions such as those described in the present study, the regulation of triglyceride-rich lipoprotein secretion in CaCo-2 cells by the addition of fatty acids to the medium can be investigated without interference from these other variables.

In the liver, the esterification rate for triglyceride synthesis is not saturable, whereas the rate of triglyceride secretion is limiting (23,24). During maximal uptake of fatty acids, therefore, there is an increase in triglyceride mass within hepatocytes. In the present study, the release of newly synthesized triglyceride in triglyceride-rich lipoproteins was also limiting despite continued uptake of free fatty acids and increased rates of triglyceride synthesis. Maximal secretion of newly synthesized triglyceride was reached at an oleic acid concentration of 250 μM in the medium. Moreover, triglyceride mass that was secreted in lipoproteins of density less than 1.006 g/ml was increased in CaCo-2 cells incubated with 250 μM of oleic acid. Despite an increase in triglyceride output, CaCo-2 cells that were incubated with the fatty acid accumulated significantly more intracellular triglyceride compared to the triglyceride content observed in control cells. Both in the hepatocyte and the CaCo-2 cell there is a limit as to the amount of triglyceride that can be secreted in response to an excess of fatty acid uptake.

Triglyceride-rich lipoprotein secretion by CaCo-2 cells did not occur immediately in response to an increase in triglyceride synthesis within the cell. Not only was there a delay in the secretion of newly synthesized triglyceride, but the rate of secretion was very slow soon after stimulation of triglyceride synthesis by oleic acid. It makes sense that there be a certain interval between the synthesis of triglycerides, the intracellular assembly of the lipoprotein particle, and its transfer to the basolateral membrane for secretion. The delay was much more pronounced in control CaCo-2 cells which had no driving force to transport triglycerides.

New protein synthesis and normal microtubular function were important factors in lipoprotein secretion in CaCo-2 cells just as they are in the intestines of lymph-fistula rats (5,6). The decrease in lipoprotein secretion that resulted from the presence of cycloheximide or colchicine in the culture medium was not secondary to a decrease in the availability of labeled triglyceride within the cell. Neither agent adversely affected the incorporation of labeled glycerol into cellular triglycerides. In fact, cells that were incubated with colchicine had increased amounts of labeled triglyceride, suggesting that the complete block of lipoprotein secretion resulted in the accumulation of this lipid within the cell. Both in the liver and intestine, the effect of colchicine on lipoprotein secretion has been shown to occur distal to the site of cellular triglyceride synthesis (25,6). Our results would agree with those data. Moreover, cycloheximide did not affect triglyceride synthesis either, as evidenced by the similar amounts of labeled triglycerides in control and cycloheximide-treated cells. This agrees with the normal rates of lipid esterification that have been observed in the intestines of animals treated with protein synthesis inhibitors (26). Therefore, the inhibition of lipoprotein secretion by cycloheximide most likely occurs between the reesterification of the lipids within the enterocyte and the egress of the lipoprotein particle from the cell.

As shown in Fig. 3, the presence of oleic acid resulted in a fourfold increase in labeled glycerol incorporation into triglyceride-rich lipoprotein lipids at 4 hr. In contrast, the amount of triglyceride mass secreted in lipoproteins of density less than 1.006 g/ml was only increased by 60% in CaCo-2 cells incubated with the fatty acid for 18 hr. This suggests, perhaps, that the secretion of labeled triglyceride may not represent the true flux of triglyceride from the cells, or that newly synthesized triglyceride was being preferentially secreted soon after stimulating triglyceride-rich lipoprotein production by oleic acid. To test this, the specific activity of secreted triglyceride in lipoproteins of density < 1.006 g/ml was compared to the specific activity of intracellular triglyceride at 4, 8, and 12 hr after incubating the cells with labeled glycerol and 250 μM oleic acid. The mass of secreted triglyceride in triglyceride-rich lipoproteins increased at each time point. The specific activity of the lipoproteins increased at each time point. The specific activity of the lipoprotein triglyceride, however, was significantly higher than the specific activity of the intracellular triglyceride at 4 hr, 1373 ± 39 cpm/μg triglyceride vs 873 ± 23 cpm/μg ($P < 0.001$). The calculated triglyceride flux as determined by the radiolabel data overestimated the mass of triglyceride flux that was measured at this time point. At the later time points of 8 and 12 hr, the specific activities of secreted and cellular triglyceride were not significantly different and the radiolabel data reflected accurately the true flux of triglyceride. The data suggest that the secreted radiolabeled triglyceride in triglyceride-rich lipoproteins does represent triglyceride flux. At times prior to 4 hr, however, there is secretion of a disproportionate amount of newly synthesized triglyceride, suggesting that the label has not fully equilibrated with all precursor pools of triglyceride within the cell.

Unlike Hep G2 cells, which are not thought to extensively remodel secreted lipoproteins (27), the present data strongly suggest that CaCo-2 cells significantly modify secreted triglyceride-rich lipoproteins. The degradation of 26.4 ng of VLDL by 24 hr probably represents a substantial proportion of the small amount of triglyceride-rich lipoproteins that are secreted. Moreover, the significant amount of hydrolysis of secreted lipoprotein triglyceride by CaCo-2 cells that was observed suggests that lipase activity is present either on the cell surface or in the medium as a result of secretion. It is clear, therefore, that measurements of lipoprotein secretion by CaCo-2 cells represent not only the secretion of nascent lipoprotein particles but also products from partial degradation and hydrolysis.

During the preparation and writing of this manuscript, two published reports have addressed the regulation of apolipoprotein B synthesis and secretion by fatty acids in CaCo-2 cells. Hughes, Ordovas, and Schaefer (9) found that fatty acids complexed to albumin did not affect the rate of apolipoprotein B secretion by CaCo-2 cells. There was, however, a significant redistribution of the apolipoprotein from low and high density lipoproteins to very low density lipoproteins. Ellsworth, Erickson, and Cooper (28) also observed a redistribution of newly synthesized apolipoprotein B from low density to very low density lipoproteins secreted by Hep-G2 cells incubated with oleic acid. We also found an increase in newly synthesized apolipoprotein B that was secreted in lipoproteins of density < 1.006 g/ml in CaCo-2 cells incubated with oleic acid supporting the previous reports (personal observa-

tions). In contrast, Traber, Kayden, and Rindler (10) found an increase in the total secretion of apolipoprotein B-100 and B-48 by CaCo-2 cells in response to the presence of oleic acid. Their experiment, however, was performed using lipoprotein-deficient serum in cells grown on porous filters, making it difficult to compare the two studies. Our observations and the results of the two former studies suggest that the increased incorporation of fatty acids into cellular lipids and their eventual secretion in triglyceride-rich lipoproteins result in a shift of apolipoprotein B from the more dense lipoproteins to lipoproteins that are responsible for transporting the triglyceride from the cell.

In the perfused rat liver, Goh and Heimberg (3) found that infusing oleic acid caused an increase in hepatic microsomal HMG-CoA reductase activity. They suggested that new cholesterol synthesis was required for lipoprotein secretion and that this was most likely related to a need for more surface material for the newly assembled lipoprotein. This did not occur in CaCo-2 cells. Over a 4-hr period, during a time when newly synthesized triglyceride secretion was increased fourfold by incubating cells with 250 μM oleic acid, the activities of HMG-CoA reductase and ACAT were unchanged compared to the activities in control cells. It is possible that the 4-hr time period used in this experiment was too short to deplete the cellular cholesterol stores utilized for lipoprotein synthesis. Thus, the enterocyte does not need to acutely regulate cholesterol metabolism. This does not explain the differences between the present study and the study by Goh and Heimberg (3), however, as reductase activity was regulated over a 4-hr period of liver perfusion in that study. The liver may be different from intestine. Cholesterol in triglyceride-rich hepatic lipoproteins may be derived largely from newly synthesized cholesterol whereas that may not be the case for the intestine. More likely, however, the basal rates of cholesterol synthesis and esterification within CaCo-2 cells are more than adequate to provide the cell with the necessary cholesterol requirements for lipoprotein assembly and secretion over this short time period.

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