Triacylglycerol-rich lipoprotein cholesterol is derived from the plasma membrane in CaCo-2 cells

F. Jeffrey Field,[1] Ella Born, and Satya N. Mathur

Department of Internal Medicine, University of Iowa and the Veterans Administration, Iowa City, IA 52242

Abstract The source for triacylglycerol-rich lipoprotein cholesterol was investigated in CaCo-2 cells grown on filters separating an upper and a lower well. Oleic acid, a fatty acid that promotes triacylglycerol-rich lipoprotein synthesis and secretion in CaCo-2 cells, increased the vesicular-mediated influx of plasma membrane cholesterol to the endoplasmic reticulum. Unesterified and esterified cholesterol derived from the plasma membrane were increased in triacylglycerol-rich lipoproteins secreted by cells incubated with oleic acid. Fatty acids, which increased the number of lipoprotein particles secreted (increased apoB secretion), increased plasma membrane cholesterol influx and secretion. Oleic acid caused a modest increase in the synthesis of cholesterol and a two-fold increase in cholesteryl esters. The amount of newly synthesized cholesterol secreted in lipoproteins of density < 1.006 g/ml represented a small fraction of that present within the cell; however, oleic acid did increase the amount of both newly synthesized cholesterol and cholesteryl esters in triacylglycerol-rich lipoproteins. Oleic acid did not affect the fraction of newly synthesized cholesterol trafficking to the plasma membrane. Compared to cholesterol delivered to cells in micelles, plasma membrane cholesterol was the much preferred substrate for acyl-CoA:cholesterol acyltransferase. Micellar cholesterol displaced cholesterol from the plasma membrane causing more of it to influx intracellularly for esterification and secretion. We propose that plasma membrane cholesterol is the major source for triacylglycerol-rich lipoprotein cholesterol in CaCo-2 cells. Micellar cholesterol and newly synthesized cholesterol replenish the plasma membrane cholesterol that is being used for the transport of lipids.—**Field, F. J., E. Born, and S. N. Mathur.** Triacylglycerol-rich lipoprotein cholesterol is derived from the plasma membrane in CaCo-2 cells. *J. Lipid Res.* 1995. **36:** 2651-2660.

Supplementary key words cholesterol • triacylglycerol • apoB • cholesterol trafficking

The mechanisms involved in the assembly and secretion of triacylglycerol-rich lipoprotein particles by the intestine are poorly understood. These large particles, which essentially transport all dietary and biliary lipids into the blood, are composed of polar lipids and apolipoproteins on the surface of the particle and neutral lipids within the core (1). It is believed that the endoplasmic reticulum (ER) is the site at which lipids and apolipoproteins, particularly apolipoprotein B, are assembled together following the translocation of apoB into the lumen of the ER (2–4). In response to an influx of fatty acid and the need to transport triacylglycerols, the intestinal cell increases the secretion of apoB, suggesting that more lipoprotein particles are being secreted to accommodate an increased flux of lipid (5–7). It is clear, therefore, that after the ingestion of fat and the promotion of lipoprotein production, the intestinal cell must also recruit or synthesize the necessary lipid components of the lipoprotein particle to ensure normal assembly and transport. Cholesterol, both unesterified and esterified, is an important component of the triacylglycerol-rich lipoprotein and is found on the surface and in the core of the particle, respectively. In the liver, it has been suggested that the synthesis of cholesterol and/or cholesteryl esters may be regulatory in the secretion of VLDL (8, 9). It is not clear in the intestine, however, where lipoprotein cholesterol originates within the absorptive cell and whether cholesterol availability affects lipoprotein synthesis and secretion.

The present study was performed in the human intestinal cell line, CaCo-2, to investigate the cellular origins of triacylglycerol-rich lipoprotein cholesterol. The results suggest that in cells incubated with micellar solutions of oleic acid and/or cholesterol, the amount of plasma membrane cholesterol that influxes to the endoplasmic reticulum is increased. Cholesterol derived from the plasma membrane is then used for the assembly and secretion of triacylglycerol-rich lipoprotein particles. The plasma membrane cholesterol pool is then, in turn, replenished by cholesterol derived from the lumen and from newly synthesized cholesterol. We can-

Abbreviations: ER, endoplasmic reticulum; VLDL, very low density lipoprotein; ACAT, acyl-CoA:cholesterol acyltransferase.

[1]To whom correspondence should be addressed.

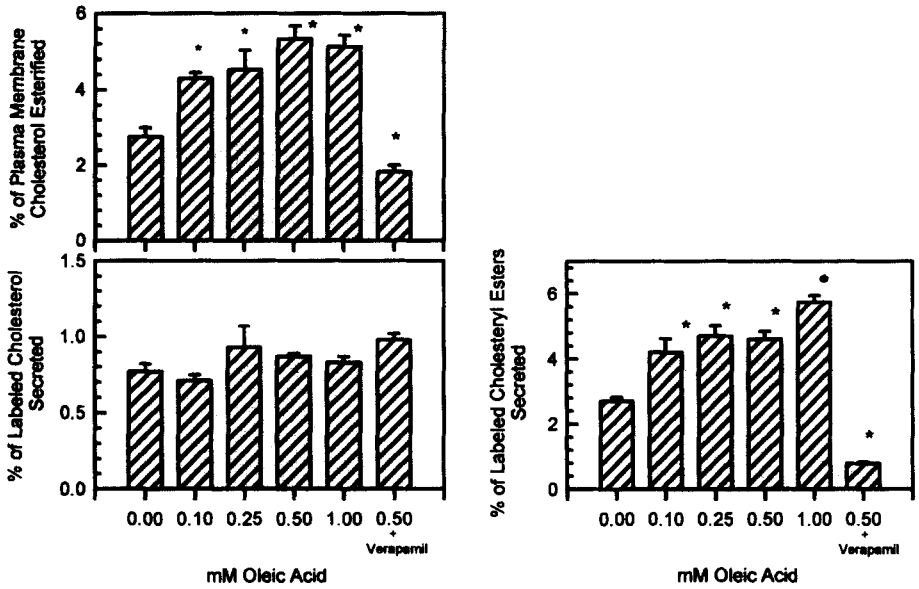

Fig. 1. Effect of oleic acid on esterification of plasma membrane cholesterol and its secretion into the basolateral medium. CaCo-2 cells were incubated with [^{3}H]cholesterol for 90 min at 4°C to label plasma membrane cholesterol as described in Methods. After the labeling, cells were incubated for 5 h at 37°C with 1 ml M199 containing 5 mM taurocholate or taurocholate and increasing concentrations of oleic acid. In one set of cells incubated with taurocholate and 0.5 mM oleic acid, verapamil 50 μM was included in the incubation. Lipids were extracted from cells and the basolateral medium and separated by thin-layer chromatography as described in Methods. Bands corresponding to unesterified and esterified cholesterol were scraped from the plate and counted. The data represent the means ± SE of four determinations for each treatment from one representative experiment of three sets of incubations; $*P < 0.05$ vs. taurocholate.

not rule out the possibility, however, that a small fraction of newly synthesized cholesterol is used directly for lipoprotein assembly without first going to the plasma membrane.

MATERIALS AND METHODS

[7-^{3}H]cholesterol, [^{14}C]cholesterol, [^{3}H]water, and [^{14}C]acetate were purchased from New England Nuclear (Boston, MA). [^{3}H]cholestenone was prepared by incubating [3H]cholesterol with cholesterol oxidase and recovering the labeled cholestenone by thin-layer chromatography. Cholesterol, cholestenone, sodium taurocholate, oleic acid, palmitic acid, linoleic acid, eicosapentaenoic acid, arachidonic acid, and verapamil were purchased from Sigma Chemical Co. (St. Louis, MO). ApoB and apoB monoclonal antibody (clone No. 1607) (immunoglobulin G 26 fraction purified by column chromatography) and apoB sheep immunopurified polyclonal antibody conjugated to horseradish peroxidase were from Biodesign International (Kennebunkport, ME). TMB Microwell Peroxidase Substrate System was from Kirkegaard and Perry Labs Inc. (Gaithersburg, MD). Ninety-six well Nunc-Immuno plates were from VWR Scientific (Batavia, IL). All other reagents were reagent grade.

Cell culture

CaCo-2 cells were grown in T-75 flasks as described previously (10). They were subcultured on polycarbonate micropore membranes inserted in Transwells (Costar, Cambridge, MA). Cells were used 14 days after plating and medium was changed every 2 days.

Esterification of plasma membrane cholesterol

Plasma membrane cholesterol was labeled by incubating cells for 90 min at 4°C with 5 μCi [^{3}H]cholesterol in 0.4 ml of M199 (medium-199/Earle's, Gibco, Grand Island, NY) containing 1% delipidated fetal calf serum. Radiolabeled cholesterol was added to this medium in ethanol. The final concentration of ethanol in the medium was 1.2%. The solution containing the labeled cholesterol was added to the top well (apical side) only. To remove unincorporated labeled cholesterol, cells were washed twice with cold M199. They were then incubated at 37°C in 1 ml of M199 containing the different solutions to be tested. After the incubation, cells were washed with cold phosphate-buffered saline and the lipids were extracted twice directly from the cells on the filter by adding 1 ml hexanes–isopropanol–water 3:2:0.1 (v/v). Unlabeled cholesterol and cholesteryl oleate were added as carriers. The lipids were separated by thin-layer chromatography, and cholesterol and cholesteryl esters were localized by authentic standards, scraped from the plate, and counted.

Using the cholesterol oxidase method (described below) to ensure that plasma membrane cholesterol was being labeled and not intracellular organelles, we observed that after labeling CaCo-2 cells for 90 min at 4°C, between 85 and 92% of labeled cholesterol was accessible to cholesterol oxidase. Thus, the labeling technique used in this study appears to label predominately cholesterol of the plasma membrane.

Oxidation of plasma membrane cholesterol

The methodology used was described by Freeman for cultured cells (11). After the incubation as described for each experiment, the cells were placed on ice. The media were removed and the cells were washed twice with ice-cold phosphate-buffered saline. After washing two more times with 10 mM sodium phosphate buffer (pH 7.4), the cells were incubated for 10 min at 4°C with water. The water was removed and the cells were fixed by incubating them for 10 min at 4°C with 1% glutaraldehyde. After removing the glutaraldehyde, the cells were washed twice at 37°C with 10 mM sodium phosphate buffer (pH 7.4) containing 310 mM sucrose. They were then incubated in this buffer for 15 min at 37°C. Cholesterol oxidase, 100 IU/ml, was added and the cells were incubated for 45 min at 37°C. The cells were again washed twice with cold phosphate-buffered saline and the lipids were extracted with hexanes–isopropanol–water 3:2:0.1 (v/v). Unlabeled cholestenone and [^{3}H]cholestenone were added as a carrier and internal standard, respectively. The hexane phase was dried under a stream of nitrogen and the lipids were saponified by heating at 80°C in an alkaline methanol solution. After reextracting the lipids in hexanes, cholesterol and cholestenone were separated by thin-layer chromatography using heptane–diethyl ether–acetic acid 85:15:1 (v/v) as an elution solvent. Bands corresponding to cholesterol and cholestenone were scraped from the plate and counted. Recoveries for cholestenone were between 92–95%.

ApoB mass measurement

The estimation of apoB mass secreted into the basolateral medium was determined as previously described (7).

Micelle solutions and cholesterol uptake and esterification

Appropriate aliquots of stock solutions of taurocholate in 95% ethanol, oleic acid in chloroform, and cholesterol in chloroform were taken and dried down under a stream of nitrogen. M199 was added and the solutions were stirred until clear. To address cholesterol uptake, cells were incubated in a micellar solution containing 5 mM taurocholate, 250 μM oleic acid, and 10 μM [^{3}H]cholesterol. At the times specified, cells were rinsed twice with cold phosphate-buffered saline; lipids were extracted twice from the cells on the filter by adding 2 ml hexanes–isopropanol–water 3:2:0.1 (v/v). Carrier lipids were added and the solvent was dried under a stream of nitrogen. The lipids were separated by thin-layer chromatography and the bands corresponding to cholesterol and cholesteryl esters were scraped from the plate and counted.

TABLE 1. Effects of fatty acids on apoB secretion and the esterification and secretion of cholesterol derived from the plasma membrane

	Plasma Membrane Cholesterol Esterified	Cellular Cholestery Esters Secreted	ApoB Secreted
	percent		*ng/well*
Taurocholate	3.0 ± 0.1	3.5 ± 0.8	125 ± 3
Taurocholate			
+ Oleic	4.5 ± 0.1[a]	5.6 ± 0.4[a]	177 ± 8[a]
+ Palmitic	2.9 ± 0.2	2.7 ± 0.3	128 ± 8
+ Linoleic	4.2 ± 0.3[a]	4.0 ± 0.3	165 ± 4[a]
+ Arachidonic	2.9 ± 0.1	2.9 ± 0.3	143 ± 10
+ Eicosapentaenoic	0.6 ± 0.0[a]	nd	35 ± 4

After the labeling of plasma membrane cholesterol as described in Methods, cells were incubated for 5 h at 37°C with 5 mM taurocholate or taurocholate containing 1 mM of oleic, palmitic, linoleic, arachidonic, or eicosapentaenoic acids. The percent of plasma membrane cholesterol esterified and the percent of cellular cholesteryl esters secreted were determined as described in Methods. In another set of cells, apoB mass secretion into the basolateral medium was estimated after a 16-h incubation with taurocholate or taurocholate containing one of the fatty acids. For plasma membrane cholesterol esterification and secretion, the data represent the mean ± SE of four individual transwells from one experiment of two, both showing similar results; nd, none detected. The data for apoB mass represents the mean ± SE of 6–8 transwells for each treatment.

[a] $P < 0.05$ vs. taurocholate.

Isolation of lipoproteins of density < 1.006 g/ml

After the incubation of cells as described in the experiment, the basolateral media from two wells were combined. Two hundred fifty μl of human plasma heated to 60°C for 10 min was added and the samples were centrifuged at 105,000 *g* for 18 h at 4°C. Lipoproteins that floated at the top were collected by slicing the tube. Lipids were extracted with chloroform–methanol 2:1 (v/v) from the density fraction < 1.006 g/ml as well as the bottom fraction. Cholesterol and cholesteryl esters were separated by thin-layer chromatography.

Estimation of cholesterol synthesis

Cholesterol synthesis was estimated by the incorporation of [^{14}C]acetate into cholesterol or [^{3}H]water into nonsaponifiable sterols as we have described previously (12, 13). In these experiments, the final acetate concentration was 10 μM with a specific activity of 120 dpm/pmol.

RESULTS

Effect of oleic acid on the esterification of plasma membrane cholesterol

In previous studies performed in CaCo-2 cells, we have shown that oleic acid increases the secretion of triacylglycerol-rich lipoproteins by increasing triacylglycerol and apoB mass secretion (7, 14). To investigate whether the plasma membrane of the cell contributes cholesterol for the normal assembly of triacylglycerol-rich lipoprotein particles, cells were incubated at 4°C with [^{3}H]cholesterol to label the plasma membrane cholesterol pool. They were then incubated at 37°C with 5 mM taurocholate or taurocholate and increasing concentrations of oleic acid. Influx of plasma membrane cholesterol to the endoplasmic reticulum was estimated by measuring the percent of plasma membrane cholesterol that was esterified by the ER-bound enzyme, ACAT. As an estimate of triacylglycerol-rich lipoprotein cholesterol secretion, the amount of labeled cholesterol and cholesteryl esters secreted into the basolateral medium was determined. **Figure 1** shows these results. In a concentration-dependent fashion, oleic acid increased the influx of plasma membrane cholesterol to the ER, plateauing at a concentration of 0.5 mM. Verapamil, an agent that we have shown to interfere with cholesterol influx without affecting ACAT activity (15), at concentrations of 50 μM, prevented the influx of plasma membrane cholesterol after the addition of oleic acid. Thus, oleic acid caused more plasma membrane cholesterol to move intracellularly to the ER. Moreover, oleic acid also caused an increase in the secretion of plasma membrane-derived cholesteryl esters, suggesting that plasma membrane cholesterol was being used for lipoprotein assembly and secretion. In the presence of verapamil, however, the secretion of cholesteryl esters was prevented. In response to oleic acid, there was a tendency for more plasma membrane-derived unesterified cholesterol to be secreted, but this was not consistent from experiment to experiment. Moreover, verapamil had no apparent effect on the secretion of labeled unesterified cholesterol. The reason for this variability became clear when triacylglycerol-rich lipoproteins were first isolated from the basolateral medium by ultracentrifugation at d 1.006 g/ml (data not shown). Of the labeled cholesteryl esters secreted into the basolateral medium from control cells incubated with taurocholate alone, 70% was found in the lipoprotein fraction that floated

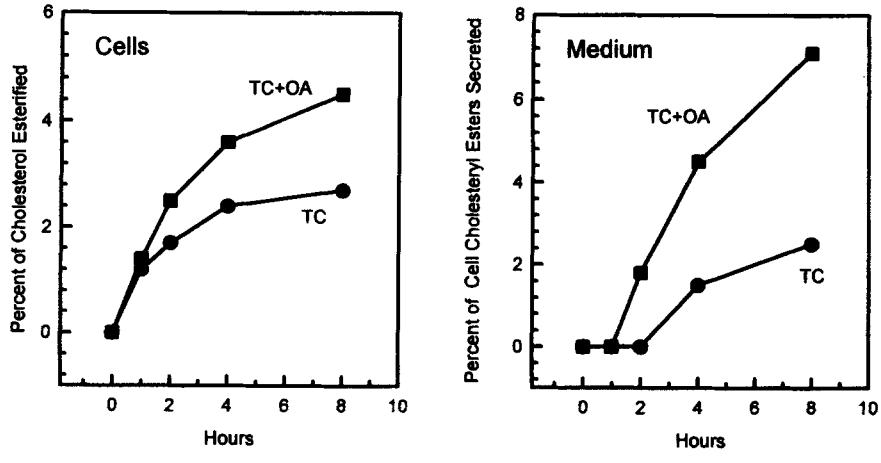

Fig. 2. Effect of oleic acid on the esterification of plasma membrane cholesterol and its secretion into the basolateral medium. The experimental design is the same as described in Fig. 1, except that cells were incubated for up to 8 h with 5 mM taurocholate or taurocholate and 0.5 mM oleic acid. The data represent the mean ± SE of three individual dishes at each time point. The error bars are smaller than the data symbols.

at d 1.006 g/ml. From cells incubated with oleic acid, a 3-fold increase in the secretion of labeled cholesteryl esters was exclusively due to an increase in cholesteryl esters recovered in the 1.006 g/ml density fraction, representing 87% of the total secreted. Thus, cholesteryl esters recovered from the basolateral medium reflected the amount of cholesteryl esters secreted as part of a triacylglycerol-rich lipoprotein particle. In contrast, in control cells and cells incubated with oleic acid, most of the secreted labeled unesterified cholesterol was recovered in the density fraction greater than 1.006 g/ml. Only 24% of the secreted unesterified cholesterol was isolated at d 1.006 g/ml which did significantly increase to 36% in medium collected from cells incubated with oleic acid. The secretion of triacylglycerol-rich lipoprotein unesterified cholesterol that was derived from the plasma membrane was increased 2-fold by oleic acid ($P < 0.01$). Thus, the initial results that were obtained without first isolating triacylglycerol-rich lipoproteins were obscured by a large background of labeled unesterified cholesterol found in the basolateral medium that was not associated with triacylglycerol-rich lipoproteins.

The effect of oleic acid on the influx and esterification of plasma membrane cholesterol was evident by 30 min and continued to increase over 6 h of incubation (**Fig. 2**). The secretion of labeled cholesteryl esters by cells incubated with oleic acid was not measurable for the first hour of incubation; thereafter, however, labeled cholesteryl esters increased linearly in the basolateral medium. In control cells that were not driven to produce lipoproteins, basolateral secretion of plasma membrane-derived cholesteryl esters was not appreciated until 4 h into the incubation and the percent of cellular cholesteryl esters secreted was significantly less than that observed in cells incubated with oleic acid.

It is believed that plasma membrane cholesterol is transported into the cell within vesicles derived from the plasma membrane (16). To investigate whether oleic acid increased the trafficking of plasma membrane cholesterol via vesicles, cells labeled with cholesterol at 4°C were further incubated at 4, 15, 22, and 37°C in the presence or absence of oleic acid. Oleic acid did not increase the influx of plasma membrane cholesterol at 4°C or 15°C, but did so at 22° and at 37°C, suggesting that the fatty acid was causing more plasma membrane cholesterol to influx to the endoplasmic reticulum within vesicles (data not shown). These data, taken together with the effects of verapamil, which prevented the increased influx of plasma membrane cholesterol by oleic acid, suggest that normal vesicular trafficking is necessary for oleic acid to promote cholesterol influx from the plasma membrane to the endoplasmic reticulum.

Effect of fatty acids on the esterification of plasma membrane cholesterol

Not all fatty acids promote triacylglycerol-rich lipoprotein production equally (14). For example, compared to oleic acid, eicosapentaenoic acid is a weak inducer of lipoprotein secretion in CaCo-2 cells (7, 17). To address a possible relationship between lipoprotein secretion and the influx of plasma membrane cholesterol, cells labeled with [^{3}H]cholesterol at 4°C, were incubated at 37°C with taurocholate or taurocholate and oleic, palmitic, linoleic, arachidonic, or eicosapentaenoic acids. The influx of plasma membrane choles-

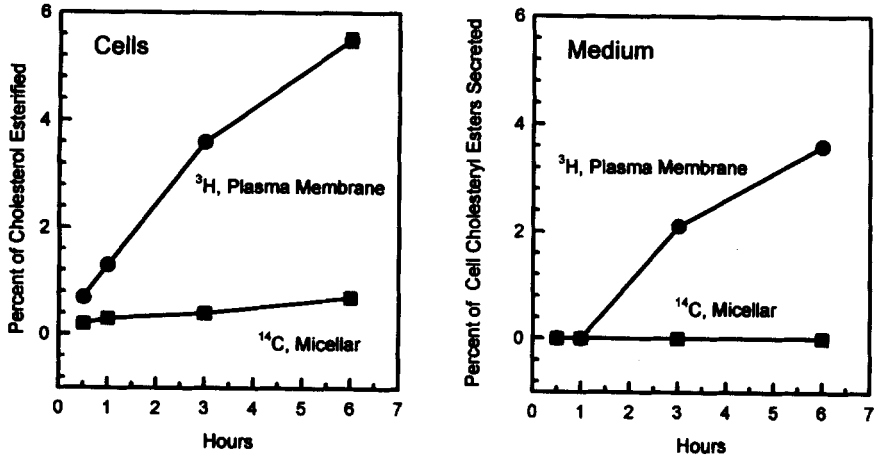

Fig. 3. Effect of oleic acid on the esterification of micellar or plasma membrane cholesterol and their secretion into the basolateral medium. Plasma membrane cholesterol was labeled at 4°C with [^{3}H]cholesterol as described in Methods. The cells were then incubated for 6 h at 37°C with 5 mM taurocholate, 250 μM oleic acid, and 10 μM [^{14}C]cholesterol. At the times indicated in the figure, lipids were extracted from the cells and the whole basolateral medium and separated by thin-layer chromatography. Bands corresponding to unesterified and esterified cholesterol were scraped from the plate and counted. The data represent the mean ± SE of three dishes at each time point from a representative experiment of two sets of incubations.

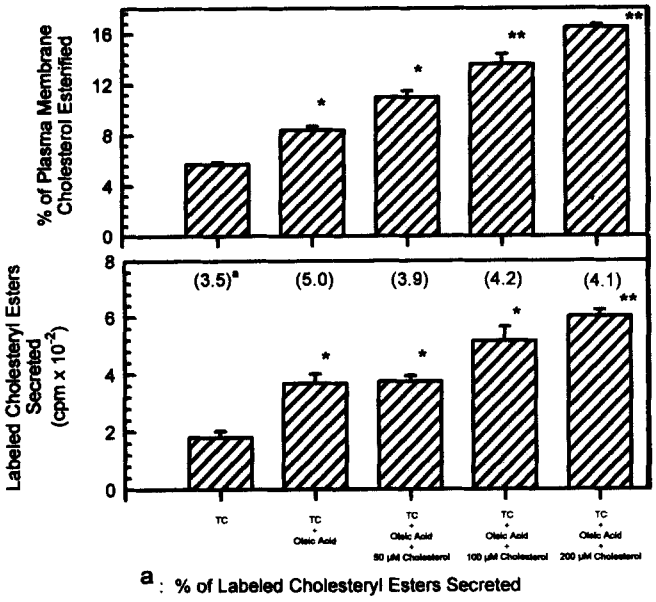

Fig. 4. Effect of oleic acid and cholesterol on the esterification of plasma membrane cholesterol and its secretion into the basolateral medium. Plasma membrane cholesterol was labeled as described in Fig. 1. Cells were then incubated for 6 h at 37°C with 5 mM taurocholate (TC), taurocholate and 0.5 mM oleic acid, or taurocholate, oleic acid and increasing concentrations of cholesterol. After the incubation, lipids were extracted from the cells and basolateral medium and separated by thin-layer chromatography. Bands corresponding to unesterified and esterified cholesterol were scraped from the plate and counted. The data represent the mean ± SE of 4 individual dishes for each treatment. A: The number in parentheses is the percent of cellular labeled cholesteryl esters secreted; $*P < 0.01$ vs. taurocholate; $**P < 0.01$ vs. taurocholate and oleic acid.

terol to the endoplasmic reticulum and the secretion of labeled cholesteryl esters into the basolateral medium were estimated and compared to the effects of these fatty acids on the secretion of apoB mass. The results are shown in **Table 1.** Oleic and linoleic acids were the only fatty acids that increased the influx of plasma membrane cholesterol and caused an increase in the secretion of apoB mass. Although there was a tendency for more plasma membrane-derived cholesteryl esters to be secreted by cells incubated with linoleic acid, only oleic acid significantly increased the secretion of labeled cellular cholesteryl esters. The n–3 fatty acid, eicosapentaenoic acid, actually caused an inhibition of plasma membrane cholesterol esterification suggesting that it interfered with cholesterol influx. Because of the small amount of labeled cellular cholesteryl esters synthesized from the plasma membrane in cells incubated with eicosapentaenoic acid, no labeled cholesteryl esters were detected in the basolateral medium. It should be noted, however, that despite interference with cholesterol influx and a decrease in the synthesis of cholesteryl esters derived from the plasma membrane, apoB mass secretion was maintained.

Esterification of plasma membrane versus micellar cholesterol

In fibroblasts, macrophages, and hepatocytes, cholesterol derived from the plasma membrane is believed to be the predominate substrate for ACAT (16, 18, 19). In intestinal cells, it has been suggested that cholesterol taken up from micelles is the preferred substrate for ACAT (20). To investigate the esterification of micellar versus plasma membrane cholesterol in CaCo-2 cells, plasma membrane cholesterol was labeled with tritium at 4°C. The cells were then incubated at 37°C with a micellar solution containing 5 mM taurocholate, 250 µM oleic acid, and 10 µM [^{14}C]cholesterol. The rate of esterification of plasma membrane-derived cholesterol ([^{3}H]cholesteryl esters) versus that derived from the uptake of micellar cholesterol ([^{14}C]cholesteryl esters) was estimated and compared during a 6-h incubation (**Fig. 3**). At each of the time points, the percent esterification of plasma membrane-derived cholesterol was significantly greater than the percent esterification of cholesterol taken up from a micellar solution. At 3 and 6 h, similar amounts of ^{3}H- and ^{14}C-labeled unesterified cholesterol were associated with the cell and yet, the esterification of plasma membrane cholesterol was much preferred over micellar cholesterol. Only labeled cholesteryl esters derived from the plasma membrane were recovered in the basolateral medium.

Effect of micellar cholesterol on the influx of plasma membrane cholesterol

To address why so little micellar cholesterol was being esterified, the effect of micellar cholesterol on the influx of plasma membrane cholesterol was investigated. After the labeling of plasma membrane cholesterol at 4°C, the cells were incubated for 5 h at 37°C with 5 mM taurocholate, taurocholate plus 500 µM oleic acid, or taurocholate and oleic acid plus increasing concentrations of cholesterol. The percent of plasma membrane cholesterol esterified was estimated as well as the basolateral secretion of plasma membrane-derived cholesteryl esters (**Fig. 4**). As demonstrated previously, oleic acid increased the influx of plasma membrane cholesterol and increased the secretion of labeled cholesteryl esters. Moreover, when cholesterol was added to the micelle, significantly more labeled cholesterol from the plasma membrane moved to the endoplasmic reticulum and was esterified. Likewise, the secretion of total cholesteryl esters derived from the plasma membrane increased as well. In contrast to oleic acid, however, which promotes triacylglycerol-rich lipoprotein secretion and increases the secretion of both total cholesteryl esters and the percent secretion of cellular cholesteryl esters (Fig. 1), the percent secretion of intracellular cholesteryl esters

was not significantly altered by micellar cholesterol (approximately 4%). This supports a previous observation in CaCo-2 cells that demonstrated a lack of effect of micellar cholesterol on triacylglycerol-rich lipoprotein secretion (21).

Effect of oleic acid on the secretion of newly synthesized cholesterol

To investigate whether newly synthesized cholesterol was used for triacylglycerol-rich lipoprotein secretion, cells were incubated with increasing concentrations of oleic acid and [^{14}C]acetate. After 6 h, the incorporation of label into cellular unesterified and esterified cholesterol was determined. The secretion of newly synthesized cholesteryl esters was estimated as the percent of intracellular labeled cholesteryl esters secreted. The results are shown in **Fig. 5.** Oleic acid significantly increased the synthesis of cholesterol, an observation that was confirmed using [^{3}H]water as substrate (data not shown). In a concentration-dependent manner, the amount of label found in cellular cholesteryl esters was increased also by oleic acid. After the saponification of cholesteryl esters, 85% of the label was incorporated into cholesterol with the remaining 15% incorporated into the fatty acid (data not shown). The basolateral secretion of labeled cholesteryl esters was not significantly altered by oleic acid, which resulted in an observed decrease in the percent of cellular cholesteryl esters secreted in response to the fatty acid. To address whether oleic acid increased the secretion of newly synthesized unesterified and esterified cholesterol in triacylglycerol-rich lipoproteins, the basolateral media were collected and lipoproteins were isolated after ultracentrifugation at d 1.006 g/ml. Similar to what was observed with the plasma membrane-derived cholesterol, oleic acid did increase the secretion of both newly synthesized cholesterol (5.3 pmol/6 h vs. 2.7, $P < 0.05$) and cholesteryl esters (2.6 pmol/6 h vs. 1.0, $P < 0.05$) in triacylglycerol-rich lipoproteins (data not shown).

Effect of oleic acid on the transport of newly synthesized cholesterol to the plasma membrane

As oleic acid increased the rate of cholesterol synthesis and increased the amount of plasma membrane cholesterol influxing to the endoplasmic reticulum, the question was asked whether more of the newly synthesized cholesterol would be transported to the plasma membrane to replenish cholesterol leaving, or instead be translocated into the lumen of the endoplasmic reticulum to be used for the assembly of the lipoprotein particle. To address this, cells were incubated with taurocholate or taurocholate and oleic acid together with

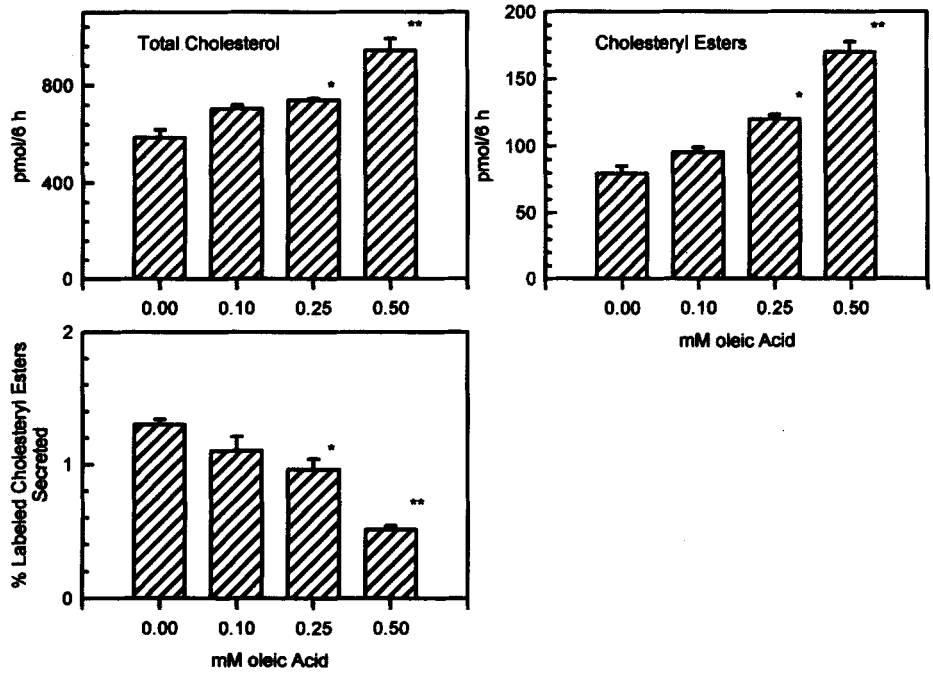

Fig. 5. Effect of oleic acid on the incorporation of [^{14}C]acetate into cholesterol within cells and that secreted into the basolateral medium. Cells were incubated for 6 h with [^{14}C]acetate and 5 mM taurocholate or taurocholate and increasing concentrations of oleic acid. At the end of the incubation, lipids were extracted from the cells and the basolateral medium and separated by thin-layer chromatography. Bands corresponding to unesterified and esterified cholesterol were scraped from the plate and counted. The data represent the mean ± SE of four dishes from a representative experiment of four; *$P < 0.05$ vs. taurocholate; **$P < 0.01$ vs. taurocholate.

[^{14}C]acetate. At intervals over a 6-h incubation, cells were fixed with glutaraldehyde and exposed to cholesterol oxidase to convert plasma membrane cholesterol to cholestenone. The percent of newly synthesized cholesterol found in the plasma membrane is estimated by measuring the amount of newly synthesized cholesterol converted to cholestenone (**Fig. 6**). As shown previously, oleic acid increased the rate of cholesterol synthesis (left panel). The amount of newly synthesized cholesterol found in the plasma membrane also increased in cells incubated with oleic acid compared to controls (right panel). As can be appreciated from the similarity of the slopes of the two lines in both panels, however, the percent of total cholesterol found in the plasma membrane was not altered by oleic acid. Thus, driving triacylglycerol-rich lipoprotein synthesis and secretion by oleic acid increases the influx of plasma membrane cholesterol to the endoplasmic reticulum but does not appreciably alter the fraction of newly synthesized cholesterol trafficking from its site of synthesis to the plasma membrane.

DISCUSSION

The results of this study clearly indicate that in CaCo-2 cells, the plasma membrane is an important intracellular source for esterified and unesterified cholesterol for the assembly of triacylglycerol-rich lipoproteins. Oleic acid, a recognized potent inducer of triacylglycerol-rich lipoprotein secretion, caused a significant increase in the movement of plasma membrane cholesterol to the ER. Results from a recent study, also performed in CaCo-2 cells, demonstrated that several recognized inhibitors of p-glycoprotein or temperatures of 15°C or below prevented the normal influx of plasma membrane cholesterol to the ER (15). In agreement with previous observations made in several different cell types, the present results suggest that trafficking of cholesterol from the plasma membrane to the ER is mediated by vesicles derived from the plasma membrane (16, 18, 22, 23). Oleic acid increased the vesicular-mediated transport of plasma membrane cholesterol to the ER, as demonstrated by the fact that verapamil, a p-glycoprotein inhibitor, or maintaining cells at a temperature that does not permit vesicular trafficking prevented oleic acid from increasing cholesterol influx. Thus, in CaCo-2 cells, the absorption of oleic acid leads to an increase in the amount of vesicular-mediated trafficking of cholesterol from the plasma membrane to the ER.

An explanation for why this should occur became clear when it was observed that both unesterified and esterified cholesterol derived from the plasma membrane were increased in triacylglycerol-rich lipoproteins isolated from cells incubated with oleic acid. In times of increased requirements for cholesterol in assembling the lipoprotein particle, the intestinal cell uses plasma membrane cholesterol to meet these immediate needs. In support of this, a correlation was observed between the ability of a particular fatty acid to drive lipoprotein secretion and its ability to cause an influx of plasma membrane cholesterol to the ER. Fatty acids that did not promote apoB secretion and, therefore, did not increase the number of lipoprotein particles secreted, did not alter trafficking of cholesterol from the plasma membrane to the ER. These results, taken together, suggest that the plasma membrane serves as a reservoir for cholesterol used for the normal assembly and secretion of triacylglycerol-rich lipoproteins.

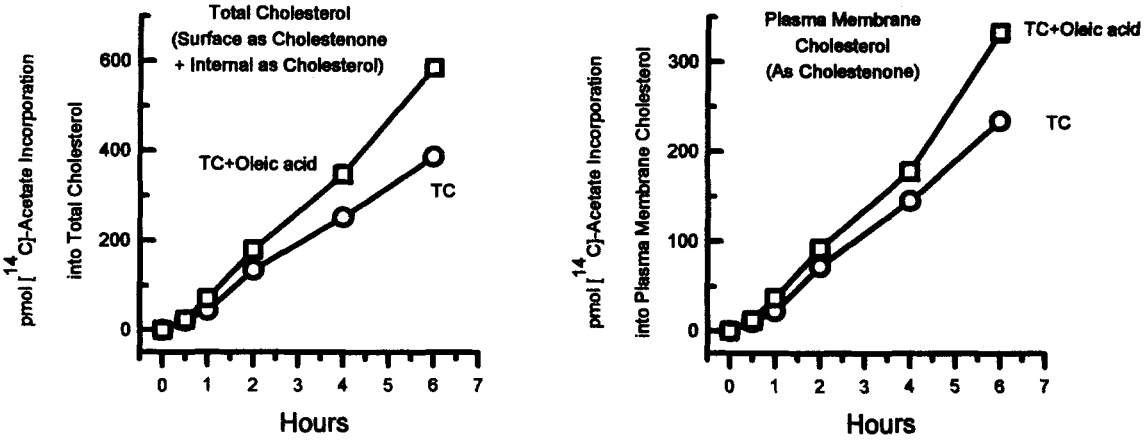

Fig. 6. Effect of oleic acid on cholesterol synthesis and its transport to the plasma membrane. Cells were incubated for 6 h with [^{14}C]acetate and 5 mM taurocholate or taurocholate and 0.5 mM oleic acid. At the times indicated, cells were fixed with glutaraldehyde and incubated with cholesterol oxidase as described in Methods. Cholesterol and cholestenone were then separated by thin-layer chromatography, scraped from the plate, and counted. The data represent the mean of two dishes at each time point from one experiment of four all showing similar results.

In earlier reports using CaCo-2 cells to investigate cholesterol absorption, we observed that only a small fraction of cholesterol that was taken up from a micellar solution was esterified, despite significant amounts of cholesterol being absorbed (10). The present data provide some insight as to why this occurred. It is clear from the results that micellar cholesterol displaces cholesterol within the plasma membrane causing more of the sterol to influx to the ER. It is also apparent that under the present experimental conditions micellar cholesterol did not equilibrate with or enrich the cholesterol pool of the plasma membrane. If this were to occur, dilution of the labeled plasma membrane pool with the unlabeled micellar cholesterol would have caused a decrease in the amount of labeled cholesteryl esters being synthesized (Fig. 4). Not only did this not occur, but more cholesteryl esters were produced from the labeled plasma membrane cholesterol pool. These results suggest that the cholesteryl esters synthesized within an intestinal cell in response to an increased flux of fatty acids or cholesterol do not come directly from cholesterol within the lumen, but rather, from the influx of plasma membrane cholesterol into the cell. These data are consistent with the observations of others suggesting that cholesterol of the plasma membrane serves as the predominate substrate for ACAT (16, 18, 19, 22).

Incubating CaCo-2 cells with oleic acid modestly but significantly increased the synthesis of cholesterol. In hepatocytes, some investigators have observed an increase in cholesterol synthesis with increased lipoprotein production (24). It has been argued that during lipoprotein synthesis, the demand for more unesterified cholesterol to package the lipoprotein particle is met by increased synthesis of cholesterol. In support of this argument, a few studies have shown that hepatic triacylglycerol-rich lipoprotein secretion can be disrupted by inhibiting the activity of HMG-CoA reductase, suggesting that newly synthesized cholesterol is required for the normal assembly and secretion of lipoproteins (8, 25). There exist other data, however, that do not support that argument (26–29). It remains unclear whether, within an intestinal cell, newly synthesized cholesterol contributes directly to lipoprotein assembly. Oleic acid did increase cholesterol synthesis and, compared to control cells, more newly synthesized cholesterol and cholesteryl esters were recovered in secreted lipoproteins of d < 1.006 g/ml. This implied that a fraction of newly synthesized cholesterol was being used for triacylglycerol-rich lipoprotein assembly. We initially speculated that this newly synthesized cholesterol was first transported to the plasma membrane prior to its incorporation into the lipoprotein particle. In data not shown, however, verapamil, which decreased the transport of plasma membrane cholesterol to the endoplasmic reticulum by some 70% (Fig. 1), had little effect on the esterification of newly synthesized cholesterol over a 6-h incubation (111 ± 3 pmol/well versus 97 ± 3, $P > 0.05$). Thus, transport of newly synthesized cholesterol to the plasma membrane was not first required for its esterification. Although we speculate that oleic acid increases cholesterol synthesis because of the cell's need to replace plasma membrane cholesterol that is moving inward and being secreted as a component of a lipoprotein particle, the data do suggest that a small fraction of newly synthesized cholesterol is shunted directly from its site of synthesis to ACAT and into the lumen of the endoplasmic reticulum. To this point, however, under similar experimental conditions that were used to show a significant fractional increase in the secretion of plasma membrane-derived cholesteryl esters (Fig. 1), we could not demonstrate an increase in the secretion of newly synthesized cholesteryl esters in response to oleic acid (Fig. 5). In fact, the fractional secretion of intracellular labeled cholesteryl esters actually decreased. This can best be explained by an expansion of the cholesteryl ester pool by unlabeled cholesteryl esters derived from the influx of plasma membrane cholesterol causing an apparent decrease in the secretion of newly labeled cholesteryl esters. Moreover, if newly synthesized cholesterol was a major source of lipoprotein cholesterol, one might speculate that during lipoprotein production, the intestinal cell would redirect the normal trafficking of newly synthesized cholesterol away from the plasma membrane and into the lumen of the endoplasmic reticulum to maintain a sufficient supply of cholesterol for assembly and secretion of the particle. This did not occur. During triacylglycerol-rich lipoprotein assembly and secretion, and with this an increased demand for unesterified cholesterol, the cell continued to deliver a similar fraction of newly synthesized cholesterol to the plasma membrane.

We would conclude from these studies that in CaCo-2 cells, the plasma membrane is the predominant source for triacylglycerol-rich lipoprotein cholesterol. This makes good sense for a cell whose needs for cholesterol are immediate to assemble and transport exogenous lipids following the ingestion of a fatty meal. By regulating the normal trafficking of the sterol within transport vesicles derived from the plasma membrane, the cell can manipulate how much plasma membrane cholesterol influxes to the endoplasmic reticulum for secretion. Although driving lipoprotein production by oleic acid does modestly increase cholesterol synthesis and a small fraction of this newly synthesized cholesterol is found in secreted triacylglycerol-rich lipoproteins, we speculate that it is the loss of plasma membrane cholesterol that

stimulates cholesterol synthesis as the cell attempts to replace that cholesterol moving to the endoplasmic reticulum to be secreted as part of a lipoprotein particle.

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REFERENCES

1. Field, F. J., and S. N. Mathur. 1995. Intestinal lipoprotein synthesis and secretion. *Prog. Lipid Res.* **34:** 185–198.
2. Borchardt, R. A., and R. A. Davis. 1987. Intrahepatic assembly of very low density lipoproteins. *J. Biol. Chem.* **262:** 16394–16402.
3. Borstrom, K., J. Boren, M. Wettesten, A. Sjoberg, G. Bondjers, O. Wiklund, P. Carlsson, and S. O. Olofsson. 1988. Studies on the assembly of apoB-100-containing lipoproteins in HepG2 cells. *J. Biol. Chem.* **263:** 4434–4442.
4. Boren, J., L. Graham, M. Wettesten, J. Scott, A. White, and S. O. Olofsson. 1992. The assembly and secretion of apoB-100-containing lipoproteins in HepG2 cells. *J. Biol. Chem.* **267:** 9859–9867.
5. Dashti, N., E. A. Smith, and P. Alaupovic. 1990. Increased production of apolipoprotein B and its lipoproteins by oleic acid in CaCo-2 cells. *J. Lipid Res.* **31:** 113–123.
6. Moberly, J. B., T. G. Cole, D. H. Alpers, and G. Schonfeld. 1990. Oleic acid stimulation of apolipoprotein B secretion from HepG2 and CaCo-2 cells occurs post-transcriptionally. *Biochim. Biophys. Acta.* **1042:** 70–80.
7. Murthy, S., E. Albright, S. N. Mathur, N. O. Davidson, and F. J. Field. 1992. Apolipoprotein B mRNA abundance is decreased by eicosapentaenoic acid in CaCo-2 cells: effect on the synthesis and secretion of apolipoprotein B. *Arterioscler. Thromb.* **12:** 691–700.
8. Khan, B., H. G. Wilcox, and M. Heimberg. 1989. Cholesterol is required for secretion of very-low-density lipoprotein by rat liver. *Biochem. J.* **259:** 807–816.
9. Cianflone, K. M., Z. Yasruel, A. Rodriguez, D. Vas, and A. D. Sniderman. 1990. Regulation of apoB secretion from HepG2 cells: evidence for a critical role for cholesteryl ester synthesis in the response to a fatty acid challenge. *J. Lipid Res.* **31:** 2045–2055.
10. Field, F. J., E. Albright, and S. N. Mathur. 1987. Regulation of cholesterol esterification by micellar cholesterol in CaCo-2 cells. *J. Lipid Res.* **28:** 1057–1066.
11. Freeman, D. A. 1987. Cyclic AMP mediated modification of cholesterol traffic in Leydig tumor cells. *J. Biol. Chem.* **262:** 13061–13068.
12. Chen, H., E. Born, S. N. Mathur, and F. J. Field. 1993. Cholesterol and sphingomyelin syntheses are regulated independently in cultured human intestinal cells: role of membrane cholesterol and sphingomyelin content. *J. Lipid Res.* **34:** 2159–2167.
13. Murthy, S., E. Albright, S. N. Mathur, and F. J. Field. 1988. Modification of CaCo-2 cell membrane fatty acid composition by eicosapentaenoic acid and palmitic acid: effect on cholesterol metabolism. *J. Lipid Res.* **29:** 773–780.
14. Field, F. J., E. Albright, and S. N. Mathur. 1988. Regulation of triglyceride-rich lipoprotein secretion by fatty acids in CaCo-2 cells. *J. Lipid Res.* **29:** 1427–1437.
15. Field, F. J., E. Born, H. Chen, S. Murthy, and S. N. Mathur. 1995. Esterification of plasma membrane cholesterol and triacylglycerol-rich lipoprotein secretion in CaCo-2 cells: possible role of p-glycoprotein. *J. Lipid Res.* **36:** 1533–1543.
16. Lange, Y. 1994. Cholesterol movement from plasma membrane to rough endoplasmic reticulum. *J. Biol. Chem.* **269:** 1–4.
17. Murthy, S., E. Albright, N. Mathur, and F. J. Field. 1990. Effect of eicosapentaenoic acid on triacylglycerol transport. *Biochim. Biophys. Acta.* **1045:** 147–155.
18. Lange, Y., F. Strebel, and T. L. Steck. 1993. Role of the plasma membrane in cholesterol esterification in rat hepatoma cells. *J. Biol. Chem.* **268:** 13838–13843.
19. Tabas, I., W. J. Rosoff, and G. C. Boykow. 1988. Acylcoenzyme A: cholesterol acyltransferase in macrophages utilizes a cellular pool of cholesterol oxidase-accessible cholesterol as substrate. *J. Biol. Chem.* **263:** 1266–1272.
20. Stange, E. F., K. E. Suckling, and J. M. Dietschy. 1983. Synthesis and coenzyme A-dependent esterification of cholesterol in rat intestinal epithelium. *J. Biol. Chem.* **258:** 12868–12875.
21. Field, F. J., E. Born, H. Chen, S. Murthy, and S. N. Mathur. 1994. Regulation of apolipoprotein B secretion by biliary lipids in CaCo-2 cells. *J. Lipid Res.* **35:** 749–762.
22. Nagy, L., and D. A. Freeman. 1990. Effect of cholesterol transport inhibitors on steroidogenesis and plasma membrane cholesterol transport in cultured MA-10 Leydig tumor cells. *Endocrinology.* **126:** 2267–2276.
23. Liscum, L., and N. K. Dahl. 1992. Intracellular cholesterol transport. *J. Lipid Res.* **33:** 1239–1254.
24. Salam, W. H., H. G. Wilcox, L. M. Cagen, and M. Heimberg. 1989. Stimulation of hepatic cholesterol biosynthesis by fatty acids. *Biochem. J.* **258:** 563–568.
25. Ginsberg, H. N., N. A. Le, P. Short, R. Ramakrishan, and R. J. Desnick. 1979. Suppression of apolipoprotein B production during treatment of cholesteryl ester storage disease with lovastatin. *J. Clin. Invest.* **80:** 1692–1697.
26. Davis, R. A., and M. Malone-McNeal. 1985. Dietary cholesterol does not affect the synthesis of apolipoproteins B and E by rat hepatocytes. *Biochem. J.* **227:** 29–35.
27. Sato, R., T. Imanaka, and T. Takano. 1990. The effect of HMG-CoA reductase inhibitor (CS-514) on the synthesis and secretion of apolipoproteins B and A-I in the human hepatoblastoma HepG2. *Biochim. Biophys. Acta.* **1042:** 36–41.
28. Ribeiro, A., M. Mangeney, C. Loriette, G. Thomas, D. Pepin, B. Janvier, J. Chambaz, and G. Bereziat. 1991. Effect of simvastatin on the synthesis and secretion of lipoproteins in relation to the metabolism of cholesterol Biochim. Biophys. Acta. **1086:** 279–286.
29. Kam, N. T. P., E. Albright, S. N. Mathur, and F. J. Field. 1990. Effect of lovastatin on acyl-CoA:cholesterol O-acyltransferase (ACAT) activity and the basolateral-membrane secretion of newly synthesized lipids by Caco-2 cells. *Biochem. J.* **272:** 427–433.