Phosphatidylcholine increases the secretion of triacylglycerol-rich lipoproteins by CaCo-2 cells

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The regulation of lipid synthesis and secretion by phosphatidylcholine was investigated in CaCo-2 cells grown on semi-permeable filters. In cells incubated with 1 mM taurocholate and 100–500 μ M phosphatidylcholine, cholesteryl ester synthesis was decreased, triacylglycerol synthesis was increased modestly, whereas phospholipid synthesis was unaffected. Acyl-CoA-cholesterol acyltransferase activity was decreased secondary to a decrease in the substrate (cholesterol) supply. The basolateral secretion of newly synthesized triacylglycerol and triacylglycerol mass was significantly increased by phosphatidylcholine, whereas cellular triacylglycerol mass decreased. This effect was not specific for phosphatidylcholine as phosphatidylethanolamine and phosphatidylserine also increased the secretion of newly synthesized triacylglycerols. Dioleoylphosphatidylcholine was as effective as egg phosphatidylcholine in increasing triacylglycerol transport. Dipalmitoylphosphatidylcholine, in contrast, was without effect.

Phosphatidylcholine also increased the basolateral secretion of apolipoprotein B (apoB) mass without altering apoB mRNA levels. Disruption of the Golgi apparatus by monensin or brefeldin A prevented the increase in apoB secretion by phosphatidylcholine. Compared with microsomes prepared from control cells, those from cells incubated with phosphatidylcholine contained more newly synthesized apoB. The percentage of new synthesized apoB isolated from the lumen of microsomes (as an estimate of apoB destined for secretion), however, was similar in the two preparations. Thus in CaCo-2 cells incubated with phosphatidylcholine, the transport of apoB and triacylglycerols is increased whereas cholesteryl ester synthesis and secretion are decreased. A normally functioning secretory pathway is required for phosphatidylcholine to increase triacylglycerol-rich lipoprotein secretion.

INTRODUCTION

There is evidence to suggest that triacylglycerol absorption is enhanced by the presence of phosphatidylcholine within the intestinal lumen. In human subjects, the duodenal infusion of phosphatidylcholine increased the production of small triacylglycerol-rich lipoproteins [1]. In lymph-fistula rats, the transport of triacylglycerol into lymph was increased after the duodenal infusion of phosphatidylcholine [2–4]. Regulation of intestinal triacylglycerol transport by phosphatidylcholine probably involves alteration of lipoprotein assembly and/or secretion, as the uptake of luminal fatty acids derived from triacylglycerol hydrolysis is not increased by the presence of phosphatidylcholine [2,3]. Since significant amounts of intact phosphatidylcholine are not thought to be absorbed across the absorptive cell membrane [5,6], investigators have suggested that the uptake of lysophosphatidylcholine or free fatty acids is responsible for the observed increase in triacylglycerol transport after infusion of phosphatidylcholine into the gut [3,4].

In a recent study, however, we demonstrated that, after the addition of intact phosphatidylcholine to the apical medium of CaCo-2 cells, a significant increase in the basolateral secretion of apolipoprotein B (apoB) occurred independently of fatty acid or lysophosphatidylcholine uptake [7]. It was postulated that phosphatidylcholine interacted with the apical membrane of the cell leading to an increase in the rate of synthesis and secretion of apoB. Whether phosphatidylcholine also altered intracellular lipid synthesis or secretion was not addressed.

The primary purpose of this study was to investigate the effects of phosphatidylcholine on the secretion of triacylglycerol-rich lipoproteins by CaCo-2 cells. To confirm and extend observations

made in our previous study [7], experiments were also performed to examine further the effects of phosphatidylcholine on apoB secretion. The results demonstrate that in CaCo-2 cells incubated with phosphatidylcholine, the synthesis and secretion of cholesteryl esters are decreased, whereas the secretion of newly synthesized triacylglycerols and triacylglycerol mass are increased 2–4-fold with a modest (22%) increase in triacylglycerol synthesis. Phosphatidylcholine also increases the secretion of apoB without altering the fraction of newly synthesized apoB destined for secretion. An intact secretory pathway is required for phosphatidylcholine to increase apoB secretion.

MATERIALS AND METHODS

Trans-[³⁵S]methionine (1100 Ci/mmol) was purchased from ICN Radiochemicals (Irvine, CA, U.S.A.). [1,2,3-³H]Glycerol, [9,10-³H]oleic acid, [7-³H]cholesterol, [cholesteryl-1,2,6,7-³H]-cholesteryl oleate and [oleoyl-1-¹⁴C]oleoyl-CoA were from New England Nuclear (Boston, MA, U.S.A.). Egg phosphatidylcholine, dioleoylphosphatidylcholine, dipalmitoylphosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, sphingomyelin, cholesterol, oleoyl-CoA, taurocholic acid and fatty acid-poor BSA were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). ApoB monoclonal antibody (clone no. 1607, IgG-2b fraction purified by column chromatography), and apoB sheep immunopurified polyclonal antibody conjugated to horseradish peroxidase were purchased from Biodesign International (Kennebunkport, ME, U.S.A.). Rabbit polyclonal antibody (IgG fraction) specific for human apoB was from Calbiochem (San Diego, CA, U.S.A.). Rabbit sera containing

antibody to human apoA 1 were graciously provided by Dr. Dennis Black, Department of Pediatrics, University of Chicago, Chicago, IL, U.S.A. Protein A bound to Sepharose was from Repli Gen (Cambridge, MA, U.S.A.). TMB Microwell Peroxidase Substrate System was purchased from Kirkegaard and Perry labs Inc. (Gaithersburg, MD, U.S.A.). Nunc-Immuno plates (96-well) were from VWR Scientific (Batavia, IL, U.S.A.). All other reagents were reagent grade.

Cell culture

CaCo-2 cells were grown in T-75 flasks as described previously [8]. They were subcultured on polycarbonate microspore membranes (0.4 μ m pore size) inserted into Transwells (Costar, Cambridge, MA, U.S.A.). Inserts of 6.5 mm diameter were used and cells were plated at an initial density of 0.2×10^5 cells per filter. Medium was changed every 2 days and the cells were used 14 days after plating.

Bile acid solutions

Taurocholic acid was dissolved in a stock solution of 95 % ethanol. Stock solutions of the phospholipids were kept in chloroform under nitrogen at 4 °C. On the day of the experiment, appropriate amounts of the stock solutions were mixed and dried under a stream of nitrogen. M199 (Medium 199/Earle's; Gibco, Grand Island, NY, U.S.A.) containing 10 mM Hepes, pH 7.4, was added to make the correct concentrations of the lipids and vortexed until clear. These solutions were added to the apical medium.

Measurement of lipid synthesis

[3 H]Oleic acid was mixed with unlabelled oleic acid and NaOH (2 mol of NaOH/mol of oleic acid). The mixture was dried under a stream of nitrogen. Water (0.2 ml) was added followed by fatty acid-poor albumin so that the ratio of oleic acid to albumin was 3:1 (mol/mol). This solution was stirred well. M199 was added to make the final concentration of oleic acid 25 μ M with a specific radioactivity of 5500 d.p.m./nmol.

After incubation with the various treatments, media were removed and the cells were washed with M199. Fresh M199 (0.6 ml) was added to the lower wells. The media in the upper wells contained the treatments and the labelled oleic acid/albumin solution. At the appropriate time, the media from the lower wells were removed and centrifuged at 380 g (2000 rev./min) for 15 min to remove any debris. The apical media were removed and the cells washed three times with M199. Lipids were extracted from the cells and the basolateral media with chloroform/methanol (2:1, v/v). The chloroform phase was taken to dryness under a stream of nitrogen. The residue was taken up in 1 ml of chloroform and 0.1 ml was taken for liquid-scintillation counting to estimate the incorporation of oleate into total lipids. The remaining 0.9 ml of chloroform was again dried down under nitrogen. The residue was dissolved in 0.125 ml of chloroform and all of it was applied to silica gel G thin-layer plates. The plates were developed with hexane/diethyl ether/methanol/acetic acid (70:30:1:1, by vol.) and the lipids were visualized by iodine vapour. The bands corresponding to cholesteryl esters, triacylglycerols and phospholipids were scraped into 5 ml of scintillation fluid and counted. Recoveries did not differ significantly among experiments and ranged from 84 to 87 %.

In other experiments, labelled glycerol was used to estimate triacylglycerol and phospholipid synthesis and secretion. Conditions were exactly as described above except that oleic acid was

not labelled and 10 μ Ci of [3 H]glycerol (specific radioactivity 450 d.p.m./pmol) was added to the apical media. After lipid extraction with chloroform/methanol, the chloroform phase was washed three times with a 1:1 methanol/water solution, pH 3.0. The chloroform phase was then dried under nitrogen and the lipids separated using the methodology described above except that only bands corresponding to triacylglycerols and phospholipids were scraped from the plates and counted.

Of the total labelled lipids and apoB recovered from apical and basolateral media, less than 15 % was found in the apical medium. The different treatments had no effect on triacylglycerol, cholesteryl ester or apoB secretion into the apical medium. Thus only data from the basolateral medium will be shown.

Na₂CO₃ release of labelled apoB

To estimate the percentage of apoB within the lumen of microsomes, the methodology described by Davis et al. [9] was used. CaCo-2 cells grown in T-75 flasks and on Transwells were incubated for 18 h with 1 mM taurocholate or 1 mM taurocholate and 500 μ M phosphatidylcholine. The next day, cells grown on the Transwells were pulsed for 3 h with 200 μ Ci of [35 S]methionine. After 3 h pulse, cells from one T-75 flask (used as carrier for isolation of microsomes and subsequent steps) and two transwells were combined and homogenized in 2 ml of 10 mM Tris/HCl buffer, pH 7.4, containing 0.25 M sucrose, 50 μ g/ml leupeptin, 1 μ g/ml aprotinin and 1 mM PMSF. The protease inhibitors were added to all buffers used in subsequent steps. After centrifugation for 20 min at 10000 g, microsomes were prepared from the resulting supernatant by centrifugation at 100000 g for 30 min. The microsomal fraction was resuspended in 0.25 ml of Tris/sucrose buffer. An aliquot (0.1 ml) was taken and diluted with 1 ml of Tris/sucrose buffer and used as a control without Na₂CO₃ treatment. An equal aliquot was then taken and mixed with 0.4 ml of water and 0.5 ml of 0.2 M Na₂CO₃, pH 11.3. After incubation on ice for 30 min, the samples without and with Na₂CO₃ treatment were centrifuged for 30 min at 100000 g. The supernatant and pellet were diluted with 1 $\times$ RIPA buffer [RIPA is 1 % (v/v) Triton-X 100, 0.1 % (w/v) SDS, 0.5 % (w/v) sodium deoxycholate, 20 μ M leupeptin and (in mM) 10 sodium phosphate (pH 7.5), 5 EDTA, 5 EGTA, 100 sodium chloride, 1 dithiothreitol, 1 PMSF and 1 cysteine] and PBS to obtain a final concentration of 0.1 $\times$ RIPA in a total volume of 1.2 ml. The pH was adjusted to 8.0 before immunoprecipitation of apoB and separation by SDS/PAGE as previously described [7,10]. The radioactivity in apoB100 and apoB48 bands was determined by scintillation counting.

ApoB mRNA

After the treatments, RNA was extracted from the cells with acid guanidinium isothiocyanate/phenol/chloroform as described by Xie and Rothblum [11]. Total RNA (0.5–4.0 μ g) was dissolved in 0.1 ml of 10 mM Tris/HCl/1 mM EDTA buffer, (pH 7.5)/20 $\times$ SSC/37 % formaldehyde (50:30:20, by vol.) where 1 $\times$ SSC is 0.15 M NaCl/0.015 M sodium citrate. After denaturation for 15 min at 60 °C, the RNA was applied to nylon membrane filters (Nytran, Schleicher and Schuell, Keene, NH, U.S.A.) using a Bio-rad dot-blot apparatus. After vacuum-drying for 4 h at 80 °C, the filter was prehybridized for 2 h at 42 °C in buffer containing 5 $\times$ SSC, 0.1 % (v/v) N-laurylsarcosine, 0.02 % (w/v) SDS and 1 % blocking reagent (DIG DNA labelling and detection kit; Boehringer-Mannheim, Indianapolis, IN, U.S.A.). ApoB cDNA probe no. 59035 was obtained from ATCC (Rockville, MD, U.S.A.), and a human muscle α -actin cDNA probe was kindly provided by Dr. Peter Rubenstein, Department of Biochemistry,

University of Iowa. The probes were labelled with DIG and the filters hybridized exactly as described by the manufacturer. The amount of apoB and α -actin mRNA on the dot-blots were quantified by MOCHA image analysis software, Jandel Scientific, San Rafael, CA, U.S.A.

Determination of cell viability, chemical analysis and assay of acyl-CoA-cholesterol acyltransferase

Cells viability was determined by measuring the release of formazan from tetrazolium dye using a cytotoxicity assay kit from Promega Corp., Madison, WI, U.S.A. The treatments used in this study did not affect viability. Similar amounts of tetrazolium dye were metabolized by cells given M199 alone, taurocholate or taurocholate and phosphatidylcholine. Compared with control cells incubated in M199, cells treated with 0.25 μ g/ml brefeldin A metabolized 55 % more tetrazolium dye, suggesting that cells were not lost from the filter during treatment with this compound. This was further confirmed by recovering similar amounts of cell protein from the filters after the above treatments. Protein was measured by the method of Lowry et al. [12]. Triacylglycerol mass was measured as previously described [13]. ACAT activity was measured as previously described [14]. The specific radioactivity of [14 C]oleoyl-CoA was 20 000 d.p.m./nmol. ApoB mass was determined by ELISA exactly as described previously [10]. The unpaired *t* test was used to analyse the data.

RESULTS

Effect of phosphatidylcholine on lipid synthesis and secretion

Although most phosphatidylcholine is hydrolysed by pancreatic phospholipase in the upper small intestine, we have been able to demonstrate qualitatively substantial amounts of intact phosphatidylcholine in jejunal fluid obtained from individuals undergoing examinations of small intestinal motility (results not shown). In a previous study, we reported that in CaCo-2 cells incubated with taurocholate and phosphatidylcholine, more apoB was secreted than in cells incubated with taurocholate alone [7]. ApoB secretion is also increased in cultured hepatocytes and CaCo-2 cells incubated with oleic acid and this is believed to be due to, or at least associated with, a rather substantial increase in intracellular triacylglycerol synthesis [10,15]. To address whether an increase in apoB secretion by cells incubated with phosphatidylcholine is associated with changes in cellular lipid metabolism, CaCo-2 cells, cultured on semipermeable filters, were incubated for 18 h in medium alone, medium containing taurocholate or medium containing taurocholate and phosphatidylcholine. Phosphatidylcholine was dissolved in a taurocholate solution to simulate more closely how phosphatidylcholine would be presented to intestinal cells *in vivo*. After the incubation, the amount of triacylglycerol mass within cells and that secreted into the basolateral medium was determined (Table 1). In contrast with taurocholate, which had no effect on triacylglycerol mass within cells or in the basolateral medium, the addition of phosphatidylcholine caused a 3-fold increase in the amount of triacylglycerols secreted basolaterally. Moreover, triacylglycerol mass within cells decreased.

To address the effects of phosphatidylcholine on the synthesis and secretion of newly synthesized lipids, cells were again incubated for 18 h in medium alone, medium containing taurocholate or medium containing taurocholate and phosphatidylcholine. The incorporation of labelled oleic acid or labelled glycerol into cellular lipids and lipids secreted into the basolateral medium was determined (Figure 1 and Table 2). Because cells incubated in medium alone were not different from cells incu-

Table 1 Effect of phosphatidylcholine on triacylglycerol mass within cells and that secreted into the basolateral medium

CaCo-2 cells were incubated for 18 h with M199 (control), M199 containing 1 mM taurocholate or M199 containing 1 mM taurocholate and 500 μ M phosphatidylcholine. After the incubation, the basolateral medium was collected, centrifuged to remove debris, and lipids were extracted. The cells were washed three times and lipids extracted as described in the Materials and methods section. Triacylglycerol mass was estimated as described in the Materials and methods section. The data represent means $\pm$ S.E.M. of six separate dishes. **P* < 0.05, ***P* < 0.01 compared with control and taurocholate.

	Triacylglycerol mass (μ g/mg of protein)	
	Cell	Medium
Control	79 $\pm$ 4	1.9 $\pm$ 0.2
Taurocholate	75 $\pm$ 2	2.4 $\pm$ 0.4
Taurocholate + phosphatidylcholine	60 $\pm$ 1*	5.5 $\pm$ 0.4**

bated in taurocholate, only data for cells incubated with taurocholate or taurocholate and phosphatidylcholine will be shown. In results not shown, the incorporation of either label into total lipids was not altered by phosphatidylcholine. Figure 1 shows a time course for the incorporation of labelled substrate into cell and medium lipids. Two important findings can be reported. First, compared with control cells, the rates of synthesis and secretion of cholesteryl esters were decreased in cells incubated with phosphatidylcholine; and second, the secretion of labelled triacylglycerols was markedly increased in cells incubated with phosphatidylcholine without major changes in the rates of triacylglycerol synthesis. Because of this second observation, several more experiments were performed to address the effects of phosphatidylcholine on triacylglycerol synthesis. From five separate experiments using 20 filters, the rate of triacylglycerol synthesis in cells incubated with phosphatidylcholine was consistently increased by approximately 22 % compared with controls [246 $\pm$ 16 versus 201 $\pm$ 14 pmol of [3 H]glycerol incorporated/4 h per filter (*P* < 0.05, randomized-block analysis of variance)]. Data in Table 2 show the effects of phosphatidylcholine concentration on the secretion of labelled lipids. An increase in the secretion of newly synthesized triacylglycerols was not observed until a concentration of 250 μ M phosphatidylcholine was reached. Phosphatidylcholine did not alter the secretion of labelled phospholipids. In contrast, cells incubated with phosphatidylcholine secreted less newly synthesized cholesteryl esters.

To explain the decrease in the synthesis of cholesteryl esters in cells incubated with phosphatidylcholine, ACAT activities were estimated in total membranes prepared from cells incubated with taurocholate or taurocholate plus phosphatidylcholine. To distinguish effects on substrate supply from direct effects of phosphatidylcholine on the enzyme, ACAT activities were measured in the presence and absence of exogenous cholesterol added to the assay mixture. Compared with that in membranes from cells incubated with taurocholate alone, ACAT activity in membranes prepared from cells incubated with taurocholate plus phosphatidylcholine was decreased by 34 % (266 $\pm$ 6 versus 177 $\pm$ 10 pmol/10 min per mg; *P* < 0.02). ACAT activity measured in the presence of exogenously added cholesterol, however, was similar in the two membrane preparations (878 $\pm$ 26 versus 881 $\pm$ 9 pmol/10 min per mg). Thus cholesteryl ester synthesis and ACAT activity were decreased in cells incubated with

Figure 1 Effect of phosphatidylcholine on lipid synthesis and secretion

The experiment was performed as described in Table 2 except that lipids were extracted from cells and basolateral media at 1, 2 and 4 h after the addition of [3 H]oleate or [3 H]glycerol. The concentration of phosphatidylcholine was 500 μ M. The data represent the means of duplicate dishes at each time point. ●, Taurocholate; ■, taurocholate and phosphatidylcholine.

Table 2 Effect of phosphatidylcholine on secretion of lipids by CaCo-2 cells

CaCo-2 cells were incubated for 18 h with M199 containing 1 mM taurocholate or 1 mM taurocholate and increasing concentrations of egg phosphatidylcholine. At the end of the incubation, media were removed from the apical (top) and basolateral (bottom) wells. The bottom wells were filled with fresh M199. The cells were washed with M199 and the treatments were added back along with either [3 H]oleic acid or [3 H]glycerol. After 4 h, the basolateral media were removed, centrifuged to remove debris, and lipids extracted. The cells were washed three times with M199 and lipids were extracted. The data are expressed as means $\pm$ S.E.M. of four dishes at each concentration. * $P < 0.05$, ** $P < 0.01$ compared with taurocholate alone.

Phosphatidyl- choline (μ M)	Lipid secreted (pmol of [3 H]/4 h per filter)			Lipid secreted (pmol of [3 H]glycerol/4 h per filter)	
	Cholesteryl esters	Triacylglycerol	Phospholipids	Triacylglycerol	Phospholipids
0	0.40 $\pm$ 0.05	11.3 $\pm$ 0.81	2.67 $\pm$ 0.12	1.42 $\pm$ 0.23	0.87 $\pm$ 0.11
10	0.37 $\pm$ 0.04	10.4 $\pm$ 0.86	2.91 $\pm$ 0.16	1.19 $\pm$ 0.19	1.29 $\pm$ 0.05
50	0.36 $\pm$ 0.03	11.3 $\pm$ 0.60	3.15 $\pm$ 0.12	1.24 $\pm$ 0.23	1.05 $\pm$ 0.21
100	0.30 $\pm$ 0.05*	11.3 $\pm$ 0.80	2.76 $\pm$ 0.26	1.17 $\pm$ 0.23	0.95 $\pm$ 0.10
250	0.21 $\pm$ 0.11*	23.0 $\pm$ 5.10**	2.42 $\pm$ 0.09	2.69 $\pm$ 0.33*	0.99 $\pm$ 0.14
500	0.14 $\pm$ 0.03*	24.4 $\pm$ 0.60**	2.07 $\pm$ 0.09	5.83 $\pm$ 0.22**	0.93 $\pm$ 0.07

phosphatidylcholine because of a decrease in the supply of cholesterol to the enzyme.

Effect of phospholipids on triacylglycerol transport

To determine if the increase in triacylglycerol secretion by cells incubated with phosphatidylcholine was specific for this phospholipid, cells were incubated with taurocholate or taurocholate and various phospholipid species. After the incubation, [3 H]glycerol incorporation into cellular and medium triacylglycerols was estimated (Table 3). The incorporation of

glycerol into cellular and medium phospholipids was unchanged by the different phospholipid species and is therefore not shown. All the phospholipids caused a modest increase in the incorporation of glycerol into cellular triacylglycerols, but their effects on the secretion of labelled triacylglycerol were variable. Phosphatidylcholine was the most potent in increasing the secretion of triacylglycerol into the medium. Phosphatidylethanolamine and phosphatidylserine significantly increased the secretion of glycerol-labelled triacylglycerols. In contrast, phosphatidylinositol and sphingomyelin had no effect on triacylglycerol secretion. Thus other phospholipid species would appear to cause an

Table 3 Effect of phospholipids on triacylglycerol synthesis and secretion

CaCo-2 cells were incubated for 18 h with M199 containing 1 mM taurocholate or 1 mM taurocholate and various phospholipids (500 μ M). After the incubation, triacylglycerol synthesis and secretion were estimated using [3 H]glycerol as described in Table 1. The data represent means $\pm$ S.E.M. of at least four dishes for each treatment. * P < 0.05, ** P < 0.01, *** P < 0.001 compared with taurocholate.

Phospholipid	Triacylglycerol (pmol of [3 H]glycerol incorporated/4 h per filter)	
	Cells	Medium
Taurocholate	203 $\pm$ 6	1.32 $\pm$ 0.10
Phosphatidylcholine	226 $\pm$ 4*	4.52 $\pm$ 0.53***
Phosphatidylethanolamine	247 $\pm$ 11*	2.35 $\pm$ 0.16**
Phosphatidylinositol	238 $\pm$ 12*	1.86 $\pm$ 0.27
Phosphatidylserine	293 $\pm$ 14**	2.78 $\pm$ 0.14***
Sphingomyelin	239 $\pm$ 5**	1.64 $\pm$ 0.07

Table 4 Effect of fatty acid composition of phosphatidylcholine on triacylglycerol synthesis and secretion

CaCo-2 cells were incubated for 18 h with M199 containing 1 mM taurocholate or 1 mM taurocholate and 500 μ M dipalmitoylphosphatidylcholine, dioleoylphosphatidylcholine or egg phosphatidylcholine. After the incubation, triacylglycerol synthesis and secretion were estimated using [3 H]glycerol as described in Table 1. The data represented means $\pm$ S.E.M. of eight dishes for each treatment. * P < 0.05, ** P < 0.001 compared with taurocholate.

	Triacylglycerol (pmol of [3 H]glycerol incorporated/4 h per filter)	
	Cells	Medium
Taurocholate	269 $\pm$ 11	1.58 $\pm$ 0.12
Dipalmitoylphosphatidylcholine	280 $\pm$ 7	1.87 $\pm$ 0.22
Dioleoylphosphatidylcholine	333 $\pm$ 11*	5.70 $\pm$ 0.27**
Egg phosphatidylcholine	325 $\pm$ 13*	5.58 $\pm$ 0.38**

increase in newly synthesized triacylglycerol transport, suggesting that this effect is not necessarily specific for phosphatidylcholine.

Table 4 shows data on the effects of altering the fatty acid

composition of phosphatidylcholine on [3 H]glycerol incorporation into cellular triacylglycerols and triacylglycerols secreted into the basolateral medium. Cells were incubated with taurocholate or taurocholate plus egg phosphatidylcholine, dioleoylphosphatidylcholine or dipalmitoylphosphatidylcholine. Clearly, only dioleoylphosphatidylcholine and egg phosphatidylcholine significantly increased the secretion of labelled triacylglycerols. There was also a small but significant increase in the incorporation of label into cellular triacylglycerol. Dipalmitoylphosphatidylcholine, however, was without effect. Again, the various phosphatidylcholines had no effect on the synthesis or secretion of phospholipids (results not shown). In other results not shown, all three phosphatidylcholine species, dioleoyl-, dipalmitoyl- and egg-phosphatidylcholine, decreased the incorporation of labelled oleate into cellular cholesteryl esters and decreased their secretion into the basolateral medium. Similar to what was observed with labelled glycerols, however, only dioleoylphosphatidylcholine and egg phosphatidylcholine increased the secretion of oleate-labelled triacylglycerols.

Effect of phosphatidylcholine on apoB secretion

We have demonstrated that compared with cells incubated with taurocholate alone, cells incubated with taurocholate and phosphatidylcholine contained significantly more apoB and secreted more apoB into the basolateral medium [7]. To examine this further, we asked whether phosphatidylcholine increased apoB secretion by altering apoB gene expression. Cells were incubated 18 h in taurocholate or taurocholate and phosphatidylcholine. After the incubation, cells and the basolateral medium were collected to measure apoB mass, and from another set of cells total RNA was extracted to determine apoB mRNA levels. Table 5 shows these results. It is clear that the increase in apoB mass observed within cells and basolateral medium of cells incubated with phosphatidylcholine and the increase in the rate of apoB synthesis observed previously [7] are secondary to a post-transcriptional event as apoB mRNA levels were unaltered by phosphatidylcholine.

An increase in the amount of apoB mass found in the basolateral medium of cells incubated with phosphatidylcholine does not necessarily exclude the possibility that by altering membrane properties, phosphatidylcholine could cause some non-specific release of apoB (and perhaps other proteins as well).

Table 5 Effect of phosphatidylcholine on ApoB mass and ApoB mRNA

Cells were incubated for 18 h with M199 containing 1 mM taurocholate or taurocholate and 500 μ M egg phosphatidylcholine. The indicated amounts of brefeldin A or monensin were added in ethanol with the treatments. In these experiments the final concentration of ethanol was 0.1%. The amount of apoB mass within cells and that secreted into the basolateral medium was estimated by ELISA. The values are means $\pm$ S.E.M. The numbers of observations are shown in parentheses. In another set of cells, RNA was isolated and the amount of apoB and actin mRNA was estimated by dot-blot as described in the Materials and methods section. For each treatment, six mRNA preparations obtained from two experiments were analysed by dot-blot. For each sample 4, 2, 1 and 0.5 μ g of mRNA was applied on the blot. The data were confirmed by Northern blot. * P < 0.001 compared with taurocholate. nd, Not determined.

	ApoB (ng/18 h per filter)			ApoB mRNA/actin mRNA
	Cells	Medium		
Taurocholate	60 $\pm$ 7	141 $\pm$ 10	(35)	1.0 (arbitrary)
Taurocholate + phosphatidylcholine	107 $\pm$ 11*	252 $\pm$ 19*	(35)	0.9
With 0.05 μ g of brefeldin A/ml				
Taurocholate	54 $\pm$ 3	107 $\pm$ 7	(6)	nd
Taurocholate + phosphatidylcholine	102 $\pm$ 2	132 $\pm$ 6	(6)	nd
With 5 μ M monensin				
Taurocholate	77 $\pm$ 9	146 $\pm$ 17	(6)	nd
Taurocholate + phosphatidylcholine	83 $\pm$ 7	158 $\pm$ 13	(6)	nd

Table 6 Effect of phosphatidylcholine on the amount of labelled apoB associated with microsomes

CaCo-2 cells grown on T-75 flasks and on 6.5 mm diameter Transwells were incubated for 18 h with 1 mM taurocholate (TC) or 1 mM taurocholate and 500 μ M phosphatidylcholine (TC + PC). Cells grown on Transwells were then pulsed for 3 h with 200 μ Ci of [35 S]methionine. Microsomes were prepared from cells of two Transwells and one flask. To monitor recovery of apoB during the subsequent procedures, an aliquot was taken for immunoprecipitation of apoB. Two equal portions of microsomes were incubated in the presence or absence of 0.1 M Na_2CO_3 to release intraluminal contents. The suspensions were then re-centrifuged and the sediments and supernatants were taken for immunoprecipitation of apoB and separation by SDS/PAGE. Values represent means $\pm$ S.E.M. of nine samples for each treatment from three experiments. * $P < 0.01$ compared with taurocholate.

	ApoB100 (d.p.m./microsomal preparation)		ApoB48 (d.p.m./microsomal preparation)	
	TC	TC + PC	TC	TC + PC
Microsomes (M) (total; sediment + supernatant)	997 $\pm$ 88	1800 $\pm$ 124*	623 $\pm$ 82	982 $\pm$ 98*
Supernatants				
Na_2CO_3				
— (A)	124 $\pm$ 10	300 $\pm$ 16	86 $\pm$ 22	109 $\pm$ 11
+ (B)	355 $\pm$ 22	775 $\pm$ 55	169 $\pm$ 43	228 $\pm$ 36
Specific release of apoB caused by Na_2CO_3 treatment:				
Lumen (B — A)	230 $\pm$ 14	475 $\pm$ 30*	82 $\pm$ 10	119 $\pm$ 15
Percentage in lumen $[100 \times (B - A)]/M$	23	26	13	12

We therefore asked whether a normal secretory process was required for phosphatidylcholine to have an effect on apoB secretion. Brefeldin A and monensin are two agents that disrupt protein secretion at separate and distinct intracellular locations [16–18]. Brefeldin A disrupts the Golgi apparatus by causing it to collapse into the endoplasmic reticulum. Monensin, in contrast, interferes with the transport of proteins through the trans-cisternae of the Golgi apparatus. Added separately to CaCo-2 cells incubated with taurocholate and phosphatidylcholine, both brefeldin A (0.05 μ g/ml) and monensin (5 μ M) completely prevented the increase in apoB secretion, suggesting that a normal secretory pathway was necessary for phosphatidylcholine to cause an increase in apoB secretion (Table 5).

Lastly we had observed that an increase in apoB secretion by cells incubated with phosphatidylcholine was associated with an increase in apoB synthesis without altering the rate of apoB degradation [7]. We therefore asked whether the fraction of newly synthesized apoB destined for the secretory pathway was altered in cells incubated with phosphatidylcholine. After a 3 h pulse with [35 S]methionine, microsomes were prepared from cells incubated with taurocholate or taurocholate and phosphatidylcholine. The amount of labelled apoB100 and apoB48 associated with intact microsomes and the amount found within the lumen after release of the intraluminal contents with Na_2CO_3 was determined. The results presented in Table 6 show that, although more labelled apoB was found in microsomes prepared from cells incubated with phosphatidylcholine and more was present within the lumen (indicating that more newly synthesized apoB was destined for secretion), the fractional amount of apoB within the lumen was similar to that observed in microsomes from control cells.

DISCUSSION

It is clear from the present results that exposure of intestinal cells to phosphatidylcholine mobilizes the secretion of cellular triacylglycerols by increasing the number of apoB-containing lipoprotein particles being secreted. These results agree with results obtained in animal studies showing that luminal phos-

phatidylcholine increases the amount of triacylglycerols transported into lymph [2–4]. It is interesting that phosphatidylcholine increased apoB and triacylglycerol secretion only when concentrations of 250 μ M or more were reached. This all or none phenomenon might suggest that a critical concentration of phosphatidylcholine is required to cause a change in the physical properties of a membrane or a membrane protein which is important for lipoprotein secretion. Evidence that interaction with the membrane may be important comes from the observation that dipalmitoylphosphatidylcholine was shown to have no effect on apoB or triacylglycerol transport. Replacing an unsaturated fatty acid with palmitic acid on position two of phosphatidylcholine alters its physical properties so that it does not spontaneously transfer to plasma membranes [19,20]. More detailed studies on the biophysical interaction of phosphatidylcholine with CaCo-2 cell plasma membranes are required for a better understanding of the possible relationship between changes in membrane properties and lipoprotein secretion.

It is recognized from data obtained in CaCo-2 and HepG-2 cells that oleic acid increases the secretion of apoB by protecting nascent apoB from intracellular degradation [10,15]. The regulation of apoB secretion by phosphatidylcholine, however, differs from that of oleic acid. First, CaCo-2 cells lack the ability to hydrolyse and release fatty acids from phosphatidylcholine added to the apical medium [7]. We have estimated that fatty acids would be present at a concentration of no more than 4 μ M in the apical medium after an 18 h incubation of cells with 500 μ M phosphatidylcholine. This amount of fatty acid is insufficient to cause triacylglycerol accumulation within the cell or triacylglycerol-rich lipoprotein production. Secondly, incubation of CaCo-2 cells with oleic acid causes the accumulation of triacylglycerol mass within cells and increases the rate of triacylglycerol synthesis by 4-fold [10,21]. Phosphatidylcholine, in contrast, depletes cells of triacylglycerol mass and increases triacylglycerol synthetic rates by a modest 22%. This suggests that, unlike oleic acid which drives lipoprotein production by increasing triacylglycerol synthesis and accumulation [10,15,21,22], the effect of phosphatidylcholine occurs at later stages of the secretory pathway, mobilizing intracellular stores of both newly

synthesized and preformed (unlabelled) triacylglycerols. This is supported by animal studies. In bile-fistula rats, O'Doherty et al. [23] demonstrated that the jejunal infusion of egg phosphatidylcholine caused a rapid clearance of triacylglycerol from intestinal mucosa. Likewise, Mansbach et al. [4] observed that, when phosphatidylcholine was included in the duodenal infusate of lymph-fistula animals, the estimated turnover rate for mucosal triacylglycerol pools was greatly enhanced. Lastly, oleic acid increases the secretion of apoB without altering rates of apoB synthesis [10,15]. In contrast with this, phosphatidylcholine increases apoB synthesis but does not appear to affect apoB degradation rates [7]. Recently, in cultured hepatocytes, very-low-density lipoprotein (VLDL) was shown to increase apoB secretion by increasing apoB synthesis, a mechanism independent of oleic acid [15]. It is possible that phosphatidylcholine in the VLDL particle, as observed here in CaCo-2 cells, was responsible for the increase in apoB synthesis. From these results with VLDL and our data, we postulate that phosphatidylcholine causes an increase in apoB synthesis. This leads to more nascent apoB entering the secretory pathway but does not alter the fraction of nascent apoB translocated into the lumen of the endoplasmic reticulum (Table 6).

It has been postulated that the availability of lipids within the lumen of endoplasmic reticulum dictates the amount of apoB eventually secreted [24–26]. There remains controversy, however, as to which lipid is important. In this regard, evidence has been provided for the importance of cholesteryl esters, triacylglycerols and phospholipids. The results of this study show quite clearly that new synthesized cholesteryl esters are not required for apoB secretion. Despite a rather marked decrease in cholesteryl ester formation within cells incubated with phosphatidylcholine, apoB and triacylglycerol secretion were increased. This agrees with previous results in CaCo-2 cells demonstrating an independence of apoB secretion from cholesteryl ester synthesis [27,28]. Since triacylglycerol synthesis was increased some 22% in cells incubated with phosphatidylcholine, we cannot exclude the possibility that a small pool of triacylglycerol, important for lipoprotein assembly, was expanded by phosphatidylcholine. Although this cannot be proved or disproved by the data, we argue that phosphatidylcholine probably has its effect, not by driving lipoprotein production, but by mobilizing existing pools of triacylglycerols for secretion. This would explain why triacylglycerol mass was depleted within CaCo-2 cells incubated with phosphatidylcholine (Table 1).

Likewise, the results do not support a role for phospholipid synthesis as a mechanism to explain the increase in triacylglycerol and apoB transport by phosphatidylcholine. Neither phospholipid synthesis nor secretion were increased in cells incubated with phosphatidylcholine. Similarly, in lymph-fistula rats, lymphatic secretion of newly synthesized phospholipids was not increased in animals whose duodenum was perfused with phosphatidylcholine [2]. Thus we postulate that phosphatidylcholine increases triacylglycerol-rich lipoprotein secretion by mechanisms that are unrelated to a general increase in lipid synthesis. Mechanisms for the mobilization by phosphatidylcholine of intracellular triacylglycerols for transport

causing more apoB to be synthesized and secreted remains to be elucidated.

In patients with chronic biliary obstruction such as primary sclerosing cholangitis and primary biliary cirrhosis, the malabsorption of fat and fat-soluble vitamins remains a significant problem in their long-term care. Since these are conditions in which luminal biliary bile salts and phosphatidylcholine are severely lacking, supplementing the diet with bile acids and phosphatidylcholine (lecithin) may improve the absorption of fat and other nutrients without concomitantly increasing the absorption of cholesterol.

This work was supported by the Veterans Administration and NIH grant HL 49264. We are grateful for the expert assistance of Joan Ockenfels and Jon Klein for typing the manuscript.

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