Role of the neutral lipid accessible pool in the regulation of secretion of apoB-100 lipoprotein particles by HepG2 cells

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Abstract The rate of secretion of apoB-100-containing lipoprotein particles by HepG2 cells is determined to an important extent by post-translational mechanisms, the mass of neutral lipids clearly playing a role in this process. Our previous data indicated that cholesteryl ester might influence the proportion of newly synthesized apoB-100 molecules that are incorporated into nascent lipoproteins rather than being catabolized intracellularly shortly after they have been synthesized. The present studies, therefore, were designed: *1)* to examine in more detail the relationship between the mass of triglyceride and cholesteryl ester in HepG2 cells and the rate of apoB-100 secretion, and *2)* to determine whether cholesteryl ester molecules that have been synthesized and stored within these cells must undergo hydrolysis and re-esterification before being secreted with newly synthesized apoB-100 molecules. Changes in apoB-100 secretion in HepG2 cells were assessed in response to changes in intracellular triglyceride and/or cholesteryl ester pool size. This was accomplished through lipid loading of the cells by incubating them overnight with exogenously supplied very low density lipoprotein (VLDL) (with lipoprotein lipase, LPL), low density lipoprotein (LDL), oleate, or LDL + oleate. The medium was changed to fresh serum-free medium and apoB-100 secretion was shown to increase over at least 8 h. After overnight incubation with VLDL, intracellular triglyceride mass increased 6-fold, while intracellular cholesteryl ester mass increased 2-fold. Medium apoB-100 increased up to 3-fold, while apoB-100 mRNA increased by only 12%. Both heparin (10 IU/ml) and lactoferrin (20 μM) independently blocked the VLDL-mediated increases in intracellular cholesteryl ester mass (-56% and -46%) without decreasing triglyceride mass. ApoB-100 secretion was also reduced by 53% and 72%, respectively. Incubation of HepG2 cells with LDL increased intracellular cholesteryl ester mass but triglyceride mass remained unchanged. In this instance, apoB-100 secretion increased 2-fold but there was no change in apoB-100 mRNA. Overall, there was little relationship between the mass of intracellular triglyceride and the rate of apoB-100 secretion (r^2 = 0.034, NS) whereas there was a strong correlation between the intracellular mass of cholesteryl ester and apoB-100 secretion (r^2 = 0.67, P < 0.0005). To examine the process of cholesteryl ester secretion, intracellular triglyceride and cholesteryl ester mass were increased after incubation with LDL + oleate. The medium was then changed to fresh serum-free medium containing an acyl-CoA:cholesterol acyltransferase (ACAT) inhibitor (Sandoz compound 58-035). Although de novo cholesteryl ester synthesis was inhibited up to 89%, cholesteryl ester mass secretion remained constant with up to 15% of total cholesteryl ester mass secreted over the 8-h period. ApoB-100 secretion also remained elevated above control, with 92% of the cholesteryl ester secreted associated with apoB-100 particles (27% with d < 1.006 g/mL particles and 65% with d 1.006–1.063 g/mL particles). Therefore, not only newly synthesized cholesteryl ester but also stored cholesteryl ester can associate with newly synthesized apoB-100 molecules and can be secreted without the necessity of an intracellular hydrolysis/re-esterification step.—**Kohen Avramoglu, R., K. Cianflone, and A. D. Sniderman.** Role of the neutral lipid accessible pool in the regulation of secretion of apoB-100 lipoprotein particles by HepG2 cells. *J. Lipid Res.* 1995. **36:** 2513-2528.

Supplementary key words hepatocyte • cholesteryl ester • triglyceride

The rate at which apoB-100 lipoprotein particles are secreted by the liver can differ considerably as can their lipid composition. These differences in composition and number are important determinants of the plasma concentration of atherogenic lipoproteins in humans and, therefore, determining the metabolic mechanisms responsible for them is of considerable clinical as well as biologic importance (1). Fortunately, understanding of the secretion of apoB-100 particles has advanced considerably in the past few years. A series of studies, principally in HepG2 cells, has shown that the secretory rate and composition of apoB-100 lipoprotein particles are a function of the type and amount of lipid substrate

Abbreviations: TLC, thin-layer chromatography; LPL, lipoprotein lipase; VLDL, very low density lipoprotein; LDL, low density lipoprotein; PBS, phosphate-buffered saline; FA, fatty acid; ACAT, acyl-CoA:cholesterol acyltransferase.

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synthesized within, or presented to, the liver, the amount and type of amino acids delivered to these cells, or the hormonal milieu in which these cells are maintained (2–7).

It is generally agreed that a major determinant of the secretory rate of apoB-100 particles results from variation in the proportion of newly synthesized apoB-100 molecules that associate with lipid, enter the lumen of the endoplasmic reticulum, and are secreted by the cell, versus the proportion of these molecules that are hydrolyzed intracellularly shortly after their synthesis. The hydrolysis of newly synthesized apoB-100 may relate to the accessibility of domains of the molecule to proteolytic enzymes within the cytosol or the endoplasmic reticulum (7–13). In contrast, delivery of amino acids, which operates at a transcriptional rather than post-translational level, does not affect the secretion efficiency of newly synthesized apoB-100 (6). Transcriptional versus post-translational regulation are not entirely distinct, however, as increased delivery of cholesterol (14) or very low density lipoprotein (VLDL) remnants (15) may affect transcription as well as secretion efficiency.

The evidence that microsomal triglyceride transfer protein is essential for assembly of an apoB-100 lipoprotein (16) is consistent with the hypothesis that core lipid must rapidly associate with the newly synthesized apoB-100 molecule if the latter is to successfully complete its translocation into the lumen of the endoplasmic reticulum. If this hypothesis is correct, then is it only the mass of the major neutral lipid (triglyceride) in a secreted VLDL particle that is important? It should be noted that microsomal triglyceride transfer protein transfers not only triglyceride, but also cholesteryl ester at comparable rates (17). Could cholesteryl ester, although quantitatively a much smaller pool, also play a role? The present studies were therefore designed to examine in more detail the role of cholesteryl ester in this process.

The data indicate that in HepG2 cells, there is a much closer relationship between the mass of intracellular cholesteryl ester and the rate of apoB-100 secretion than there is with the mass of intracellular triglyceride. We have previously shown a relationship between increases in de novo synthesized cholesteryl ester and apoB-100

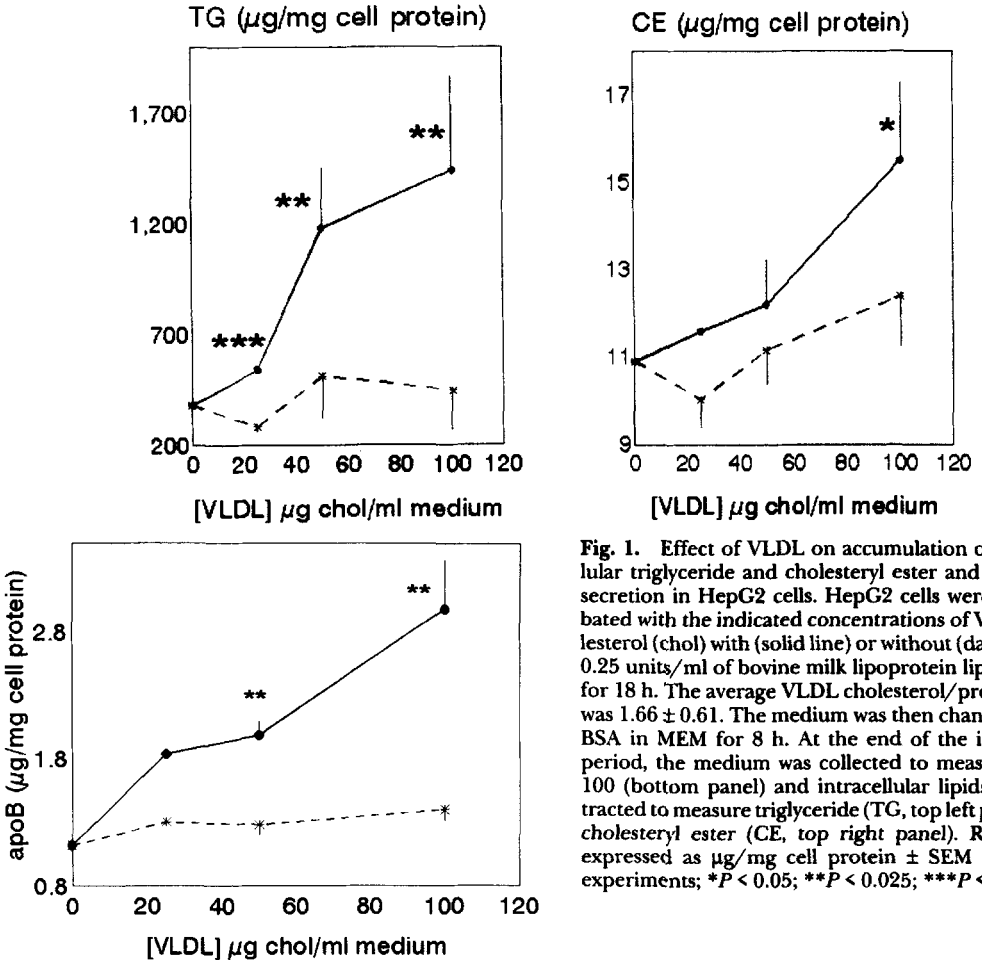

Fig. 1. Effect of VLDL on accumulation of intracellular triglyceride and cholesteryl ester and apoB-100 secretion in HepG2 cells. HepG2 cells were preincubated with the indicated concentrations of VLDL cholesterol (chol) with (solid line) or without (dashed line) 0.25 units/ml of bovine milk lipoprotein lipase (LPL) for 18 h. The average VLDL cholesterol/protein ratio was 1.66 ± 0.61. The medium was then changed to 1% BSA in MEM for 8 h. At the end of the incubation period, the medium was collected to measure apoB-100 (bottom panel) and intracellular lipids were extracted to measure triglyceride (TG, top left panel) and cholesteryl ester (CE, top right panel). Results are expressed as μg/mg cell protein ± SEM for n = 3 experiments; $*P < 0.05$; $**P < 0.025$; $***P < 0.01$.

secretion in a HepG2 cell challenged with fatty acid (9). The present study extends these findings by demonstrating that it is not only newly synthesized cholesteryl ester but also stored cholesteryl ester mass that can increase apoB-100 secretion. This effect of cholesteryl ester is achieved without the necessity of an intracellular hydrolysis/re-esterification step prior to secretion of cholesteryl ester associated with an apoB-100 particle.

MATERIALS AND METHODS

Materials

All reagents used were obtained from Fisher Scientific (Montreal, Quebec) and Sigma (St. Louis, MO) unless otherwise indicated. Bradford protein assay kit and bovine serum albumin standard were obtained from Bio-Rad Laboratories (Mississauga, Ontario). HepG2 cells were obtained from the American Tissue Culture Collection (Rockville, MD). All tissue culture media and supplies were from Gibco (Burlington, Ontario) or Flow Laboratories (Mississauga, Ontario). Lactoferrin and lipoprotein lipase (LPL) were purchased from Sigma (St. Louis, MO). Heparin was purchased from Organon Pharmaceuticals Canada LTD. TLC plates were obtained from Mandel Scientific (Rockwood, Ontario). Sodium oleate was obtained from Sigma Chemicals (St. Louis) and [9,10-^{3}H(N)]oleic acid (sp act = 10 Ci/mmol) from DuPont NEN. Oleate was complexed to bovine serum albumin (BSA) as described by Van Harken, Dixon, and Heimberg (18).

Lipoprotein isolation

Fasting blood was obtained from hypertriglyceridemic individuals visiting the Lipid Clinic at the Royal Victoria Hospital. These studies were approved by the Royal Victoria, Hospital Ethics Committee and all subjects gave informed consent. The blood was collected into tubes containing EDTA to a final concentration of 0.15% (w/v). The plasma fraction was immediately isolated by a 20-min centrifugation at 2,000 rpm at 4°C. The plasma obtained was layered under an equal volume of solution of density 1.006 g/mL consisting of 0.195 M NaCl, 1 mM Tris, pH 7.4, 1 mM EDTA, and 3 mM sodium azide.

Triglyceride-rich VLDL were isolated by centrifugation at 40,000 rpm (100,000 *g*) for 2 h at 12°C (19). The collected supernatant was recentrifuged for 18 h at 40,000 rpm (100,000 *g*) at 12°C and collected in as small a volume as possible. The isolated VLDL was filtered through a 0.45-μm pore syringe filter into a sterile tube. Low density lipoprotein (LDL) was isolated by layering one volume of plasma under two volumes of solution of d 1.019 g/mL and centrifuging for 18 h at 40,000 rpm at 12°C in order to remove VLDL (19). The infranate was collected and its density was increased to 1.063 g/mL and recentrifuged for 20 h at 40,000 rpm and

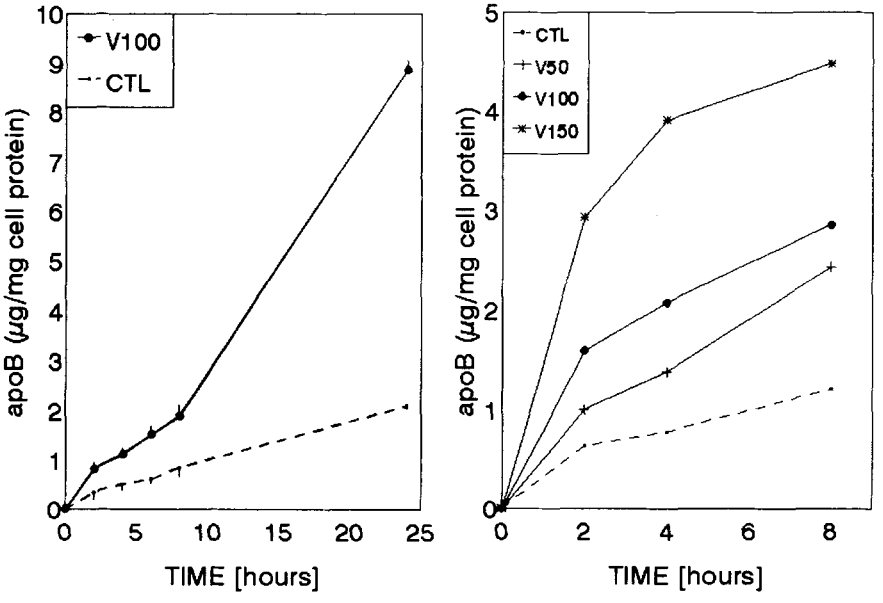

Fig. 2. Time course of apoB-100 secretion in HepG2 cells effect of VLDL. HepG2 cells were preincubated with the indicated concentrations of VLDL (V) cholesterol (chol) (50, 100, or 150 μg VLDL cholesterol/ml medium) with LPL (0.25 units/ml) for 18 h. The medium was then changed to 1% BSA in MEM and medium apoB-100 was measured at the indicated times. Left panel: Results are expressed as μg/mg cell protein ± SEM for n = 3 experiments over 24 h. Right panel: Results are expressed as μg/mg cell protein for a representative experiment over 8 h.

12°C. LDL was dialyzed against phosphate-buffered saline (PBS), sterile-filtered, and kept at 4°C. A modified Lowry assay was used to measure protein content of the isolated lipoproteins (20) using BSA as a standard. Total cholesterol and triglyceride in the lipoprotein fractions were measured by colorimetric enzymatic kits (CHOD-PAP and triglyceride-without-free-glycerol, respectively, from Boehringer Mannheim Canada, Laval, Quebec). The average cholesterol/protein ratio for 18 different VLDL preparations was 1.66 ± 0.61, and in 8 different LDL preparations was 1.53 ± 0.60.

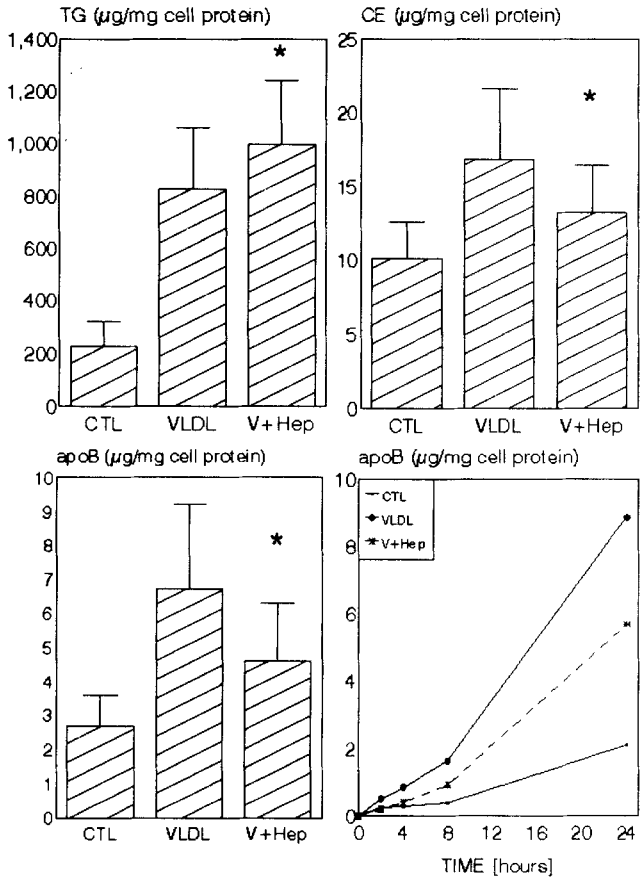

Fig. 3. Effect of heparin on VLDL-induced accumulation of triglyceride and cholesteryl ester and apoB-100 secretion in HepG2 cells. HepG2 cells were preincubated with VLDL (V) (100 μg cholesterol/ml) and LPL (0.25 units/ml) with or without heparin (Hep) (10 units/ml) for 18 h. The medium was then changed to 1% BSA in MEM for 8 h. At the end of the incubation period, medium was collected to measure apoB-100 (bottom left panel) and intracellular lipids were extracted to measure triglyceride (TG, top left panel) and cholesteryl ester (CE, top right panel). Results are expressed as μg/mg cell protein ± SEM for n = 5 experiments; $*P < 0.05$. Significance was calculated by paired *t*-test for incubations containing VLDL + heparin as compared to VLDL alone. Bottom right panel: A representative experiment measuring apoB-100 secretion over 24 h after preincubation with VLDL or VLDL + heparin.

HepG2 cell culture

HepG2 cells were grown in 75-mm² flasks in minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS). The cells were subcultured every 7 days with a split ratio of 1:3. At confluency, cells were plated out in 24-well plates at a density of 1.3×10^4 cells per cm² for experiments. Cells were preincubated overnight in MEM supplemented with 1% BSA. Known amounts of lipoprotein as well as lipoprotein lipase (LPL) or inhibitors were then added to the medium (MEM with 1% BSA) and the cells were incubated for an additional 18 h. Lipoproteins were added to the cells based on cholesterol content in μg cholesterol/mL culture medium. LPL was added at a concentration of 0.25 units/mL culture medium where one unit is defined as 1 μmol of free fatty acid released per hour. The amount of LPL used was based on previous work done by Cianflone et al. (21) and by Evans et al. (22, 23). In all cases, there was no evidence of any change in cell morphology or variations in the total amount of cell protein following the incubations.

To measure apoB-100 secretion into the medium after incubation under various conditions, the medium containing lipoproteins or other additions was first removed, the cells were washed 3 times with 1 mL of 37°C PBS, and a 0.5-mL aliquot of fresh MEM containing 1% BSA was added for an additional incubation of 8 h. After this incubation, cells were washed 3 times with 1 mL ice-cold PBS and the intracellular lipids were extracted twice with 1 mL heptane–isopropanol 3:2 (v/v) at room temperature for 30 min and the extracts were pooled. Cell protein was solubilized using 1 mL of 0.1 N NaOH and quantified by the method of Bradford (24) using BSA as a standard.

Determination of intracellular and secreted lipids

Cell lipid extracts were concentrated and separated by thin-layer chromatography (Silica Gel G) using a solvent system consisting of hexanes–ethyl ether–acetic acid 75:25:1 (v/v/v). Lipid spots were identified by exposure to iodine vapor and compared to reference lipids. Triglyceride spots were scraped into tubes and the lipid was extracted by adding 0.5 mL isopropanol; mass was determined by the method of Neri and Frings (25) using a standard curve of 0–52 μg/mL triolein. Cholesteryl ester spots were similarly scraped into tubes and cholesteryl ester was measured by the method of Rudel and Morris (26) using a standard curve of 0–14 μg/mL cholesteryl oleate. Conditioned medium was extracted with 3 volumes isopropanol–heptane 1:1 (v/v). The lipid extract was then analyzed as described above for intracellular cholesteryl ester and triglyceride.

Determination of apolipoprotein B-100

An indirect competitive type enzyme-linked immunosorbent assay (ELISA) was used to quantify the apoB-100 secreted by the HepG2 cells. Rabbit antihuman apoB-100 antibody was generated in white male New Zealand rabbits. To do so, human plasma LDL was separated by 3–15% polyacrylamide gradient SDS gel electrophoresis under denaturing conditions as described by Laemmli (27). The gel was stained with Coomassie blue, and the band corresponding to apoB-100 was excised, emulsified with Freund's adjuvant, and injected subcutaneously. Microtiter plates were coated with a 3 μg/mL solution of LDL protein in PBS, (pH = 7.2) overnight at 4°C and blocked with 1.5% BSA in PBS for 2 h at room temperature. Standards containing 0.06–4 μg/mL LDL-derived apoB-100 were prepared. Rabbit anti-human apoB-100 polyclonal antibody (diluted 1:6000 in PBS containing 0.5 mL/L Tween 20) was added to each sample (standards and HepG2 media) and incubated overnight. Then 100 μL of each sample was added in triplicate to the microtiter plate for 2 h at 37°C. After this incubation, plates were washed and 100 μL anti-rabbit IgG conjugated to horseradish peroxidase (diluted in 4% polyethylene glycol, 0.5 mL/L Tween 20 in PBS) was added to each well and incubated at 37°C for 30 min. Plates were again washed and a colorimetric reaction was initiated by adding 100 μL 1 mg/mL *o*-phenylenediamine dihydrochloride in 0.018% H_2O_2 in 100 mM sodium citrate, pH 5.0. The color reaction was stopped with 50 μL 4 N H_2SO_4 and the plate was read spectrophotometrically at 490 nm. A log-log plot of apoB-100 concentration (μg/mL) versus absorbance at 490 nm was constructed and linear least squares analysis of the data was carried out. ApoB-100 controls (Boehringer Mannheim, Laval, Quebec, Canada) of known concentration were run with each analysis to ensure reproducibility of the results.

Lipoprotein fractionation of HepG2 medium

Medium from HepG2 cells was fractionated as described for plasma lipoproteins. HepG2 cell medium was layered under a salt solution of d 1.006 g/mL and centrifuged at 100,000 *g* for 18 h and the top 2 mL was collected. The remaining solution was adjusted to d 1.063 g/mL and recentrifuged for 20 h at 100,000 *g*. Apolipoprotein B-100, cholesteryl ester mass, and triglyceride mass were measured on each fraction.

Slot-blot analysis of HepG2 mRNA

HepG2 cells in 75 mm^2 flasks were preincubated in MEM supplemented with 1% BSA overnight. Known amounts of lipoprotein (VLDL or LDL) and fatty acids as well as LPL were then added to the medium containing 1% BSA and the cells were incubated for an additional 18 h. The cells were washed rapidly with ice-cold PBS and then immediately lyzed with a commercial extraction solvent (TRIzol, Gibco BRL Life Technologies) and the RNA was isolated. Aliquots of RNA (5, 10, and 20 μg) were applied to a nylon membrane, using a

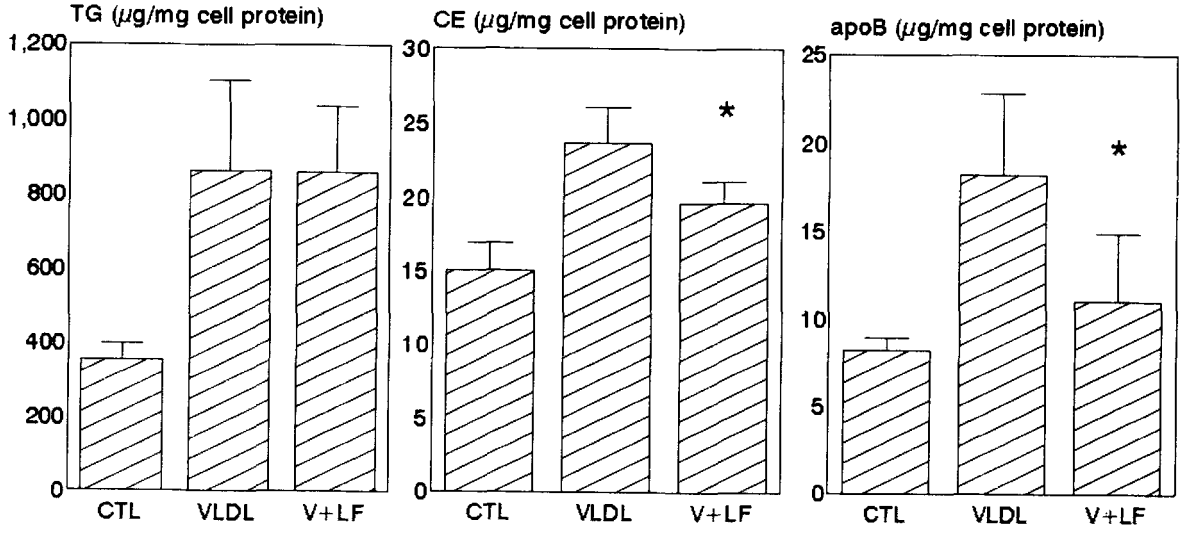

Fig. 4. Effect of lactoferrin on VLDL-induced accumulation of triglyceride and cholesteryl ester and apoB-100 secretion in HepG2 cells. HepG2 cells were preincubated with VLDL (V) (100 μg cholesterol/ml) and LPL (0.25 units/ml) with or without lactoferrin (LF) (20 μM) for 18 h. The medium was changed to 1% BSA in MEM for 8 h. At the end of the incubation period, medium was collected to measure apoB-100 (right panel) and intracellular lipids were extracted to measure triglyceride (TG, left panel) and cholesteryl ester (CE, center panel). Results are expressed as μg/mg cell protein ± SEM for n = 4 experiments; $*P < 0.05$. Significance was calculated by paired *t*-test for incubations containing VLDL + heparin as compared to VLDL alone.

vacuum manifold apparatus, and cross-linked by ultraviolet light. Biotinylated cDNA probes were prepared using a nonradioactive PCR labeling system (Gibco BRL Life Technologies #10200-012) with biotin-14d-CTP as the label. Primers for apoB-100 were: sense (5′ GAC CAC AAG CTT AGC TTG G) antisense (5′ GGG TGG CTT TGC TTG TAT G) and for glyceraldehyde-3-phosphate: sense (5′GGT GAA GGT CGG AGT CAA CGG ATT TGG), antisense (5′GGC CAT GAG GTC CAC CAC CCT GTT) yielding products of 320 bp and 978 bp, respectively (28, 29). Membranes were hybridized overnight and apoB-100 RNA was visualized using an enzymatic chemiluminescent method (Photogene #81925A Gibco BRL Life Technologies). The exposed film was scanned on a densitometer. The membrane was then stripped and reprobed for glyceraldehyde-3-phosphate. Glyceraldehyde-3-phosphate was used as a "housekeeping" gene, as it does not change under the incubation conditions (30). Results are expressed in arbitrary units as a ratio of apoB-100/glyceraldehyde-3-phosphate.

Statistical analysis of data

Each experiment is the average of triplicate determinations for each point and is expressed per mg soluble cell protein. Results are expressed as the average of a number of experiments (n) as indicated in the figure legends. Deviation is expressed as the standard error of the mean (SEM). Statistical significance was calculated using a paired t test with significance set at $P < 0.05$, where NS is not significant.

RESULTS

Two series of experiments were performed. The object of the first was to determine whether the rate of apoB-100 secretion related more closely to the mass of triglyceride or cholesteryl ester within the cells. The object of the second was to determine whether preformed molecules of cholesteryl ester could associate with apoB-100 molecules and be secreted. This would indicate that both preformed cholesteryl ester as well as newly synthesized molecules of cholesteryl ester could associate with newly synthesized apoB-100 molecules.

Experiments to differentiate the effects of triglyceride versus cholesteryl ester mass on apoB-100 secretion by HepG2 cells

These experiments were conducted as follows. In all cases, there was an initial 18-h preincubation period during which the cells were maintained in MEM with 1% BSA plus any of the specific additions, which will be noted. Next, the preincubation medium was removed, MEM with 1% BSA added, and after a further 8-h incubation, the concentration of apoB-100 in the medium was measured. The first point to note is that addition of VLDL to the preincubation medium increased neutral lipid mass in HepG2 cells only when LPL was also present (**Fig. 1,** top panels) as has been demonstrated previously by Evans and colleagues (22) and by our laboratory (21). In the absence of LPL, preincubation for 18 h with VLDL did not significantly affect the mass of either triglyceride (top left panel) or cholesteryl ester (top right panel) within the cells. By contrast, addition of LPL to the preincubation medium with increasing concentrations of VLDL (up to 100 μg VLDL cholesterol/mL medium where the average VLDL cholesterol/protein ratio was 1.66 ± 0.61, n = 18) was associated with a dose-dependent increase in both triglyceride and cholesteryl ester mass in the HepG2

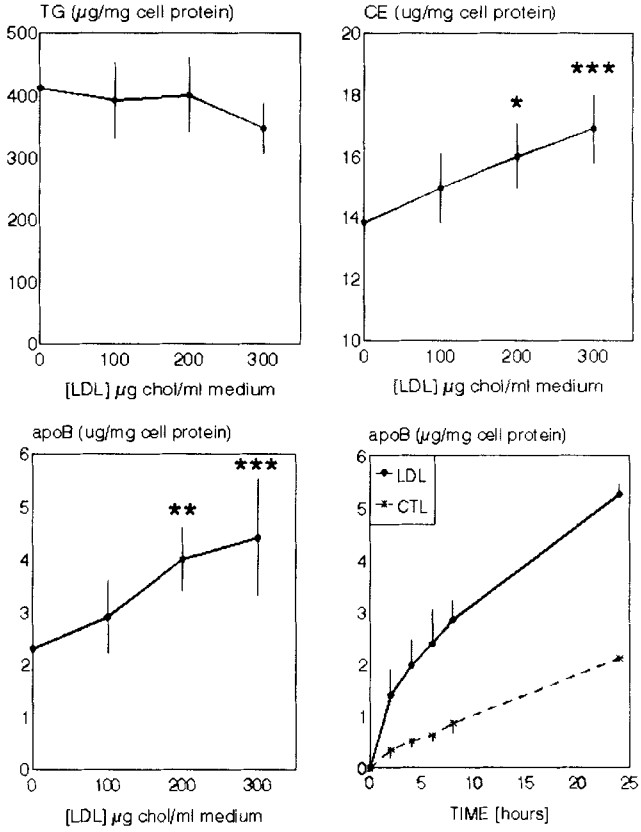

Fig. 5. Effect of LDL on accumulation of intracellular cholesteryl ester and apoB-100 secretion in HepG2 cells. HepG2 cells were preincubated with the indicated concentrations of LDL cholesterol (chol) for 18 h. The medium was changed to 1% BSA in MEM for 8 h. At the end of the incubation period, the medium was collected to measure apoB-100 (bottom left panel) and intracellular lipids were extracted to measure triglyceride (TG, top left panel) and cholesteryl ester (CE, top right panel). Results are expressed as μg/mg cell protein ± SEM for n = 5 experiments; $*P < 0.05$; $**P < 0.025$; $***P < 0.0025$. Bottom right panel: Time course of apoB-100 secretion over 24 h after preincubation with LDL (300 μg cholesterol/ml) for 18 h. Results are expressed as μg apoB-100/mg cell protein ± SEM for n = 3 experiments.

cells. In these experiments, there was a substantial increase in the mass of apoB-100 secreted from the HepG2 cells during the incubation period whereas this did not occur when LPL had not been added (Fig. 1, bottom panel). Indeed at the highest concentration of VLDL (100 μg/mL cholesterol), there was almost a 3-fold increase in apoB-100 secretion. Clearly, an increase in lipid mass within the HepG2 cells during the preincubation period was associated with increased apoB-100 accumulation in the medium during the subsequent 8-h observation period.

There are two assumptions made in the previous experiment (Fig. 1). First, that the apoB-100 secretion from the cells after the preincubation is increased for at least 8 h; and second, that the lipid mass remaining in the HepG2 cells 8 h after the preincubation period is an accurate reflection of the amount of lipid mass present immediately after lipid loading. As shown in **Fig. 2,** left panel, basal apoB-100 secretion increased for at least 24 h. After lipid loading of the cells with VLDL (100 μg cholesterol/mL) and LPL for 18 h, the medium was changed and apoB-100 secretion under these conditions was also increased for at least 24 h (Fig. 2, left panel). We then examined the effects of varying concentrations of VLDL + LPL on apoB-100 secretion over an 8-h period. As shown in Fig. 2, right panel, apoB-100 secretion in preloaded cells increased with time up to 8 h at all concentrations of VLDL + LPL tested. The rate of secretion of apoB-100 (for all VLDL concentrations) measured as the slope of the line from 2 to 8 h by linear regression was 2.0- to 2.5-fold that of control. This excludes the probability that any of the LDL secreted was preformed during the inhibition period (6). In parallel experiments, we measured the amount of lipid in HepG2 cells immediately after the preincubation period, and compared it to the amount of lipid remaining after a further 8-h period. In control cells, 87% ± 7% of triglyceride mass and 96% ± 2% of cholesteryl ester mass remained within the cell (n = 3 experiments). Similarly in VLDL + LPL-loaded cells, 96% ± 12% of triglyceride mass and 92% ± 8% of cholesteryl ester mass remained after the 8-h incubation (n = 3 experiments). Therefore, it was felt that an 8-h observation period allowed us to measure the effect of the lipid states of the cell on apoB-100 secretion accurately without a significant change in the lipid mass of the cells over that time period.

As increases in intracellular triglyceride and cholesteryl ester mass were both observed after incubation with VLDL + LPL, it is not possible to judge the relative importance of either lipid on the increased apoB-100 secretion that resulted. Accordingly, the uptake process of the VLDL was modified by the addition of agents with the goal of selectively diminishing either triglyceride or cholesteryl ester mass accumulation within the HepG2 cells.

The first set of experiments was designed to manipulate the mass of cholesteryl ester with little change in the

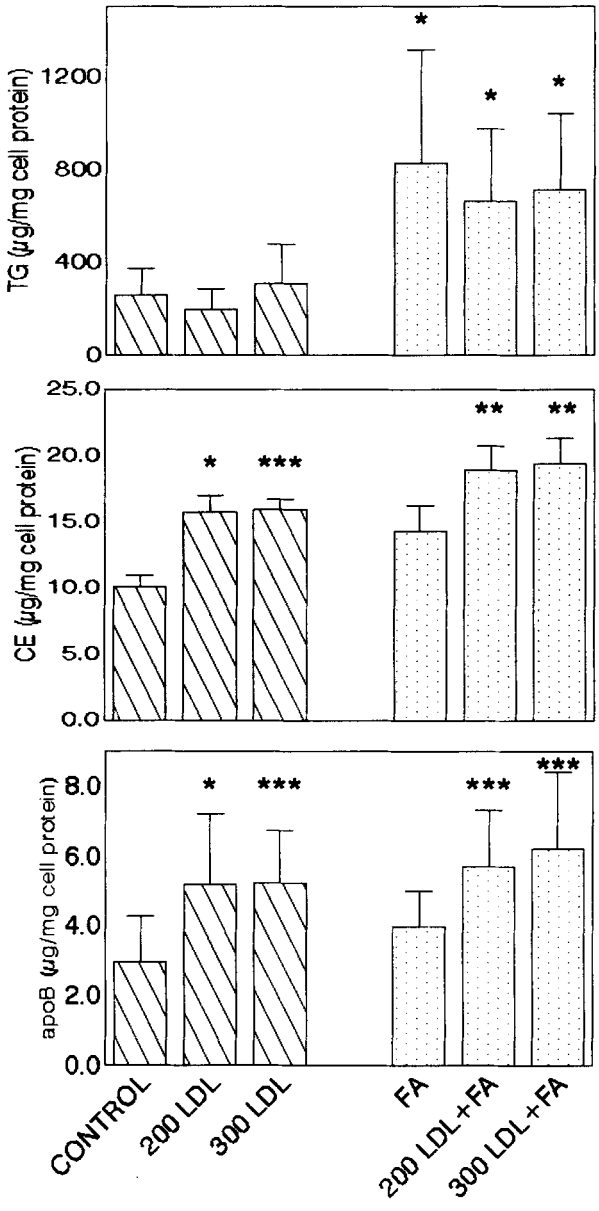

Fig. 6. Effect of LDL and fatty acid on accumulation of intracellular triglyceride, cholesteryl ester, and apoB-100 secretion in HepG2 cells. HepG2 cells were preincubated with the indicated concentrations of LDL cholesterol (200 or 300 μg/ml) for 18 h in the absence (bars) or presence (dots) of 800 μM oleic acid complexed to BSA (FA). The LDL cholesterol/protein ratio was 1.53 ± 0.60. The medium was changed to 1% BSA in MEM for 8 h. At the end of the incubation period, the medium was collected to measure apoB-100 (bottom panel) and intracellular lipids were extracted to measure triglyceride (TG, top panel) and cholesteryl ester (CE, center panel). Results are expressed as μg/mg cell protein ± SEM for n = 4 experiments; $*P < 0.05$; $**P < 0.025$; $***P < 0.01$.

mass of triglyceride. To do so, either heparin or lactoferrin was present during the preincubation period in addition to VLDL + LPL. Lipoprotein interaction with the extracellular matrix in the presence of LPL is the key to increased binding of lipoprotein particles and heparin (a proteoglycan) has been shown to interfere with this process (31–37). Lactoferrin has also been shown to bind chondroitin sulfate proteoglycans and to inhibit interaction with the LRP receptor (38, 39). The effects of these additions are shown in Figs. 3 and 4. In each instance, concurrent controls were processed, consisting of addition of VLDL + LPL alone. Additions of heparin or lactoferrin alone to HepG2 cells had no effect on basal levels of intracellular triglyceride, cholesteryl ester, or basal apoB-100 secretion. With respect to heparin, as shown in **Fig. 3,** top left panel, the mass of triglyceride within the HepG2 cells was even greater when heparin was present than when it was not (228 ± 95 basal, 825 ± 235 with VLDL + LPL alone vs. 998 ± 242 with heparin + VLDL + LPL expressed as μg triglyceride/mg cell protein, $P < 0.005$ with vs. without heparin). By contrast, there was less cholesteryl ester within the cell when heparin was present than when it was not (10.1 ± 2.5 basal vs. 16.8 ± 4.8 with VLDL + LPL alone vs. 13.2 ± 3.2 with heparin and VLDL + LPL μg cholesteryl ester/mg cell protein, $P < 0.05$ with vs. without heparin, Fig. 3, top right panel). Heparin, therefore, favored the accumulation of triglyceride and diminished the accumulation of cholesteryl ester. The effect on apoB-100 secretion is shown in Fig. 3, bottom left panel. Preincubation with VLDL + LPL caused an increase in apoB-100 secretion; however, this was decreased significantly upon incubation with heparin, paralleling the changes in cholesteryl ester but not triglyceride (2.7 ± 0.9 basal vs. 6.7 ± 2.5 with VLDL + LPL alone vs. 4.6 ± 1.7 with heparin + VLDL + LPL expressed as μg apoB-100/mg cell protein $P < 0.05$ with vs. without heparin). Note that this effect of heparin on apoB-100 secretion was evident up to 24 h (Fig. 3, bottom right panel).

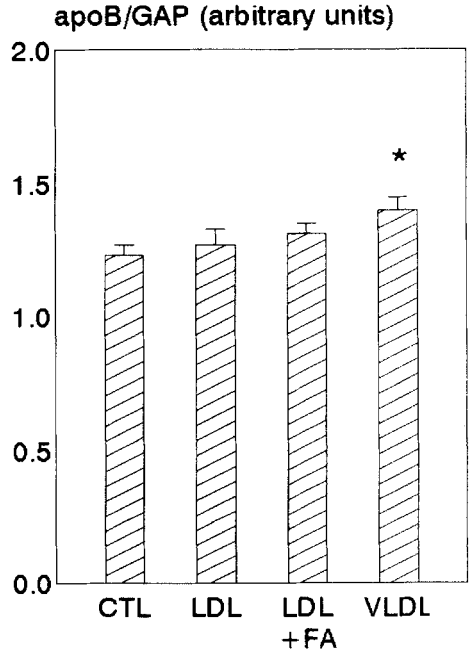

Fig. 7. Slot-blot analysis of apoB-100 mRNA and glyceraldehyde-3-phosphate mRNA in HepG2 cells. HepG2 cells were preincubated with VLDL (100 μg cholesterol/ml with 0.25 units/ml LPL) or LDL (300 μg cholesterol/ml) with or without 800 μM oleic acid complexed to BSA (FA) for 18 h. RNA was then extracted from cells and 5, 10, and 20 μg was blotted onto nylon membrane. RNA was then hybridized to a biotinylated apoB-100 probe, followed by incubation with streptavidin/enzyme conjugate and visualization by chemiluminescence. Blots were stripped and reprobed with a glyceraldehyde-3-phosphate (GAP) probe. Results are expressed in arbitrary units as apoB-100/GAP ratio as average ± SEM for n = 6 where *$P < 0.05$.

The same pattern was evident in the experiment in which lactoferrin was added during the preincubation period (**Fig. 4**). Although VLDL + LPL caused a significant increase in triglyceride (355 ± 42 basal vs. 863 ± 241 with VLDL + LPL expressed as μg triglyceride/mg cell protein, $P < 0.05$), there was no significant difference in triglyceride mass with the addition of lactoferrin (859 ± 176 μg triglyceride/mg cell protein) whereas the cholesteryl ester mass accumulation was significantly inhibited (15.0 ± 1.9 basal vs. 23.7 ± 2.4 with VLDL + LPL vs. 19.7 ± 1.4 with lactoferrin and VLDL + LPL expressed as μg cholesteryl ester/mg cell protein, $P <$ 0.05 with vs. without lactoferrin, Fig. 4, middle panel). Accumulation of apoB-100 in the medium during the 8-h period was also substantially decreased when lactoferrin was present in the preincubation medium (8.2 ± 0.7 basal vs. 18.2 ± 4.6 VLDL + LPL alone vs. 11.0 ± 3.9 with lactoferrin and VLDL + LPL expressed as μg apoB100/mg cell protein, $P < 0.05$ with vs. without lactoferrin, Fig. 4, right panel).

Experiments were also performed to increase predominantly the mass of cholesteryl ester with or without accompanying increasesn in triglyceride. This was achieved by the addition of LDL, fatty acids, or both, during the preincubation period. As with the experiments with VLDL, LDL was added to the HepG2 cells and these were incubated overnight in order to increase the intracellular cholesteryl ester mass. LDL was added on the basis of μg cholesterol/mL medium where the average cholesterol/protein ratio was 1.53 ± 0.60 for n = 8 preparations. After lipoprotein incubation, the cells were then washed 3 times with warm PBS, fresh medium was added, and the incubation was continued for a further 8 h. To verify that the apoB-100 measured did not result from nonspecific carry-over from the preincubation, control cells were preincubated for 30 min in

the presence of the highest concentration of LDL, and apoB-100 secretion was measured over the same 8-h period. There was no change in intracellular triglyceride (216 vs. 200/μg/mg cell protein), intracellular cholesteryl ester (9.4 vs. 8.7 μg/mg cell protein), or medium apoB-100 (7 vs. 6 μg/mg cell protein) in basal vs. 30 min-LDL treated cells (n = 6 experiments). With the addition of LDL alone during the preincubation period, cholesteryl ester mass was increased at the end of the preincubation period in a dose-dependent fashion (**Fig. 5,** top right panel). In contrast, triglyceride mass did not change (Fig. 5, top left panel). As also shown in Fig. 5 (bottom left panel), increase in the cholesteryl ester mass during the preincubation period markedly increased apoB-100 secretion during the subsequent 8-h incubation (up to 192% of basal, $P < 0.0025$). If triglyceride were the sole determinant of apoB-100 secretion, no such change should have occurred. This effect of LDL on subsequent HepG2 apoB-100 secretion was demonstrable for up to 24 h after lipid loading (Fig. 5, bottom right panel). The rate of apoB-100 secretion during the 8 h, measured as the slope from 2 to 8 h determined by linear regression, was 2.9-fold the slope for the control incubation. At 8 h incubation, the amount of lipid remaining in the cells represented 91% ± 12% (triglyceride) and 87% ± 0% (cholesteryl ester) of the initial lipid mass after the LDL loading period (n = 3 experiments). This was no different from the amount in the control cells (87% ± 7% and 96% ± 2% for triglyceride and cholesteryl ester, respectively).

Addition of oleate to the preincubation mixture in addition to LDL also caused the mass of triglyceride within the HepG2 cells to increase significantly (to 322% of control, $P < 0.05$) (**Fig. 6,** top panel), but only marginally affected the mass of cholesteryl ester formed during the preincubation period as compared to LDL alone (Fig. 6, middle panel). There was little effect of fatty acid on apoB-100 secretion over and above the effect of LDL alone (Fig. 6, bottom panel). This is another instance, therefore, in which apoB-100 secretion and accumulation of triglyceride within the HepG2 cells were dissociated.

The effect of VLDL, LDL, and LDL + oleate (FA) loading on apoB-100 mRNA was also examined. As shown in **Fig. 7,** there was only a slight increase in apoB-100 mRNA after VLDL loading (112% ± 9%, n = 6, $P < 0.05$) with no change in apoB-100 mRNA after LDL or LDL + FA incubation (103% ± 11% and 107% ± 8% respectively, NS, n = 6). This contrasts with the marked increases in secreted apoB-100 and suggests that the changes occur at a post-translational level.

The overall relationships between triglyceride mass and apoB-100 secretion and cholesteryl ester mass and apoB-100 secretion for the series of experiments shown in Figs. 1–6 are shown in **Fig. 8.** For triglyceride, a correlation coefficient of $r^2 = 0.034$ (P = NS) between triglyceride mass and apoB-100 secretion was obtained indicating there was no significant relation between the two. By contrast, the correlation coefficient between intracellular cholesteryl ester mass and apoB-100 secretion was $r^2 = 0.670$ ($P < 0.0005$) pointing to a close relation between these parameters.

Experiments to differentiate the effects of newly synthesized from preformed cholesteryl ester on apoB-100 secretion

Studies from other laboratories have demonstrated that in short term experiments when the synthesis of cholesteryl ester, as measured by radioactive [^{3}H]oleate incorporation into cholesteryl ester, was acutely inhibited, there was no drop in apoB-100 secretion (40–42). In contrast, our original data using radioactive tracers

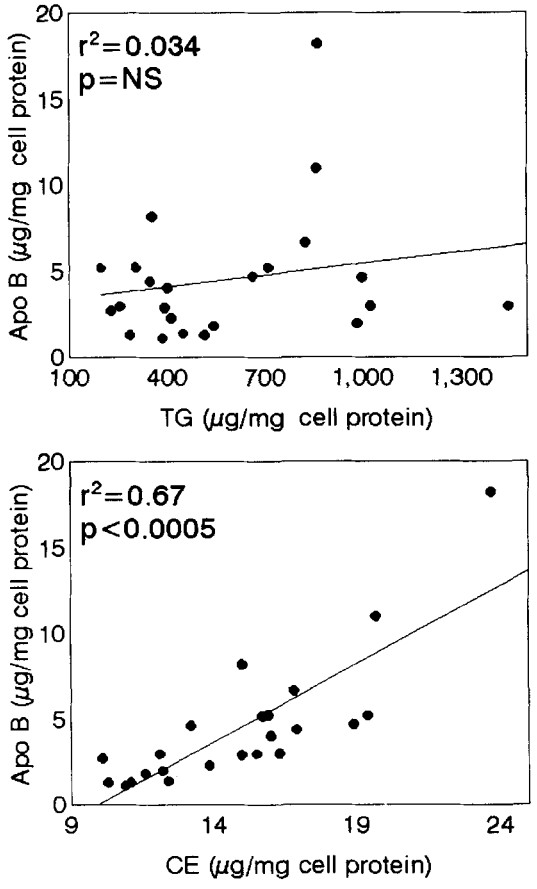

Fig. 8. Correlation of intracellular triglyceride and cholesteryl ester with apoB-100 secretion in HepG2 cells. Data from Figs. 1 to 6 were compiled to correlate intracellular triglyceride (TG, top panel) and cholesteryl ester (CE, bottom panel) with secreted apoB-100. For triglyceride: $y = 0.0022 \times +3.213$, $r^2 = 0.034$, P = NS. For CE: $y = 0.908 \times -9.024$, $r^2 = 0.670$, $P < 0.0005$.

to determine cholesteryl ester synthesis demonstrated a decreased cholesteryl ester synthesis accompanied with decreased apoB-100 secretion when assayed over a much longer time course (24 h) (9). This was confirmed by cholesteryl ester mass determinations in our experiments. Based on these data, we hypothesized that it was not simply newly synthesized molecules of cholesteryl ester that could associate with newly formed apoB-100 molecules but also the total mass of intracellular cholesteryl ester that might have an effect. To test this hypothesis, triglyceride and cholesteryl ester mass of HepG2 cells were increased by preincubation with LDL and fatty acids. ^{14}C-labeled oleate was present during this time as well, such that the cholesteryl ester formed would incorporate this label. After the preincubation, the cells were washed and the medium was changed to a serum-free medium without LDL or fatty acid for a further 8-h incubation. An acyl-CoA:cholesterol acyltransferase (ACAT) inhibitor, Sandoz compound 58-035, was also present in the medium at the indicated concentrations at this point. Tracer [^{3}H]oleate was added as well, so that ACAT activity, and thus the effectiveness of the inhibitor, could be monitored.

The data demonstrate that during the 8-h incubation period, the ACAT inhibitor effectively blocked the synthesis of cholesteryl ester as measured by [^{3}H]oleate incorporation into intracellular cholesteryl ester (**Fig. 9,** left panel). Cholesteryl ester synthesis was inhibited 85%–89% at either inhibitor concentration (2.5 or 5 mg/mL) even in those cells preicubated with LDL. In contrast, the inhibitor had no effect on triglyceride mass or triglyceride synthesis (measured as [^{3}H]oleate incorporation into triglyceride) over the same time period (data not shown). Even so, this profound ACAT inhibition resulted in only a small but significant decrease in the total intracellular cholesteryl ester mass at the end of the 8-h observation period (Fig. 9, right panel).

Notwithstanding this, cholesteryl ester continued to be secreted into the medium as documented both by the appearance of cholesteryl ester mass (**Fig. 10,** top left panel) and [^{14}C]cholesteryl ester (not shown). The results for [^{14}C]cholesteryl ester (cholesteryl ester labeled in the preincubation period) paralleled the results obtained for cholesteryl ester mass; therefore, only the results for intracellular and secreted cholesteryl ester mass are shown in Figs. 9 and 10. Cholesteryl ester secretion from the cells preincubated with LDL and fatty acid (solid line) was substantially greater than in HepG2 cells preincubated with fatty acid alone (dashed line) consistent with the increased intracellular cholesteryl ester mass shown in Fig. 9. Over this 8-h period, HepG2 cells secreted up to 15% of their total cholesteryl ester mass. Moreover, addition of either 2.5 or 5.0 μg/mL compound 58-035 had no effect on the cholesteryl ester mass secreted in spite of almost complete ACAT inhibition. The effectiveness of the ACAT inhibition is demonstrated by a sharp decrease in secreted ^{3}H-labeled cholesteryl ester, as shown in Fig. 10

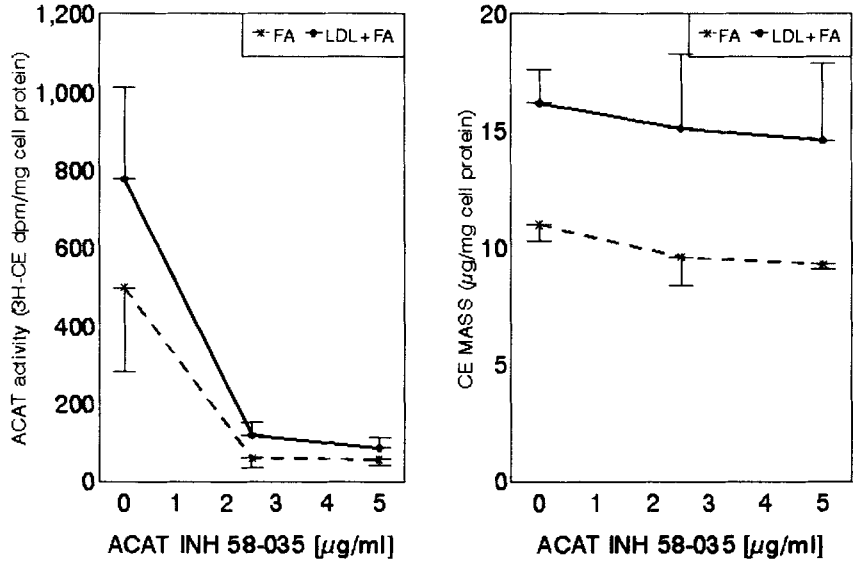

Fig. 9. Effect of compound 58-035 on intracellular ACAT activity and intracellular cholesteryl ester mass in HepG2 cells. HepG2 cells were preincubated overnight in the presence of 800 μM [^{14}C]oleic acid complexed to BSA (FA) with (solid line) or without (dashed line) LDL cholesterol (300 μg/ml) for 18 h. The cells were then changed to 1% BSA medium containing [^{3}H]oleate tracer and the indicated concentrations of ACAT inhibitor, Sandoz compound 58-035, for a further 8 h. After incubation, the medium was removed and intracellular lipids were extracted. ACAT activity (left panel) was measured as [^{3}H]oleate incorporation into cholesteryl ester (CE, dpm/mg cell protein). CE mass (right panel) is expressed as μg/mg cell protein. Results are shown as average ± SEM for n = 4 experiments.

(top right panel). Therefore, virtually all of the cholesteryl ester mass secreted (90%) was preformed cholesteryl ester, whereas only 10% of the cholesteryl ester was potentially newly synthesized cholesteryl ester based on the extent of ACAT inhibition.

Finally, apoB-100 secretion was greater in the cells that had been preincubated with LDL compared to those that had not (Fig. 10, bottom panel), a result which is again consistent with the relative cholesteryl ester mass in the cells after the preincubation period. Again, addition of the ACAT inhibitor had no effect over the 8-h incubation period. Density gradient ultracentrifugation of HepG2 medium demonstrated that the cholesteryl ester mass was indeed secreted in association with apoB-100 particles. As shown in **Fig. 11,** left panel, up to 92% ± 3% of secreted cholesteryl ester was present in either d < 1.006 g/mL particles or in d 1.006–1.063 g/mL particles with only 8.3% ± 3% on average present in d > 1.063 g/mL particles (n = 5 experiments). This was true under basal, LDL, LDL + oleate, or LDL + oleate + 58-035 conditions. In these experiments 87% ± 2% of apoB-100 was recovered in the d < 1.063 g/mL range (n = 5 experiments).

Clearly, it is important to determine whether the increased apoB-100 secretion after LDL preincubation is a result of apoB lipoprotein particles preformed before the medium is changed. This is particularly important with respect to the ACAT inhibitor effect. If the majority of apoB-100 secreted is in the period immediately following the addition of ACAT inhibitor (less than 1 h), then this may be due to preformed lipoprotein particles. Accordingly, HepG2 cells were preincubated with LDL for 18 h and the medium was then changed to 1% BSA with or without ACAT inhibitor. As shown in Fig. 11, apoB-100 secretion was increased after preincubation with LDL even at time points after 1 h. The rate of apoB-100 secretion between 1 and 4 h (measured as the slope of the line) was 3.25-fold greater than the control. Even in the presence of ACAT inhibitor this increased rate of secretion was 3.90-fold greater than the control.

These data establish clearly that, during a time in which cholesteryl ester cannot be formed (i.e., in the presence of ACAT inhibitor), cholesteryl ester can still be mobilized from the cell and there is a direct relation between the mass of cholesteryl ester within the HepG2 cells and the rate of apoB-100 secretion from them.

DISCUSSION

We believe the data in the present study amplify understanding of the regulation of hepatic apoB-100 secretion and the role of cholesteryl ester in this process. To begin, it must be emphasized that regulation of this process is complex and that no single molecule will account for all the variation that occurs. Effects on transcription as well as post-translational effects on apoB-100 have been documented in HepG2 cells (6–8, 14, 15). The intracellular mass of lipid substrate clearly affects secretion of apoB-100, but its secretion is also influenced by a variety of other factors such as the hormonal milieu as well as the amino acid and albumin concentrations in the medium (2–6). These experiments were stimulated by reports of other investigators that cholesteryl ester played no apparent role in determining apoB-100 secretion rates in HepG2 cells (40–42). The present data, however, again demonstrate that there is always a much clearer relation between the mass of cholesteryl ester within the cell and the rate of apoB-100 secretion than there was with triglyceride. Moreover, no evidence for significant transcriptional regulation was obtained.

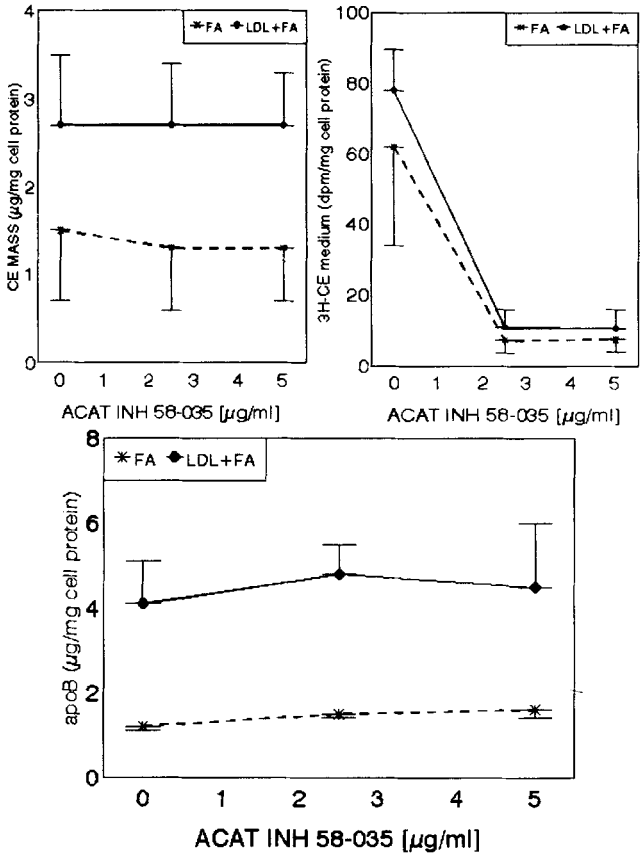

Fig. 10. Effect of compound 58-035 on secreted cholesteryl ester mass, ^{3}H-labeled cholesteryl ester, and apoB-100. HepG2 cells were preincubated overnight in the presence of 800 µM [^{14}C]oleic acid complexed to BSA (FA) with (solid line) or without (dashed line) LDL cholesterol (300 µg/ml) for 18 h. The cells were then changed to 1% BSA medium containing [^{3}H]oleate tracer and the indicated concentrations of ACAT inhibitor Sandoz compound 58-035 for a further 8 h. After incubation, the medium was collected to measure apoB-100 and secreted lipids were extracted and quantified. Secreted cholesteryl ester (CE) mass (top left panel) and secreted apoB-100 (bottom panel) are expressed as µg/mg cell protein. Secreted ^{3}H-labeled CE (top right panel) is expressed as dpm/mg cell protein. Results are the average ± SEM for n = 4 experiments.

It should be noted that many investigators have studied a variety of both in vivo and in vitro models and have also found evidence that cholesterol and/or cholesteryl ester modulate secretion of apoB-100 particles by the liver. Heimberg and his colleagues (43–46), in a series of studies that antedate ours, showed that cholesterol is an essential factor for hepatic lipoprotein secretion and that the rate at which this occurs can be influenced by administration of an HMG-CoA reductase inhibitor. Tanaka et al. (47, 48) showed that cholesterol loading of rabbit hepatocytes increased apoB-100 secretion and this stimulatory effect could be diminished by administration of an HMG-CoA reductase inhibitor which reduced overall hepatic cholesteryl ester mass. These observations extended earlier work by Fuki et al. (49) and by Kosykh et al. (50) which also demonstrated apoB-100 secretion to be related to the cholesterol content of HepG2 cells. The studies of Craig and Cooper (51, 52) provide evidence in support of the hypothesis as well. Though they did not comment on it, they showed that addition of rabbit chylomicron remnants and beta-VLDL both caused equivalent increases in triglyceride synthesis in HepG2 cells. Beta-VLDL, however, which contains more cholesteryl ester than do chylomicron remnants, resulted in a greater increase in cholesteryl ester within the hepatocytes and was associated with a much greater increase in apoB-100 secretion than was the addition of chylomicron remnants. In vivo and perfused liver studies by Krause and his colleagues (53, 54), by Carr, Hamilton, and Rudel (55), and by Huff and his associates (56) have all shown that administration of agents to inhibit ACAT reduced secretion of apoB-100 particles by the liver. Moreover, Dashti (14) demonstrated that delivery of 25-hydroxycholesterol to HepG2 cells led to increased cholesteryl ester mass and increased apoB-100 secretion, a sequence that could be inhibited by progesterone, acting as an ACAT inhibitor. Finally, administration of an HMG-CoA reductase inhibitor has been shown in a wide variety of animal and human studies to diminish the rate of secretion of hepatic apoB-100 particles and this, of course, adds powerful support for the model (43, 47, 48, 57–66).

On the other hand, a number of other laboratories (40–42) have shown that acute inhibition of the synthesis of cholesteryl ester does not result in a sudden drop in apoB-100 secretion, a finding we confirm in the present studies and one which, on the face of it, would be difficult to reconcile with the model we have suggested (9). Reviewing these studies, however, it became apparent that with few exceptions most of the data dealt with the relation between the simultaneous rates of acute cholesteryl ester synthesis as measured by radiolabel incorporation and apoB-100 secretion. We speculated, therefore, that it was not only the newly synthesized

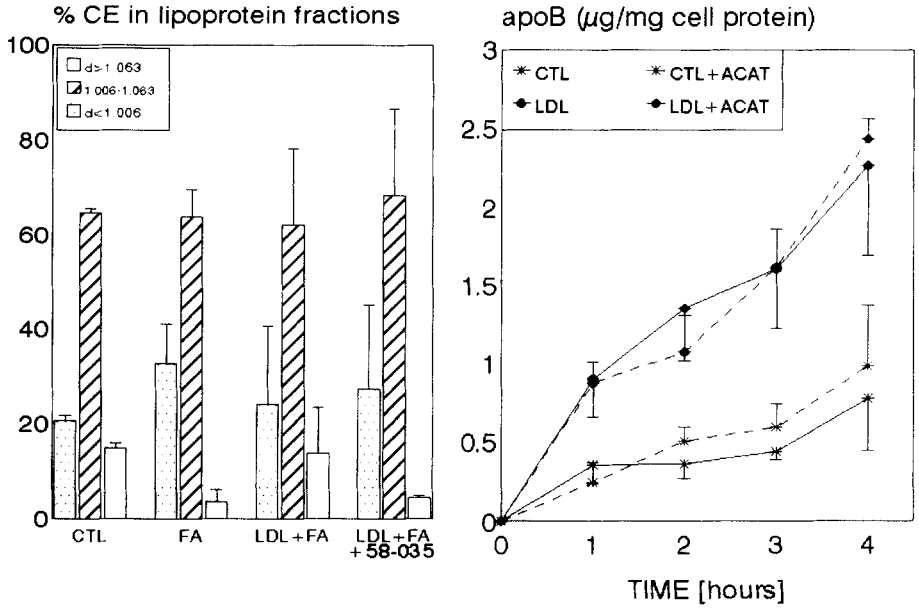

Fig. 11. Effect of compound 58-035 on secreted apoB-100 and cholesteryl ester lipoprotein distribution. Left panel: Cells were incubated as described in Fig. 10. The medium was then fractionated by ultracentrifugation into density (d) < 1.006 g/mL (dots); d 1.006-1.063 g/mL (lines); and d > 1.063 g/mL (□). Cholesteryl ester (CE) mass and apoB-100 (not shown) were measured in each lipoprotein fraction and the results are expressed as % distribution ± SEM for an average of n = 5 experiments. Right panel: HepG2 cells were preincubated overnight with (●) or without (*) 300 μg/mL LDL for 18 h. The cells were then changed to 1% BSA medium in the absence (solid line) or presence (dashed line) of 5 μg/mL ACAT inhibitor Sandoz compound 58-035 for 1 to 4 h. After incubation, the medium was collected to measure apoB-100 and results are expressed as μg/mg cell protein for an average ± SEM for n = 4 experiments.

cholesteryl ester molecules that played a role in influencing apoB-100 release into the lumen of the endoplasmic reticulum, but that much of the total mass of cholesteryl ester already stored within the hepatocyte might participate in this process, and indeed, this is precisely what the data in the present study establish. That is, when the mass of cholesteryl ester within the HepG2 cells was increased, and then its synthesis was inhibited, apoB-100 secretion persisted at levels above basal, as did secretion of cholesteryl ester, as determined both by radiolabeling studies and by measurement of mass. Preformed cholesteryl ester, therefore, associates with apoB-100 molecules and is secreted from these cells establishing that it is not just newly synthesized cholesteryl ester but rather a much larger pool of performed cholesteryl ester that can affect apoB-100 secretion.

In this respect, it is important to appreciate that cholesteryl ester could be mobilized directly without the necessity of being hydrolyzed and reesterified prior to association with apoB-100. Even when de novo cholesteryl ester synthesis was effectively blocked by 90% with an acyl-CoA:cholesterol acyltransferase inhibitor, up to 15% of the performed cholesteryl ester could be secreted over an 8-h time period. On the other hand, intracellular metabolism of triglyceride appears to differ considerably. Most of the triglyceride is in lipid droplets within the cytosol and is not associated with the endoplasmic reticulum. A number of investigators have shown that triglyceride within the cytosol is first hydrolyzed and the fatty acids that are released are reformed into triglycerides on the endoplasmic reticulum before being secreted as apoB-100 lipoproteins (67–71).

With respect to the role of lipids in altering the secretion efficiency of apoB-100, the data suggest that the critical events take place shortly after synthesis of the apoB-100 molecule, most likely while it is still associated with the rough endoplasmic reticulum. Our hypothesis has been that the initial association of core lipids with the newly synthesized apoB-100 molecule is the critical physiologic event that determines whether an apoB-100 molecule goes on to form part of a nascent lipoprotein particle. Although we have focused on the potential role of cholesteryl ester in this process, as already noted, none of the data excludes a similar function for triglyceride, at least for the triglyceride that is synthesized in the rough as opposed to the smooth endoplasmic reticulum (72).

In this regard, the work of Elovson and his colleagues (73, 74) and of Hamilton and Havel (75) is of interest. Both have proposed that much of the triglyceride enters the lumen of the endoplasmic reticulum not associated with apoB-100 and that it is only afterwards that a triglyceride-phospholipid vesicle fuses with the nascent apoB-100 lipoprotein particle. If so, the triglyceride that enters the lumen of the endoplasmic reticulum unassociated with apoB-100 would, of course, not be able to influence the fate of the newly synthesized apoB-100 molecules on the rough endoplasmic reticulum. Recently, Swift (76) has reported that B-48 particles isolated from rat liver rough endoplasmic reticulum participate in a two-step assembly and secretion process, and both precursor lipid-poor particles as well as subsequent lipid-rich particles could be demonstrated. This was not as evident for apoB-100 particles. On the other hand, he did demonstrate that although apoB-100 lipoprotein particles isolated from the rough endoplasmic reticulum contained both cholesteryl ester and triglyceride, these lipoproteins had less triglyceride than those isolated from the Golgi apparatus or from plasma. These data are in contrast to those obtained by Rusiñol, Verkade, and Vance (77), but if correct, they are consistent with the concept that VLDL particles, particularly with respect to their triglyceride content, are formed sequentially, though the site of these secondary additions has not been determined.

These issues are of clinical as well as physiologic importance. An elevated LDL plasma particle number due to increased secretion of apoB-100 particles by the liver is one of the commonest dyslipoproteinemias associated with premature coronary artery disease (78, 79). To the extent that modulation of cholesteryl ester mass affects apoB-100 secretion, the use of HMG-CoA reductase inhibitors or ACAT inhibitors to diminish this becomes a rational therapeutic extension of these fundamental physiologic observations.

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