Effects of chylomicron remnants and β-VLDL on the class and composition of newly secreted lipoproteins by HepG2 cells

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Abstract The regulation of lipoprotein secretion in the cell line HepG2 was studied. HepG2 cells were preincubated with chylomicron remnants (triglyceride- and cholesterol-rich) or with beta very low density lipoproteins (β-VLDL) (cholesterol-rich). The medium was removed and the cells were incubated for an additional 24 hr in a lipoprotein-free medium that contained either [2-^{3}H]glycerol or DL-[2-^{3}H]mevalonate. Cells and media were harvested, and lipoproteins were separated and fractionated. The mass and radioactivity of the lipids in cells and in the lipoproteins were measured. The activities of cellular acyl-CoA:cholesterol acyltransferase (ACAT) and 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase were also determined. Preincubation with chylomicron remnants induced an increase in cellular triglyceride and stimulated both HMG-CoA reductase and ACAT. Preincubation with β-VLDL induced an increase in cellular free and esterified cholesterol, inhibited HMG-CoA reductase and stimulated ACAT. Although the absolute amount of VLDL is small, chylomicron remnants induced large relative increases in the amount of triglyceride and phospholipid secreted in VLDL and decreases in the amount of triglyceride secreted in low density (LDL) and high density (HDL) lipoproteins as well as a decrease in the amount of phospholipid secreted in HDL. In contrast, preincubation with β-VLDL did not affect triglyceride secretion, but markedly stimulated the amount of phospholipid secreted in HDL. ■ Comparison of the mass of glycerolipid actually secreted with that calculated from the cellular specific activity suggested that glycerolipids are secreted from single, rapidly equilibrating pools. Cholesterol and cholesteryl ester secretion were affected differently. Preincubation with chylomicron remnants increased the amount of free cholesterol secreted in both VLDL and LDL, but did not alter cholesteryl ester secretion. Preincubation with β-VLDL increased free cholesterol secretion in all lipoprotein fractions and increased cholesteryl ester secretion in VLDL and LDL, but not HDL. Comparison of isotope and mass data suggested that the cholesteryl ester secreted came primarily from a preformed, rather than a newly synthesized, pool. In summary, these data provide insight to the mechanism whereby a liver cell regulates the deposition of exogenous lipid.—**Craig, W. Y., and A. D. Cooper.** Effects of chylomicron remnants and β-VLDL on the class and composition of newly secreted lipoproteins by HepG2 cells. *J. Lipid Res.* 1988. **29:** 299–308.

Supplementary key words triglyceride • cholesterol • phospholipid • liver

The factors influencing the relative amounts and composition of different lipoproteins secreted by the liver are largely unknown. Davis et al. (1) suggested that apoB synthesis limits the rate of lipoprotein secretion and that the cellular composition of neutral lipids determines the core composition of secreted hepatic very low density lipoprotein (VLDL). Miller and Lane (2) suggested that the lipid content of VLDL secreted by cultured chick hepatocytes determines the apoprotein content. Thus, understanding the determinants of the lipid content of lipoproteins is potentially of fundamental importance in understanding how serum lipoprotein and lipid levels are determined.

It is difficult, if not impossible, to study the regulation of nascent lipoprotein secretion in vivo. Accordingly, a variety of model systems have been used. The isolated perfused liver has provided much information about the composition and characteristics of nascent lipoprotein, but this system is not readily manipulated. More recently, cultured animal hepatocytes have proved useful in elucidating some basic aspects of lipoprotein formation. Most recently, the human hepatoma-derived cell line HepG2 has been used to gain insights into the nature of the lipoproteins secreted by human liver. Although one must be cautious about extrapolating to normal liver, it has been shown that these cells synthesize apoA-I, apoE and apoB (3, 4). The synthesis of apoA-I (5) is under hormonal regulation. The accumulation of a high density lipoprotein-like particle in the media of HepG2 cells has been documented (6). In previous work from this laboratory,

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Abbreviations: VLDL, very low density lipoproteins; HDL, high density lipoproteins; LDL, low density lipoproteins; MEM, minimum essential medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline; TLC, thin-layer chromatography; ACAT, acyl-CoA:cholesterol acyltransferase; HMG, 3-hydroxy-3-methylglutaryl; MVA, mevalonic acid; LCAT, lecithin: cholesterol acyltransferase.

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the ability of the cells to secrete VLDL-like particles and a triglyceride-rich low density lipoprotein (LDL) particle was reported (7), while in other work, we found that the synthesis of apoB, apoE, and apoA-I could be induced by loading the cells with cholesterol-containing lipoproteins (8).

The purpose of the present work was to examine the determinants of lipoprotein lipid composition secreted by HepG2 and to assess whether specific pools of lipids were utilized preferentially.

To study the effect of modulating cellular lipid levels on lipoprotein lipid secretion, we have investigated the effects of incubating HepG2 cells with lipoproteins of defined lipid composition. Chylomicron remnants are rich in triglyceride as well as cholesterol and cholesteryl ester while β-VLDL contain predominately cholesteryl ester, although both particles have similar apoprotein components. Our data demonstrate that lipid substrate supply is indeed important in determining both the composition and amount of hepatic lipoproteins secreted. Loading HepG2 cells with chylomicron remnants caused a redistribution of glycerolipid secretion to favor production of VLDL-like particles. When β-VLDL were delivered to the cells, the secretion of free cholesterol and cholesteryl ester in lipoprotein was increased, and their distribution seemed to be independently regulated. Moreover, preformed, rather than newly synthesized cholesterol, was preferentially secreted.

MATERIALS AND METHODS

Materials

Minimal essential medium (MEM), L-glutamine, penicillin-streptomycin solution, trypsin/EDTA (10×), Earle's balanced salt solution, and fetal bovine serum (FBS) were obtained from Gibco (Santa Clara, CA). [2-^{3}H]Glycerol was from New England Nuclear (Boston, MA), and DL-[2-^{3}H]mevalonic acid lactone was from Amersham (Arlington Heights, IL). HepG2 cells were a gift from Dr. Barbara Knowles. Male Sprague-Dawley rats were from Simonsen Laboratories (Gilroy, CA).

Cell culture

HepG2 cells were grown in 25 cm^3 tissue culture flasks as previously described (7). Confluent monolayers of cells were split 1:3 by trypsinization (day 0) into 5 ml of MEM containing 10% fetal bovine serum (FBS), 100 U/ml penicillin-streptomycin and supplemented with 0.3 mg/ml L-glutamine. On day 4, the cells had reached confluence and were refed with 5 ml of media. Experiments were always started on day 4. Cells were split on a 7-day cycle.

Preparation of lipoproteins

To prepare β-VLDL, four male Sprague-Dawley rats (350 g) were fed an atherogenic diet (9) for 4 weeks. The animals were fasted for 24 hr to enable the clearance of chylomicron remnants and then exsanguinated. Serum was separated, and EDTA, sodium azide (0.04%), and gentamycin (1.25 μl/ml) were added to inhibit bacterial growth. The serum was transferred to ultracentrifuge tubes and overlaid with PBS A (PBS, 0.4% EDTA, NaN_3, pH 7.4, d 1.006 g/ml). Tubes were spun in a Beckman SW41 rotor for 24 hr at 4°C, 40,000 rpm, and the opaque layer of β-VLDL was collected.

Chylomicron remnants were prepared from rat chylomicrons as previously described (10). Chylomicrons were collected by drainage from a lymph fistula in a rat continuously fed egg emulsion. The chylomicrons were injected into the bloodstream of an eviscerated rat. After 2 hr, the rat was exsanguinated, and the chylomicron remnants were centrifuged through a layer of PBS A for 2 hr at 4°C at 35,000 rpm in a Beckman SW41 rotor. The supernatant was further purified by chromatography through a Bio-Gel A50 (Bio-Rad, Richmond, CA) column (2.5 × 50 cm) at 4°C.

Prior to use in the cell culture system, lipoproteins were dialyzed against PBS (pH 7.4, 0.02% EDTA). All lipoproteins were analyzed for protein, cholesterol, and triglyceride as described below. Their protein composition was analyzed by electrophoresis on 10% SDS-polyacrylamide gels and found to be similar to that previously reported by this laboratory (9) for these lipoprotein classes.

Thin-layer chromatography (TLC) of lipids

Following extraction of lipids by the method of Folch, Lees, and Sloane Stanley (11), individual species were separated by TLC. Samples were applied to silica gel H plates which were developed with a two-solvent system. Phospholipids were separated by an initial development in chloroform-methanol-glacial acetic acid-water 65:25:8:4, to the middle of the plate, and then apolar lipids were separated from the same sample by a second development in hexanes-diethyl ether-glacial acetic acid 86:16:1.

For quantitation of radiolabeled lipids, bands were scraped into 10 ml of toluene-based scintillation fluid. When phospholipid was present, 80 μl of methanol was added to the fluid. Radioactivity was quantitated in a Beckman LS150 liquid scintillation counter.

Measurement of cellular and lipoprotein lipid radioactivity, mass, and specific activity

HepG2 cells were grown to confluence in 75 cm^3 tissue culture flasks. On day 4, the media were changed, and cells were incubated at 37°C for 24 hr with 3 ml of MEM (containing 10% FBS) in the presence or absence of added lipoprotein. The lipoproteins were added at a concentra-

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tion of 40 μg of lipoprotein protein/ml of media. The media were then removed, the cell monolayer was washed with MEM (no FBS), and the cells were incubated for a further 24 hr in 3 ml of MEM (no FBS) containing 50 μCi of radioactive lipid precursor. DL-[2-^{3}H]mevalonic acid lactone was utilized to label cholesterol and cholesteryl ester and [2-^{3}H]glycerol was used to label glycerolipids. Media were then pooled from three flasks and dialyzed against PBS A to remove unincorporated radiolabel. Cells were harvested, pooled as for the media, and washed twice in PBS (pH 7.4, 4°C) prior to assay of cell protein. Aliquots of the cell suspension were extracted by the method of Folch et al. (11) for measurement of lipid mass and for separation of radiolabeled lipids by thin-layer chromatography for counting. [2-^{3}H]Glycerol incorporation into media triglyceride and phospholipid increased for at least 21 hr, but plateaued by 28 hr. Thus, cellular lipid mass and specific activity could be determined in the same samples.

For some experiments, lipoprotein classes were separated from the medium by sequential ultracentrifugation according to the method of Havel, Eder, and Bragdon (12). The density ranges were adjusted by addition of KBr: VLDL, d < 1.019 g/ml; LDL, 1.019-1.063 g/ml; HDL, 1.063-1.21 g/ml. Lipoproteins were spun for 24 hr (VLDL, LDL) or 48 hr (HDL) at 40,000 rpm in a Beckman SW 41 rotor. Lipoprotein fractions were extracted by the method of Folch et al. (11) for lipid mass measurement and for thin-layer chromatographic separation and counting of radiolabeled lipid. Thus, as with cellular lipid, mass and specific activity were determined.

In the case of phospholipid mass measurement, cells were washed and resuspended in phosphate-free buffer (10 mM Tris, 150 mM NaCl, pH 7.4), and lipoprotein fractions were dialyzed against this buffer prior to lipid extraction and mass measurement.

When total lipoprotein lipid was to be investigated, the density of dialyzed media from preincubated and labeled HepG2 cells was adjusted to 1.21 with KBr and the media were spun for 24-48 hr at 40,000 rpm in a Beckman SW41 rotor. Total lipoproteins were then aspirated from the top of the centrifuge tube.

In those experiments in which only lipoprotein-lipid radioactivity was measured, the lipoproteins were separated by the method of Redgrave, Roberts, and West (13). The cell medium was first dialyzed against PBS A to remove free radiolabel; then made up to a volume of 3.75 ml with PBS A, and the density was adjusted to 1.21 g/ml with KBr. This was overlayered with a discontinuous gradient of PBS A, containing KBr at densities of 1.063, 1.019, and 1.006 g/ml. After centrifugation, gradients were fractionated using a Buchler (Saddle Brook, NJ) Auto Densiflow fractionator. Density was calculated from refractive index, and protein content was estimated by measuring absorbance at 280 nm. Aliquots of fractions were counted for radioactivity to localize lipoprotein peaks. The fractions comprising a discrete lipoprotein class were then pooled and extracted by the method of Folch et al. (11). Lipids were separated by TLC and radiolabel incorporation was determined.

To assess whether there was re-uptake or alteration of newly secreted VLDL, normal serum VLDL was isolated and iodinated. This was incubated with HepG2 cells, media in which confluent HepG2 cells had been grown, or fresh media. The lipoproteins were then re-isolated by the density gradient method (13) and radioactivity was quantitated. In separate experiments, the amounts of VLDL added were chosen to equal or be five times the amount isolated from control HepG2 cultures. In both instances, the presence of HepG2 cells or HepG2 cell-conditioned media did not affect the recovery (always greater than 70% of the VLDL in control media incubation) or distribution of recovered lipoprotein. This result suggests that re-uptake or lipolytic remodeling of the newly secreted particles is not a major problem (8).

Enzyme assays

HepG2 cells were grown to confluence, washed, and incubated with either β-VLDL or chylomicron remnants at 40 μg/ml media for 24 hr in media containing 10% FBS for 24 hr. They were harvested in enzyme buffer (0.04 M $K_xH_xPO_4$, 0.1 M sucrose, 0.05 M KCl, 0.02 M EDTA, pH 7.2) at 4°C and sonicated 2 × 15 sec. For the assay of ACAT, 350 mg of cell protein was made up to a final volume of 300 μl with enzyme buffer. The membrane suspension was preincubated at 37°C for 10 min with gentle agitation, and 5 nmol of [^{14}C]oleoyl CoA (sp act 50-80,000 dpm/nmol) was added. After a 30-min incubation, the reaction was stopped, and lipids were extracted by the addition of 5 ml chloroform-methanol 2:1 and 1 ml of acidic water. [^{3}H]Cholesteryl oleate (3000 cpm) was added as internal standard, and the phases were allowed to separate overnight at 4°C. The aqueous layer was then aspirated, and the organic layer was dried down under nitrogen, taken up in chloroform, and cholesteryl esters were separated by thin-layer chromatography on silica gel plates developed in hexanes-diethyl ether-glacial acetic acid 86:16:1. Separated cholesteryl ester was scraped into 10 ml of toluene scintillation fluid, and radioactivity was counted in a Beckman liquid scintillation counter.

To assay HMG-CoA reductase, each sample contained 500 μg of cell protein, glucose 6-phosphate (100 mM), NADP (20 mM), glucose 6-phosphate dehydrogenase (1.7 U), dithiothreitol (100 mM), and NaCl (700 mM) in enzyme buffer to a final volume of 1 ml. This membrane suspension was preincubated at 37°C for 10 min with gentle agitation, and 60 nmol of [3-^{14}C-glutaryl]HMG-CoA (sp act 10-12,000 dpm/nmol) was added. After a 30-min incubation, 0.1 ml of 10 M NaOH was added, and the incubation was continued for a further 15 min.

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Finally, 0.2 ml of concentrated HCl, 6000 cpm of DL[2-^{3}H]mevalonic acid lactone (internal standard), and 0.1 ml of 200 mM MVA (carrier) were added. The samples were left overnight at 4°C, then 0.5 g of Na_2SO_4 was added to each tube, and the samples were extracted with 3 × 10 ml of diethyl ether. The ether extracts were taken to dryness under nitrogen, taken up in 100 μl of acetone, and radiolabeled MVA was separated by thin-layer chromatography on Kodak 13179 silica gel sheets developed in acetone-benzene 1:1. Radioactivity was determined by scraping the MVA back into Aquasol II scintillation fluid and counting in a Beckman liquid scintillation counter.

Chemical assays

Phospholipid was measured by the method of Bartlett (14), protein by the method of Lowry et al. (15), and lipoprotein and cellular triglyceride by an enzymatic kit (Sigma, St. Louis, MO). Cholesterol and cholesteryl ester from HepG2 cells and lipoproteins were measured by gas-liquid chromatography as previously described (7), and rat lipoprotein total cholesterol was measured by an enzymatic kit from Sigma (St. Louis, MO). Statistical significance was assessed by Student's *t*-test.

RESULTS

The effect of preincubation of HepG2 cells with chylomicron remnants or β-VLDL on regulation of cellular lipid metabolism

Cells were preincubated with either chylomicron remnants (triglyceride-cholesterol-protein 27:3:1) or with β-VLDL (triglyceride-cholesterol-protein 0.8:3.8:1) and their effect on cellular lipid content and on glycerolipid synthesis was determined. When cells were preincubated with 40 μg of chylomicron remnant protein/ml media for 24 hr, the cells became visibly laden with lipid droplets, and cellular triglyceride levels rose from 93 ± 10 μg/mg of cell protein to 517 ± 101 μg/mg of cell protein (**Fig. 1**). In addition, there was an increase in the incorporation of [^{3}H]glycerol into triglyceride from 16432 ± 6675 to 83447 ± 34084 cpm of [^{3}H]glycerol incorporated/mg of cell protein ($P < 0.05$) (**Fig. 2**). Cellular levels of cholesterol and cholesteryl ester were not significantly altered (Fig. 1), although increases were seen in some experiments. There was also stimulation of both HMG-CoA reductase and ACAT (**Fig. 3**). In contrast, β-VLDL, which contain a high percentage of cholesterol, increased both cellular free and esterified cholesterol levels (Fig. 1). This effect was dose-dependent. At 10 μg/ml, cellular cholesteryl ester was 8.33 μg/mg of cell protein; at 20 μg/ml, it was 14.72, and at 40 μg/ml, it was 17.0 μg/mg of cell protein compared to 4.8 μg/mg of cell protein in control. Since 40 μg/ml lipoprotein is about ten times the K_d for the uptake of

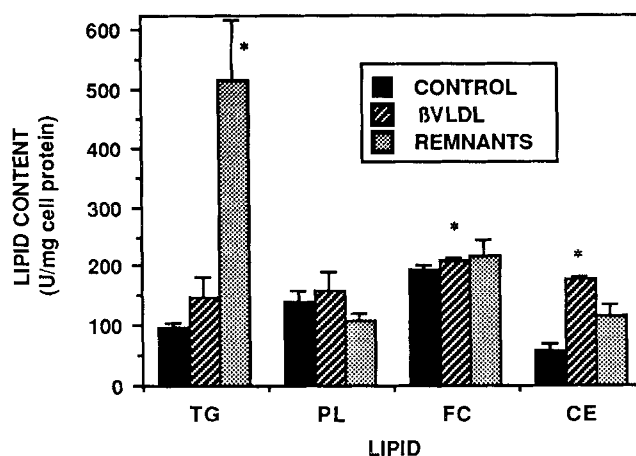

Fig. 1. Effect of preincubation with chylomicron remnants or β-VLDL on hepatic lipid content. HepG2 cells were cultured to near confluence. The medium was changed to one containing 10% FCS alone or including 40 μg/ml of the indicated lipoprotein. After 24 hr, the media were removed, and the cells were cultured an additional 24 hr in FBS-free media that contained 50 μCi of [2-^{3}H]glycerol or DL-[2-^{3}H]mevalonate. After an additional 24 hr, the cell media were removed, and the lipids were extracted and quantified as described in Methods; TG, triglyceride; PL, phospholipid; FC, free cholesterol; CE, cholesteryl ester. The units (U) are μg for TG, μmol × 10^3 for PL, and μg × 10 for C and CE. Means ± SEM of eight determinations are shown. *Different from control, $P < 0.05$.

these particles and appeared to have nearly saturated the dose-response curve, this was used in all future experiments. The increase in free cholesterol was slight but significant, and cholesteryl ester levels rose dramatically (from 5.8 to 17.8 μg/mg of cell protein), suggesting there was a large increase in the cellular level of stored cholesterol (Fig. 1). β-VLDL did not affect the mass of cellular triglyceride (Fig. 1) or the rate of incorporation of [^{3}H]glycerol into triglyceride (Fig. 2). ACAT was markedly stimulated and HMG-CoA reductase was inhibited (Fig. 3) by β-VLDL. Neither β-VLDL nor chylomicron remnants altered the mass or rate of synthesis from glycerol of phospholipid in HepG2 cells (Figs. 1, 2).

Effect of preincubation of HepG2 cells with chylomicron remnants or β-VLDL on total glycerolipid secretion

The effect of preincubation with remnants and β-VLDL on the secretion of phospholipid and triglyceride in total lipoprotein over the subsequent 24 hr was determined. The incubation medium also contained [^{3}H]glycerol during the final 24-hr incubation period. The mass of glycerolipid secretion was both measured directly and calculated from the cellular lipid specific activity and the radioactivity in the secreted lipids. In control, chylomicron remnant and β-VLDL-treated cells, the values calculated from the specific activity data were reflective of true mass secreted (**Table 1**). This suggests that these moieties in the lipoproteins are derived from precursor pools that have reached equilibration within the time

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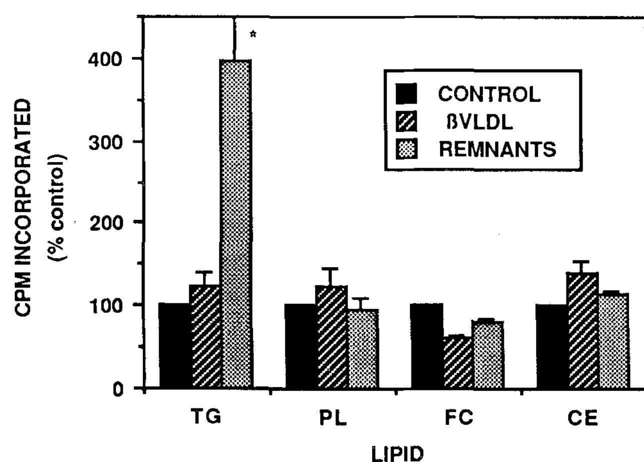

Fig. 2. Effect of preincubation with chylomicron remnants or β-VLDL on [2-^{3}H]glycerol incorporation into triglyceride and phospholipid or DL-[2-^{3}H]mevalonate into cholesterol and cholesteryl ester in HepG2 cells. The experimental protocol is the same as in Fig. 1. Radioactivity incorporation into each lipid class is shown. Mean ± SEM of incorporation relative to control incorporation × 100 is shown; n = 12 for triglyceride and 5 for all others. *Different from control, $P < 0.05$.

frame of the experiments, and justifies the use of radioisotope measurement in subsequent investigation of lipoprotein triglyceride and phospholipid secretion. Neither chylomicron remnants nor β-VLDL induced an increase in total triglyceride secretion. However, β-VLDL did induce an increase in phospholipid secretion.

Effect of preincubation of HepG2 cells with chylomicron remnants or β-VLDL on glycerolipid secretion in various lipoprotein classes

Following a 24-hr preincubation of HepG2 cells with either chylomicron remnants or β-VLDL (40 μg of lipoprotein protein/ml of media), the incorporation of [^{3}H]glycerol into glycerolipids in various lipoprotein classes was measured, as described in Methods. The results, relative to the control for that class, are plotted in **Fig. 4.** Relative to total secretion in controls, VLDL had 2%, LDL had 78%, and HDL had 20% of the triglyceride, and VLDL had 3%, LDL had 26%, and HDL had 71% of the phospholipid. Combined with the data of Table 1, mass rates of secretion in each class can be calculated.

Chylomicron remnants induced a redistribution of lipid with a dramatic increase in VLDL triglyceride secretion and decreases in triglyceride in the other fractions (Fig. 4a). This is similar to the effect of free fatty acids on HepG2 cells previously reported by this laboratory (7). There was a similar pattern of redistribution of lipoprotein phospholipid with an increase of VLDL phospholipid secretion to 242% of control values (Fig. 4b). There were no significant changes in LDL phospholipid secretion, but chylomicron remnants caused a decrease in HDL phospholipid secretion (Fig. 4b).

In contrast, β-VLDL had no effect on either the amount of lipoprotein triglyceride secreted by HepG2 cells or on its distribution among different lipoprotein fractions (Fig. 4a). There was, however, a change in phospholipid secretion following preincubation of HepG2 with β-VLDL, with a significant increase in phospholipid secreted in HDL (Fig. 4b).

Measurement of cholesterol and cholesteryl ester specific activity in HepG2 cells and secreted lipoprotein

For both triglyceride and phospholipid, there appeared to be single cellular pools, with regard to the glycerol moiety, in which lipid of exogenous origin was in equilibrium with newly synthesized, endogenous lipid. However, this was not always the case for cholesterol and cholesteryl ester. After preincubation with lipoproteins for 24 hr, and then incubation with DL[2-^{3}H]mevalonic acid lactone for 24 hr to label the newly synthesized cholesterol pool, the specific activities of free and esterified cholesterol were determined in the cell and in total secreted lipoproteins. The data are shown in two ways: in **Table 2a,** the actual specific activities are given; in **Table 2b,** the measured secretion is compared to the secretion calculated from the cellular specific activity. In this case, the calculated mass will be less than the secreted mass to the extent that there is preferential secretion of preformed cholesterol. When there were no lipoproteins in the preincubation media, the cellular and secreted lipoprotein specific activities were quite similar. However, when β-VLDL were present in the preincubation media, this was not the case. First,

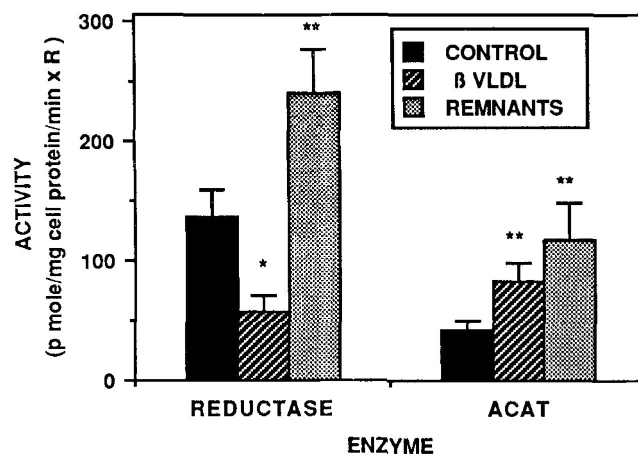

Fig. 3. Effect of preincubation with chylomicron remnants and β-VLDL on HMG-CoA reductase and ACAT in HepG2 cells. HepG2 cells were grown to confluence, and the medium was changed to one containing 10% FBS alone or including 40 μg/ml of the indicated lipoprotein. After 24 hr, the cells were washed and harvested in incubation buffer and enzyme activity was assayed as described in Methods. Results are means ± SEM of eight determinations where R = 10 for HMG-CoA reductase and 100 for ACAT. *Different from control, $P < 0.02$. **Different from control, $P < 0.05$.

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TABLE 1. Comparison of glycerolipid secretion measured by mass and calculated from cellular specific activity

	Measured Mass	Calculated Mass
	µg triglyceride/mg cell protein	
Triglyceride		
Control (n = 6)	10.61 ± 2.06	14.69 ± 2.59
Chylomicron remnant (n = 6)	12.60 ± 1.49	13.77 ± 2.66
Control (n = 2)	21.82	23.00
β-VLDL (n = 2)	21.59	23.15
	nmol phospholipid/mg cell protein	
Phospholipid		
Control (n = 6)	2.8 ± 0.4	2.6 ± 0.5
β-VLDL (n = 4)	5.3 ± 1.2[a]	4.7 ± 1.2
Control (n = 2)	4.0	4.4
Chylomicron remnant (n = 2)	3.2	3.4

HepG2 cells were grown to confluence, and fresh media with 10% FBS alone (control) or containing the indicated lipoprotein (40 µg of protein/ml) were added and incubation was continued for 2 hr. The media were then removed, and the cells were washed and cultured in lipoprotein-free media (no FBS) containing 50 µCi of [2-^{3}H]glycerol. After 24 hr additional incubation, the media were collected, and free radioactivity was removed by dialysis. The density was adjusted to 1.21 g/ml, and the total lipoprotein fraction was isolated. This was analyzed for phospholipid and triglyceride mass and radioactivity. The cells were harvested and the radioactivity and mass of triglyceride and phospholipid were also determined; n = number of determinations. Note that the β-VLDL experiments were done on different days and with different passage cells than the chylomicron remnant experiments.

[a]Significantly different from control, $P < 0.05$.

both the cellular and lipoprotein specific activities fell, as would be expected, from the expansion of the endogenous pool (Table 2a). However, the specific activities of the secreted cholesterol and cholesteryl ester were significantly lower than those of cellular values. The specific activity of cellular free cholesterol decreased by 35% while that of lipoprotein free cholesterol decreased by 55% (Table 2a). The decreases in cellular and lipoprotein cholesteryl ester specific activity were 43% and 75%, respectively (Table 2a). The data of Table 2b suggest that about one-third of the secreted cholesterol is preferentially derived from the exogenous as compared to the cellular pool.

Following preincubation with chylomicron remnants, a similar, but less profound, effect was noted with a significant decrease in lipoprotein free cholesterol specific activity, but not in cellular free cholesterol specific activity, and a fall in cellular cholesteryl ester specific activity, with no change in lipoprotein cholesteryl ester specific activity (Table 2). It should be borne in mind that in the absence of β-VLDL preincubation, there is very little cholesteryl ester secretion. These data indicate that preformed cellular cholesterol is preferentially utilized as a precursor for lipoprotein free cholesterol and cholesteryl ester secretion. They also suggest that, in contrast to the case of glycerolipid, precursor incorporation is not a reliable measure of the rate of cholesterol secretion.

Effect of preincubation of HepG2 cells with β-VLDL or chylomicron remnants on cholesterol secretion in various lipoprotein classes

The mass of free and esterified cholesterol secreted by HepG2 cells into each lipoprotein class over the 24-hr period, following a 24-hr preincubation in lipid free media or media containing chylomicron remnants or β-VLDL, was determined. Chylomicron remnants caused an increase in free cholesterol secretion into both VLDL and LDL (**Fig. 5a**). The absolute increase was greater in the LDL range, but the relative increase was greater in

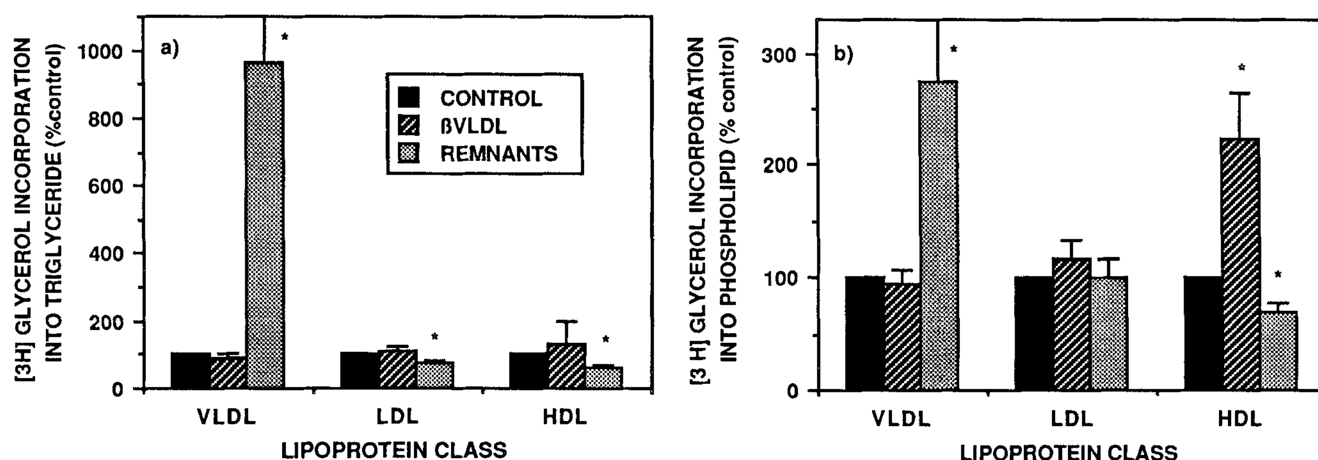

Fig. 4. Effect of preincubation with chylomicron remnants or β-VLDL on [2-^{3}H]glycerol incorporation into triglyceride and phospholipid in the different lipoprotein classes secreted by HepG2 cells. The experiment was conducted exactly as described in the legend to Fig. 1. After the final incubation period, the media were collected and dialyzed to remove unincorporated radioactivity. Lipoprotein classes were separated by density gradient centrifugation and lipids were isolated as described in Methods; a) triglyceride, b) phospholipid. Results are expressed as means ± SEM of percent of control for that class, n = 8. In the lipoprotein from control cells, 2, 78, and 20% of the triglyceride were in VLDL, LDL, and HDL, respectively, and 3, 26, and 71% of the phospholipid were in VLDL, LDL, and HDL, respectively. Absolute values for total mass secreted are given in Table 1. Together these data allow calculation of mass secretion in each class. *Different from control, $P < 0.01$. **Different from control, $P < 0.05$.

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TABLE 2a. Effect of β-VLDL and chylomicron remnants on the specific activity of cholesterol and cholesteryl ester in HepG2 cells and in total secreted lipoproteins

	Effect of β-VLDL (n = 13)	
	Cell	Total Lipoproteins
	cpm/μg lipid	
Cholesterol		
Control	5978 ± 1401	5603 ± 1164
β-VLDL	3889 ± 918[a]	2543 ± 587[a,c]
Cholesterol ester		
Control	1772 ± 376	2001 ± 430
β-VLDL	917 ± 237[a]	497 ± 100[a]
	Effect of Chylomicron Remnants (n = 5)	
	Cell	Total Lipoproteins
	cpm/μg lipid	
Cholesterol		
Control	1569 ± 80	1396 ± 73
Chylomicron remnants	1728 ± 89	1078 ± 31[d]
Cholesteryl ester		
Control	609 ± 45	564 ± 114
Chylomicron remnants	370 ± 52[b]	763 ± 355

The experiment was carried out exactly as described in Table 1, except 50 μCi of DL-[2-^{3}H]mevalonate was included in the media of the last incubation. Cholesterol and cholesteryl ester were isolated and determined as described in Methods; n = number of flasks assayed.
[a]Significant difference compared to control, $P < 0.01$.
[b]Significant difference compared to control, $P < 0.05$.
[c]Significant difference compared to cellular value, $P < 0.05$
[d]Significant difference compared to cellular value, $P < 0.01$.

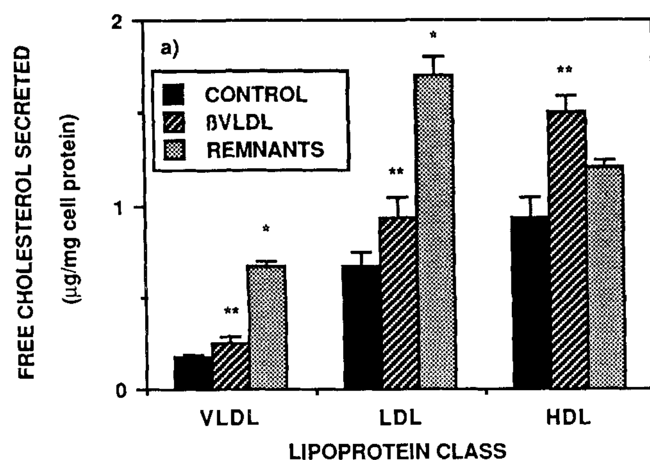
TABLE 2b. Comparison of cholesterol and cholesteryl ester secretion measured by mass and calculated from cellular specific activity

	Free Cholesterol	
	Measured	Calculated
	μg secreted/mg cell protein	
Control (n = 20)	1.86 ± 0.12	1.58 ± 0.15
Chylomicron remnants (n = 5)	3.56 ± 0.08[a]	2.27 ± 0.24[c]
β-VLDL (n = 15)	3.04 ± 0.19[a]	1.92 ± 0.15[d]
	Cholesteryl Ester	
	Measured	Calculated
	μg secreted/mg cell protein	
Control (n = 20)	0.70 ± 0.07	0.63 ± 0.23
Chylomicron remnant (n = 5)	0.99 ± 0.34	0.69 ± 0.17
β-VLDL (n = 15)	1.84 ± 0.31[b]	1.20 ± 0.20[a]

The cellular specific activity data from Table 2a were used to calculate the mass secreted and this was compared to the measured mass; n = number of flasks.
[a]Difference compared to control, $P < 0.01$.
[b]Difference compared to control, $P < 0.02$.
[c]Difference compared to Measured value, $P < 0.05$
[d]Difference compared to measured value, $P < 0.01$.

the VLDL range (3.9-fold compared to 2.5-fold). There was no change in the amount of free cholesterol in the HDL range (Fig. 5a) nor was there any change in the amount of cholesteryl ester secretion into any lipoprotein class (Fig. 5b).

Following preincubation with β-VLDL, there was an increase in free cholesterol secretion into all three lipoprotein classes (Fig. 5a). This increase was relatively uniform among the classes (1.4-fold in VLDL, 1.4-fold in LDL, and 1.6-fold in HDL). The total increase in free cholesterol secretion was lower than with chylomicron remnants (1.79 μg/mg of cell protein with chylomicron remnants compared to 0.91 μg/mg of cell protein with β-VLDL). Preincubation with β-VLDL also stimulated cholesteryl ester secretion in both VLDL and LDL (Fig. 5b), but not in HDL.



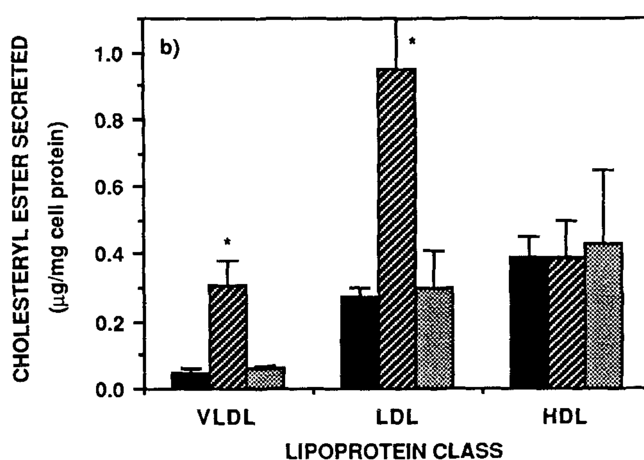


Fig. 5. Effect of preincubation with chylomicron remnants or β-VLDL on cholesterol and cholesteryl ester secretion in the different lipoprotein classes by HepG2 cells. The experiment was carried out exactly as described in the legend to Fig. 4, except that no radioisotope was present in the media of the second incubation. Cholesterol and cholesteryl ester in the lipoprotein classes were measured by gas-liquid chromatography as described in Methods; a) free cholesterol, b) cholesteryl ester. Data are means ± SEM; n = 13 for control, 5 for chylomicron remnants, and 8 for β-VLDL. *Different from control, $P < 0.01$. **Different from control, $P < 0.05$.

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To ascertain that the increased lipoprotein cholesterol seen was not derived from lipoprotein remaining following the initial incubation period, we carried out the following experiment. ^{125}I-Labeled β-VLDL was incubated with HepG2 cells for 24 hr in 3 ml of MEM (containing 10% FBS) using unlabeled β-VLDL to give a final concentration of 40 μg of β-VLDL protein/ml of media. The specific activity of β-VLDL with regard to cholesterol was known. The cells were then washed with 3 ml of MEM and reincubated for 24 hr in 3 ml of MEM prior to the isolation of total lipoproteins as described under Methods. ^{125}I-Labeled β-VLDL cpm in total lipoprotein were counted in a gamma counter, and the amount of cholesterol this represented was calculated. The experiment was repeated twice, in duplicate, and the data indicated that only 0.057 μg of total cholesterol/mg of cell protein in lipoprotein could be attributed to β-VLDL from the initial incubation period. In β-VLDL-treated cells, 4.88 μg of total cholesterol/mg of cell protein is secreted in total lipoprotein; therefore, the contribution of β-VLDL-cholesterol remaining in the media is insignificant. In separate experiments it was found that the amount of LCAT activity in the medium was very low and probably not sufficient to account for the cholesteryl ester content of the secreted lipoproteins.

DISCUSSION

There are two possible ways for the liver to respond to the varying load of lipids which it must metabolize. One is to secrete a lipoprotein of relatively constant composition at a variable rate, and the second is to secrete a spectrum of lipoproteins of differing composition. The results of this investigation provide further evidence for the second mechanism, and by using the human hepatoma-derived HepG2 cell line, suggest that this may be how the process is regulated in man.

When interpreting these experiments, two facts should be borne in mind. First, the lipoproteins that accumulate in the media may have been modified after secretion and thus, may not be truly nascent lipoprotein. The lack of a major alteration of lipoprotein added to the media suggests that remodeling and re-uptake are not extensive (8). However, we refer to these as secreted lipoproteins, implying that we are studying the net effect of secretion, rather than a nascent or newly secreted particle. Secondly, we do not believe that loading with different classes of lipoprotein per se causes the changes seen; rather they are most certainly due to the different lipid substrates delivered and, particularly to the different cholesterol-to-triglyceride ratios. Accordingly, we chose to use lipoproteins of similar apoprotein composition, but with widely varied cholesterol-to-triglyceride ratio. We used an amount of lipoprotein designed to result in near maximal uptake. Had we used lesser amounts of lipoprotein or lipoproteins of less disparate composition, we would probably have seen smaller differences. We chose not to do this since the results might have been difficult to interpret. In fact, the amount of cholesterol delivered by the two lipoproteins used was not very different. Thus, as in our previous publications (16, 17), the principal variable was the amount of triglyceride delivered per molecule of cholesterol. This has certain practical implications for understanding how dietary perturbations will ultimately affect lipoprotein homeostasis.

Preincubation with lipoproteins of varied lipid composition had the expected effects on cellular lipid composition with significant accumulation of the stored neutral lipids, triglyceride (chylomicron remnants) and cholesteryl ester (β-VLDL), and little or no accumulation of the membrane components, phospholipid and free cholesterol. The lipoproteins under investigation also affected the enzymes of intracellular lipid metabolism. Both β-VLDL and chylomicron remnants stimulated ACAT activity in HepG2 cells, presumably due to the delivery of cholesterol substrate to the enzyme. However, there were different effects on HMG-CoA reductase, the rate-limiting step of cholesterol biosynthesis. This enzyme was inhibited by β-VLDL and stimulated by chylomicron remnants. HMG-CoA reductase is known to be inhibited by feedback control by cholesterol (18) and thus, the cholesterol content of β-VLDL would explain its inhibition by this lipoprotein. Chylomicron remnants also deliver cholesterol to the cell, and yet cause a stimulation of reductase activity. This could be due to the presence of triglyceride in chylomicron remnants, which stimulates VLDL secretion (19, 20), and thus, the obligatory secretion of lipoprotein cholesterol. Presumably, insufficient cholesterol is delivered to the cell to accommodate the demands of lipoprotein secretion and, therefore, HMG-CoA reductase is stimulated to produce the extra amount required (16, 21). In addition, we have noted that cholesteryl ester, when delivered by chylomicron remnants, is degraded slowly compared to that delivered by other lipoproteins, and this may help account for the paradoxical effect of these lipoproteins on HMG-CoA reductase (7).

VLDL are quantitatively the principal class of lipoprotein secreted by normal human liver, but not by HepG2 cells. In previous experiments reported from this laboratory, oleic acid alone was found to stimulate VLDL secretion, and this occurred by a recruitment of components from the LDL range without inducing new apoB synthesis (7). This agrees with most (22), but not all, other reports (23). In the presence of chylomicron remnants, a triglyceride stimulus that also supplied cholesterol, there was an increase in apoB synthesis (8), and, as was found in the present investigation, the other components of lipoprotein also increased. Interestingly, the phospholipid necessary to form lipoprotein surface apparently came from recruit-

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ment from the HDL range, and this may help account, at least in part, for the reciprocal relationship between HDL and VLDL. In contrast, free cholesterol, the other surface component of lipoproteins, was derived preferentially from preformed, exogenous sources.

When stimulated by a high cholesterol load in the absence of triglyceride, in the form of β-VLDL, there was also a change in VLDL secretion, but to a lesser degree. Although there was no change in triglyceride secretion, there was increased free cholesterol secretion and a large increase in secretion of cholesteryl ester in the core. ApoB secretion in VLDL was also increased (8). Thus, most likely, there were more particles secreted, but most notable was the change in core composition. An analogous substitution of cholesteryl ester for triglyceride was reported in rat liver cells (1), but has not been established in a human tissue to date. In man, most cholesteryl ester in the circulation is generated by the LCAT reaction. However, human liver does contain ACAT (24), albeit at a lower level than the rat; thus, it appears that ACAT-derived cholesteryl ester could contribute to circulating VLDL and LDL cholesteryl ester.

In cultured cells, the secretion of LDL appears to be a phenomenon of triglyceride deprivation or cholesterol overload. The particle secreted in this range always contains more triglyceride than serum LDL (7); thus, either remodeling in the circulation must occur or this is a particle peculiar to HepG2 cells. In man, direct secretion of LDL appears to occur only under pathological conditions where there is cholesterol overloading (25), although in other species it may be a normal metabolic event (26, 27).

The issue of HDL secretion is more complex. HDL apolipoproteins are secreted by liver, and their rate of secretion is regulated (28). Nascent HDL have been reported in the hepatic secretory mechanism by some (29), but not most, investigators (30). Thus, the possibility that some HDL are formed in sinusoids as a result of free apolipoprotein secretion must also be considered. Similarly, cholesteryl ester-poor apoE-rich particles have been found in the serum of LCAT-deficient patients (31) and in perfusate of monkey livers (28) and orotic acid-fed rat livers (30) when LCAT has been inhibited. The fact that β-VLDL induced the secretion of free, but not esterified, cholesterol in the HDL range is compatible with the formation of this particle after secretion. Thus, free apoE or apoA-I might associate with phospholipid and free cholesterol to form a disk. Had there been core material in the particle, this might have argued for intrahepatic assembly of the particle.

In the present experiments, the endogenous lipids were labeled with radioactive precursors, and the specific activities of cellular and secreted lipids were compared. The finding of a uniform specific activity of cellular and secreted triglyceride supports the concept that stored and secreted triglycerides are derived from a common precursor pool and that exogenous triglycerides are all hydrolyzed before utilization of the fatty acid. We found that the same was true for phosphoglycerolipids, using [2-^{3}H]glycerol to label the endogenous pool. Vance and Vance (32) have reported that phosphotidylcholine secreted into lipoproteins was preferentially derived from de novo synthesis. Thus, although there may be discrete phospholipid pools for various functions, it appears that, over the time course of these experiments, the predominant phospholipid moieties in these pools have reached equilibrium.

A different result was obtained for cholesterol. In the unstimulated state, where there is little exogenous lipid, a single pool seems to be used for the secretion of both free and esterified cholesterol in lipoproteins. However, when stimulated by exogenous cholesterol in the absence of substantial triglyceride, as delivered by β-VLDL, there was preferential secretion of preformed (unlabeled) cholesterol, both free and esterified, compared to newly synthesized cholesterol. It has recently been reported (33) that there is a mechanism for shunting cholesterol from endoplasmic reticulum to plasma membrane, but not the reverse. The present result suggests that exogenously derived cholesterol does not rapidly enter that pathway, but may constitute a somewhat distinct pool whose metabolic use is controlled separately.

Taken together with our previous work (16, 17), these results begin to clarify the responses that the liver may make to dietary lipid loads of varying composition. They suggest that there is a direct link between the composition of lipoproteins reaching the liver and the composition and class of lipoprotein secreted by the liver.

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