Hepatic metabolism and secretion of a cholesterol-enriched lipoprotein fraction

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Abstract A potentially important source of cholesterol secreted in bile is cholesterol-rich lipoproteins. However, the fate of the cholesterol carried in these lipoproteins after hepatic uptake has not been investigated. We harvested an apoE- and cholesterol-rich lipoprotein fraction (d 1.02–1.06 g/ml) from hypercholesterolemic rats and examined the acute effects of these lipoproteins on hepatic cholesterol metabolism, very low density lipoprotein (VLDL) secretion, and biliary lipid secretion. Administration of a lipoprotein bolus (20 mg of cholesterol) to rats resulted in a significant decrease in 3-hydroxy-3-methylglutaryl coenzyme A reductase activity and a significant increase in acyl-coenzyme A:cholesterol acyltransferase activity over controls at 1 hr. Hepatic cholesteryl ester content increased 400% with no change in hepatic free cholesterol content or biliary cholesterol secretion. These cholesterol-rich lipoproteins delivered in the isolated perfused liver effected a fivefold increase in hepatic VLDL secretion with no change in composition. Therefore, cholesterol-rich lipoproteins do not acutely alter biliary cholesterol secretion. Rather, the majority of the cholesterol delivered to the liver in these lipoproteins is either esterified and stored as cholesteryl ester or resecreted as free and esterified cholesterol in hepatic VLDL—**Stone, B. G., D. Schreiber, L. D. Alleman, and C-Y. Ho.** Hepatic metabolism and secretion of a cholesterol-enriched lipoprotein fraction. *J. Lipid Res.* 1987. **28:** 162–172.

Supplementary key words biliary cholesterol secretion • reverse cholesterol transport • apoprotein E • very low density lipoproteins • cholesterol-induced high density lipoproteins

The liver is thought to be the most important organ of the body in maintaining cholesterol homeostasis (1). It is the major site of cholesterol synthesis, the principal site of lipoprotein cholesterol uptake and secretion, and the major site of cholesterol elimination from the body by biliary secretion. Although there exists a significant amount of research concerning hepatic cholesterol metabolism, the precise determinants of the mode of egress of a cholesterol molecule from within the hepatocyte is not yet understood.

Three possible modes exist. First, the cholesterol molecule can be secreted into the serum as a component of a lipoprotein particle. The principle lipoprotein secreted by the liver is very low density lipoprotein (VLDL) which consists of free cholesterol as a surface component and cholesteryl esters contained within the lipid core of the particle (2–4). The cholesteryl esters secreted in nascent VLDL are derived from intrahepatic esterification of free cholesterol by the microsomal enzyme acyl-coenzyme A:cholesterol acyltransferase (ACAT) (5, 6). Approximately 90% of the VLDL secreted by the liver is converted into remnant particles in the serum and subsequently cleared by lipoprotein receptors located on the liver (7, 8). Once taken up by the liver, the lipoprotein cholesteryl esters are rapidly hydrolyzed to free cholesterol (8).

Second, hepatic cholesterol can be stored within the cytoplasm as cholesteryl ester. In the liver, free cholesterol is derived both from new synthesis and serum lipoproteins, and free cholesterol from either source can subsequently undergo esterification by ACAT (9). The newly formed cholesteryl esters are thought to represent an inert storage form but are also available for secretion in VLDL (6, 10). Conversion of cytoplasmic cholesteryl esters to free cholesterol in the hepatocyte is catalyzed by a neutral cholesteryl ester (CE) hydrolase (11).

Finally, hepatic cholesterol can be secreted in the bile either as biliary cholesterol or, after conversion, as bile salts. In the bile, cholesterol is only found in the free form as intracellular cholesteryl esters are not thought to be available for direct biliary secretion. Prior work has demonstrated that the rates of biliary bile salt and phospholipid secretion are important regulators of biliary cholesterol secretion (12–14). More recent work has demonstrated that intrahepatic cholesterol synthesis and esterification rates alone and together also serve to regulate biliary cholesterol secretion in the face of a constant bile salt and phospholipid output (15). This is thought to occur by regulation of a biliary precursor pool of free cholesterol at some critical site in the cell. Moreover, these studies demonstrated that an acute increase in intracellular cholesterol resulting from

Abbreviations: HDL, high density lipoproteins; VLDL, very low density lipoproteins; HDL_c, cholesterol-induced high density lipoproteins; ACAT, acyl-coenzyme A:cholesterol acyltransferase; HMG, 3-hydroxy-3-methylglutaryl; CE, cholesteryl ester; apoE, apoprotein E; apoB, apoprotein B; SDS, sodium dodecyl sulfate; GLC, gas–liquid chromatography

new synthesis is balanced by an increase in cholesterol esterification. These changes served to maintain biliary cholesterol output constant by shunting the newly formed free cholesterol into the unavailable ester form.

However, it has been demonstrated that only 10–20% of cholesterol secreted in the bile is derived from new synthesis (16, 17). Another important source of biliary cholesterol is thought to be cholesterol delivered to the liver via serum lipoproteins. Free cholesterol contained in high density lipoproteins (HDL) appears to be preferentially secreted into the bile (18). A subpopulation of HDL is increased with cholesterol feeding (19). This HDL has the ability to accept cholesterol from peripheral tissues (20), and is efficiently cleared from the plasma by a hepatic receptor (21). This particle, termed HDL_c, is rich in apoprotein E (apoE) and cholesterol but lacks significant amounts of apoprotein B (apoB) and triglycerides. Furthermore, it has been speculated that this particle plays the important role of transporting "excess" cholesterol from the peripheral tissues to the liver for elimination from the body via biliary secretion (22). It is therefore feasible to suggest that cholesterol-rich lipoproteins are potentially important sources of cholesterol secreted in the bile.

The mechanism of transport of cholesterol from lipoproteins to the bile micelle has not been determined. However, one suggestion is that there is a direct transport of plasma cholesterol to the bile without this cholesterol entering the cell interior (23). This direct transport of lipoprotein cholesterol would effectively bypass the various intrahepatic processes that have been previously demonstrated to control biliary cholesterol secretion (15). Alternatively, if the cholesterol contained within these particles is internalized, it might be subject to the same intrahepatic regulation as newly synthesized cholesterol. To date, the acute effect of cholesterol-rich lipoproteins on hepatic cholesterol synthesis and esterification, biliary cholesterol secretion, or hepatic VLDL secretion has not been reported.

MATERIALS

Chemicals

[1,2-^{3}H]Cholesterol (40–60 Ci/mmol), 3-hydroxy-3-methyl-[3-^{14}C]glutaryl coenzyme A (57.6 mCi/mmol), [(9,10-^{3}H)]oleic acid (2–10 Ci/mmol), and D,L-[5-^{3}H]mevalonolactone (13.8 Ci/mmol) were obtained from New England Nuclear (Boston, MA). [1-^{14}C]Oleoyl coenzyme A (56–60 mCi/mmol) and cholesteryl [1-^{14}C]oleate (53.1 mCi/mmol) were from Amersham (Arlington Hills, IL). Oleoyl coenzyme A, cyanogen bromide-activated Sepharose 4B, 3-hydroxy-3-methylglutaryl coenzyme A and 3 α-hydroxysteroid dehydrogenase were from Sigma (St. Louis, MO). Acrylamide, N-N-bis-methylene-acrylamide, N,N,N', N'-tetramethylethylenediamine (TEMED), sodium dodecyl sulfate, bromophenol blue, and Coomassie Blue G-250 were from Biorad (Rockville Center, NY).

Animals

Male Sprague-Dawley rats (Zivic Miller, Pittsburgh, PA), fed standard lab chow and housed under normal lighting conditions, were used in all experiments. For preparation of the cholesterol-rich lipoproteins, retired breeders were fed an atherogenic diet (ICN Nutritional Biochemicals, Cleveland, OH) consisting of 5% lard, 1% cholesterol, 0.1% propothiouracil, and 3% taurocholic acid. Liver donors for in situ liver perfusion weighed between 260–340 g at the time of perfusion. Bile fistula rats weighed between 330–380 g at the time of use.

METHODS

Cholesterol-rich lipoprotein preparation

A cholesterol-rich lipoprotein fraction (d 1.02–1.06 g/ml) was obtained by a modification of HDL_c preparation as described by Mahley and Holcombe (19). Groups of 25–30 retired breeders were fed an atherogenic diet for a 3-wk period. After an overnight fast, the rats were killed by aortic puncture and the blood was allowed to clot at 4°C. The red blood cels were isolated by low speed centrifugation and discarded. To the resultant plasma were added disodium EDTA (4 mg/ml plasma), sodium azide (4 mg/ml plasma), and gentamycin (62.5 μg/ml plasma). After density adjustment with KBr, the d < 1.02 g/ml fraction was harvested by ultracentrifugation at 3.7×10^6 *g*-hr using a Sorvall T-865 rotor (Wilmington, DE). This lipoprotein fraction was subsequently utilized for apoprotein E isolation (see below).

The d 1.02–1.06 g/ml fraction was then harvested by density adjustment and ultracentrifugation at 3.95×10^6 *g*-hr. The resultant top layer was removed, washed by resuspension, and reisolated by ultracentrifugation. The isolated lipoproteins were concentrated and extensively dialyzed against phosphate-buffered saline (pH 7.4) using a negative pressure microprotein dialysis/concentrator (Bio-Molecular Dynamics, Beaverton, OR) to a final cholesterol concentration of 16.5–53.3 mg/ml. This cholesterol-enriched lipoprotein fraction was used within 1 week of preparation.

In selected preparations, [5-^{3}H]mevalonolactone (13.8 Ci/mmol) in normal saline was administered intragastrically to retired breeders fed an atherogenic diet for 3 weeks. After a 12-hr incorporation period, a cholesterol-enriched lipoprotein fraction was prepared as described above.

Sodium dodecyl sulfate (SDS) gel electrophoresis

Apoprotein E was obtained from the d < 1.02 g/ml lipoprotein fraction of rats fed an atherogenic diet. After

delipidation, the apoE was isolated by heparin-Sepharose 4B affinity chromatography exactly as described by Shelborne and Quarfordt (24). Human HDL and LDL were obtained by ultracentrifugation with progressive density adjustment (25).

All lipoproteins were delipidated twice with 10 volumes of ethanol–ether 3:1 at 4°C and washed with 10 volumes of ether. The resultant protein precipitates were dissolved in 62.5 mM Tris buffer (pH 6.8) containing 1% SDS, 15% glycerol, 1% 2-mercaptoethanol, and 0.001% bromophenol blue to a final concentration of approximately 1 mg/ml. The protein samples were incubated at 37°C for 2 hr to reduce possible apoE–A-II complexes (26), and 10–40 μg of protein was applied to each lane of either a 6% or 10% SDS-polyacrylamide gel as described by Laemmli (27). After electrophoresis, the gels were fixed and stained overnight with 25% isopropanol, 10% acetic acid, and 0.02% Coomassie brilliant blue. The gels were photographed and sliced, and the individual lanes were scanned at 550 nm with a Gilford spectrophotometer (Oberlin, OH) equipped with a 20-cm gel scanner. The areas under the peaks were calculated using planimetry.

Animal preparation for in vivo studies

Groups of age- and weight-matched rats were fitted with biliary catheters, intraduodenal tubes, and femoral vein catheters; the experimental protocol was as previously described (15). A bile replacement solution (24 mM taurocholate, 3 mM lecithin, and 0.45 mM cholesterol) was constantly infused at a rate 1.4 ml/hr to maintain the enterohepatic circulation. After a 21-hr recovery period, a 1-hr bile sample was obtained and then either 20 mg of lipoprotein cholesterol or the normal saline vehicle alone was administered as an intravenous bolus through the femoral vein catheter. After injection, hourly bile samples were obtained for the 2 successive hours. On the subsequent day, the experiment was repeated except the solution not given the previous day was administered. Whether the animal initially received the cholesterol-rich lipoproteins or the normal saline vehicle was determined randomly. Therefore each animal served as its own control.

Hepatic ACAT, HMG-CoA reductase, and CE hydrolase activity

Separate groups of age- and weight-matched animals were administered a 20-mg bolus of either lipoprotein cholesterol or the vehicle alone through a femoral vein catheter. After 1 hr, the animals were killed and the livers were removed. The harvested livers were rinsed in iced buffer (0.25 M sucrose, 1 mM EDTA, pH 7.2) and homogenized with four vol of buffer/g of liver. Microsomes were prepared and assayed for ACAT and 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase activities as described by Erickson et al. (28, 29).

The 105,000 *g*-hr supernatant (S-105) was defatted by passage through glass wool and utilized for CE hydrolase activity determination (30). CE hydrolase activity was measured by the addition of a nonlimiting amount (40 nmol) of cholesteryl [1-^{14}C]oleate (3.3 dpm/pmol in absolute ethanol) to 0.2 ml of the S-105 (protein concentration of approximately 20 mg/ml). The mixture was incubated at 37°C for 60 min and the reaction was terminated by the addition of 3 ml of a 0.1 mM oleate solution of benzene–chloroform–methanol 2:1:2.4. After the addition of [^{3}H]oleate (approximately 20,000 dpm) to assess recovery, 0.7 ml of 3 M NaOH was added and the sodium oleate was extracted by vigorous mixing. The layers were separated by low speed centrifugation and 1 ml of the upper aqueous layer containing the sodium oleate was counted in Aquasol II (New England Nuclear, Boston, MA). The CE hydrolase activity was calculated from the amount of (1-^{14}C]oleate formed after correcting for recovery and subtracting the [1-^{14}C]oleate recovered in a blank assayed without added S-105 protein.

Liver perfusion

After the donor rats were anesthetized with pentobarbital and common bile duct cannulation was performed, the livers of donor rats were perfused by the recirculating methods of Mortimer (31) at a rate of 1.1 ml/min per g of liver. The perfusate (40–70 ml, pH 7.4) consisted of 21–25% washed human red cells suspended in Earle's balanced salt solution (Grand Island Biochemical Co., Grand Island, NY) supplemented with 3 g/dl of fatty acid-free bovine serum albumin (Sigma, St. Louis, MO). Liver oxygenation was maintained by circulating the RBC-containing perfusate through a 95% O_2, 5% CO_2 gas mixture using a silastic "lung" as described by Hamilton et al. (32). Liver and perfusate temperatures were strictly maintained between 36–37°C. No significant change in perfusate hematocrit or pH occurred over the 1.5 or 2 hr recirculation period (data not shown).

Bile flow was maintained over the entire recirculation period by infusion of a bile salt replacement solution. The replacement solution (32 mM taurocholate in Earle's balanced salt solution with 3 g/dl bovine serum albumin) was delivered directly into the portal vein cannula at a rate of 15.2 μmol/100 g body weight per hr. Liver viability was monitored by color, oxygen extraction, and maintenance of bile flow (assessed every 0.5 hr) over the entire perfusion period.

After an initial 5-min period to establish viability of the isolated perfused liver, either 20 mg of lipoprotein cholesterol or the same volume (0.4–1.2 ml) of normal saline was added to the reservoir and allowed to recirculate for 1.5 hr. At the end of this period, the entire perfusate was collected, the hematocrit and volume was determined, and the red cells were isolated by low speed centrifugation and

discarded. The newly secreted VLDL (d < 1.006 g/ml) was harvested by ultracentrifugation, washed by resuspension in phosphate-buffered saline (pH 7.4), and reisolated for subsequent lipid determinations.

Selected experiments utilized a perfusate exchange protocol (**Fig. 1**) adapted from Van Zuiden, Erickson, and Cooper (33). After 0.5 hr of recirculation, the cholesterol-rich lipoprotein-containing perfusate was removed from the collecting reservoir and discarded. During the exchange period, viability of the isolated liver preparation was maintained by the delivery of 50 ml of fresh oxygenated perfusate via a single pass perfusion technique. This also served to wash any remaining lipoproteins from the liver preparation (34). The reservoir was filled with fresh perfusate which was recirculated for an additional 1.5 hr. The newly secreted VLDL was then harvested from the perfusate as outlined above.

Sample analysis

Triglycerides were enzymatically determined by the method of Kreutz (35) using the triglyceride assay kit from Sigma (St. Louis, MO). VLDL and hepatic cholesterol content (total and free) were assayed by gas–liquid chromatography as described previously (15). Cholesteryl ester content was determined as the difference between the total and free cholesterol content in the same sample. Biliary cholesterol was determined colorimetrically according to Mann (36) after saponification of the bile samples (37). Phospholipid content was determined by the choline oxidase method (38) utilizing the PL kit-K from Nippon-Shoji Kaisha, Ltd. (Osaka, Japan). This method measured all bile phospholipid species (39) and all but approximately 5% of VLDL phospholipids (40). Protein was determined by the method of Lowry et al. (41) or by the biuret method (42) using bovine albumin as a reference standard. Bile salts were measured according to Turley and Dietschy (43). All results, unless otherwise stated, are expressed as the mean ± standard error.

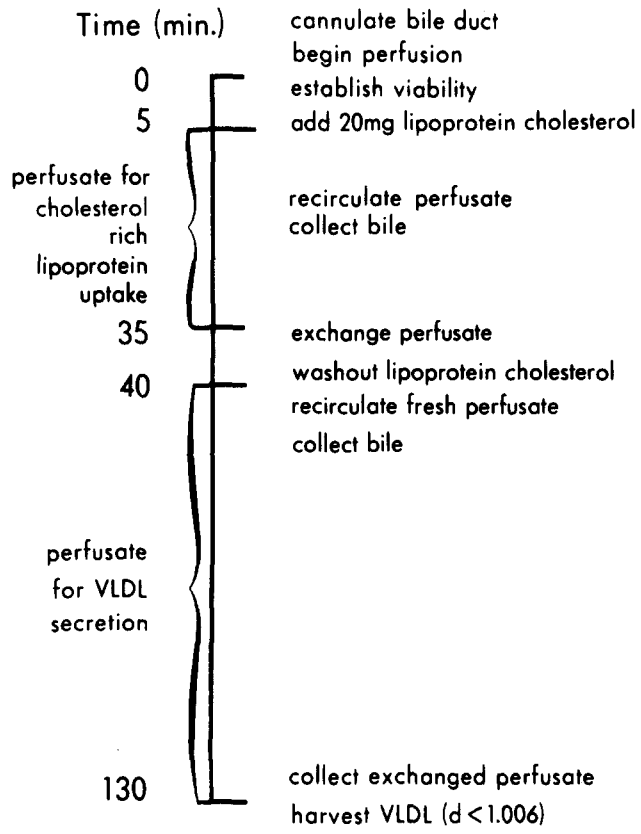

Fig. 1. Experimental protocol for in situ liver perfusion with perfusate exchange. After bile duct cannulation and isolated liver perfusion were established, 20 mg of lipoprotein cholesterol was added to the perfusate and allowed to recirculate for 0.5 hr. Then the cholesterol-rich lipoprotein-containing perfusate was exchanged for fresh lipid-free perfusate. During the exchange period, viability of the isolated liver was maintained by single-pass perfusion of 50 ml of oxygenated perfusate, which also serves to flush the liver of any remaining lipoproteins. After exchange, the fresh perfusate was allowed to recirculate for an additional 1.5 hr. This perfusate was collected and the newly secreted VLDL was harvested from the d < 1.006 g/ml fraction.

RESULTS

Characterization of the cholesterol-rich lipoprotein fraction

To prepare a cholesterol-rich lipoprotein fraction, rats were fed an atherogenic diet for a 3-week period. After 3 weeks, the cholesterol level of pooled rat serum averaged 604 ± 192 mg/dl (n = 4). In addition, feeding of this diet to rats also results in a fourfold increase in the total apoE plasma concentration with the majority of the apoE found in the d 1.02–1.06 g/ml density interval (19). Characterization of this density interval by SDS gel electrophoresis using either 6% or 10% polyacrylamide (**Fig. 2**) demonstrated that apoE was the major apoprotein (80 ± 3% of the total stained apoprotein, n = 4). There was a modest amount of apoB (14 ± 4%, n = 4) while only trace amounts of other apoproteins (6 ± 1%, n = 4) were identified. The lipoproteins contained within the d 1.02–1.06 g/ml interval were demonstrated to have the following composition by weight: 52.0 ± 8.9% cholesterol, 2.2 ± 0.5% triglyceride, 30.4 ± 6.2% phospholipid, and 15.3 ± 4.9% protein, (n = 3). The majority of the cholesterol in this lipoprotein fraction was in the form of cholesteryl esters (81% ± 2%, n = 3). The apoprotein content and lipid composition were very similar to that previously published for HDL_c (19, 21) suggesting that the major lipoprotein contained within the d 1.02–1.06 g/ml density interval was HDL_c. Further purification of HDL_c from the d 1.02–1.06 g/ml lipoprotein fraction requires Geon-Pevikon block electrophoresis (19). Since the goal of these experiments was to harvest a cholesterol-rich fraction, we elected to use the d 1.02–1.06 g/ml lipoprotein fraction (subsequently termed

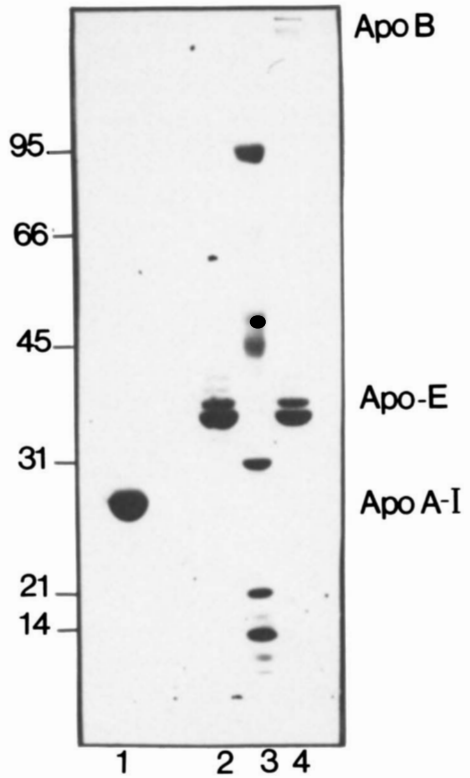

Fig. 2. Apoprotein characterization of the cholesterol-rich lipoprotein fraction. Human HDL (lane 1), heparin-Sepharose 4B-purified rat apoE (lane 2), molecular weight markers (lane 3), and a delipidated sample of the d 1.02–1.06 g/ml fraction (lane 4) of rats fed an atherogenic diet were analyzed by gel electrophoresis on a 10% SDS-polyacrylamide gel. Between 10–40 μg of protein was applied to each gel. Very little apoB could be identified in the d 1.02–1.06 g/ml fraction (lane 4), suggesting that it contained primarily apoE. This lack of apoB was confirmed by apoprotein analysis using a 6% SDS-polyacrylamide gel (data not shown).

cholesterol-rich lipoproteins) without further purification in subsequent experiments.

Effect of cholesterol-rich lipoproteins on biliary lipid secretion

Previous work has suggested the existence within the hepatocyte of a biliary precursor pool of free cholesterol whose size is determined in part by a balance between the rates of cholesterol synthesis and esterification (15). Other authors have suggested that cholesterol-rich lipoproteins (particularly HDL_c) may be a major source of the cholesterol secreted into the bile (22). To date, it is not known whether an increase in intrahepatic cholesterol derived from cholesterol-rich lipoproteins influences biliary cholesterol secretion. Two effects are possible. If the cholesterol carried in these lipoproteins is rapidly and directly secreted into the bile, administration of a bolus of lipoprotein cholesterol should result in an increase in biliary cholesterol secretion. Alternatively, if this lipoprotein cholesterol is subject to the same regulatory mechanisms (changes in synthesis and esterification) as has been previously demonstrated for newly synthesized cholesterol (15), no change in biliary cholesterol secretion should be expected. To examine these possibilities, we delivered a 20-mg intravenous bolus of cholesterol-rich lipoproteins or the vehicle alone on successive days to rats with bile fistulas and rigidly maintained enterohepatic circulations. Neither 20 mg of the lipoprotein cholesterol nor the vehicle alone induced a change in bile flow (data not shown), bile salt, cholesterol, or phospholipid secretion (**Fig. 3**). The failure of the cholesterol-rich lipoproteins to induce an acute increase in biliary cholesterol secretion suggests that regulatory mechanisms must have compensated for the increased cholesterol load to the liver, and thus maintained constant the precursor pool of biliary cholesterol. Therefore, we examined the parameters of hepatic cholesterol metabolism previously shown to regulate biliary cholesterol secretion. One hour after administration of the bolus of lipoprotein cholesterol, there was a 62% decrease in HMG-CoA reductase activity, a 45% increase in ACAT activity, and no change in CE hydrolysis activity compared to age- and weight-matched controls given only the normal saline vehicle (**Table 1**).

In order to eliminate the possibility of inter-lipoprotein cholesterol transfer, the isolated perfused liver was utilized to investigate the effects of this cholesterol-rich lipoprotein fraction on biliary cholesterol secretion and hepatic cholesterol content. The addition of 20 mg of lipoprotein cholesterol had no effect on biliary lipid secretion in the isolated perfused liver (**Table 2**) confirming the in vivo findings (Fig. 3). In response to the lipoprotein cholesterol bolus, there was a fourfold increase in hepatic cholesteryl ester content without a significant change in the free cholesterol content (**Table 3**). The increase over control livers in hepatic cholesteryl ester content averaged 10.68 mg of cholesteryl ester/liver and represented a significant proportion (approximately 53%) of the 20 mg of lipoprotein cholesterol added to the perfusate. These results suggest that the majority of the lipoprotein cholesterol delivered to the liver was esterified by ACAT rendering it unavailable for biliary secretion.

Finally, it is possible that the cholesterol derived from the administered lipoproteins is not available for biliary secretion because it does not contribute to the intrahepatic biliary precursor pool. To test this alternative, a cholesterol-rich lipoprotein fraction containing radiolabeled cholesterol was prepared as described in the Methods section and administered in the isolated perfused liver system. One-half hour after the administration of this lipoprotein bolus, radioactive cholesterol was detected in the secreted bile and 13.4% of the biliary cholesterol mass secreted over the 1.5-hr perfusion period was derived from the cholesterol-rich lipoproteins. However, the total radioactive cholesterol secreted in the bile over the 1.5-hr period represented less than 1% of the lipoprotein cholesterol removed from the perfusate. This assumes

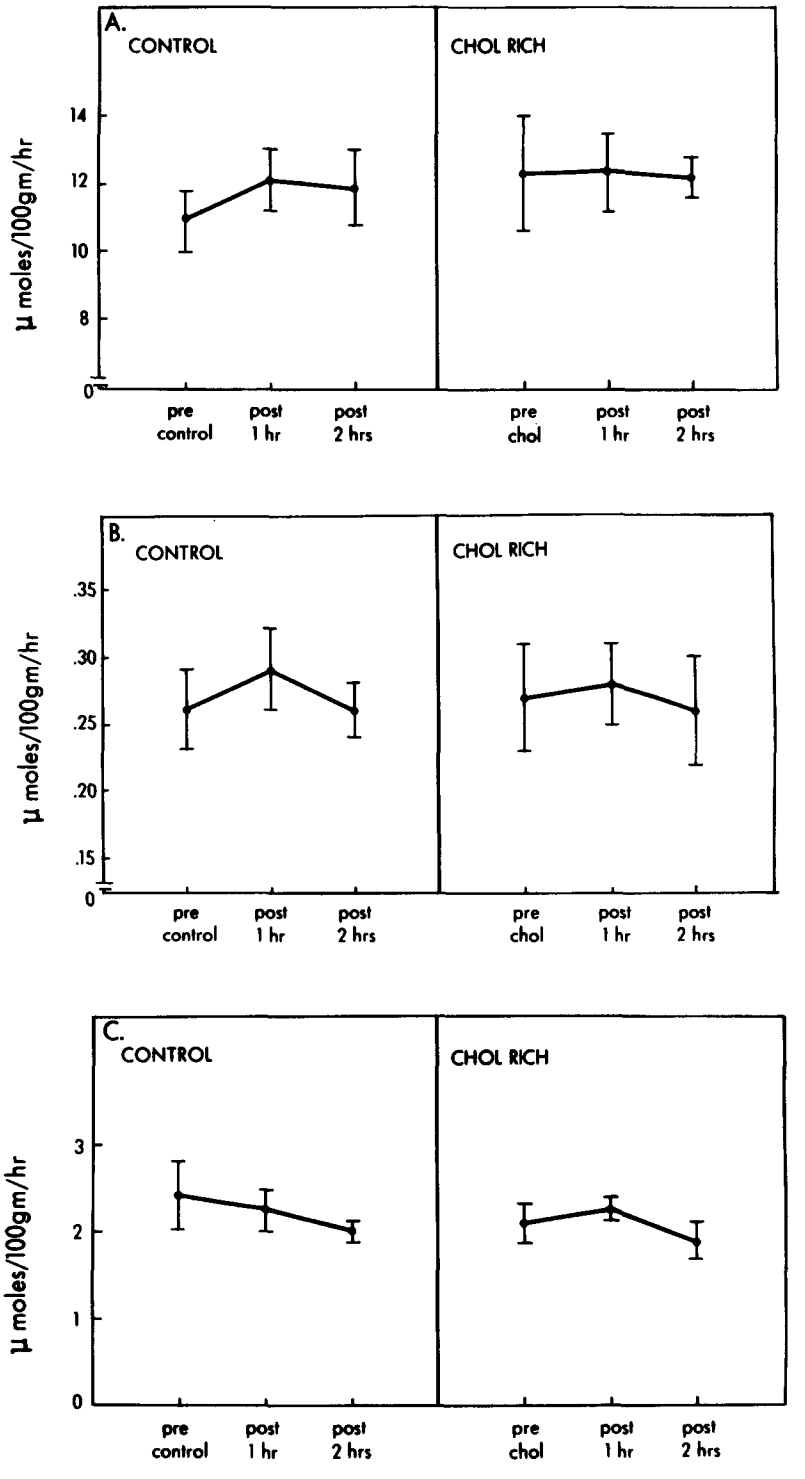

Fig. 3. Effect of cholesterol-rich lipoproteins on biliary lipid output in vivo. Rats were surgically fitted with biliary drainage catheters for bile sampling and intraduodenal tubes for bile replacement in order to maintain the enterohepatic circulation. The animals were placed in restraining cages and allowed free access to food and water. After a 21-hr recovery period, a 1-hr bile sample (pre) was obtained. Then either the normal saline vehicle (control) or cholesterol-rich lipoprotein (20 mg of cholesterol) was administered as a bolus through a femoral vein catheter. Two consecutive 1-hr bile samples (post) were then collected. The next day the sequence was repeated except the solution not given the day before was administered. Whether the animal first received the cholesterol-rich lipoprotein or the normal saline vehicle was determined randomly so that each animal served as his own control. The 1-hr bile samples (pre and post) were analyzed for bile flow and bile salt, cholesterol, and phospholipid output in response to the cholesterol-rich lipoproteins (right) or the normal saline control (left). A, Effect on bile salt secretion; B, effect on cholesterol secretion; C, effect on phospholipid secretion. Each point represents the mean ± standard error of the biliary lipid outputs of five animals. No significant differences were demonstrated.

TABLE 1. The effect of cholesterol-rich lipoproteins on hepatic HMG-CoA reductase, ACAT, and CE hydrolase activities

	HMG-CoA Reductase (pmol of mevalonic acid/min per mg)	ACAT (pmol of CE/min per mg)	CE Hydrolase (pmol of oleate/min per mg)
Cholesterol-rich (8)	38 ± 8[a]	188 ± 14[a]	13.6 ± 0.9
Control (vehicle alone) (5)	101 ± 27	129 ± 20	14.1 ± 0.8

HMG-CoA reductase and ACAT activities were determined in hepatic microsomes 1 hr after intravenous delivery of the cholesterol-rich lipoproteins (20 mg of cholesterol) or the vehicle alone as described in Methods. After the microsomes were pelleted, the resultant 105,000 *g*-hr supernatant was assayed for CE hydrolase activity. The number of determinations is in parentheses and each determination represents a single control or experimental animal. The values are expressed as the mean ± standard error.

[a]Different from control as judged by grouped *t* test, $P < 0.05$.

a constant specific activity of the added lipoprotein cholesterol over 1.5-hr perfusion period. Although this may not be a valid assumption, this experiment does demonstrate the ability of the lipoprotein cholesterol to enter the biliary cholesterol precursor pool, but not in quantities large enough to expand this pool and increase biliary cholesterol secretion.

Effect of cholesterol-rich lipoproteins on hepatic VLDL composition and secretion

In the rat, the majority of the cholesteryl esters found in plasma VLDL is derived from the intrahepatic esterification of free cholesterol by ACAT (5, 6, 44). Furthermore, the pharmacologic manipulation of ACAT enzyme activity in turn regulates the amount of cholesteryl esters in newly secreted VLDL (6). Therefore, cholesteryl esters derived from intrahepatic esterification of free cholesterol delivered in the cholesterol-rich lipoproteins should also be available for secretion in nascent VLDL. However, it is not known whether expansion of the intrahepatic pool of cholesteryl ester by lipoprotein-derived cholesterol will result in an acute increase in VLDL cholesterol secretion. We therefore examined the effect of the cholesterol-rich lipoproteins on hepatic VLDL secretion using the isolated perfused liver. After 1.5 hr of recirculation of the added lipoproteins, there was a significant increase in hepatic VLDL secretion compared to the amount of VLDL secreted by control livers (**Table 4**). This increase could be demonstrated in all components of the secreted VLDL so that no substantial change in VLDL composition occurred. Furthermore, the increase in VLDL cholesterol output was a result of an absolute increase in both free cholesterol and cholesteryl ester secretion without a change in the relative proportion of each (**Table 5**).

Routinely, 940 ± 360 μg (n = 3) of triglyceride was added to the perfusate as a component of the cholesterol-rich lipoproteins. However, the increase in triglyceride output between the lipoprotein-stimulated VLDL and that of the control averaged 7.93 mg over the 1.5-hr perfusion period. Thus, significantly more triglyceride was secreted in the VLDL than was supplied in the cholesterol-rich lipoproteins, suggesting that the added lipoproteins stimulated VLDL triglyceride synthesis as well as secretion. This is in accord with the observation that isolated hepatocytes from cholesterol-fed rats demonstrate increased triglyceride synthesis compared to control hepatocytes (45). In distinction, the increase in VLDL cholesterol and phospholipid secretion can be accounted for completely by the amount of these lipids delivered in the cholesterol-rich lipoproteins added to the medium.

TABLE 2. The effect of cholesterol-rich lipoproteins on biliary lipid secretion in the isolated perfused liver

	Output		
Added to Perfusate	Bile Salt	Cholesterol	Phospholipid
		μmol/100 g body weight per hr	
Cholesterol-rich lipoproteins (5)	10.3 ± 1.4	0.24 ± 0.04	1.13 ± 0.09
Cholesterol-rich lipoproteins with perfusate exchange (6)	7.7 ± 0.2	0.27 ± 0.02	1.32 ± 0.07
Control (vehicle only) (7)	10.7 ± 1.3	0.24 ± 0.02	1.21 ± 0.17

Bile salts were directly infused into the liver through the portal vein cannula at a rate of 15.2 μmol/100 g body weight per hr. After addition of the cholesterol-rich lipoproteins (20 mg of lipoprotein cholesterol) or vehicle to the perfusate, bile was collected in preweighed vials over the 1.5 hr in which the lipoproteins were recirculated. In the perfusate exchange experiments, the bile samples were collected for 1.5-hr period after fresh perfusate was substituted for the lipoprotein-containing perfusate (see Fig. 1). There were no significant changes in biliary lipid output observed among the three groups. The number of livers perfused in each group is in parentheses. The data are expressed as mean ± standard error of the biliary lipid outputs from the three groups of perfused livers.

TABLE 3. Cholesterol content of isolated perfused livers following recirculation of the cholesterol-rich lipoproteins

Added to Perfusate	Hepatic Cholesterol Content	
	Free Cholesterol	Cholesteryl Ester
	mg/g liver	
Cholesterol-rich lipoproteins (7)	1.9 ± 0.2	1.2 ± 0.2[a]
Cholesterol-rich lipoproteins with perfusate exchange (6)	1.4 ± 0.1	0.9 ± 0.2[a]
Control (vehicle only) (7)	1.5 ± 0.1	0.3 ± 0.1

The hepatic cholesterol contents were determined by GLC after recirculation of the cholesterol-rich lipoproteins or vehicle alone for a 1.5-hr period. In the perfusate exchange experiments, the added lipoprotein cholesterol was washed out after 0.5 hr and fresh perfusate was substituted for the cholesterol-rich lipoprotein-containing perfusate. The cholesteryl ester contents were calculated as the difference between the total cholesterol and free cholesterol contents in the same sample and expressed as mg cholesteryl ester/g liver. The number of perfused livers is in parentheses and the values are expressed as mean ± standard error of each of the three groups of perfused livers.

[a]Different from control by grouped t test, $P < 0.001$.

Since the nascent VLDL was harvested from recirculated perfusate containing the cholesterol-rich lipoproteins, it is possible that an extrahepatic transfer of lipid components occurred between added lipoproteins and the newly secreted VLDL. However, in distinction to other species, the rat does not appear to contain a significant serum cholesteryl ester transfer activity (46). Furthermore, Hamilton et. al. (4) have demonstrated that the composition of nascent VLDL secreted from the perfused liver is not altered by the action of lecithin:cholesterol acyltransferase (LCAT), the enzyme thought to be responsible for cholesteryl ester formation in serum. Therefore, the changes in VLDL secretion are presumably a result of hepatic uptake of the cholesterol-rich lipoproteins, intracellular esterification of free cholesterol, and resecretion of "excess" free cholesterol and cholesteryl ester in hepatic VLDL.

To confirm this point, a perfusate exchange system as described in the Methods section (Fig. 1) was utilized. In this system the cholesterol-rich lipoprotein-containing perfusate was recirculated for 0.5 hr, after which the perfusate was replaced with lipid-free perfusate. This experimental design did not allow exposure of the originally added lipoproteins to the newly secreted VLDL (harvested from the fresh perfusate after an additional 1.5 hr of recirculation). After 0.5 hr of cholesterol-rich lipoprotein recirculation, 58% of the orginally added cholesterol (20 mg) was present in the perfusate. This result demonstrates the rapid uptake of these apoE- and cholesterol-rich lipoproteins by the liver, and agrees with determinations of hepatic uptake (64% within 20 min) of other cholesterol- and apoE-rich particles (HDL_c) (47). In the subsequent 1.5 hr of recirculation of fresh perfusate, the cholesterol-rich lipoproteins effected a twofold increase in hepatic VLDL output with no change in composition (Table 5 and **Table 6**). These results suggest that the increase in hepatic VLDL secretion resulted from the uptake and subsequent resecretion of the lipid components of the cholesterol-rich lipoproteins. In these experiments there was again no significant change in biliary lipid output (Table 2). However, there was a significant increase in hepatic cholesteryl ester content with no significant change in hepatic free cholesterol content (Table 3). This increase in cholesteryl ester content

TABLE 4. The effect of cholesterol-rich lipoproteins on hepatic VLDL output and composition

	Output		Composition	
	Cholesterol-rich (6)	Control (5)	Cholesterol-rich (6)	Control (5)
	μg/g liver per hr		*% by weight*	
Triglyceride	516 ± 38[a]	109 ± 23	59 ± 1	61 ± 1
Cholesterol (total)	59 ± 8[a]	13 ± 1	7 ± 1	9 ± 2
Phospholipid	107 ± 9[a]	17 ± 5	12 ± 1[b]	9 ± 1
Protein	182 ± 13[a]	38 ± 8	21 ± 1	22 ± 1

Cholesterol-rich lipoproteins (20 mg of cholesterol) or an identical volume of normal saline was added to the perfusate and allowed to recirculate for 1.5 hr in an isolated liver perfusion preparation. After this period, hepatic VLDL was harvested from the d < 1.006 g/ml fraction by ultracentrifugation. After washing by resuspension, the VLDL was reisolated and analyzed as described in Methods. The number of liver perfusions in each group is given in parentheses and the values are expressed as the mean ± standard error of each of the two groups.

[a]Different from control by grouped t test, $P < 0.001$.

[b]Different from control by grouped t test, $P < 0.02$.

TABLE 5. Effect of cholesterol-rich lipoproteins on VLDL cholesterol output and composition

Added to Perfusate	Output		Composition	
	Free	Ester	Free	Ester
	μg cholesterol/g liver per hr		*% by weight*	
Cholesterol-rich lipoprotein (6)	39 ± 7[a]	25 ± 3[a]	60 ± 4	40 ± 4
Cholesterol-rich lipoprotein with perfusate exchange (6)	16 ± 3[b]	10 ± 2[b]	61 ± 4	39 ± 4
Control vehicle only (5)	8 ± 1	5 ± 1	66 ± 5	34 ± 5

Secretion of VLDL cholesterol by the isolated liver was determined by GLC after recirculation of the cholesterol-rich lipoproteins or the control vehicle as described in Methods. The cholesteryl ester output was calculated as the difference between the total cholesterol and the free cholesterol in the same samples and is expressed as μg of cholesterol/g liver per hr. The number of liver perfusions in each group is in parentheses and the values are expressed as the mean ± standard error of each of the three groups of perfused livers.

[a]Different from control by grouped *t* test, $P < 0.01$.

[b]Different from control by grouped *t* test, $P < 0.05$.

persisted even though the livers had not been perfused with the cholesterol-rich lipoproteins for a period of 1.5 hr.

DISCUSSION AND CONCLUSION

As of yet the source of cholesterol secreted in the bile has not been completely elucidated. Ten to twenty percent of biliary cholesterol is derived from cholesterol, newly synthesized within the liver (16, 17). Another potential source of biliary cholesterol is cholesterol-rich lipoproteins. Work by several authors has demonstrated that HDL can acquire cholesterol from a variety of peripheral tissue sources (20, 48, 49). The aquisition of cholesterol is accompanied by an increase in lipoprotein size and apoE content (20). These apoE- and cholesterol-rich particles are rapidly cleared by the liver, thus rendering the cholesterol contained within these lipoproteins potentially available for biliary secretion and subsequent elimination from the body. The process of acquiring cholesterol from the peripheral tissues and delivering it to the liver for elimination has been termed "reverse cholesterol transport" (22). We therefore harvested and characterized a cholesterol-rich lipoprotein fraction (d 1.02–1.06 g/ml) from rats fed an atherogenic diet. These lipoproteins had a cholesterol–triglyceride ratio of 25:1 and contained apoE as the major apoprotein. We subsequently investigated the acute effect of these cholesterol-rich lipoproteins on biliary cholesterol secretion. Our work does not support a role for cholesterol-rich lipoproteins in the rapid and direct transport of cholesterol into the bile. Both in vivo and in the perfused liver, we found no significant change in biliary cholesterol secretion in response to a bolus of cholesterol-rich lipoproteins. Although a significant proportion of the added lipoprotein cholesterol was demonstrated to be in the liver after the 1.5 hr of lipoprotein recirculation, the percentage of biliary cholesterol derived from these cholesterol-rich lipoproteins still remained small. These findings suggest that cholesterol carried in apoE-rich lipoproteins does not directly and acutely contribute to the biliary cholesterol precursor pool in the hepatocyte. However, it must be noted that these are short-term experiments. With time, the lipoprotein-derived cholesterol

TABLE 6. Effect of cholesterol-rich lipoproteins on hepatic VLDL output and composition after perfusate exchange

	Output		Composition	
	Exchange (6)	Control (5)	Exchange (6)	Control (5)
	μg/g liver per hr		*% by weight*	
Triglyceride	276 ± 52[a]	109 ± 23	63 ± 3	61 ± 1
Cholesterol (total)	26 ± 4[a]	13 ± 1	6 ± 2	9 ± 2
Phospholipid	24 ± 7	16 ± 5	7 ± 1	9 ± 1
Protein	95 ± 10[a]	38 ± 8	23 ± 2	22 ± 1

Cholesterol-rich lipoproteins were added to the perfusate and allowed to recirculate for 0.5 hr. After this period, the liver was flushed and the lipoprotein-containing perfusate was exchanged with fresh perfusate as outlined in Fig. 1. After an additional 1.5 hr, the perfusate was harvested and newly secreted VLDL was isolated, washed, and reisolated from the d < 1.006 g/ml fraction. The number of livers perfused is given in parentheses and the values are expressed as the mean ± standard error of each of the two groups.

[a]Different from control by grouped *t* test, $P < 0.05$.

may eventually equilibrate with the biliary precursor pool of free cholesterol allowing a greater proportion to be secreted in the bile.

Although administration of this cholesterol-rich lipoprotein fraction did not effect biliary cholesterol secretion, significant changes in hepatic cholesterol metabolism did occur. The resultant increase in intracellular cholesterol acutely decreases hepatic cholesterol synthesis as reflected by a decrease in HMG-CoA reductase activity. In addition, "excess" free cholesterol derived from these lipoproteins is either promptly esterified and stored within the hepatocyte or secreted as free cholesterol and cholesteryl ester in newly formed VLDL. These acute changes in hepatic cholesterol metabolism in response to a bolus of lipoprotein cholesterol appear to protect the hepatocyte from accumulation of excess free cholesterol, which is thought to be toxic to the cell (9). In addition, changes in hepatic cholesterol synthesis and esterification influence biliary cholesterol secretion by regulating the amount of free cholesterol available in a biliary precursor pool (15). These studies suggest that VLDL cholesterol secretion may also serve to regulate the size of this biliary precursor pool.

Finally, an important finding from these studies is that cholesterol originating from cholesterol-rich lipoproteins is rapidly resecreted in hepatic VLDL. To our knowledge, the finding that a bolus of cholesterol-rich lipoproteins can acutely regulate hepatic VLDL secretion has not been demonstrated. Goh and Heimberg (50) have demonstrated that an infusion of fatty acids in the perfused liver acutely increases hepatic triglyceride synthesis, hepatic triglyceride content, and VLDL triglyceride secretion. Since cholesterol is an obligatory component of the VLDL particle, they suggested that the increase in VLDL triglyceride secretion, in turn, was responsible for the observed increase in hepatic cholesterol synthesis and VLDL cholesterol secretion. The authors concluded that VLDL triglyceride secretion, in part, regulates hepatic cholesterol synthesis and VLDL cholesterol secretion (50). From our data, we suggest that the reverse is also true. We observed an increase in VLDL triglyceride secretion in response to an increase in hepatic cholesterol content and VLDL cholesterol secretion. Furthermore, the increase in hepatic cholesterol content from the cholesterol-rich lipoproteins must also stimulate hepatic triglyceride synthesis in order to maintain constant the composition of the secreted VLDL. In distinction, chronic cholesterol feeding results in an increase in secreted cholesteryl ester substituted for triglyceride in the VLDL particle resulting in a change in VLDL composition (51, 52). The basis for the difference between the acute and chronic responses to an increase in cholesterol delivered to the liver may prove a fruitful area for further research.

In summary, these experiments demonstrate that the administration of a cholesterol-rich lipoprotein bolus increases hepatic cholesterol content, which in turn evokes a decrease in HMG-CoA reductase activity and an increase in cholesterol esterification. In addition, the cholesterol-rich lipoprotein fraction stimulates hepatic cholesteryl ester accumulation and increases the rate of hepatic VLDL secretion. These changes serve to maintain constant the amount of hepatic free cholesterol as well as biliary cholesterol secretion.

The authors wish to thank Ms. Lynda Rager for preparing the manuscript and Dr. Allen Cooper for his thoughtful discussions and suggestions.

Manuscript received 21 March 1986, revised form 25 June 1986, and in re-revised form 6 October 1986.

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