Hepatic lipase promotes the uptake of HDL esterified cholesterol by the perfused rat liver: a study using reconstituted HDL particles of defined phospholipid composition

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Abstract The role of hepatic triacylglycerol lipase (H-TGL) in promoting the liver uptake of high density lipoprotein (HDL) free and esterified cholesterol was studied in a recirculating rat liver perfusion, a situation where the enzyme is physiologically expressed and is active at the vascular bed. For this purpose, reconstituted HDL of defined phospholipid composition were prepared, containing either 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine, a substrate for H-TGL, or 1-*O*-hexadecyl-2-oleoyl-*sn*-glycero-3-phosphocholine, a non-hydrolyzable analog. Reconstituted HDL were then used in the perfused rat liver system. The main results are the following. *1)* Reconstituted HDL were obtained by sonication of lipids and apolipoproteins and isolated by ultracentrifugation in the 1.07–1.21 g/ml density interval. Reconstituted HDL containing either diacylphosphatidylcholine or alkyl-acyl-phosphatidylcholine were similar in terms of chemical composition, apparent size, and apolipoprotein A-I immunoreactivity, and were comparable to native HDL_3. *2)* Reconstituted HDL were labeled with free [^{14}C]cholesterol and [^{3}H]cholesteryl ether, a non-hydrolyzable tracer of esterified cholesterol, and were perfused through the rat liver. Liver uptake of [^{3}H]cholesteryl ether was 2.5-fold higher from reconstituted HDL containing diacylphospholipid than from HDL reconstituted with alkyl-acyl-phospholipids. Liver uptake of free [^{14}C]cholesterol was identical in both cases. *3)* H-TGL-depleted rat livers were obtained by a 12-min preperfusion in the presence of heparin, displacing 90% of the enzymatic activity. The residual activity in the perfusate was inhibited by a specific antibody directed against rat H-TGL. Liver uptake of [^{3}H]cholesteryl ether from reconstituted HDL containing diacylphospholipid was reduced by 35% in hepatic lipase-depleted livers compared to controls. On the other hand, hepatic lipase depletion had no effect on the liver uptake of esterified cholesterol from HDL reconstituted with alkyl-acyl-phospholipids. The above findings support a role for the phospholipase A_1 activity of H-TGL in stimulating the delivery of HDL esterified cholesterol to liver cells.—**Marques-Vidal, P., C. Azéma, X. Collet, C. Vieu, H. Chap, and B. Perret.** Hepatic lipase promotes the uptake of HDL esterified cholesterol by the perfused rat liver: a study using reconstituted HDL particles of defined phospholipid composition. *J. Lipid Res.* 1994. **35:** 373–384.

Supplementary key words hepatic triacylglycerol lipase • free cholesterol • liver perfusion • cholesteryl ether • alkyl-acyl-phospholipids

High density lipoproteins (HDL) deliver cholesterol to liver cells which then secrete bile acids and cholesterol (1). Experimentally, uptake of HDL free and esterified cholesterol has been demonstrated in liver perfusions and in cultured hepatocytes or hepatoma cells (2–5). The mechanisms underlying the delivery of HDL cholesterol to hepatic cells are still unclear and may not be unequivocal. Specific binding of HDL apolipoproteins A-I and A-II to liver cells and membranes has been described by several groups (6–9), but cultured hepatocytes show a selective uptake of HDL esterified cholesterol, exceeding the amount accounted for by the uptake of HDL apolipoproteins (10, 11). Furthermore, a role has been proposed for hepatic triacylglycerol lipase (H-TGL) in facilitating the hepatic uptake of HDL cholesterol (12). H-TGL is a lipolytic enzyme synthesized by hepatocytes and present in liver sinusoid capillaries (13). It exerts triglyceride lipase and phospholipase A_1 activities on circulating HDL (14), leading to a reduction in the particle size (15) and to an increased exposure of certain domains of apolipoprotein A-I at the surface of HDL (16, 17). Moreover, pretreatment of sterol-rich HDL_2 with hepatic lipase or purified phospholipases A_1 and A_2 induces an increased transfer of HDL free cholesterol to Fu5AH hepatoma cells, which can accommodate large amounts of cholesterol (18), or to granulosa cells, which convert cholesterol into progesterone (19). Working with rat hepatocytes, we recently observed that phospholipid hydrolysis in HDL selectively

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Abbreviations: H-TGL, hepatic triacylglycerol lipase; HDL, high density lipoproteins; TLC, thin-layer chromatography; rHDL, reconstituted HDL; TFA, total fatty acid; MDA, malondialdehyde; CE, cholesteryl ester; Alkyl rHDL, HDL particles reconstituted with 1-*O*-hexadecyl-2-oleoyl-*sn*-glycero-3-phosphocholine; Acyl rHDL, HDL particles reconstituted with 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine

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stimulates the transfer of esterified cholesterol (20) and leads to an increased secretion of bile acids by the cultured cells (21).

In order to verify this observation under more physiological conditions, we turned to rat liver perfusion, a situation where H-TGL is present not in a free form but bound to the luminal surface of sinusoid capillaries. Reconstituted HDL of defined chemical composition were used in the perfused liver. Their phospholipids contained sphingomyelin and either diacyl phosphatidylcholine, a substrate lipid for H-TGL, or 1-*O*-alkyl-2-acyl phosphatidylcholine, which cannot be hydrolyzed by the phospholipase A_1 activity of H-TGL.

Our results show that reconstituted HDL of defined phospholipid composition and mimicking the characteristics of native human HDL can be prepared. HDL particles bearing phospholipid substrates for H-TGL deliver 2.5 times more cholesteryl linoleate than non-substrate particles. Experiments with H-TGL-depleted rat livers confirm that, in situ, the enzyme facilitates the disposal of HDL esterified cholesterol.

A preliminary report of this study was presented at the 1990 Meeting of the European Atherosclerosis Society in Lisbon (Portugal).

MATERIALS AND METHODS

Materials

Bovine serum albumin (BSA), leupeptin, pepstatin, trasylol, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (referred to as diacyl-phosphatidylcholine), 1-*O*-hexadecyl-2-oleoyl-*sn*-glycero-3-phosphocholine (referred to as alkyl-acyl-phosphatidylcholine), bovine brain sphingomyelin, cholesteryl linoleate, and free cholesterol were from Sigma (St. Louis, MO). The purity of the lipids was ascertained by thin-layer chromatography (TLC) on silica gel G60 plates (Merck, Darmstadt, Germany), using the solvent system of Skipski, Peterson, and Barclay (22) for phospholipids and petroleum ether–diethylether–acetic acid 165:35:2 (v/v/v) for neutral lipids. [$1\alpha,2\alpha$(n)-^{3}H]cholesteryl linoleyl ether and [4-^{14}C]cholesterol were from Amersham (Les Ulis, France). Chromatography gel AcA 22 was from Pharmacia-LKB (Bromma, Sweden). Hank's medium was from Biochrom KG (Berlin, Germany). Standard heparin was from Choay (Paris, France). Anti-hepatic lipase antibody was a generous gift from Dr. S. Griglio (INSERM U 177, Paris, France). It was raised in goats against H-TGL highly purified from rat liver perfusates and the IgG were purified. One ml antibody inhibited 1.9 units of triacylglycerol hydrolase activity of H-TGL. Monoclonal antibodies 5F6 and 3G10 directed against apolipoprotein A-I were a generous gift from Dr. Ross Milne and Dr. Yves Marcel (23), (Institut de Recherches Cliniques de Montréal, Montréal, Canada); the monoclonal antibodies 4A12 and 2G11 were from SANOFI (Labège, France).

Isolation and delipidation of HDL

High density lipoproteins (HDL, d 1.085–1.190 g/ml) were isolated from the plasma of healthy volunteer donors by sequential ultracentrifugation and were washed at their lower density limit. Lipoproteins were extensively dialyzed against 10 mM Tris-HCl buffer (pH 7.40), containing 150 mM NaCl, 0.01% (w/v) sodium azide, and 0.25 mM EDTA (Tris-NaCl buffer), and were kept under nitrogen, in the dark, prior to delipidation.

HDL were delipidated by successive extractions with diethyl ether–ethanol, according to the method of Brown, Levy, and Fredrickson (24). The delipidated apolipoproteins were solubilized in a Tris-NaCl buffer containing 3 M urea. Apolipoprotein composition was assessed by polyacrylamide (15%, w/v) gel electrophoresis in the presence of sodium dodecyl sulfate (0.1%, w/v) prior to HDL reconstitution.

HDL reconstitution

Reconstituted HDL particles were prepared according to Pittman et al. (25), using human instead of rat apolipoproteins. Briefly, the lipid moiety, consisting of 1.3 μmol diacyl- or alkyl-acyl-phosphatidylcholine, 0.3 μmol sphingomyelin, 1.3 μmol cholesteryl linoleate, 0.3 μmol free cholesterol, and the radioactive compounds was brought to dryness under nitrogen in a glass scintillation vial. Five ml of 10 mM Tris-HCl buffer (pH 8.60), containing 150 mM NaCl, 0.01% (w/v) sodium azide, and 2.5 mM EDTA was added at a temperature of 52°C, and the mixture was quickly vortexed for 30 sec. The lipids were then sonicated in 2-min bursts, with a 1-min pause between the bursts, using a Cell Disruptor Sonicator (Heat Systems, Plainview, NY) equipped with a microtip, at a power setting of 40 watts, and under a nitrogen flow. Temperature was kept constant at 52°C using a water bath. After 40 min of sonication time, the temperature was decreased to 42°C and 1.5 mg of delipidated HDL apolipoproteins was added dropwise, with continuous sonication. The mixture was then sonicated for another 10 min, with 2-min bursts. In some experiments, the solution pH and absorbance at 400 nm were monitored throughout the reconstitution procedure. After apolipoprotein addition, the absorbance decreased from 1.2 to 0.2 in a 10-min sonication. Finally, the solution was cooled to 4°C and tungsten particles were eliminated by centrifugation at 4,000 *g* for 20 min. HDL particles reconstituted with 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine, a substrate for H-TGL, will be referred to as Acyl rHDL, while HDL reconstituted with 1-*O*-hexadecyl-2-oleoyl-*sn*-glycero-3-phosphocholine, which is non-hydrolyzable by H-TGL, will be referred to as Alkyl rHDL. For further perfusion experiments, the protocol described above was repeated 8

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times and the different preparations were pooled.

The reconstituted HDL particles were then isolated by sequential ultracentrifugation between densities 1.07 and 1.21 g/ml. Prior to perfusion, the labeled reconstituted HDL were dialyzed against Hank's medium containing 10 mM Na-phosphate (pH 7.40).

Characterization of HDL particles

The electrophoretic mobility of native and reconstituted HDL was checked by electrophoresis on 2-3% polyacrylamide gradient gels (Sebia, Issy-les-Moulineaux, France). The particle size distribution was assessed by gel filtration on AcA 22 gel and by electron microscopy using native human HDL as a reference. For electron microscopy, reconstituted and native HDL particles were negatively stained with potassium phosphotungstate on carbon-coated grids and photographed at a magnification of 25,000 using an AEI Corinth 275 electron microscope at a power setting of 60 kV. The diameter of 50 particles was determined after a fourfold enlargement of the photograph.

The fatty acid composition of reconstituted HDL particles was determined by gas-liquid chromatography of the methyl esters (26) after lipid extraction and transmethylation. Malondialdehyde, indicative of lipid peroxide formation, was determined by the method of Yagi (27) using tetraetoxypropane as a standard. Apolipoprotein composition was assessed by SDS-polyacrylamide (0.1%-15%, w/v) gel electrophoresis. The absence of apolipoprotein degradation was verified by Western blotting using different monoclonal antibodies (mAbs) directed against apoA-I or apoA-II, kindly provided by Dr. Y. Marcel, and used at a 1:500 dilution. As a secondary antibody, an alkaline phosphatase-conjugated rabbit immunoglobulin, directed against mouse immunoglobulins, was used (Sigma, St. Louis, MO).

Immunoreactivity of apoA-I epitopes in reconstituted HDL was followed by competitive enzyme-linked immunosorbent assay (C-ELISA) using four monoclonal antibodies, 4A12, 2G11, 3G10, and 5F6. The epitopes recognized by the different antibodies have been described elsewhere (16). The plates were coated with 4 μg/ml HDL_3-apolipoproteins, diluted in 0.015 M Na_2CO_3 and 0.035 M $NaHCO_3$, pH 9.60, containing 0.3 mM NaN_3, as a solid phase antigen, and competition was measured with apoA-I present in the different HDL preparations. The antibodies and HDL preparations were diluted in Na-phosphate buffer, 0.01 M, pH 7.20, containing 0.15 M NaCl, 0.3 mM NaN_3, and gelatin 0.5% (w/v). The dilution used for each monoclonal antibody was selected so as to obtain 65% of the maximum binding in absence of competing antigen. The dilutions were between 1:800 and 1:5000. Binding of the mAbs to solid phase apoA-I was revealed using the second antibody described above. Values of ED_{50} were calculated using Log-Logit regression.

Liver perfusion

Rat liver perfusion. Male Wistar rats (350-420 g) were used. Animals were maintained on a standard chow (UAR, France) and were anesthetized with sodium pentobarbital (100 mg/kg) prior to surgical procedures.

In situ recycling liver perfusions were performed according to MacKinnon et al. (3). After a 20-min washing period with preoxygenated Hank's medium, supplemented with 10 mM sodium phosphate buffer (pH 7.40, 37°C), the recirculating perfusion was started with 60-80 ml of fresh medium supplemented with 1% BSA (w/vol), 5 mM glucose, and containing the labeled reconstituted lipoproteins. The average apolipoprotein concentration in the perfusate was 100 μg/ml. The perfusate was oxygenated with a mixture of 95% O_2 and 5% CO_2, and recirculated at a flow rate of 10 ml/min. Temperature was maintained at 37°C throughout the perfusion period. At different time points, 1-ml aliquots of the perfusate were withdrawn for measurement of radioactivity. The viability of the perfused liver was monitored by following the release of intracellular enzymes into the perfusate. Activities of alanine aminotransferase, aspartate aminotransferase, creatine phosphokinase, lactate dehydrogenase, and alkaline phosphatases were measured in a centrifuge analyzer (Cobas Bio, Roche, Basel, Switzerland), using commercial kits (Boehringer, Mannheim, Germany). The levels were very low and did not increase during the procedure. Secretion of glucose was regular with time, ranging between 0.8 and 1.8 μmol/min per g liver. In addition, after 60-min perfusion in absence of HDL, the secretion of triacylglycerol was 0.055-0.060 μmol per gram, reflecting de novo lipoprotein synthesis. In one experiment, the bile duct was cannulated during the perfusion; in 60 min the bile production was 57 μl/g liver, containing 189 ng cholic acid, 88 ng β-muricholic acid, 64 ng chenodeoxycholic acid, and 5 ng deoxycholic acid, as quantified by gas-liquid chromatography of the acetylated derivatives (21).

After a 60-min perfusion in presence of reconstituted HDL, the livers were flushed with lipoprotein-free medium until no circulating radioactivity was found. The organ was then removed, weighed, and cut into 1-g pieces. The biopsies were homogenized in ice-cold Hank's medium by four strokes, using an Ultraturrax homogenizer, and the homogenates were assayed for protein and lipid radioactivity. One gram liver corresponded to 189 ± 23 mg protein.

Hepatic lipase-depleted rat livers. The same procedure of rat liver perfusion was used except that livers were first depleted of hepatic lipase activity. Prior to the recirculating perfusion with labeled lipoproteins, livers were perfused for 12 min using Hank's medium supplemented with 10 mM sodium phosphate buffer (pH 7.40), containing BSA (0.5%, w/v) and heparin (20 IU/ml) (28). During the perfusion, aliquots of the perfusate were withdrawn

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for assay of the enzymatic activity released by heparin. At the end of the heparin chase, hepatic lipase-depleted rat livers were washed for 10 min and the recycling perfusion was then started with the reconstituted lipoproteins, as above, but in presence of 800 μl anti-HTG-L antibody. The recycling perfusion was carried out as described above. Heparin treatment did not alter glucose or triglyceride secretion by the perfused livers, and no increase in the intracellular enzymes was observed. Control perfusions were performed in a similar manner, except for the absence of heparin in the initial perfusion (12 min) medium, and the absence of antibody in the recirculating medium. At the end of the perfusion, biopsies (1 g) were taken and homogenized in 6 ml of an ice-cold medium designed for the solubilization and stabilization of hepatic lipase. This medium contained 25 mM ammonium buffer (pH 8.20), 5 IU/ml heparin, 10 μg/ml leupeptin, 1 μg/ml pepstatin, 25 KIU/ml trasylol, 5 mM EDTA, and 4 mM CHAPS. The homogenates were further centrifuged at 4°C for 20 min at 3,000 *g* and the supernatant containing the solubilized enzyme was assayed for hepatic lipase activity (28).

Measurements of HDL cholesterol uptake

Lipids were extracted from homogenates of 1-g liver samples according to Bligh and Dyer (29). Liver uptake of free cholesterol from reconstituted HDL was derived from the ^{14}C radioactivity recovered in liver samples and the specific radioactivity of [^{14}C]unesterified cholesterol in the perfused HDL. [^{3}H]cholesteryl linoleyl ether was considered as a (non-hydrolyzable) tracer of cholesteryl linoleate present in reconstituted HDL. However, trace amounts of free [^{3}H] cholesterol were always present in [^{3}H]cholesteryl linoleyl ether, so that uptake of ^{3}H radioactivity had to be corrected for free [^{3}H] cholesterol. This was done as follows. The $^{3}H/^{14}C$ ratio for HDL free cholesterol (HDL-IsR) was determined in the perfused reconstituted HDL and in the perfusate at different time points after extraction and thin-layer chromatography of the lipids. The contribution of HDL-free [^{3}H] cholesterol to the total liver uptake of ^{3}H radioactivity was calculated as liver [^{14}C] dpm × HDL-IsR.

H-TGL activity measurements and immunotitrations

Hepatic lipase activity was assayed according to Nilsson-Ehle and Ekman (30) using [^{3}H]triolein (2.66 mM) as a substrate, in the presence of 1 M NaCl. The enzymatic activity was expressed as IU (μmol of free fatty acids released per min). The hepatic lipase assay was linear in the concentration range of 1–3 mIU per tube. Each measurement was performed in duplicate and similar assays were conducted in parallel, after a 60-min preincubation at 0–4°C with a specific polyclonal antibody against hepatic lipase. Preincubations included, in equal volume, the enzymatic source and either a 1:10 dilution of a specific anti-H-TGL antibody in Tris-HCl buffer, 0.2 M, pH 9.00, containing 1% BSA (w/v), or the Tris-BSA buffer alone. The amount of antibody used was calculated to inhibit 4–5 mIU per assay tube. Ninety percent inhibition was achieved in postheparin liver perfusates.

Analytical procedures

Neutral lipids were separated by TLC using petroleum ether–diethyl ether–acetic acid 165:35:2 (v/v/v). Individual spots were visualized by iodine vapors, scraped off, and assayed for radioactivity using a Packard Tri-carb 4530 liquid scintillation counter with automatic quenching correction (Packard Instrumentation International, Zurich, Switzerland). Protein was measured by the procedure of Lowry et al. (31) using BSA as a standard. Total and unesterified cholesterol were determined enzymatically using commercial kits (Boehringer, Mannheim, Germany) (32). Phospholipids were estimated as the lipid phosphorus content according to Böttcher, Van Gent, and Pries (33). ApoA-I was measured by rocket immunoelectrodiffusion using commercial kits (Sebia, Issy-Les-Moulineaux, France).

Statistical analysis

All results are expressed as means ± SE. Statistical comparisons were performed using Students' *t* test unless otherwise stated.

RESULTS

Characterization of reconstituted HDL

In preliminary experiments, different protocols of HDL reconstitution were tested: various pH values (between 7.00 and 9.05) and temperature conditions were used as well as different sequences of lipid and apolipoprotein addition. It appeared that a first step of lipid emulsion (30 min) prior to apolipoprotein addition and a working temperature above 40°C were critical to avoid aggregates and to obtain optically clear solutions. Reconstituted particles were then isolated in the 1.07–1.21 g/ml density interval. **Table 1** displays the chemical composition of the HDL-like particles obtained at two different pH values. A pH of 8.65 during the sonication gave the most reproducible results between different experiments and between preparations containing either diacylphosphatidylcholine or alkyl-acyl-phosphatidylcholine. In these conditions, the contribution of surface components (apolipoproteins, free cholesterol, and phospholipids) was rather close to that obtained in native HDL_3, with, however, a greater participation of phospholipids (34% in rHDL versus 24% in HDL_3) at the expense of apolipoproteins (42.5% in rHDL versus 51.1% in HDL_3). As well, the relative contributions of free and esterified cholesterol were similar in rHDL and in HDL_3. In

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TABLE 1. Chemical composition of Acyl and Alkyl rHDL compared with native human HDL

HDL	n	Apolipoprotein	Cholesteryl Ester	Free Cholesterol	Phospholipids
			%		
Acyl rHDL					
pH 7.40	3	39.06 ± 8.99	27.51 ± 2.81	1.80 ± 0.37	31.64 ± 6.16
pH 8.65	9	42.39 ± 3.28	21.99 ± 1.29	2.09 ± 0.35	33.61 ± 1.93
Alkyl rHDL					
pH 7.40	3	36.91 ± 5.19	18.77 ± 1.35	1.34 ± 0.36	42.98 ± 3.99
pH 8.65	5	42.53 ± 4.76	21.03 ± 1.21	2.05 ± 0.74	34.30 ± 4.29
Native HDL					
HDL_2	8	37.09 ± 1.59	26.48 ± 1.65	4.56 ± 0.55	30.29 ± 1.65
HDL_3	8	51.06 ± 2.26	21.96 ± 1.28	2.22 ± 0.30	23.63 ± 2.39

The percent weight composition was calculated with the estimated molecular mass of 649 for cholesteryl linoleate, 386 for free cholesterol, and 755 for phospholipids. Triacylglycerols are not taken into account. They represented 1.6% and 1.0% of the total mass in HDL_2 and HDL_3, respectively, and were absent in reconstituted HDL. For the reconstituted HDL, two different pH values were tested. The results are expressed as mean ± SEM; n = number of determinations.

general, the contributions of the different components in isolated rHDL were almost similar to their proportions in the initial sonication mixture, except for unesterified cholesterol. Indeed, the latter accounted for 2.1% weight in the reconstituted HDL compared to 3.1% in the initial mixture. Increasing the amount of free cholesterol in the starting material could not enhance its contribution to isolated rHDL. As regards phospholipids in isolated rHDL, the proportion of sphingomyelin measured in four preparations was 13.8% ± 3.0% versus 18.8% in the sonicated material, which may reflect a lower incorporation of sphingomyelin compared to phosphatidylcholine. Finally, it is noteworthy that these artificial particles were devoid of triacylglycerol and thus contained only phosphatidylcholine as a potential substrate towards H-TGL. The overall yield of the reconstitution procedure, including sonication, ultracentrifugation, and dialysis, was about 40%, calculated on apoprotein recovery.

The apolipoprotein composition of the reconstituted HDL particles was determined by SDS/polyacrylamide electrophoresis after Coomassie Blue staining. In all preparations, apoA-I was predominant and bands corresponding to apoA-II and apoCs were detected. Apolipoprotein A-I represented 60 ± 3% of total proteins (n = 3) as assessed by immunological measurements of apoA-I and total protein determination. Immunoblotting of overloaded gels with several monoclonal antibodies directed against apoA-I, apoA-II, and apoE revealed the absence of the latter in reconstituted HDL, as well as the absence of immunoreactive degradation fragments from apoA-I or apoA-II (not shown).

The fatty acid composition of rHDL was analyzed after lipid extraction, acid hydrolysis, and transmethylation of the fatty acid esters (**Table 2**). As the amide bond of sphingomyelin is resistant to hydrolysis in this procedure, the recovered methyl esters originate from phosphatidylcholine and cholesteryl esters. The results were very similar whether the reconstitution procedure was carried out at pH 7.40 or at pH 8.65. In Acyl rHDL, the contributions of palmitic, oleic, and linoleic acids were comparable, which is in good agreement with their almost equal proportions in the lipids of the sonication mixture. Al-

TABLE 2. Fatty acid composition of Acyl rHDL and Alkyl rHDL

HDL	n	C16:0	C18:0	C18:1 n-9	C18:2 n-6	Others
				%		
Acyl rHDL						
pH 7.40	3	28.57 ± 0.29	1.87 ± 0.04	31.68 ± 0.15	35.95 ± 0.33	1.94 ± 0.29
pH 8.65	4	26.65 ± 1.37	1.64 ± 0.08	31.01 ± 1.25	39.31 ± 2.63	1.39 ± 0.11
Alkyl rHDL						
pH 7.40	2	3.50–3.99	7.68–8.13	36.08–36.53	49.73–49.74	2.11–2.51
pH 8.65	3	7.67 ± 3.58	5.02 ± 1.35	36.11 ± 1.86	47.51 ± 4.75	3.69 ± 1.21

Lipids from reconstituted HDL were extracted and the fatty acid methyl esters were analyzed by gas-liquid chromatography. Two different pH values were used for the reconstitution procedure. Results are expressed as percent total fatty acids and are the means ± SD from n determinations; when n = 2, the two individual values are given. The column "others" represents the sum of C16:1 n-7, C17:0, C18:3 n-3, and C20:4 n-6.

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together, these three fatty acids amount to 96-97% of the recovered methyl esters. In Alkyl rHDL, only low amounts of palmitic acid were measured, which confirms the nature of the 1-*O*-ether bond in phosphatidylcholine, non-cleavable by acid hydrolysis. Trace amounts of longer fatty acids were present in the preparations, but no oxidized derivatives, eluting at the beginning of the capillary column, were detected. To further control that lipid peroxidation did not occur during the reconstitution procedure, malondialdehyde (MDA) was measured in the lipid extracts from reconstituted HDL, in parallel to total fatty acids (TFA). A maximum value of 1.9×10^{-4} was calculated for the MDA/TFA ratio (mol/mol).

The size distribution of reconstituted HDL was estimated from the elution patterns in large pore gel filtration (**Fig. 1**) and electron microscopy. Two different columns were run in parallel for Acyl rHDL and Alkyl rHDL so that the elution volumes cannot be superimposed, but an identical preparation of native HDL (d 1.085-1.19 g/ml) was passed through the two columns for comparison. Reconstituted HDL eluted at the same position as native HDL, although the peak distribution was broader and somewhat diplaced towards larger apparent sizes. This may reflect a greater heterogeneity in the size spectrum of reconstituted HDL compared to native HDL. In all preparations, a small peak of radioactive cholesterol, never exceeding 5% of total, eluted in the void volume, which may represent particle aggregates. Finally, the elution patterns of Alkyl rHDL and Acyl rHDL were comparable. When a preparation of rHDL was run every second week on a gel filtration column, the elution profile was similar after 2 weeks, whereas after 1 month, a displacement towards larger sizes was evident. For this reason, reconstituted HDL were used in further perfusion experiments within 10 days. Reconstituted HDL were also electrophoresed in 2-3% polyacrylamide gel electrophoresis, and banded at the same position as native HDL with, however, a broader distribution of the spot. Electron microscopy showed that the reconstituted particles and native HDL had mean diameters of 18.5 ± 2.1 nm and 13.35 ± 1.72 nm (mean ± sem, n = 50), respectively.

To ensure that the expression of different apoA-I epitopes was preserved at the surface of reconstituted HDL,

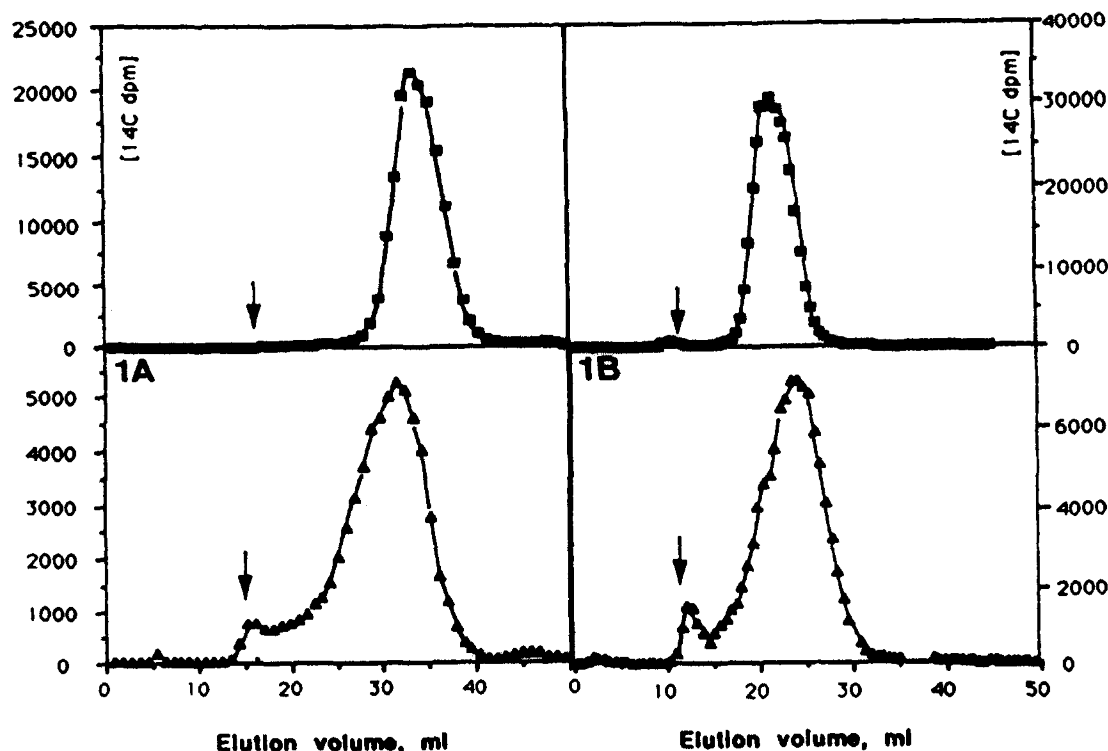

Fig. 1. Gel filtration of native and reconstituted HDL particles. Reconstituted HDL particles containing either diacylphosphatidylcholine (1A, ▲) or alkyl-acylphosphatidylcholine (1B, ▲) were labeled with free [^{14}C]cholesterol and submitted to gel filtration chromatography on AcA 22 (lower part). About 1 mg HDL-protein was added to the column. Two different columns were run in parallel (25 cm × 0.95 cm for Acyl rHDL and 20 cm × 0.95 cm for Alkyl rHDL). In both cases, native human HDL (d 1.085-1.19 g/ml) labeled in the same way were passed through the same column for comparison (upper part, ■). The flow rate was 3.8 ml/h and 0.5-ml fractions were collected. The radioactivity recovered in the elution fractions is displayed. Although different amounts of ^{14}C radioactivity were added, the recovery was always around 95%. The arrows indicate the column void volume.

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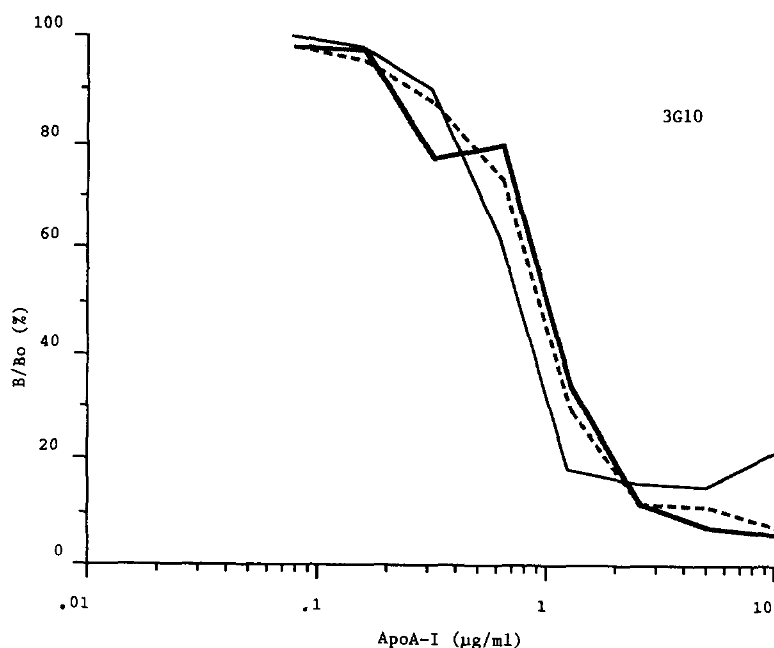

Fig. 2. Immunoreactivity of apolipoprotein A-I in reconstituted HDL particles and in native HDL_3. The immunoreactivity of native HDL_3 (——) and reconstituted HDL, containing either diacylphosphatidylcholine (Acyl rHDL, ━━) or alkyl-acylphosphatidylcholine (Alkyl rHDL, ---) towards monoclonal antibodies directed against human apoA-I was assayed by competitive ELISA. The plates were coated with 4 µg/ml HDL_3-apolipoprotein as a solid phase antigen, and competition was allowed with apoA-I present in the different HDL preparations. The figure shows a representative experiment with monoclonal antibody 3G10.

the immunoreactivity of apoA-I was assayed in C-ELISA towards several monoclonal antibodies (mAbs). **Fig. 2** displays the displacement curve obtained with 1 of these mAbs (3G10) in a typical experiment. The epitope for 2G11 is located in the N-terminal half, is discontinuous, and extends over four separate sequences, while the 4A12 epitope has been mapped between residues 186 and 195, close to the C terminal end. The 3G10 and 5F6 epitopes belong to the center of the molecule, residues 98–103 and 118–141, respectively, and are constituted by β-turn and α-helix fragments. In all cases, the displacement curves obtained with Acyl or Alkyl rHDL were comparable to those obtained with native HDL_3, and this is reflected in the comparison of the ED_{50} values (**Table 3**).

Liver uptake of free [^{14}C] cholesterol and [^{3}H]esterified cholesterol from reconstituted HDL

Reconstituted HDL labeled with free [^{14}C] cholesterol and [^{3}H]cholesteryl ether were perfused for 60 min into the isolated rat liver, in a recycling mode. Two kinds of reconstituted particles were introduced: Acyl rHDL which contain a substrate phospholipid towards H-TGL, and Alkyl rHDL which are not substrates, as the 1-*O*-ether bond of phosphatidylcholine cannot be hydrolyzed by the phospholipase A_1 activity of the enzyme (**Table 4**). The apolipoprotein concentration was 100 µg/ml, which represents about 4 times the K_D of the binding of human HDL on rat hepatocytes (20). Transfer of free [^{14}C] cholesterol from HDL to the liver is referred to as "uptake," although most of it must represent a pure exchange phenomenon, proportional to the pools of exchangeable free cholesterol in HDL and in the cell membranes. The uptake values of HDL free [^{14}C] cholesterol from Acyl rHDL and from Alkyl rHDL were not different. By contrast, the delivery of [^{3}H]cholesteryl ether from Alkyl rHDL to liver cells was only 40% of that obtained from Acyl rHDL ($P < 0.001$). Expressed as percent of the perfused dose by taking into account the liver weight and the perfusate volume, about 30% of the free [^{14}C] cholesterol from HDL was recovered in the liver. The recovery of [^{3}H]cholesteryl ether was 9.5% from Acyl rHDL versus 3.3% from Alkyl rHDL ($P < 0.02$).

TABLE 3. Immunoreactivity of Acyl rHDL and Alkyl rHDL towards different monoclonal antibodies

HDL	n	5F6	3G10	2G11	4A12
Acyl rHDL	2	1.12–1.31	0.44–1.01	0.73–0.91	0.59–0.77
Alkyl rHDL	2	1.16–1.14	1.02–1.08	0.78–0.75	0.74–0.88
HDL_3	3	1.56 ± 0.18	0.96 ± 0.18	0.92 ± 0.04	0.57 ± 0.10
HDL_2	2	6.83–9.18	0.97–2.22	0.45–0.63	0.85–1.15

Immunoreactivity was assessed by competitive enzyme-linked immunosorbent assay (C-ELISA) using four monoclonal antibodies. The dilution used for each monoclonal antibody was selected so as to obtain 65% of the maximum binding in absence of competing antigen. Results are expressed as ED_{50} (µg/ml), determined in a Log/Logit regression. Values are expressed as means ± SD for HDL_3 and as individual values for the other particles (n = 2). The reconstituted particles had an immunoreactivity close to that of native HDL_3.

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TABLE 4. Hepatic uptake of esterified and free cholesterol from Acyl rHDL and Alkyl rHDL

		Esterified Cholesterol		Free Cholesterol	
HDL	n	nmol/g	%	nmol/g	%
Acyl rHDL	4	25.17 ± 1.36	9.47 ± 1.33	9.93 ± 1.99	26.50 ± 0.50
Alkyl rHDL	4	9.29 ± 0.18[a]	3.34 ± 0.20[b]	12.69 ± 3.29[NS]	33.63 ± 6.82[NS]

Reconstituted HDL containing either diacylphosphatidylcholine (Acyl rHDL) or alkyl-acyl phosphatidylcholine (Alkyl rHDL) and labeled with [^{3}H]cholesteryl linoleyl ether and free [^{14}C]cholesterol were perfused in the isolated rat liver for 60 min, using 100 μg apoHDL /ml perfusate. [^{3}H]cholesteryl linoleyl ether was considered representative of HDL cholesteryl esters and its uptake was corrected for free [^{3}H]cholesterol as described in Methods. Results are expressed as nmol cholesterol uptake per gram tissue, and as percent of the perfused dose, taking into account the perfusate volume and the liver weight. Statistical comparisons using the Student's *t*-test: $^aP < 0.001$; $^bP < 0.02$; NS, not significant.

Distribution of H-TGL activity in the perfused rat liver

In order to confirm the role of hepatic lipase in promoting the liver uptake of HDL esterified cholesterol, experiments were performed in H-TGL-depleted rat livers. A preperfusion of the liver with heparin (20 IU/ml) induced a rapid release of hepatic lipase activity, which was maximal after 4 min and then leveled off (not shown). The average activity released from the liver after 12 min was 9.7 IU, compared to 1.5 IU in absence of heparin (**Table 5**). After an extensive washout of heparin from the perfused liver, the recycling perfusion was initiated with the reconstituted HDL and in presence of 0.8 ml of an antibody directed against rat H-TGL. At the end of the perfusion, H-TGL activity was measured in liver biopsies. Control livers were treated likewise except for the absence of heparin in the preperfusion and the omission of the antibody in the recycling perfusion. In control perfusions, 5.6 IU hepatic lipase activity was measured in the liver tissue and 1.8 IU in the recycling perfusate. The corresponding figures in hepatic lipase-depleted perfusions were 0.9 IU in the liver tissue and less than 0.1 IU in the perfusate. The recoveries of H-TGL activity under both conditions were comparable. Hence, about 90% of the liver-associated lipase activity can be displaced by a prior heparin exposure.

Uptake of HDL ^{14}C/^{3}H cholesterol in the H-TGL-depleted rat liver

Acyl rHDL labeled with free [^{14}C] cholesterol and [^{3}H]cholesteryl ether were perfused in control or H-TGL-depleted rat livers as defined in Table 4. The same preparation of reconstituted HDL was used in two parallel experiments, in a control and a hepatic lipase-depleted perfused liver. The recycling perfusions were conducted as described above. The uptake of free [^{14}C] cholesterol was almost unaffected by the removal of H-TGL from the liver vascular bed and the further inhibition of the residual activity (**Table 6**). On the other hand, the uptake of [^{3}H]cholesteryl ether in hepatic lipase-depleted livers was reduced by 35%, compared to control perfusions. Hence, removal of H-TGL led to a significant decrease ($P < 0.05$) in the liver uptake of HDL esterified cholesterol.

When Alkyl rHDL were perfused in control and

TABLE 5. Distribution of H-TGL activity in the perfused rat liver

	H-TGL Activity (IU)		
Treatment	Preperfusion	Recycling Perfusion	Liver Tissue
Heparin + antibody	9.70 ± 1.15 (90%)	0.09 ± 0.05 (0.9%)	0.88 ± 0.59 (8.2%)
Control	1.49 ± 1.08 (16.2%)	1.78 ± 0.53 (20.2%)	5.56 ± 1.41 (63.6%)

Isolated rat livers were preperfused for 12 min either without (control) or with heparin (20 U/ml). After extensive washing, the livers were perfused in a recycling mode as described in Table 3, in the presence of Acyl rHDL, 100 μg/ml apoHDL. When the preperfusion was performed in the presence of heparin, 0.8 ml of an antibody directed against rat H-TGL was added to the recycling perfusate. At the end of the perfusion, the liver was weighed, and 1-g biopsies were taken for lipase activity measurements. The H-TGL activity recovered was calculated taking into account the liver weight and the perfusate volumes. The percentages of total activity recovered are in parentheses. Results are the means ± SD of three determinations.

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TABLE 6. Hepatic uptake of esterified and free cholesterol from Acyl rHDL in control and H-TGL-depleted rat livers

H-TGL	n	Esterified Cholesterol nmol/g	Esterified Cholesterol %	Free Cholesterol nmol/g	Free Cholesterol %
H-TGL present	4	23.43 ± 5.02	8.68 ± 1.86	10.89 ± 2.98	28.91 ± 7.91
H-TGL-depleted	4	15.22 ± 5.70[a]	5.63 ± 2.12[a]	8.93 ± 2.39[NS]	23.71 ± 6.34[NS]

Isolated rat livers were preperfused either in the presence (H-TGL-depleted) or in the absence (H-TGL present) of 20 U/ml heparin. Then, reconstituted HDL, containing diacylphosphatidylcholine (Acyl rHDL) and labeled with [^{3}H]cholesteryl linoleyl ether and free [^{14}C]cholesterol, were perfused in the isolated rat liver in a recycling mode as described in Table 3. During the latter perfusion, H-TGL-depleted rat livers received a 0.8 ml dose of an antibody directed against rat hepatic lipase. Results are expressed as nmol cholesterol uptake per gram tissue, and as percent of the perfused dose, taking into account the perfusate volume and the liver weight. Statistical comparisons using the Student's *t*-test: [a]$P < 0.05$; NS, not significant.

hepatic lipase-depleted livers, no difference was found for [^{3}H]cholesteryl ether uptake: 8.2 versus 9.5 nmol/g for control and hepatic lipase-depleted livers, respectively, $P > 0.32$ (Mann-Whitney test). Also, the uptake of HDL free cholesterol was unchanged: 3.20 versus 3.60 nmol/g of liver for control and hepatic lipase-depleted livers, respectively, $P > 0.40$ (Mann-Whitney test).

DISCUSSION

The objective of the present study was to follow the hepatic uptake of HDL free and esterified cholesterol using reconstituted HDL particles that would be substrates or nonsubstrates for hepatic lipase.

The chemical composition of our reconstituted HDL closely resembles that previously reported by other groups, who obtained spherical particles by sonication of lipids and apoA-I (25, 34). With this procedure, substantial amounts of esterified cholesterol can be incorporated into the lipid core, giving a mass contribution of up to 24% of total, almost similar to that in human HDL_3. Other procedures have been used to prepare spherical HDL recombinants, such as the cholate dialysis method followed by the action of lecithin:cholesterol acyltransferase in the presence of LDL, as a source of free cholesterol (35). Compared to the sonication procedure, this technique enables a higher yield of the HDL components, but a lower incorporation of esterified cholesterol. Moreover, as we wanted to control the phospholipid composition of our reconstituted HDL, any incubation with LDL was to be avoided due to the possible exchange of phospholipids between lipoproteins (36). It is noteworthy that the relative contribution of free cholesterol could not be raised over 2% of total mass. In an earlier study, Jonas and Krajnovich (37) also demonstrated that the incorporation of cholesterol into apoA-I/phospholipid disks was limited, and that excess sterol may impede the interaction between apolipoprotein and phosphatidylcholine.

The expression of four apolipoprotein A-I epitopes was assessed using a panel of monoclonal antibodies spanning different regions of apoA-I, which react differently with lipid-free apoA-I and with particles of different sizes or lipid contents. For instance, the 3G10 epitope, located between residues 98 and 105, is constituted by a β-turn and by part of the adjacent down-stream α-helix (16). This segment is much more immunoreactive at the surface of HDL particles than in free apoA-I, which probably reflects a stabilization of its conformation when present in a lipoprotein complex. Conversely, the 5F6 epitope, which includes one α-helix in the center of the molecule, is expressed less in HDL than in free apoA-I. Furthermore, its immunoreactivity is fairly dependent on the particle size or on the free cholesterol/phospholipid ratio of the HDL surface (16). Hence, the relative exposition of those different epitopes at the surface of HDL is not homogenous, and can be considered as an indirect reflect of the conformation of apoA-I in a particle of a given composition. The displacement curves obtained with our reconstituted HDL and with native HDL_3 were comparable. Although these results cannot establish that the overall conformation of apoA-I is similar in reconstituted and native HDL particles, they indicate that the reconstitution procedure does not significantly alter the apoA-I conformation at the surface of the reconstituted HDL.

ApoA-I represented 60% of total protein in the reconstituted HDL particles, the remaining being represented by apoA-II and apoCs. Hence, the rHDL appear to be compositionally similar to HDL containing apoA-I and apoA-II, and not to HDL with apoA-I alone. ApoA-II has been shown to stimulate H-TGL activity (38), and a recent report indicates that LpA-I:A-II particles are more reactive towards H-TGL than LpA-I (39). Nevertheless, these results have not been confirmed by other authors (40, 41). Interestingly, Pittman and coworkers (25) reconstituted HDL with rat apolipoproteins, which are poor in apoA-II (less than 5%), and these particles were also able to selectively transfer more esterified cholesterol than

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apoA-I towards cultured hepatocytes. Hence, the exact role of apoA-II towards H-TGL and in the liver uptake of HDL components remains to be assessed.

The role of hepatic lipase as a factor involved in the delivery of HDL cholesterol to the liver has been postulated for more than a decade (12). Experiments carried out in cultured cells have indeed demonstrated that pretreatment of HDL subfractions with purified H-TGL or phospholipases A stimulates the delivery of HDL free or esterified cholesterol, depending on the cell model studied (18-20). The present study lends further support to this hypothesis in a situation closer to the physiological conditions. In a previous work (28), we observed that the perfused rat liver is able to hydrolyze phosphatidylcholine, phosphatidylethanolamine, and triacylglycerol from human HDL, and to take up the generated free fatty acids. Although triacylglycerol behaves as a better substrate towards H-TGL compared to phosphatidylcholine (14), due to their relative proportions in human HDL, comparable amounts of both substrates were degraded (28). In this study, we prepared reconstituted HDL containing only one substrate for H-TGL, i.e., diacylphosphatidylcholine. In parallel experiments, this phospholipid was replaced by an alkyl-acyl analog, bearing the same fatty chain length and unsaturation, but non-hydrolyzable by H-TGL. We observed that the substrate HDL particles deliver 2.5-fold more esterified cholesterol than the non-substrate particles. The role of hepatic lipase in this process was further confirmed by the observation that H-TGL-depleted rat livers take up 35% less esterified cholesterol from substrate HDL particles. In this work, the fact that the uptake values from diacyl rHDL in H-TGL-depleted livers were somewhat higher than the values obtained with alkyl-acyl rHDL in control livers is unexplained. It is, however, possible that the 10% of H-TGL activity remaining in the perfused liver, following heparin displacement and immunological inhibition, may play a significant role in the uptake of esterified cholesterol from diacyl rHDL.

Liver cells, as well as adrenal or ovarian cells, are able to take up HDL cholesterol for further conversion into secreted steroids (2-5, 21, 42). As it has been reported in rat or rabbit hepatocytes and in adrenal cells (5, 11, 20), the transfer of HDL esterified cholesterol exceeds by 2- to 8-fold the amount accounted for by the uptake of the whole HDL particle, calculated from the uptake of apoA-I. In the perfused rabbit liver, a selective transfer of HDL esterified cholesterol was also demonstrated, and after 90 min, some 25% of the esterified cholesterol from perfusate HDL were recovered in the liver tissue over a wide range of HDL concentrations (3). In the present work, only 10% of the HDL cholesteryl ether was taken up by the rat liver, after a 60-min perfusion. These figures are in the same range, but species differences may be invoked regarding the amplitude of the uptake of esterified cholesterol. Alternatively, the selective transfer of HDL cholesteryl esters may depend on the particle size and density, being greater with denser particles (11, 25).

The present study is additional experimental evidence of the role of hepatic lipase in promoting the delivery of HDL esterified cholesterol to liver cells. Although the present results do not explain the mechanism by which the liver takes up CE from HDL, it is possible that hepatic lipase may bind to the amphipathic phospholipid/apolipoprotein interface of the particle (43), thus leading to a local concentration of HDL that will facilitate its further interaction with cells. However, the lower uptake of HDL cholesterol from alkyl-acyl rHDL, particles which also provide an amphipathic interface, suggests that the phospholipase A_1 catalytic function of H-TGL is of importance in triggering the transfer of esterified cholesterol. A limited phospholipid hydrolysis for instance, might uncover certain domains of apoA-I as we reported earlier (16), and these domains would in turn interact with specific cell receptors. In favor of this hypothesis, several apoA-I epitopes are better exposed on small-sized HDL_3 than in larger HDL_2, e.g., 3G10, 5F6 (16). The selective transfer of HDL esterified cholesterol was also reported to be greater with particles of decreasing sizes (11, 25). However, attempts to inhibit the transfer of HDL cholesterol to hepatic cells with anti-apoA-I monoclonal antibodies led to only partial inhibition (44), and further research is needed to elucidate which domains of apoA-I are involved in HDL-hepatic cells interactions.

In conclusion, the use of HDL recombinants of a controlled phospholipid composition combined with the liver perfusion system demonstrate that, in situ, the phospholipase A_1 activity of hepatic lipase promotes the uptake of HDL esterified cholesterol by the liver.

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